Available online at www.sciencedirect.com
ScienceDirect
Metabolism Clinical and Experimental 59 (2010) 359–366
Metabolism
Clinical and Experimental
www.metabolismjournal.com

Glimepiride induces proliferation and differentiation of rat osteoblasts via the PI3-kinase/Akt pathway

Pan Ma[a,b], Bin Gu[a], Junli Ma[a], Lingling E[a], Xia Wu[a], Junkai Cao[a], Hongchen Liu[a,*]

[a]*Institute of Stomatology, Chinese General Hospital of PLA, Beijing 100853, PR China*
[b]*Huangsi Aesthetic Plastic Surgery Hospital, Beijing 100011, PR China*

Received 11 February 2009; accepted 4 August 2009

Abstract

Glimepiride is a third-generation sulfonylurea agent and is widely used in the treatment of type 2 diabetes mellitus. In addition to the stimulatory effects on pancreatic insulin secretion, glimepiride has also been reported to have extrapancreatic functions including activation of PI3 kinase (PI3K) and Akt in rat adipocytes and skeletal muscle. PI3-kinase and Akt are important signaling molecules in the regulation of proliferation and differentiation in various cells. This study investigated the actions of glimepiride in rat osteoblasts and the role of PI3K/Akt pathway. Cell proliferation was determined by measuring absorbance at 550 nm. Supernatant assay was used for measuring alkaline phosphatase activity. Western blot analysis was used for determining collagen I, insulin receptor substrate–1/2, PI3K/Akt, and endothelial nitric oxide synthase expression. We found that glimepiride significantly enhanced proliferation and differentiation of osteoblasts and led to activation of several key signaling molecules including insulin receptor substrate–1/2, PI3K/Akt, and endothelial nitric oxide synthase. Furthermore, a specific inhibitor of PI3K abolished the stimulatory effects of glimepiride on proliferation and differentiation. Taken together, these observations provide concrete evidence that glimepiride activates the PI3K/Akt pathway; and this activation is likely required for glimepiride to stimulate proliferation and differentiation of rat osteoblasts.

1. Introduction

Diabetes mellitus has been associated with specific bone metabolism alterations [1,2]. Sulfonylurea has been widely used in the treatment of type 2 diabetes mellitus. Investigations also found that long-term use of sulfonylurea is responsible for a significantly decreased risk of fracture, which may attribute to the increase of bone density [3,4]. These changes may result from the direct effects of sulfonylurea on osteoblasts or from changes in the bone microenvironment. Experimental evidence to distinguish these possibilities is currently lacking.

Glimepiride is a third-generation sulfonylurea agent. In addition to the stimulatory effects on pancreatic insulin secretion, glimepiride has also been shown to play extrapancreatic roles in tyrosine phosphorylation of insulin receptor substrate–1/2 (IRS-1/2) and activation of the PI3 kinase (PI3K)/Akt pathway in rat adipocytes and skeletal muscle [5-9]. In endothelial cells, it is suggested that glimepiride induces endothelial nitric oxide synthase (eNOS) phosphorylation with a dependent mechanism of PI3K/Akt [10,11]. PI3 kinase is a heterodimeric enzyme important for growth and proliferation, and Akt is a downstream serine-threonine kinase that transmits survival signals from growth factors [12,13]. Previously, the PI3K/Akt pathway was shown to be involved in osteoblastic cell proliferation and differentiation [14,15]. LY294002 is a specific, reversible inhibitor of PI3K and is useful in investigating the functional and regulatory mechanisms mediated by PI3K [16]. To date, direct elucidation of the role of glimepiride on osteoblast cell growth, however, has not been reported.

In the current study, we examined the effects of glimepiride on proliferation and differentiation of osteoblasts and investigated a putative regulatory mechanism involving the PI3K/Akt pathway.

* Corresponding author. Tel.: +86 10 66937949; fax: +86 10 66936254.
E-mail address: Liuhc2009@yahoo.com.cn (H. Liu).

0026-0495/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2009.08.003

2. Materials and methods

2.1. Materials

Sprague-Dawley rats aged 6 to 8 weeks were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China). Glimepiride and 3-(4,5-dimethylithiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma (St Louis, MO). Antibodies against Akt, phospho-Akt (P-Akt, Ser-473), IRS-1, and IRS-2 were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). LY294002 and antibodies against phosphor-IRS-1, phospho-IRS-2, and PI3K (P85) were from Cell Signaling Technology (Danvers, MA). Anti–collagen I antibody was kindly provided by Dr Larry W Fisher (NIDCR, Bethesda, MD).

2.2. Primary osteoblast cultures

Primary osteoblasts were prepared as previously described [17-19]. In brief, primary osteoblasts were obtained from the mandible of 6- to 8-week-old Sprague-Dawley rats. To avoid other cell types presented in these cultures, we minced the mandibles into fragments after removal of the condylar cartilage, connective tissue, and alveolar socket including periodontal ligament. To further avoid particularly periodontal ligament cells, the supernatant was removed after mandibles were treated with 0.1% type I collagenase solution for 10 minutes at 37°C. The next two 20-minute sequential collagenase digestions were then pooled and filtered through 100-μm nylon filters. The cells were plated in plastic cell culture dishes and incubated in a 37°C incubator with 5% CO_2. L-Dulbecco modified Eagle medium (DMEM) cell culture medium was supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The resultant osteoblasts were characterized for morphology, alkaline phosphatase (ALP) expression, and calcification nodule staining. Osteoblast cells were subsequently divided into 4 experimental groups: (1) the control group: L-DMEM alone, (2) the glimepiride group: L-DMEM + glimepiride (10 μmol/L), (3) the LY294002 group: L-DMEM + LY294002 (10 μmol/L), and (4) the glimepiride + LY294002 group: L-DMEM + glimepiride (10 μmol/L) + LY294002 (10 μmol/L). Culture medium was changed every 3 days. The concentrations for glimepiride and LY294002 were chosen according to previous reports [5-9,20-22]. Glimepiride stock solution (10 mmol/L) was prepared by dissolving the powder in distillate water. Before use, the stock solution was adjusted with 0.03 N NaOH and heated to 56°C.

