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Leptin Effect on RANKL and OPG Expression in MC3T3-E1 Osteoblasts

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Abstract Recent studies have suggested that leptin hormone may play a pivotal role on bone remodeling through a direct effect by modulating positively the OPG/RANKL balance. Here, we investigate the effect of leptin hormone on RANKL and OPG expression in MC3T3-E1 osteoblasts using RT-PCR and ELISA measurements. We have at first identified the expression of Ob-Rb and Ob-Ra leptin receptor isoforms in MC3T3-E1 and observed that these cells respond to mrleptin treatments. We then investigated the effect of mrleptin on RANKL and OPG expression. We show that mrleptin dose-dependently regulated the expression of RANKL mRNA with complete inhibition observed at concentrations higher than 12 ng/ml. This effect was confirmed with sRANKL protein measurements. However, the exposure of MC3T3-E1 to mrleptin had no effect on OPG mRNA. Taken together, these results suggest that leptin modulates positively OPG/RANKL balance by inhibiting the expression of RANKL gene. J. Cell. Biochem. 98: 1123–1129, 2006. © 2006 Wiley-Liss, Inc.

Key words: osteoblasts; MC3T3-E1; leptin; Ob-Ra; Ob-Rb; RANKL; OPG

Leptin, a 167 amino acid peptide encoded by the obese gene (*ob*), is a circulating hormone secreted mainly by white adipose tissue. By inhibiting the secretion of neuropeptide Y (NPY) in the hypothalamus, leptin acts to influence body weight by regulating food intake and energy expenditure [Elmquist, 2001]. Accumulating evidence implicates leptin as a regulating factor in a variety of physiological processes, including bone metabolism. Two opposing mechanisms have been suggested in leptin effect on regulation of bone metabolism. Leptin may work locally to promote the development of osteoprogenitors cells and stimulate osteoblasts to make new bone [Thomas et al., 1999; Reseland et al., 2001; Cornish et al., 2002]. In contrast to these findings, others have suggested a more centrally mediated mechanism where leptin, through the central nervous system, decreases osteoblast activity [Ducy et al., 2000; Elefteriou et al., 2005].

Bone is constantly resorbed by osteoclasts and then replaced by osteoblasts in a physiological process called bone remodeling. Osteoblasts and osteoclasts maintain the integrity of bone during this remodeling process, with tight regulation and coordination of their activities. Receptor activator of NFκB ligand (RANKL) is a member of TNF family of cytokines that is indispensable to osteoclast differentiation. This molecule is produced as a membrane-bound protein on osteoblast, bone marrow stromal cells and T lymphocytes, and cleaved into a soluble form by metalloprotease [Lacely et al., 1998; Nagai and Sato, 1999]. The stimulatory effect of RANKL in the bone microenvironment is neutralized by the secreted decoy OPG (osteoprotegerin). OPG is produced by osteoblasts and other cell types [Simonet et al., 1997]. The balance between OPG and RANKL secretion is critical to the regulation of bone remodeling [Horwood et al., 1998; Nagai and Sato, 1999]. It has been suggested that the ratio between OPG and RANKL expression levels in osteoblastic cells is a key factor in bone resorption. Extrinsic or intrinsic perturbations of this OPG/RANKL balance will directly result in the imbalance of bone remodeling leading to

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skeletal diseases such as osteoporosis [Horwood et al., 1998; Nagai and Sato, 1999].

Holloway and co-workers have suggested that leptin is implicated in bone remodeling through an inhibition of osteoclast generation [Holloway et al., 2002]. It was also suggested that leptin could modulate bone remodeling in favor of better balance [Burguera et al., 2001; Martin et al., 2005]. These findings led us to hypothesize that leptin might regulate the expression of RANKL.

For this purpose we analyzed the effect of leptin hormone on RANKL and OPG expression by MC3T3-E1 osteoblasts. We have at first attempted to identify the presence of Ob-Ra (short leptin receptor isoform involved in leptin transport) and Ob-Rb (long signal-transducing isoform) in MC3T3-E1 osteoblasts and analyzed whether these cells respond to leptin treatments by measuring the alkaline phosphatase activity, shown to be modulated by leptin treatment in human marrow stromal cells [Thomas et al., 1999].

After these receptors were shown to be expressed in MC3T3-E1 osteoblasts we investigated the effect of leptin on the expression on RANKL and OPG by these cells.

