Up-regulation of cyclinD1 and Bcl2A1 by insulin is involved in osteoclast proliferation

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ABSTRACT

Aims: Insulin receptor signaling in osteoblasts has been well established, but the effects of insulin on osteoclast proliferation are poorly explored. The objective of this study was to investigate the roles and the mechanisms of insulin on osteoclast proliferation.

Main methods: After insulin treatment to primary osteoclast precursors, BrdU incorporation assay was performed and the expression of cell cycle- and apoptosis-related genes was determined by real-time PCR and immunoblotting. Apoptosis was analyzed using a FACScan flow cytometer.

Key findings: Insulin activated insulin receptor and promoted the proliferation of osteoclast precursors in time- and dose-dependent manners. However, the expression of insulin receptor was not changed by it during that time. Insulin remarkably induced the expression of cyclinD1, a cell cycle marker, and Bcl2A1, an anti-apoptotic oncogene, whereas cdk1 and cdk4 were not affected by it. The expression of Bcl2l11 and Bax, both apoptotic markers, was reduced or not changed in osteoclast precursors. Bcl2A1/Bax ratio was also increased in protein levels. Treatment with obatoclax, a Bcl2 family inhibitor, significantly induced the apoptosis of osteoclast precursors in the presence of insulin. These results demonstrate that insulin promotes osteoclast proliferation by increasing cell cycle and suppressing apoptosis through specific gene regulation.

Significance: These data provide a basis for understanding and ultimately treating several bone-related metabolic diseases.

Introduction

Bone is a dynamic tissue and continuously renewed by bone remodeling process, which is accompanied by two cellular events: One is bone formation by osteoblasts and the other is resorption of the mineralized bone matrix by osteoclasts. Bone homeostasis is affected by the balance of these two types of cells (Harada and Rodan, 2003; Teitelbaum, 2000). Especially, the elevation in numbers and/or activity of osteoclasts derived from hematopoietic progenitors of the monocyte/macrophage lineage causes a variety of bone disorders such as osteoporosis (Boyle et al., 2003; Rodan and Martin, 2000).

Recently, several studies demonstrated that bone remodeling and energy metabolism are reciprocally regulated. Osteocalcin secreted from osteoblasts improved glucose handling by regulating insulin secretion and activity (Lee et al., 2007; Lee and Karsenty, 2008). Conversely, insulin receptor signaling in osteoblasts regulated bone density and finally metabolic function. Mice lacking insulin receptors in osteoblasts developed postnatal osteopenia and an impairment of osteoblast proliferation and differentiation (Fulzele et al., 2010). Direct treatment of insulin promoted osteoblast proliferation, collagen synthesis, and alkaline phosphatase production (Ferron et al., 2010; Fulzele et al., 2010; Yang et al., 2010). Osteoblast-specific insulin receptor-deficient mice showed a decreased bone mass by the decline of osteoblast proliferation although had a reduction of CathespinK (Ctsk) and T-cell immune receptor1 (Tirc1) genes, related to osteoclast bone resorption, without a decrease of osteoclast number (Ferron et al., 2010). Thus, roles of insulin signaling in osteoblasts have been defined by many groups. However, there have been little studies on whether insulin regulated osteoclast proliferation directly. If it does, the direct regulatory mechanism of insulin on osteoclast proliferation needs to be elucidated.

Upon insulin binding, the insulin receptor undergoes a series of autophosphorylation events that control metabolism, growth, survival, and differentiation (Hubbard, 1997; Schlessinger, 2000; Wei et al., 1995; Yang et al., 2010). The CCND1, a gene encoding the protein CyclinD1, is up-regulated as an important promoter of cell cycle (Baldin et al., 1993; Soh and Weinstein, 2003). The balance between Bcl2, an anti-apoptotic protein, and Bax, a pro-apoptotic protein, determines cell fate. Whereas an increase in Bax activates caspase3 and, finally, facilitates apoptosis, an enhancement of Bcl2 promotes cell proliferation (Hockenbery et al., 1993; Yamashita et al., 2008). Anti-apoptotic Bcl2 family member BCL2A1 is recurrently amplified in ~30% of melanomas (Haq et al., 2013). An increase of Bcl2A1/Bax ratio results in cell proliferation rather than in apoptosis.