2.3. The MTT assay

We used MTT as an indicator of cell viability. Osteoblasts were plated onto 96-well culture plates at a density of 1.5×10^4 cells per well in 10% fetal bovine serum/L-DMEM. After being cultured for 24 hours, the cells of 4 groups were serum-starved overnight in L-DMEM with 0.5% bovine serum albumin and then were cultured for another 7 days. On each day during this period, 20 μL MTT was added and incubated for 4 hours at 37°C. Subsequently, the supernatants were removed; and the blue crystals in viable cells were solubilized with 150 μL dimethylsulfoxide. The supernatants were then transferred to a 96-well plate, and the absorbance of each well was measured at 550 nm using an ELx800uv reader (Bio-Tek Instruments, Winooski, VT).

2.4. The ALP assay

Alkaline phosphatase is a marker for differentiated osteoblasts. The supernatants of the above groups were collected at 3 periods: days 1 to 7, days 8 to 14, and days 15 to 21. The supernatant from each well was collected twice a week. The ALP activities in supernatants were determined by measuring hydrolysis of *p*-nitrophenyl phosphate according to the manufacturer's instructions (Sigma). The supernatant was incubated with 30 mmol/L *p*-nitrophenyl phosphate in an alkaline buffer (pH 10.5) for 60 minutes in a 37°C water bath. The reaction was terminated by adding 2 mL of ice-cold 0.1 N NaOH. The amount of *p*-nitrophenyl phosphate released was measured at 410 nm by using an ELX800 microplate reader (Bio-Tek Instruments).

2.5. Western blot analysis

Cells were plated at 2×10^5 cells in 24-well culture plates and serum-starved for 24 hours before incubation with different doses of glucose. The levels of collagen I were measured in 4 groups on days 1, 7, and 14. To analyze protein levels of IRS-1/2, PI3K, and Akt/P-Akt, cells were cultured with glimepiride for 0, 15, 30, 60, and 120 minutes and subsequently washed with PBS for 3 times before cell lysis was carried out for 30 minutes at 4°C to prepare cell extracts. The electrophoresis was performed on 8% to 12% sodium dodecyl sulfate gels, followed by protein transfer to Immobilon-P (polyvinylidene difluoride) membranes (Bedford, MA) at 4°C overnight. Membranes were blocked with 5% nonfat milk in TBS-T (Tris-Buffered Saline with Tween 20) for 1 hour at room temperature (RT) and then incubated with primary antibodies diluted in TBS-T for 3 hours at RT. After 3 washes in TBS-T, membranes were incubated with a sheep anti-rabbit peroxidase-conjugated secondary antibody (1:10 000 dilution) for 1 hour at RT. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS films (Rochester, NY). As a control, the same blot was also probed with rabbit anti-rat β-actin antibody (1:1000 dilution). Signal band intensity was measured by quantifying optical density (Leica, Wetzlar, Germany). The ratio of targeted band to actin was considered as the relative intensity of targeted band.

2.6. Statistical analysis

An SPSS (Chicago, IL) 11.0 software package was used for statistical analysis. Data were expressed as mean ± SD,

and $P < .05$ was considered statistically significant. To evaluate the differences between groups, least significant difference test and Scheffe test were conducted. All experiments were repeated at least 3 times, and representative experiments are shown.

3. Results

3.1. Glimepiride enhanced proliferation and differentiation of osteoblasts

We first asked whether glimepiride may affect proliferation of osteoblasts. To this end, we measured viable cells via the MTT assay and plotted growth curves for 4 groups of cells (Fig. 1). The number of viable cells in the control group and the glimepiride group increased gradually from day 1. From days 5 to 7, the glimepiride group exhibited significantly more growing cells than the control group (Fig. 1A), with 11.1% higher MTT measurement (0.60 ± 0.03 vs 0.54 ± 0.04, $P < .05$) on day 7.

We next observed the effect of glimepiride on differentiation of osteoblasts by measuring the levels of ALP and collagen I at 3 intervals: days 1 to 7, 8 to 14, and 15 to 21. During each of these time intervals, the ALP levels in the glimepiride group were significantly higher than those of the control group (days 1-7: 8.02 ± 0.3 vs 6.06 ± 0.4, $P < .05$; days 8-14: 12.48 ± 0.3 vs 10.88 ± 0.2, $P < .05$; days 15-21: 8.87 ± 0.4 vs 7.58 ± 0.4, $P < .05$) (Fig. 1B). In addition, the expression of collagen I was analyzed by Western blot on days 1, 7, and 14 (Fig. 1C). Significantly higher expression of collagen I was again observed in the glimepiride group than the control group on day 7 (35.33%, 10.61 ± 0.3 vs 7.84 ± 0.2, $P < .05$) and day 14 (41.54%, 8.79 ± 0.3 vs 6.21 ± 0.2, $P < .05$). These results together indicate that glimepiride significantly increased rat osteoblast proliferation and differentiation.

