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Insulin effect on RANKL and OPG expression in human osteoblast-like MG63 cells

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Aim: To investigate the effect of insulin on receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG) expression in human osteosarcoma MG63 cells using real-time polymerase chain reaction.

Materials and methods: MG63 cells were exposed to different concentrations of human recombinant insulin (1, 10, and 100 nM) for 24 h, and then the expression of RANKL and OPG mRNA was identified. After that, MG63 cells were exposed to tensile stress combined with 0 or 10 nM insulin respectively for 0, 3, 6, and 12 h, and then the expression of RANKL and OPG mRNA was identified. Human monocytes were cultured with the collected culture media from MG63 cells plates that were exposed to different concentrations of human recombinant insulin (0 and 10 nM) for 7 days, and then were stained with an acid phosphatase kit.

Results: After 24 h of treatment with 1, 10, and 100 nM doses of insulin, the expression of RANKL and OPG mRNA was suppressed ($P < 0.05$), and the ratio of OPG to RANKL also was suppressed ($P < 0.05$). Application of tensile stress to MG63 cells induced a decrease in RANKL mRNA expression and an increase in OPG mRNA expression at 6 and 12 h ($P < 0.05$); 10 nM insulin decreased the mechanical stress-induced RANKL and OPG mRNA expression in MG63 cells at 6 and 12 h ($P < 0.05$). The ratio of OPG to RANKL increased at 6 and 12 h in the 2 groups ($P < 0.05$), but insulin decreased the mechanical stress-induced OPG/RANKL ratio at 12 h ($P < 0.05$). For the group of monocytes cultured with DMEM collected from MG63 cells plates that were cultured with 0 nM human recombinant insulin, osteoclast formation was not observed. Osteoclast formation was observed in monocytes cultured with DMEM collected from MG63 cells plates that were cultured with 10 nM human recombinant insulin.

Conclusion: These results suggest that insulin can modulate osteoclast differentiation via altering the OPG and RANKL expression in osteoblasts. The deficiency in insulin may decrease the number of osteoclasts and osteoclast-mediated bone resorption in patients with type 1 diabetes mellitus.

Key words: Insulin, MG63, OPG, RANKL, mechanical stimulus

1. Introduction

It is well established that type 1 diabetes mellitus (T1DM), which is insulin-dependent, is associated with retinopathy, cardiovascular symptoms, sexual dysfunction, and other complications (1,2). Recent studies also found that alteration in bone metabolism (low bone-mineral density, osteopenia, osteoporosis, etc.) is common in T1DM patients (3–5), which suggests that insulin acts as a regulator in bone metabolism. The accumulating evidence implies that insulin can regulate bone metabolism by facilitating bone formation through promoting osteoblast proliferation and differentiation (6,7), but whether insulin can regulate osteoclast-mediated bone resorptive activity remains unclear.

Besides being involved in bone formation, osteoblasts can modulate osteoclast differentiation by expressing a specific osteoclast differentiation factor, the receptor activator of nuclear factor kappa-B ligand (RANKL), and its decoy receptor, osteoprotegerin (OPG). RANKL is indispensable in osteoclast differentiation (8). Acting as a decoy receptor by blocking the interaction of RANKL with its functional receptor RANK, OPG can inhibit osteoclast differentiation and osteoclastogenesis (9). The ratio between OPG and RANKL expression levels in osteoblasts determines the osteoclast differentiation (8–10).

Mechanical loading is a key factor in regulating bone remodeling (11). Besides mechanical loading, numerous factors (including growth factors and systemic hormones) can influence bone remodeling (12). Osteoblasts may exhibit distinct responses to mechanical stimulus under various in vivo conditions, as we can see in defective cellular responses to mechanical loading in patients with musculoskeletal diseases, for example the patients with disuse osteoporosis, senile osteoporosis, or osteoarthritis

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(13,14). In vitro studies demonstrate that mechanical stimulus reduces RANKL expression but up-regulates the OPG expression in osteoblasts (15–17). However, whether insulin modulates mechanical stress-induced RANKL and OPG expression in osteoblasts remains unclear.

The aim of this study was to investigate the effects of insulin on the RANKL and OPG expression in osteoblasts for the purpose of making clear whether the decrease of insulin can impact the RANKL and OPG expression in osteoblasts and consequently influence osteoclast differentiation in patients with T1DM.

2. Materials and methods

2.1. Materials

Human osteosarcoma MG63 cell was obtained from ATCC (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco-BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT, USA). Insulin was from Sigma (St. Louis, MO, USA). The First Strand cDNA Synthesis Kit was from Fermentas UAB (Vilnius, Lithuania). The tartrate-resistant acid phosphatase (TRAP) kit was from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The LightCycler 480 SYBR Green I Master was purchased from Roche Applied Science (Branford, CT, USA).