We show here that insulin promotes osteoclast proliferation by increasing cell cycle and suppressing apoptosis through the expression of insulin in osteoclast precursors.

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up-regulation of cyclinD1 and Bcl2a1, respectively. These findings provide a basis for understanding and ultimately treating several bone-related metabolic diseases.

**Materials and methods**

**Cell culture and Isolation of bone marrow precursors**

RAW264.7 monocyte/macrophage cells (Korean Cell Line Bank) were maintained in DMEM (Hyclone) containing 10% FBS with antibiotics. Isolation of bone marrow precursors and in vitro osteoclastogenesis experiment were performed as described previously (Choi et al., 2013). In brief, bone marrow cells were flushed out from the femur of 4–6-week-old C57BL/6 mice with a sterile 21-gauge syringe and incubated in alpha-MEM media containing 10% FBS and 10 ng/ml M-CSF (R&D Systems). After 24 h, non-adherent cells were harvested and cultured in the presence of M-CSF (20 ng/ml) for 3 days. After washing out the non-adherent cells, adherent cells were used as bone marrow-derived monocytes/macrophages (BMMs).

**BrdU incorporation assay**

After 24 h of seeding with 2 × 10⁴ cells/well in 96-well plates, the cells were treated with insulin (Sigma, St. Louis, MO). Cell proliferation was assayed using BrdU incorporation assay kit (Cell Signaling Technology) according to the manufacturer’s instruction. BrdU was added 12 h before culture termination. At the end of culture, cells were fixed with fixing solution for 30 min at RT, rinsed twice with PBS, and incubated with monoclonal anti-BrdU antibody for 1 h, followed by anti-mouse secondary antibody for 30 min. After the final wash, a substrate was added to the wells and then a stop solution was provided. The proportion of total cells incorporating BrdU into the nucleus was determined by reading the absorbance on a microplate reader (Bio-Rad) at 450 nm to calculate cell viability.

**Real-time PCR**

Total RNAs isolated from primary preosteoclasts were reverse transcribed using SuperscriptIII reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. Real-time PCRs were performed with the Brilliant UltraFast SYBR Green QPCR Master Mix (Agilent Technologies) and specific primers for target genes and HPRT (for endogenous control) from QIAGEN in triplicates on an MX3000 instrument (Agilent Technologies). HPRT was used for the normalization of all quantitation.

**Western blot analyses**

Western blotting was performed as described previously (Choi et al., 2013). In brief, primary preosteoclasts stimulated with insulin were lysed in lysis buffer and the supernatants were prepared by centrifugation, electrophoresed on a 10% SDS-polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane. Immunoblotting was performed with antibodies (Abs) specific to insulin receptor (InsR), phosphorylated InsR (Tyr1150/1151), Bcl2A1, and β-actin (as a loading control) (Cell Signaling Technology, Beverly, MA), cyclinD1 and Bax (Santa-Cruz), followed by HRP-conjugated secondary antibodies and enhanced using an ECL detection kit (Amersham Biosciences).

**Apoptosis assay**

Apoptosis was measured using the Annexin V Apoptosis Kit (BD Biosciences) according to the manufacturer’s instruction. Primary preosteoclasts were seeded with 2 × 10⁶ cells/well in 96-well plates and then treated with obatoclax (Selleck Chemicals) for 48 h in the presence of insulin. The cells were then washed with cold PBSs, resuspended in 1× Annexin V binding buffer (BD Biosciences), and labeled with 10 μL Annexin V-APC (BD Biosciences) for 15 min in the dark. After incubation, 1× binding buffer was added. The analyses were performed using a FACScan flow cytometer (Becton Dickinson).

**Statistical analysis**

The results are presented by mean ± SD of triplicates from at least three independent experiments. Statistical analyses were determined using Student’s t test. P < 0.05 was considered significant.

**Results**

**Insulin regulates osteoclast proliferation**

To determine whether insulin regulates osteoclast proliferation, insulin was added to RAW264.7 cells or primary osteoclast precursors isolated from the bone marrow of mice femurs. BrdU incorporation assay showed that insulin increased proliferation of both cell types in dose- and time-dependent manners. The most obvious effect was observed after 72 h of treatment with 100 nM insulin in both cell types in which more than 30% increased (Fig. 1A and B). These results indicate that insulin regulates osteoclast proliferation.