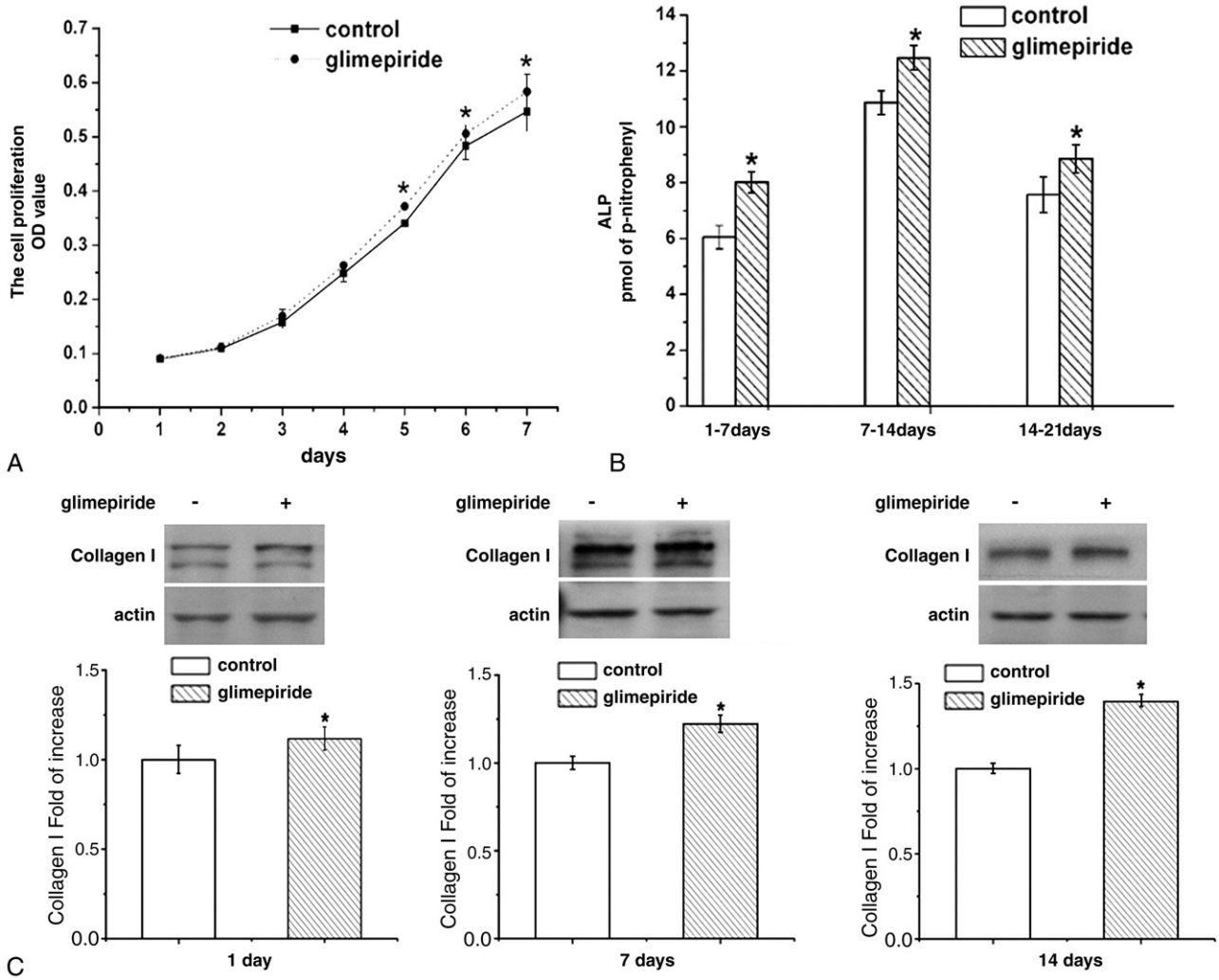

Fig. 1. Glimepiride enhanced proliferation and differentiation of rat osteoblasts. Osteoblasts were incubated in L-DMEM with or without glimepiride. A, The viability was tested by MTT assay. B, The ALP activities in osteoblasts as measured by hydrolysis of *p*-nitrophenyl phosphate in the supernatant. C, Collagen I expression determined by Western blotting. The membranes were stripped and reprobed with anti-actin antibodies. Data are presented as mean ± SEM. The MTT measurements and the ALP activity measurements were repeated 6 times. Collagen I measurements were repeated at least 3 times. *$P < .05$ vs the control group.

3.2. The PI3K/Akt signaling pathway participated in the regulation of proliferation and differentiation of osteoblasts by glimepiride

To determine whether glimepiride can activate the PI3K/Akt pathway in rat osteoblasts, osteoblasts were cultured with glimepiride for 15, 30, 60, and 120 minutes. Western blot analysis was performed to determine IRS-1/2, PI3K, Akt, and eNOS expression.

We found that expression of the P85 subunit of PI3K was rapidly induced by glimepiride, with a substantial increase observed after 15 minutes; further accumulation of P85