2.2. Cell culture

Cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. After reaching confluence, these cells were trypsinized and seeded into 6-well plates at a density of 1×10^5 cells/well. The cells were left undisturbed in the incubator for 24 h, and then the medium was changed to FBS-free medium for 24 h for equalizing cell growth prior to experiments. The cells were then exposed to different concentrations of human recombinant insulin for 24 h.

Human monocytes were isolated from freshly collected buffy coats discarded from healthy adult donors by Ficoll-Hypaque density gradient centrifugation. The separated lymphomonocytes were washed with PBS and resuspended in DMEM supplement with 10% FBS and 1% penicillin/streptomycin.

2.3. Applying mechanical stress

Cells were seeded on the force-loading plate at a density of 1×10^4 cells/cm^2. The force-loading plates were made by using a method described previously (18). The cells were incubated in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin for 48 h. The medium was then changed to FBS-free medium for 24 h for equalizing cell growth prior to experiments. The cells were then loaded with tensile stress at 2000 u-strain and 0.5 Hz, which has been characterized and described in detail elsewhere (18). In the control group, cells were subjected to similar treatment but without mechanical stress stimulus.

2.4. RNA isolation and real-time PCR

After the cells were harvested, total RNA was extracted and quantified using the method described previously (18). RNA was reverse-transcribed using the First Strand cDNA Synthesis Kit following the manufacturer's instructions. The cDNA was then amplified through real-time polymerase chain reaction (PCR) using LightCycler. Sequences of all PCR primers are shown in the Table. Data were analyzed using the $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen (19), using the housekeeping gene β-actin to calculate the ΔCT, and using the control at each time point to calculate the –ΔΔCT.

2.5. TRAP staining

MG63 cells were cultured with different concentrations of human recombinant insulin (0 nM, 10 nM) for 24 h, and then the culture media were collected.

After reaching confluence, the monocytes were trypsinized and seeded into the 6-well plates at a density of 1×10^4 cells/well. The cells were cultured with the collected culture media as mentioned above. The culture media were completely removed and replaced every 2 days thereafter. On day 7, the cells were stained for TRAP with an acid phosphatase kit as instructed in the product manual. Cells containing 3 or more nuclei were considered a multinuclear cell.

2.6. Statistical analyses

Results are shown as mean ± SD. Statistical analysis was performed with SPSS using the independent Student's t-test for 2 groups of data and analysis of variance (ANOVA). The difference was considered significant at $P < 0.05$.

3. Results

3.1. Effect of insulin on RANKL and OPG mRNA expression

MG63 cells were treated with different doses of insulin (1, 10, 100 nM) for 24 h. RANKL (Figure 1A) and OPG (Figure 1B) mRNA expression was determined by real-time PCR. Data are presented as percentage changes relative to the unstimulated control cells (normalized to β-actin gene expression). As shown in Figures 1A and 1B, after 24 h of treatment with 1, 10, and 100 nM doses of insulin, the expression of RANKL and OPG mRNA was suppressed ($P < 0.05$), and the ratio of OPG to RANKL was also suppressed ($P < 0.05$) (Figure 1C).

3.2. Insulin modulates tensile stress-induced OPG and RANKL mRNA expression in MG63 cells

MG63 cells were exposed to tensile stress combined with 0 or 10 nM insulin respectively for 0, 3, 6, and 12 h. Data are presented as percentage changes relative to the unstimulated control cells (normalized to β-actin gene expression). As shown in Figures 2A and 2B, application

Table. Primer sequences used for real-time PCR.

Gene name	Primer sequence	Size (bp)	Annealing Temperature (Celsius)
RANKL	AGAGCGCAGATGGATCCTAA TTCCTTTTGCACAGCTCCTT	180	56
OPG	GCAGCGGCACATTGGAC CCCGGTAAGCTTTCCATCAA	69	60
β-actin	AAATCGTCCGTGACATCAAG GGAAGGAAGGCTGG AAGA GA	180	60