![Fig. 1. Effect of insulin on cell viability. (A and B) The indicated concentrations of insulin were treated into RAW264.7 cells (A) or primary preosteoclasts (B) for 24 h, 48 h, and 72 h. Then, cell proliferation was measured using the BrdU incorporation assay kit. Results are representative of at least three independent experiments. *p < 0.05 vs. non-treated cells.](image-url)
Insulin activates insulin receptor in osteoclast precursors

Prior to investigate how insulin regulates osteoclast proliferation, we first examined whether the activation and/or expression of insulin receptor is affected by insulin stimulation during osteoclast proliferation. Stimulation with insulin effectively activated insulin receptor within 5 min and the activity lasted for 30 min in primary preosteoclasts (Fig. 2A). Real-time PCR and Western blot analysis showed that neither mRNA nor protein level of the insulin receptor was changed upon insulin stimulation up to 72 h in primary preosteoclasts.

Fig. 2. The activation of insulin receptor by insulin. (A) Primary preosteoclasts were treated with 100 nM of insulin for indicated times and then total proteins were isolated. Extracts were then subjected to Western blot analysis using phosphorylated insulin receptor- or insulin receptor-specific antibodies. Protein bands were quantified by densitometry, and phosphorylated levels were normalized to levels of insulin receptor. (B and C) Total RNAs or proteins were isolated from primary preosteoclasts treated with 100 nM of insulin for indicated times. Extracts were then subjected to real-time PCR (B) or Western blot analysis (C) using insulin receptor-specific primers or antibodies, respectively. All quantitation was normalized to an HPRT (B). Protein bands were quantified by densitometry and levels of insulin receptor was normalized to levels of β-actin. The fold increase in the stimulated cells compared with untreated cells is shown (C).

Fig. 3. The increase of cyclinD1 expression by insulin stimulation. (A–C) Primary preosteoclasts were treated with 100 nM of insulin for 24 h (A), 100 nM for indicated times (B), or 48 h with indicated concentrations (C), respectively. Then total RNAs were isolated from cultured samples and reverse transcribed. Synthesized cDNAs were then subjected to real-time PCR as described in Fig. 2A except cyclinD1 primers. (D) Primary preosteoclasts were treated with 100 nM of insulin for indicated times and then to Western blot analysis as described in Fig. 2B except cyclinD1 antibodies. *p < 0.05 and **p < 0.005 vs. non-treated cells.
Fig. 2B and C). This result suggests the possibility that insulin may regulate osteoclast proliferation not by affecting the expression but through the activation of insulin receptor.

**Insulin regulates cyclinD1 expression**

Cell proliferation is mainly controlled by cell cycle regulators. To identify mechanisms involved in cell proliferation by insulin treatment, the expression of cyclinD1, cdk1, and cdk4 after insulin stimulation was determined. We found that insulin specifically regulates the expression of cyclinD1. Whereas cdk1 and cdk4 were not induced, the expression of cyclinD1 was increased 1.7 fold by insulin (Fig. 3A). Insulin significantly increased the expression of cyclinD1 in time- and dose-dependent manners. At 24 h and 48 h of insulin treatment, insulin effectively induced the expression of cyclinD1 (Fig. 3B). The same was true when treated with 10 nM and 100 nM of insulin for 24 h (Fig. 3C). In the protein level, the expression of cyclinD1 was increased by 1.8 fold and 2.3 fold at 48 h and 72 h of insulin treatment, respectively (Fig. 3D). These results strongly suggest that insulin regulates osteoclast proliferation by inducing the expression of cyclinD1 but not of cdk1 and cdk4 in osteoclast precursors.

**Insulin suppresses apoptosis of osteoclast precursors**

To verify that the increase of osteoclast proliferation by insulin stimulation is also involved in the apoptosis in part, we examined whether apoptosis-related genes are affected by insulin. Interestingly, whereas Bcl2A1, an anti-apoptotic oncogene, was remarkably induced by insulin stimulation, Bcl2I1 and Bax, both apoptotic markers, were reduced or not changed by it in osteoclast precursors, respectively (Fig. 4A). Especially, treatment with insulin failed to induce the expression of Bcl2, an anti-apoptotic oncogene (Fig. 4A).