MATERIALS AND METHODS

Cell Culture

MC3T-E1 cells, established as an osteoblastic cell line from normal mouse calvaria were grown in alpha minimal medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 0.5% (v/v) gentamicin (Invitrogen), 1% (v/v) fungizone (Invitrogen), 50 μg/ml Vitamin C (Sigma chemical), and 10 mM β glycerophosphate (Sigma chemical) in 5% CO_2 at 37°C. The medium was changed twice weekly. At confluence, the cells were trypsinized and seeded into 6-well plates to be used at different experiments. Cells were left undisturbed in the incubator for 24 h and then exposed to different concentrations of mouse recombinant leptin (mrleptin, R&D system). In all experiments the exposure time to mrleptin was 24 h.

Alkaline Phosphatase Activity

To assay alkaline phosphatase activity, cells were lysed in 1% (v/v) Triton X-100 in phosphate-buffered saline (PBS). The cell lysate was incubated in bicarbonate buffer 0.2 M, pH 10, 0.05% Triton X-100, 4 mM $MgCl_2$, and 2 mM *p*-nitrophenol phosphate for 60 min at 37°C. The reaction was stopped by adding 1 M NaOH. The concentration of generated *p*-nitrophenylphosphate was quantified by spectrophotometry at 405 nm. Enzyme activity was normalized to cell protein content measured using the bicinchoninic acid assay (Pierce, IL).

RNA Isolation

Total RNA was extracted using an RNeasy kit (Qiagen). Concentration and purity of the RNA were determined by measuring the absorbance at 260 and 280 nm. Only samples displaying satisfactory quality were used for analysis.

RT-PCR

RT-PCR was performed using Titan One Tube RT-PCR (Roche). The RT reaction using 2 μg of total RNA was incubated at 45°C for 60 min and the template was denaturated at 94°C for 2 min.

PCR was performed with DNA Thermal Cycler (Whatman Biometra®). Optimal conditions for each primer pair were predetermined in preliminary experiments.

Amplification reactions were performed for the following cDNAs: Ob-Ra, Ob-Rb, RANKL, and OPG. The house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as a control for RNA loading and variations in cDNA synthesis efficiency.

Primers used were Ob-Ra and Ob-Rb common upstream:

5′-ACACTGTTAATTTCACACCAGAG-3′, Ob-Ra downstream, 5′-AGTCATTCAAACCATAGTTTAGG-3′, Ob-Rb downstream, 5′-TGGATAAACCCTTGCTCTTCA-3′, RANKL upstream: 5′-TATGATGGAAGGCTCATGGT-3′ and downstream: 5′-TGTCCTGAACTTTGAAAGCC-3′, OPG upstream 5′-AAAGCACCCTGTAGAAAACA-3′ and downstream 5′-CCGTTTTATCCTCTCTACACTC-3′, GAPDH upstream 5′-TCCACTCACGGCAAATTCAACG-3′, downstream 5′TAGACTCCACGACATACTCAGC-3′.

PCR was performed with the following thermal cycling parameters: Ob-Ra and Ob-Rb: 94°C for 10 s, 55°C for 30 s, and 68°C for 45 s for 10 cycles. This was followed by an additional 25 cycles with thermal cycling 94°C for 30 s, 55°C for 30 s, and 68°C for 45 s. The reaction was completed with final extension at 68°C for

7 min. RANKL: 94°C for 10 s, 60°C for 30 s, and 68°C for 45 s for 10 cycles followed by an additional 25 cycles with thermal cycling 94°C for 30 s, 60°C for 30 s, and 68°C for 45 s. The reaction ended with final extension at 68°C for 7 min. OPG: 94°C for 10 s, 60°C for 30 s, and 68°C for 45 s for 10 cycles followed by an additional 20 cycles with thermal cycling 94°C for 30 s, 60°C for 30 s, and 68°C for 45 s. The reaction ended with final extension at 68°C for 7 min. GADPH: 94°C for 10 s, 55°C for 30 s, and 68°C for 45 s for 10 cycles followed by an additional 15 cycles with thermal cycling 94°C for 30 s, 55°C for 30 s, and 68°C for 45 s. The reaction ended with final extension at 68°C for 7 min.

The RT-PCR products were then loaded in a 2% TAE-buffered agarose gel and analyzed after gel electrophoresis by EtBr-staining and UV light illumination.

Assay of RANKL Protein Production

The concentration of sRANKL in the culture media of MC3T3-E1 treated with different mrleptin concentrations was performed using mouse RANKL immunoassay (Quantikine; R&D Systems) according to the manufacturer's instructions.

Statistical Analysis

ANOVA tests were used to evaluate differences between the sample of interest and its respective control. A P-value ≤ 0.05 was considered statistically significant.