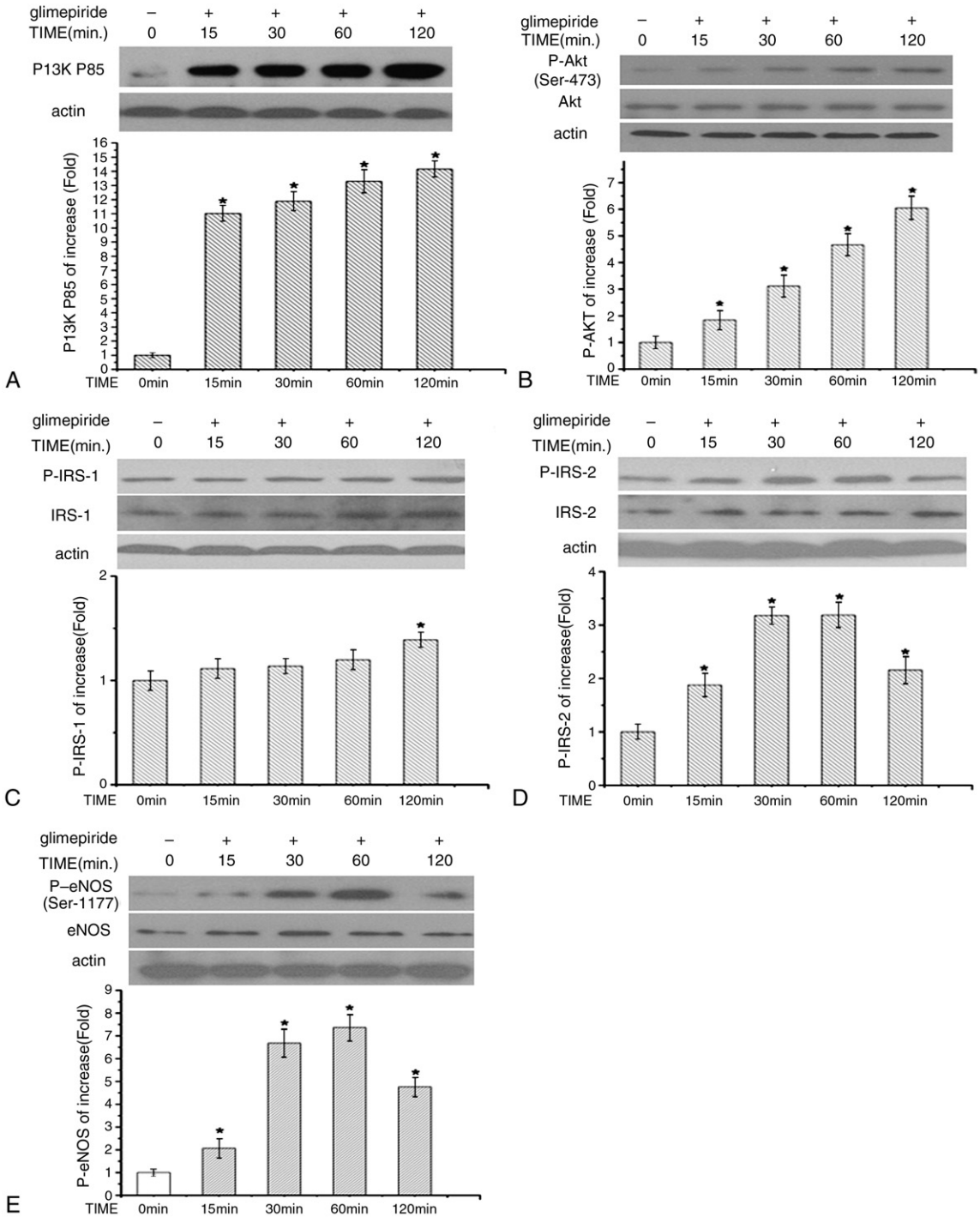

Fig. 2. Activation of the PI3K/Akt pathway in rat osteoblasts by glimepiride. Osteoblasts were incubated with glimepiride (10 μmol/L) for up to 120 minutes. Western blot was performed to detect (A) PI3K p85, (B) P-Akt (Ser-473) and total Akt, (C) phosphor-IRS-1 and total IRS-1, (D) phospho-IRS-2 and total IRS-2, and (E) P-eNOS (Ser-1177) and total eNOS. Actin served as the internal control. Data are presented as mean ± SEM from 3 separate experiments. The lower panels of parts A to E are normalized densitometer plots. *$P < .05$ vs the control group.

continued afterward, reaching approximately 14.2-fold of the baseline level (time 0) at 120 minutes (Fig. 2A). Whereas the total expression of Akt was unaltered throughout the time course, the levels of P-Akt (Ser-473) increased gradually, accumulating to a level 6-fold higher than the baseline level after 120 minutes of incubation (Fig. 2B). Likewise, the levels of phosphorylated IRS-1 and IRS-2 showed 1.4-fold increase after 120 minutes and 3.1-fold increase after 60 minutes, respectively, despite near-constant levels of total IRS-1 and IRS-2 proteins (Fig. 2C, D). Because eNOS is a downstream regulator of the PI3K/Akt pathway, we also examined eNOS expression in response to glimepiride treatment. Glimepiride markedly induced the level of phospho-eNOS (P-eNOS) (Ser-1177), with more than 7.3-fold increase at 60 minutes; on the other hand, total eNOS levels were only slightly enhanced (Fig. 2E). These results strongly indicate that glimepiride treatment activated the PI3K/Akt signaling pathway.

Finally, osteoblasts were preincubated for 30 minutes with LY294002. The results showed that LY294002 abolished glimepiride-dependent Akt and eNOS activation (Fig. 3).

3.3. LY294002 inhibited the enhanced proliferation and differentiation of osteoblasts induced by glimepiride

To further address the question of whether the PI3K/Akt signaling pathway participated in the enhanced proliferation and differentiation of osteoblasts induced by glimepiride, we next investigated the effects of LY294002, a specific PI3K inhibitor, on osteoblasts in the presence or absence of glimepiride treatment. As shown in Fig. 4A, the number of viable osteoblast cells in the LY294002 group was clearly reduced relative to the control group, comprising only 40.74% of the control group on day 7 (0.22 ± 0.02 vs 0.54 ± 0.04, $P < .05$) (Fig. 4A). Likewise, the glimepiride + LY294002 group also exhibited similarly marked decreases compared with the glimepiride group, with 58.62% decrease on day 7 (0.24 ± 0.03 vs 0.58 ± 0.03, $P < .05$) (Fig. 4B). No significant differences were detected between the LY294002 group and the glimepiride + LY294002 group.