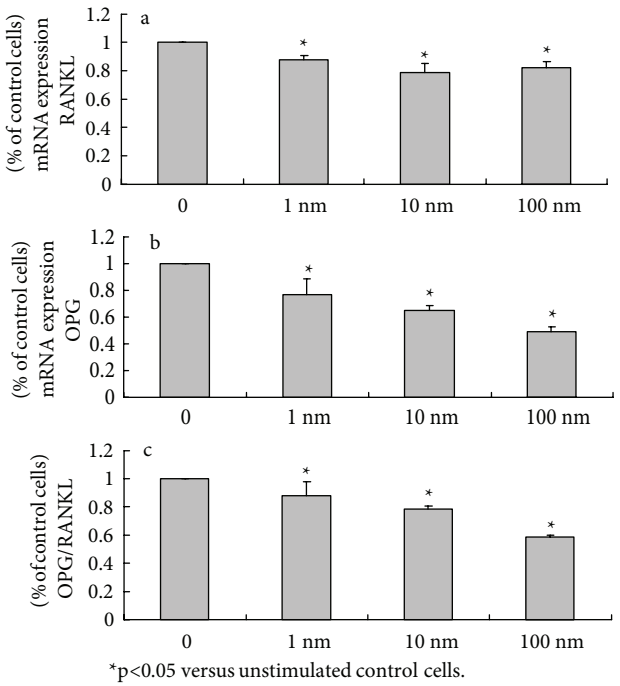

Figure 1. Effect of insulin on RANKL (A) and OPG (B) mRNA expression, and the ratio of those expressions (C). *: P < 0.05 versus unstimulated control cells.

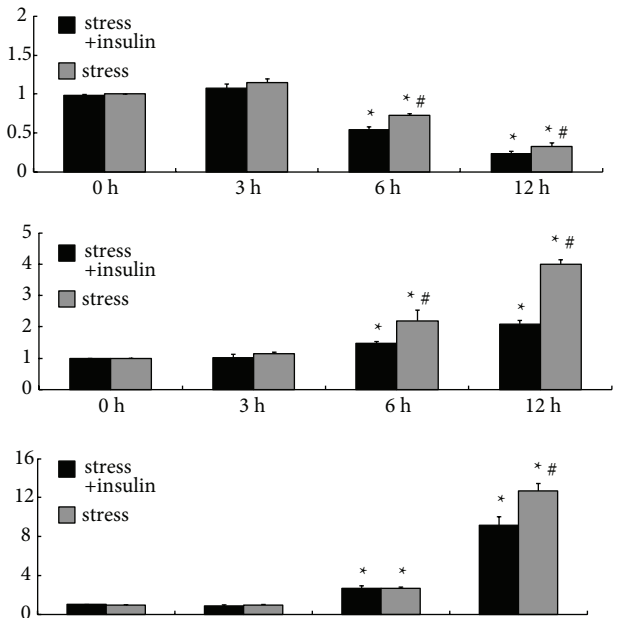

Figure 2. Effect of insulin on mechanical stress-induced RANKL (A) and OPG (B) mRNA expression, and the ratio of those expressions (C). *: P < 0.05 versus unstimulated control cells. #: P < 0.05 versus the cells that were treated with 10 nM insulin and exposed to tensile stress for equal time.

of tensile stress to MG63 cells induced a decrease in RANKL mRNA expression and an increase in OPG mRNA expression at 6 and 12 h (P < 0.05); 10 nM insulin decreased the mechanical stress-induced RANKL and OPG mRNA expression in MG63 cells at 6 and 12 h (P < 0.05). The ratio of OPG to RANKL increased at 6 and 12 h in the 2 groups (P < 0.05), but insulin decreased the mechanical stress-induced OPG-to-RANKL ratio at 12 h (P < 0.05) (Figure 2C).

3.3. Effect of insulin on osteoclast differentiation

For the group of monocytes cultured with DMEM collected from the MG63 cell plates that were cultured with 0 nM human recombinant insulin, osteoclast formation was not observed (Figure 3A). Osteoclast formation was observed in monocytes cultured with DMEM collected from MG63 cell plates that were cultured with 10 nM human recombinant insulin (Figure 3B).

4. Discussion

Controversy exists as to whether insulin can affect bone resorptive activity. Some previous studies indicated a decrease in the number of osteoclasts in type 1 diabetic rodent (20–22). On the other hand, several groups have reported higher levels of TRAP activity and an increased number of osteoclasts was observed in type 1 diabetic rodent (23–26).

In this regard, this study aimed to investigate the potential effect of insulin on RANKL and OPG expression in osteoblasts for the purpose of investigating the effect of insulin on osteoclast differentiation. We found that insulin suppressed RANKL mRNA expression in a dose-dependent manner. These results indicate that insulin modulates RANKL expression in osteoblasts and the deficiency in insulin may decrease the RANKL level in patients with T1DM. However, the potential cellular mechanism remains unclear. Several studies have demonstrated that insulin receptor substrates (IRS-1 and IRS-2) that mediate intracellular signaling by insulin and IGF are essential for the RANKL expression in osteoclasts (27–29). To make clear the potential cellular mechanism of RANKL expression regulated by insulin, future studies can take into account these 2 substrate-mediated signaling pathways activated by insulin in osteoblasts.