RESULTS

Expression of Leptin Receptor mRNA in MC3T3-E1 Osteoblasts

To study whether MC3T3-E1 osteoblasts express leptin receptor, RT-PCR was performed using the primer sets for Ob-Ra and Ob-Rb leptin receptor isoforms. Expression of mRNAs encoding the receptor splice variants Ob-Ra and Ob-Rb were detected (Fig. 1; lanes 1 and 6). No significant changes were observed in Ob-Ra and OB-Rb expression following MC3T3-E1 osteoblasts incubation with different concentrations of recombinant leptin for a period of 24 h. The level of expression of these isoforms was not affected by the mrleptin (Fig. 1; lanes 2, 3, 4, 5, 7, 8, 9, and 10).

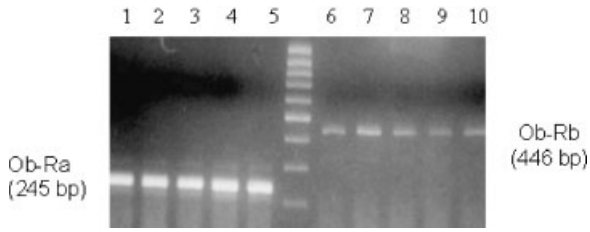

Fig. 1. Expression of Ob-Ra (**lanes 1–5**) and Ob-Rb (**lanes 6–10**) isoforms by MC3T3-E1 osteoblasts in absence or presence of mrleptin. Control: lanes 1 and 6, 1 ng/ml: lanes 2 and 6, 12 ng/ml: lanes 3 and 8, 24 ng/ml: lanes 4 and 9, 200 ng/ml: lanes 5 and 10.

Effect of Leptin on Alkaline Phosphatase Activity

In order to functionally demonstrate the existence of leptin receptor in MC3T3-E1 osteoblasts, ALP activity was measured after cells treatment by mrleptin for 24 h. As shown in Figure 2, significant increase of ALP activity was observed in presence of 12 and 20 ng/ml of mrleptin when compared to the control. However, after being incubated with 200 ng/ml of mrleptin the ALP activity of MC3T3-E1 osteoblasts was significantly decreased.

Effect of Mrleptin on RANKL Expression

To determine if mrleptin could modulate RANKL expression in MC3T3-E1 osteoblasts, both mRNA expression and protein levels were analyzed. As shown in Figure 3, RT-PCR experiment reveals that RANKL transcript was detected in MC3T3-E1. No alteration was observed after 24-h treatment with 0.2 and 1 ng/ml mrleptin. In contrast, following exposure to 12 ng/ml mrleptin increase of RANKL mRNA

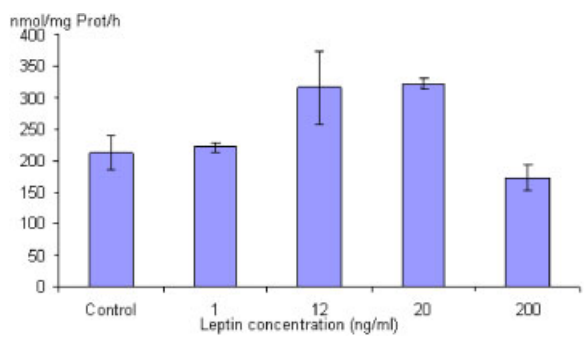

Fig. 2. Effect of leptin on alkaline phosphatase activity. MC3T3-E1 osteoblasts cells were cultured in standard medium in absence or presence of different concentration of mrleptin (1, 12, 20, and 200 ng/ml) for 24 h. Alkaline phosphatase activity was quantified as nanomoles of *p*-nitrophenylphosphate released per hour per mg of total protein.

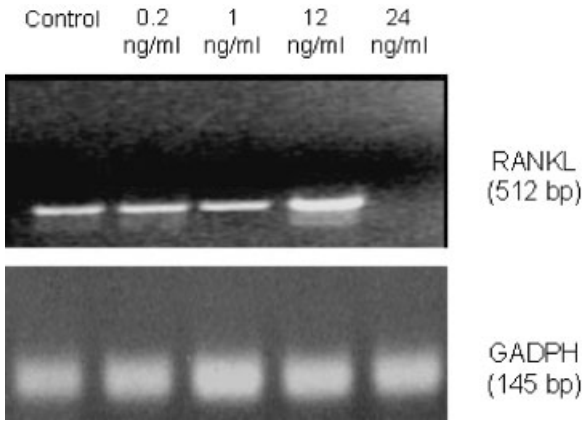

Fig. 3. Effect of leptin treatment on RANKL gene expression. MC3T3 E1 osteoblasts cells were treated with different concentration of mrleptin (0.2, 1, 12, 24 ng/ml) for 24 h, and then the expression of RANKL mRNA was examined by RT-PCR. RT-PCR was carried out with the same amount of RNA under the same conditions. The GADPH gene was used as an endogenous internal control.

expression was obtained. However, 24 ng/ml rmleptin induced an inhibition of RANKL mRNA expression. The RANKL protein quantification, using ELISA analysis, showed a result similar to that for gene expression (Fig. 4). RANKL protein production was inhibited in the presence of 24 ng/ml mrleptin.