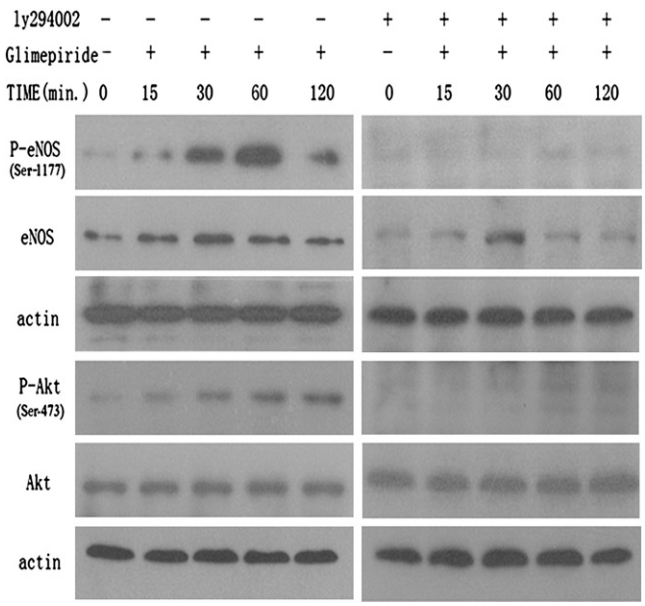

Fig. 3. LY294002 inhibited glimepiride-induced phosphorylation of Akt and eNOS in osteoblasts. Osteoblasts were treated with glimepiride (10 μmol/L) for the indicated times in the absence (left panels) or presence (right panels) of LY294002. Total cell extracts were subjected to Western blotting analysis. The blots shown here are representative of at least 3 independent experiments.

We also examined changes in ALP activity in response to LY294002. The ALP activities in the LY294002 group were significantly lower than those in the control group (days 1-7: 2.54 ± 0.3 vs 6.06 ± 0.4, $P < .05$; days 8-14: 3.20 ± 0.2 vs 10.88 ± 0.2, $P < 0.05$; days 15-21: 2.94 ± 0.4 vs 7.58 ± 0.4, $P < .05$). Similarly, ALP activities in the glimepiride + LY294002 group were also greatly reduced compared with the glimepiride group (days 1-7: 2.86 ± 0.2 vs 8.02 ± 0.3, $P < .05$; days 8-14: 3.50 ± 0.3 vs 12.48 ± 0.3, $P < .05$; days 15-21: 3.3 ± 0.4 vs 8.87 ± 0.4, $P < .05$) (Fig. 4C). Furthermore, collagen I levels in the LY294002 group and the glimepiride + LY294002 group were found to be significantly decreased, with 65.43% and 65.05% reduction on day 14 ($P < .05$) compared with the control group and the glimepiride group, respectively (Fig. 4D). No significant differences in ALP activity and collagen I level were observed between the LY294002 group and the glimepiride + LY294002 group (data not shown). These observations indicate that LY294002 inhibited proliferation and differentiation of osteoblasts regardless of glimepiride.

4. Discussion

Glimepiride is the third generation of sulfonylureas. Besides accelerating the excreting of insulin [23], glimepiride has also been reported to have extrapancreatic functions [5-9]. Previously, Vestergaard et al [3,4] reported that long-term use of sulfonylurea agents could significantly decrease risk of bone fracture [3,4]. The effect of glimepiride on insulin excretion might partially explain this effect. However, could glimepiride exert direct effects on bone metabolism; or more precisely, could glimepiride itself affect the proliferation and differentiation of bone cell, like osteoblast? Based on this speculation, we examined the effects of glimepiride on rat osteoblast proliferation, ALP activity, and collagen I production. Our results clearly demonstrated stimulatory effects of glimepiride on proliferation and differentiation of rat osteoblasts, thus providing a possible mechanism for the beneficial effect of glimepiride on bone fracture that Vestergaard et al reported. Because in vitro culture conditions represent only a rudimentary approximation of the metabolism environment in vivo, future research is needed to test the influences of glimepiride on bone growth in a more physiologic setting.