Insulin increased the expression of the Bcl2A1 gene, dose-dependently (Fig. 4B). This result was also confirmed by immunoblotting, in which the Bcl2A1/Bax ratio was significantly increased (Fig. 4C). These results demonstrate that insulin suppresses apoptosis of osteoclast precursors.

**Obatoclax induces apoptosis of osteoclast precursors in the presence of insulin**

To further confirm that insulin-induced Bcl2A1 is involved in the apoptosis of osteoclast, a Bcl2 family inhibitor, obatoclax, was treated to osteoclast precursors. In the presence of insulin, obatoclax stimulated apoptosis of osteoclast precursors (Fig. 5A). Taken together, these results indicate that insulin promotes osteoclast proliferation by increasing cell cycle and suppressing apoptosis through up-regulation of cyclinD1 and Bcl2A1 genes, respectively (Fig. 6A).
Insulin has been proposed to be an anabolic agent in bone metabolism (Ferron et al., 2010; Fulzele et al., 2010; Thrailkill et al., 2005; Yang et al., 2010) but its direct role on osteoclast proliferation and its underlying mechanism remain unclear. Here, our study demonstrated direct regulatory mechanisms of osteoclast proliferation by insulin.

Stimulation with insulin significantly activated insulin receptor and showed a remarkable positive effect on proliferation of both RAW264.7 cells and primary osteoclast precursors in a time- and dose-dependent manner. However, it failed to regulate the expression of insulin receptor up to 72 h of treatment. These results imply that insulin may regulate osteoclast proliferation through insulin receptor signaling. Further studies using knock-down systems such as insulin receptor shRNA are needed to examine the direct involvement of insulin receptor signaling.

Insulin regulated osteoclast proliferation through two distinct mechanisms: On one hand, by increasing the expression of a cell cycle marker, cyclinD1, and on the other hand, by up-regulating an anti-apoptotic oncogene, Bcl2A1, and decreasing Bcl211 that stimulates apoptosis, thus decreasing apoptosis. Bcl2A1, an anti-apoptotic factor, was identified as a unique melanoma oncogene located on chromosome 15q (Haq et al., 2013). Bcl2 seems to play a critical anti-apoptotic role in osteoclasts but not osteoblasts since Bcl2−/− mice showed an increased bone mass by the reduced numbers of osteoclasts whereas proliferation and differentiation of osteoclasts were normal (Yamashita et al., 2008). In our present study, Bcl2 and Bax, a pro-apoptotic oncogene, as well as other cell cycle markers, cdk1 and cdk4, were not affected by insulin.

Obatoclax binds to all anti-apoptotic Bcl-2 family members, including Bcl-2 and Bcl2A1, as a synthetic derivative of bacterial prodiginines (Kim et al., 2014; Konopleva et al., 2008). It has been widely tested and has shown a promising activity in both preclinical and early human clinical studies for various cancers including chronic lymphocytic leukemia in which Bcl2 has been regulated (Campa’s et al., 2006; Kim et al., 2014; Konopleva et al., 2008; Trudel et al., 2007). Treatment with obatoclax effectively suppressed osteoclast proliferation by insulin and induced their apoptosis. This is the first study to examine the effect of a Bcl2 inhibitor on osteoclast proliferation.

Here, we focused on the changes of cell cycle markers and apoptosis-related molecules critical to determine the cell proliferation by insulin. Although, how insulin receptor signaling in osteoclasts results in the activation of cell cycle proteins and whether signaling pathways increasing cyclinD1 could activate the anti-apoptotic cascade or inhibit apoptotic cascade are still the subjects of further research (Korulu et al., 2013) for the survival of osteoclasts, these findings may provide a basis for the treatment of bone-related metabolic diseases through combining Bcl2 inhibitors and cell cycle inhibitors. Taken together, our results would ultimately give hints toward understanding of diabetic osteoporosis and novel targets for the treatment.

Conflict of interest

There are no conflicts of interest in the research.

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References