Effect of Mrleptin on OPG Expression

Because the decoy receptor OPG has been described to be a crucial negative regulator of RANK–RANKL interactions, the effect of leptin on OPG mRNA expression was analyzed by RT-PCR. Unlike RANKL, Figure 5 shows no alteration of the basal, unstimulated, OPG mRNA expression in MC3T3-E1 osteoblasts following 24 h treatment by different concentrations of mrleptin.

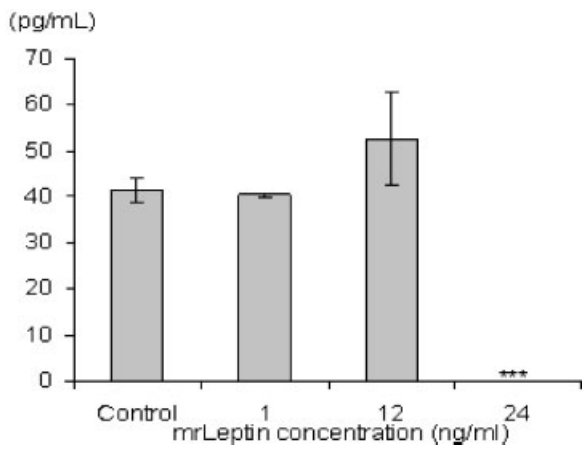

Fig. 4. Effect of leptin treatment on sRANKL protein secretion. MC3T3 E1 osteoblasts cells were cultured in medium containing mrleptin (1, 12, and 24 ng/ml) for 24 h sRANKL was measured using mouse RANKL ELISA kit.

DISCUSSION

Controversy exists whether leptin can affect bone metabolism by a direct or indirect mechanism. Several groups have reported the presence of a functional leptin receptor in cells within the osteoblast lineage [Steppan et al., 2000; Reseland et al., 2001; Cornish et al., 2002; Lee et al., 2002; Wamoto et al., 2004]. One other group, however, was unable to show the presence of leptin receptors and/or a functional response to leptin in osteoblast cultures [Ducy et al., 2000].

Therefore, we first analyzed, using RT-PCR, the gene expression of Ob-Ra (short isoform involved in leptin transport) and Ob-Rb (long signal-transducing isoform) by MC3T3 osteoblasts. mRNA expression of Ob-Rb, and Ob-Ra, was detected in MC3T3 osteoblasts. The level of expression of these isoforms is not affected by the mrleptin. This result is in agreement with those obtained by Reseland et al. suggesting that the absence of the autoregulation of leptin receptor by leptin in primary cultures human osteoblasts [Reseland et al., 2001]. Then, to investigate whether MC3T-E1 cell line responds to mrleptin hormone we examined their level of alkaline phosphatase activity after 24 h mrleptin treatment. We observed that the exposure of MC3T-E1 cells to different concentrations of mrleptin led to a variation of the enzyme activity. Previous in vitro studies reported that leptin treatment affects ALP mRNA expression and activity in a dose-dependant manner [Thomas et al., 1999; Parhami et al., 2001; Gordeladze et al., 2002; Nakajima et al., 2003]. However, it is important to point out that this effect could be cell type, cell culture stage, leptin dose and time-response dependent. Thomas et al. reported that 3 days incubation of hMS2-12 cells in the presence of graded dosages (0.075, 0.15, 0.3, 0.6, 1.2, and 2.4 μg/ml) of leptin revealed that 0.6 μg/ml was the optimal concentration resulting in a maximal increase of ALP activity [Thomas et al., 1999]. At early confluent stage, 4 days treatment of cultured growth plate chondrocytes by 5 ng/ml rat recombinant leptin resulted in an increase of ALP activity whereas 50 ng/ml leptin did not increase ALP activity. In other hand, at