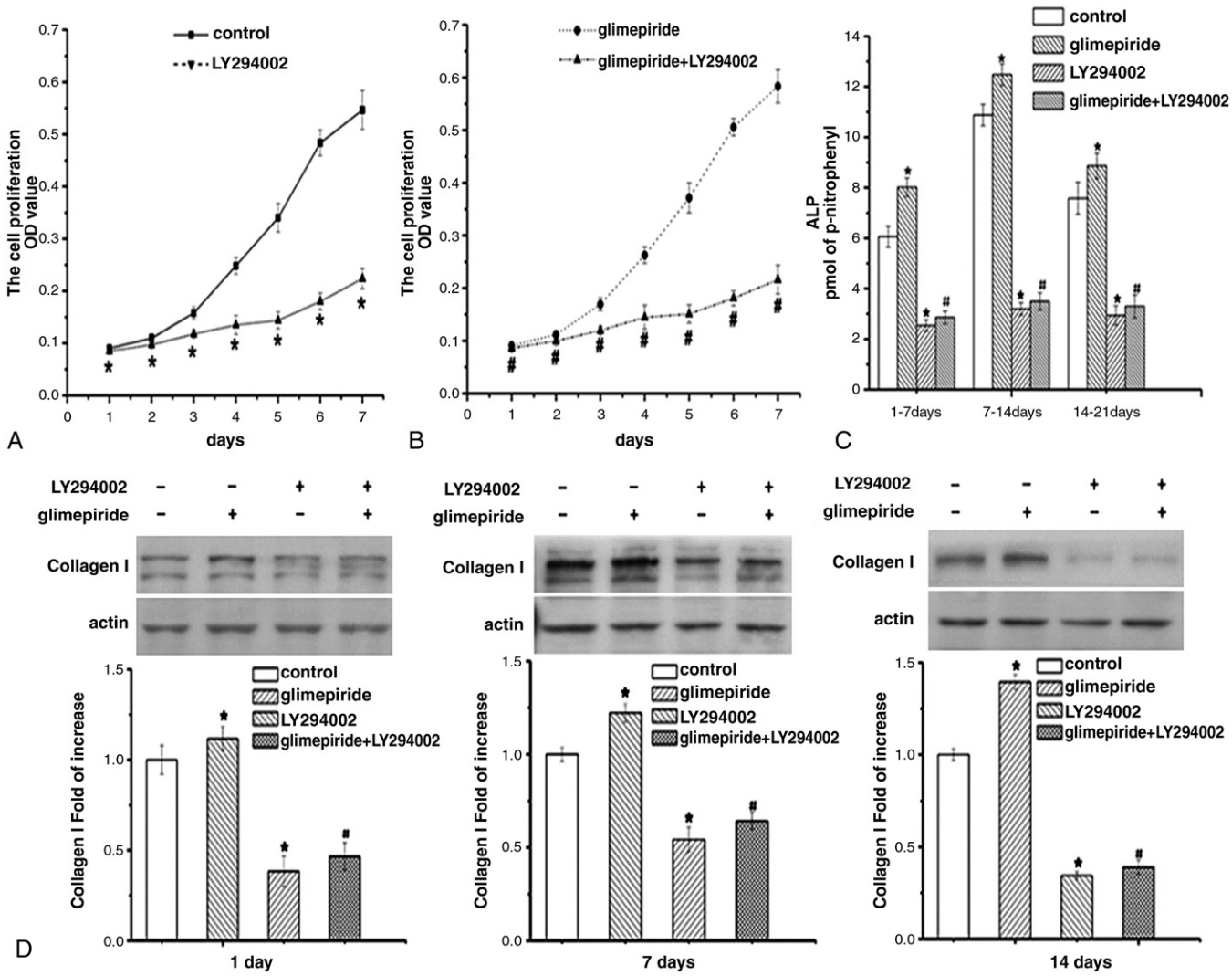

Fig. 4. Pharmacologic inhibition of PI3K/Akt inhibits proliferation and differentiation of rat osteoblasts. Cells were cultured incubated in L-DMEM with or without LY294002 (10 μmol/L) or glimepiride. A and B, The cell proliferation was tested by MTT assay. C, The ALP activities in osteoblasts as measured by hydrolysis of *p*-nitrophenyl phosphate in the supernatant. D, Collagen I expression determined by Western blotting. The membranes were stripped and reprobed with anti-actin antibodies. Data are presented as mean ± SEM. The MTT measurements and the ALP activity measurements were repeated 6 times. Collagen I measurements were repeated at least 3 times. $^{*}P < .05$ vs the control group; $^{\#}P < .05$ vs the glimepiride group.

A number of studies have provided evidence for extrapancreatic functions of glimepiride, including tyrosine phosphorylation of IRS-1/2 and activation of the PI3K/Akt pathway in rat adipocytes, muscle cells, and endothelial cells [5-11]. However, putative effects of glimepiride on the PI3K/Akt pathway in osteoblasts were not reported. In this study, glimepiride was shown to induce accumulation of PI3K P85 protein and activation of Akt 473 site in phosphorylated protein fraction in rat osteoblasts. Furthermore, tyrosine phosphorylation of IRS-1/2, an upstream regulator of PI3K/Akt, was enhanced after glimepiride treatment. Finally, glimepiride also appeared to activate eNOS phosphorylation at Ser-1177 in rat osteoblasts, a downstream regulator of the PI3K/Akt pathway. Taken together, our findings strongly suggest that glimepiride activates the PI3K/Akt pathway in osteoblasts.

Extensive research has indicated that activation of the PI3K/Akt pathway enhances proliferation and differentiation of osteoblastic cells [14,15,24-28]. We hypothesized that the PI3K/Akt pathway may also play a key role in glimepiride-induced proliferation and differentiation of rat osteoblasts. To test this hypothesis, we conducted experiments using LY294002, a PI3K-specific inhibitor [16].

Consistent with previous observations that the enhanced proliferation and differentiation of osteoblastic cells by PI3K activators were largely blocked by LY294002 [24,26,27], 10-μmol/L LY294002 treatment abolished glimepiride-stimulated Akt and eNOS activation, and significantly blocked glimepiride-induced proliferation and differentiation of rat osteoblasts. One explanation for this effect was that glimepiride-induced proliferation and differentiation of rat osteoblasts were mediated by the PI3K/Akt pathway;

another possible reason might be that LY294002 was toxic for osteoblasts, which covered the effect of glimepiride-induced proliferation and differentiation via other pathways. In our view, the possibility of the second explanation was very weak. LY294002 is a specific, reversible inhibitor of the adenosine triphosphate binding site of PI3K and has been widely used in investigating the functional and regulatory mechanisms mediated by PI3K since 1994 [16]. In previous studies, the toxicity of LY294002 itself has been extensively discussed [16,29-32]. We conducted experiments using LY294002 at the concentration of 10 μmol/L as described in previous studies [21,22,24,26]. Thomas et al [24] have shown that, at this concentration, LY294002 was nontoxic for osteoblastic cells.