OPG, acting as a decoy receptor, plays a crucial role in inhibiting osteoclast differentiation by blocking the RANK–RANKL interaction (9). We therefore tested whether the expression of OPG mRNA was affected by insulin. We found that insulin decreased the OPG mRNA expression in MG63 cells in a dose-dependent manner. The results imply that the deficiency in insulin increases the OPG level in patients with T1DM. This may support previous in vivo studies that demonstrated that patients with T1DM have a significant increase of OPG serum levels and that it can be normalized after insulin treatment (30,31). As OPG is known to exert an antiresorptive effect on bone, and many patients with T1DM have osteoporosis (3,5), the insulin-induced decrease of OPG expression in MG63 cell was unexpected. Further studies are needed to determine the underlying mechanisms.

Although insulin decreases both the RANKL and OPG mRNA expression, the ratio of OPG to RANKL decreased in a dose-dependent manner in our study. As described in Section 1, the ratio of OPG to RANKL expressed in the osteoblasts is a key factor in osteoclast differentiation. These results imply that insulin promotes osteoclast differentiation by decreasing the ratio of OPG to RANKL in osteoblasts and indicate that the deficiency in insulin decreases the number of osteoclasts and the level of TRAP activity in patients with T1DM. TRAP staining also supported the hypothesis. These results are consistent with data from experiments in which the number of osteoclasts decreases in rodents with T1DM (20–22).

In this study, we found that tensile stress increased the ratio of OPG to RANKL in MG63 cells. This result agrees with the findings of Kaneuji, who found that mechanical loading inhibited osteoclastogenesis by increasing the ratio of OPG to RANKL in osteoblasts (32). We demonstrated that 10 nM insulin decreased the mechanical stress-induced RANKL and OPG mRNA expression (at 6 and 12 h) and the ratio of OPG to RANKL (only at 12 h) in MG63 cells. These results suggest that compared with normal people, the deficiency in insulin results in a smaller number of osteoclasts induced by osteoblasts under mechanical condition in patients with T1DM. These results also indicated that temporal change of insulin level has no effects on RANKL and OPG mRNA expression or their ratio in osteoblasts under mechanical conditions.

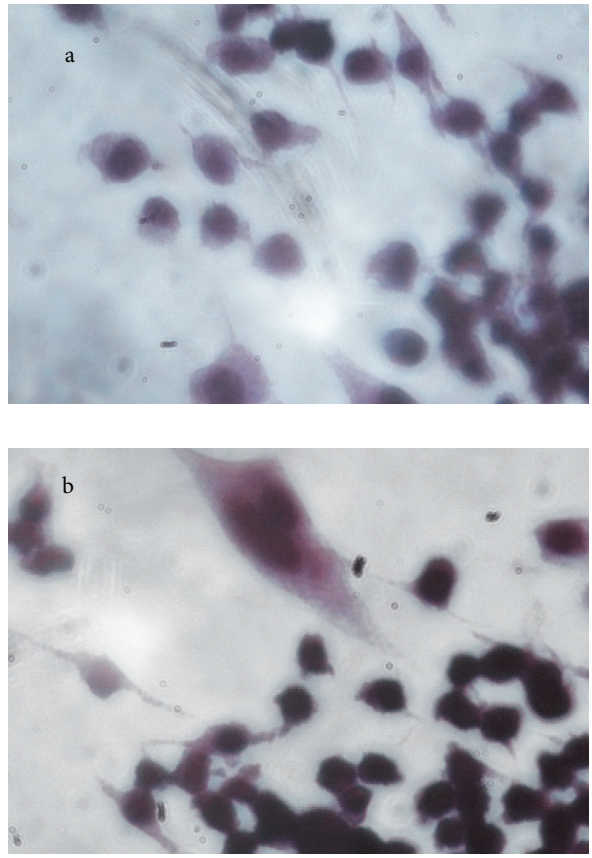

Figure 3. Effect of insulin on osteoclast differentiation: A) 0 nM insulin, B) 10 nM insulin, 40× magnification. Cells containing 3 or more nuclei were considered as a multinuclear cell.

In summary, the data presented here support the hypothesis that insulin can influence bone remodeling though regulating bone resorption by changing the ratio of OPG to RANKL expressed in osteoblasts. We found that insulin decreased the ratio of OPG to RANKL in MG63 cells. These results imply that the deficiency in insulin may decrease the number of osteoclasts and osteoclast-mediated bone resorption in patients with T1DM. The etiopathogenesis of osteoporosis in these patients may be a reduction of bone formation but not an increase in bone resorption. These results suggest that insulin can modulate osteoclast differentiation via altering the OPG and RANKL expression in osteoblasts. The deficiency in insulin may decrease the number of osteoclasts and osteoclast-mediated bone resorption in patients with T1DM.

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