We did not observe any significant difference between the LY294002 group and the glimepiride + LY294002 group. These results demonstrated that the PI3K/Akt pathway appears to play a predominant role in glimepiride-induced proliferation and differentiation of rat osteoblasts. However, we still could not exclude additional signaling pathways involved in this process, which needs to be identified in future study.

In our research, we noticed that glimepiride enhanced the levels of not only P-eNOS but also total eNOS. Katharine et al [33] also found that estrogen up-regulated the expression of total eNOS in human osteoblastic cells. Interestingly, Salani et al [10] observed activation of P-eNOS, but not total eNOS, in glimepiride-treated endothelial cells, perhaps reflective of cell-type specificity. In vitro studies previously indicated that eNOS could regulate bone metabolism. For example, estrogen was known to enhance proliferation and differentiation of osteoblastic cells by stimulating eNOS activities [34-37]. Given these precedents, we hypothesized that glimepiride might enhance bone metabolism partially through induction of eNOS activity in osteoblasts. In the future, we need to refine our understanding of the role of eNOS on glimepiride-induced osteoblast proliferation and differentiation.

In summary, our results provide direct evidence that glimepiride could enhance rat osteoblast proliferation and differentiation, in which the PI3K/Akt pathway plays a key role. We also noticed that the effects of glimepiride on proliferation and differentiations of rat osteoblasts were relatively small, compared with other biological factors regulating bone metabolism, like insulin and insulin-like growth factor–1 [38-42]. Evaluation of the direct effects of glimepiride on proliferation and differentiation of rat osteoblasts in the complex mechanism needs to be further explored.

Acknowledgment

We thank Dr Huixia He for critically reading the manuscript. We also thank Drs Li Zang and Kang Chen for their unselfish technical assistance.

References

[1] Kemink SA, Hermus AR, Swinkels LM, Lutterman JA, Smals AG. Osteopenia in insulin-dependent diabetes mellitus; prevalence and aspects of pathophysiology. J Endocrinol Invest 2000;23:295-303.
[2] Suzuki K, Sugimoto C, Takizawa M, et al. Correlations between bone mineral density and circulating bone metabolic markers in diabetic patients. Diabetes Res Clin Pract 2000;48:185-91.
[3] Vestergaard P, Rejnmark L, Mosekilde L. Relative fracture risk in patients with diabetes mellitus, and the impact of insulin and oral antidiabetic medication on relative fracture risk. Diabetologia 2005;48: 1292-9.
[4] Vestergaard P, Rejnmark L, Mosekilde L. [Osteoporosis and fractures associated with drugs]. Ugeskr Laeger 2008;170:1550-5.
[5] Muller G, Geisen K. Characterization of the molecular mode of action of the sulfonylurea, glimepiride, at adipocytes. Horm Metab Res 1996; 28:469-87.
[6] Kramer W, Muller G, Geisen K. Characterization of the molecular mode of action of the sulfonylurea, glimepiride, at beta-cells. Horm Metab Res 1996;28:464-8.
[7] Muller G. The molecular mechanism of the insulin-mimetic/sensitizing activity of the antidiabetic sulfonylurea drug Amaryl. Mol Med 2000; 6:907-33.
[8] Rodriguez E, Pulido N, Romero R, et al. Phosphatidylinositol 3-kinase activation is required for sulfonylurea stimulation of glucose transport in rat skeletal muscle. Endocrinology 2004;145:679-85.
[9] Muller G, Jung C, Wied S, Welte S, Frick W. Insulin-mimetic signaling by the sulfonylurea glimepiride and phosphoinositolglycans involves distinct mechanisms for redistribution of lipid raft components. Biochemistry 2001;40:14603-20.
[10] Salani B, Repetto S, Cordera R, Maggi D. Glimepiride activates eNOS with a mechanism Akt but not caveolin-1 dependent. Biochem Biophys Res Commun 2005;335:832-5.
[11] Jojima T, Suzuki K, Hirama N, Uchida K, Hattori Y. Glimepiride upregulates eNOS activity and inhibits cytokine-induced NF-kappaB activation through a phosphoinoside 3-kinase-Akt–dependent pathway. Diabetes Obes Metab 2009;11:143-9.
[12] Duronio V, Scheid MP, Ettinger S. Downstream signalling events regulated by phosphatidylinositol 3-kinase activity. Cell Signal 1998; 10:233-9.
[13] Shepherd PR, Withers DJ, Siddle K. Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling. Biochem J 1998;333 (Pt 3):471-90.
[14] Martelli AM, Borgatti P, Bortul R, et al. Phosphatidylinositol 3-kinase translocates to the nucleus of osteoblast-like MC3T3-E1 cells in response to insulin-like growth factor I and platelet-derived growth factor but not to the proapoptotic cytokine tumor necrosis factor alpha. J Bone Miner Res 2000;15:1716-30.
[15] Carpio L, Gladu J, Goltzman D, Rabbani SA. Induction of osteoblast differentiation indexes by PTHrP in MG-63 cells involves multiple signaling pathways. Am J Physiol Endocrinol Metab 2001;281:E489-499.
[16] Vlahos CJ, Matter WF, Hui KY, Brown RF. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J Biol Chem 1994;269:5241-8.
[17] Abe Y, Abe T, Aida Y, Hara Y, Maeda K. Follistatin restricts bone morphogenetic protein (BMP)–2 action on the differentiation of osteoblasts in fetal rat mandibular cells. J Bone Miner Res 2004;19: 1302-7.
[18] Abe Y, Aida Y, Abe T, et al. Development of mineralized nodules in fetal rat mandibular osteogenic precursor cells: requirement for dexamethasone but not for beta-glycerophosphate. Calcif Tissue Int 2000;66:66-9.
[19] Abe Y, Akamine A, Aida Y, Maeda K. Differentiation and mineralization in osteogenic precursor cells derived from fetal rat mandibular bone. Calcif Tissue Int 1993;52:365-71.
[20] Brenneisen P, Wlaschek M, Schwamborn E, et al. Activation of protein kinase CK2 is an early step in the ultraviolet B–mediated increase in

interstitial collagenase (matrix metalloproteinase–1; MMP-1) and stromelysin-1 (MMP-3) protein levels in human dermal fibroblasts. Biochem J 2002;365:31-40.

[21] Ulici V, Hoenselaar KD, Gillespie JR, Beier F. The PI3K pathway regulates endochondral bone growth through control of hypertrophic chondrocyte differentiation. BMC Dev Biol 2008;8:40.

[22] Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 2000;351:95-105.

[23] Langtry HD, Balfour JA. Glimepiride. A review of its use in the management of type 2 diabetes mellitus. Drugs 1998;55:563-84.

[24] Thomas JE, Venugopalan M, Galvin R, et al. Inhibition of MG-63 cell proliferation and PDGF-stimulated cellular processes by inhibitors of phosphatidylinositol 3-kinase. J Cell Biochem 1997;64:182-95.

[25] Osyczka AM, Leboy PS. Bone morphogenetic protein regulation of early osteoblast genes in human marrow stromal cells is mediated by extracellular signal–regulated kinase and phosphatidylinositol 3-kinase signaling. Endocrinology 2005;146:3428-37.

[26] Hou CH, Hou SM, Tang CH. Ultrasound increased BMP-2 expression via PI3K, Akt, c-Fos/c-Jun, and AP-1 pathways in cultured osteoblasts. J Cell Biochem 2009;106:7-15.

[27] Zhang Y, Zeng X, Zhang L, Zheng X. Stimulatory effect of puerarin on bone formation through activation of PI3K/Akt pathway in rat calvaria osteoblasts. Planta Med 2007;73:341-7.

[28] Raucci A, Bellosta P, Grassi R, Basilico C, Mansukhani A. Osteoblast proliferation or differentiation is regulated by relative strengths of opposing signaling pathways. J Cell Physiol 2008;215:442-51.

[29] Sanchez-Margalet V, Goldfine ID, Vlahos CJ, Sung CK. Role of phosphatidylinositol-3-kinase in insulin receptor signaling: studies with inhibitor, LY294002. Biochem Biophys Res Commun 1994;204: 446-52.

[30] Dey A, Cederbaum AI. Geldanamycin, an inhibitor of Hsp90 increases cytochrome P450 2E1 mediated toxicity in HepG2 cells through sustained activation of the p38MAPK pathway. Arch Biochem Biophys 2007;461:275-86.

[31] Luconi M, Torcia S, Grillo D, et al. Enhancement of mouse sperm motility by the PI3-kinase inhibitor LY294002 does not result in toxic effects on preimplantation embryo development. Hum Reprod 2005; 20:3500-4.

[32] Shingu T, Yamada K, Hara N, et al. Synergistic augmentation of antimicrotubule agent-induced cytotoxicity by a phosphoinositide 3-kinase inhibitor in human malignant glioma cells. Cancer Res 2003;63: 4044-7.

[33] Jun SS, Chen Z, Pace MC, Shaul PW. Estrogen upregulates cyclooxygenase-1 gene expression in ovine fetal pulmonary artery endothelium. J Clin Invest 1998;102:176-83.

[34] Armour KE, Ralston SH. Estrogen upregulates endothelial constitutive nitric oxide synthase expression in human osteoblast-like cells. Endocrinology 1998;139:799-802.

[35] Ernst M, Heath JK, Rodan GA. Estradiol effects on proliferation, messenger ribonucleic acid for collagen and insulin-like growth factor–I, and parathyroid hormone–stimulated adenylate cyclase activity in osteoblastic cells from calvariae and long bones. Endocrinology 1989;125:825-33.

[36] Oursler MJ, Cortese C, Keeting P, et al. Modulation of transforming growth factor–beta production in normal human osteoblast-like cells by 17 beta-estradiol and parathyroid hormone. Endocrinology 1991; 129:3313-20.

[37] Simoncini T, Hafezi-Moghadam A, Brazil DP, et al. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature 2000;407:538-41.

[38] Kim SJ, Chun JY, Kim MS. Insulin stimulates production of nitric oxide via ERK in osteoblast cells. Biochem Biophys Res Commun 2000;278:712-8.

[39] Xie H, Tang SY, Luo XH, et al. Insulin-like effects of visfatin on human osteoblasts. Calcif Tissue Int 2007;80:201-10.

[40] Zambonin G, Grano M, Greco G, Oreffo RO, Triffit JT. Hydroxyapatite coated with insulin-like growth factor 1 (IGF1) stimulates human osteoblast activity in vitro. Acta Orthop Scand 1999;70:217-20.

[41] Schmid C, Steiner T, Froesch ER. Insulin-like growth factor I supports differentiation of cultured osteoblast-like cells. FEBS Lett 1984;173:48-52.

[42] Yamaguchi M, Ozaki K, Hoshi T. Simulated weightlessness and bone metabolism: impairment of insulin effect on alkaline phosphatase activity in bone tissue. Res Exp Med (Berl) 1991;191:113-9.