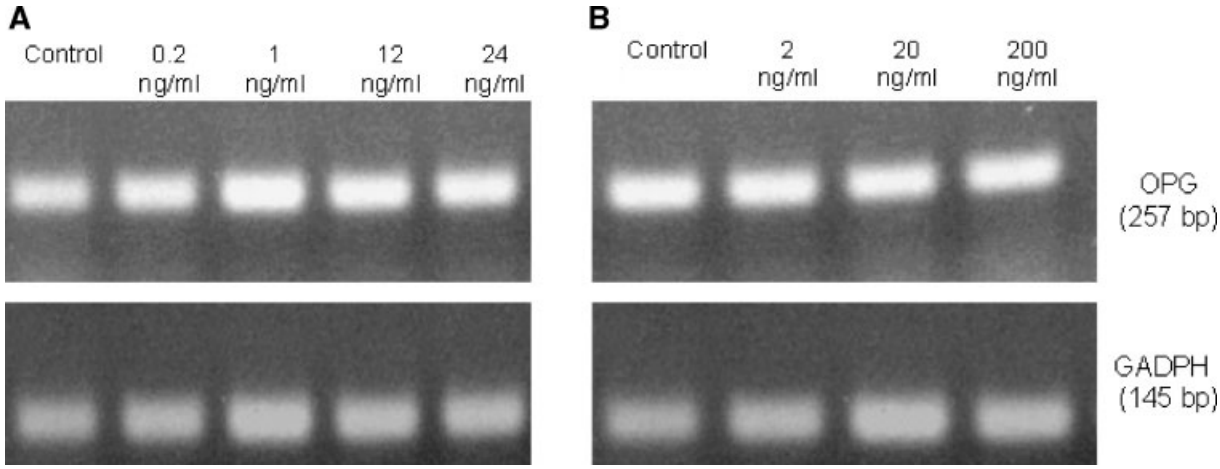

Fig. 5. Effect of leptin treatment on OPG gene expression. MC3T3 E1 osteoblasts cells were treated with two ranges of mrleptin concentrations 3A: 0.2, 1, 12, and 24 ng/ml and 3B: 2, 20, and 200 ng/ml for 24 h, and then the expression of RANKL mRNA was examined by RT-PCR. RT-PCR was carried out with the same amount of RNA under the same conditions. The GADPH gene was used as an endogenous internal control.

post confluence stage, the incubation of theses cells with concentration range (2, 5, 10, 20, 50 ng/ml) of leptin did not increase ALP activity [Nakajima et al., 2003]. Our own dose response experiment shows that ALP activity of MC3T3-E1 osteoblasts was significantly increased after 24 h treatment by 12 and 20 ng/ml mrleptin while in the presence of higher concentration (200 ng/ml) the level of the enzyme activity decreased significantly.

Accumulating evidence suggests that leptin hormone is able to directly alter bone remodeling by modulating both osteoblast and osteoclast activities [Thomas et al., 1999; Holloway et al., 2002; Wamoto et al., 2004; Martin et al., 2005]. Because RANKL and OPG are involved in the interaction between osteoblasts and osteoclasts [Udagawa et al., 2000] and consequently are considered as key regulators of bone remodeling we analyzed whether leptin can modulate the mRNA expression of these proteins in MC3T3-E1 osteoblasts.

We found that RANKL mRNA expression increases when cells are exposed to 12 ng/ml of mrleptin. However, the treatment with higher concentrations 24 and 200 ng/ml (data not shown) leads to an inhibition of RANKL mRNA expression. ELISA-based protein measurements for sRANKL further support the RT-PCR results. In fact, the level of sRANKL protein showed an increasing tendency in the presence of 12 ng/ml rmleptin. In contrast, sRANKL protein secretion was significantly inhibited by 24 ng/ml of rmleptin. These results are consistent with data from experiments in which leptin decreases significantly RANKL mRNA expression by human marrow stromal cells in vitro [Burguera et al., 2001]. The cellular mechanism by which leptin mediates the inhibitory effect on RANKL expression by osteoblasts remain unknown. Several studies have demonstrated the induction of RANKL expression by hormones and factors such as vitamin D, PTH [Lee and Lorenzo, 1999; Lee et al., 2002a; Suda et al., 2003], and IGF-I [Rubin et al., 2002b] as well as mechanical strain, reported to inhibit RANKL expression in vitro and in vivo [Kanematsu et al., 2002; Rubin et al., 2002]. Recently, it has been demonstrated that the basal transcription of *RANKL* gene in osteoblasts and stromal cells is regulated by two *cis*-elements located close to the transcription start site which bind transcription factor Sp1 and Sp3 [Liu et al., 2005]. Understanding RANKL regulation by leptin will require further analysis taking into account these two transcription factors.

Because the decoy receptor OPG has been described to be a crucial negative regulator of RANK–RANKL interactions [Yasuda et al., 1998], we tested whether the expression of OPG mRNA was affected by leptin. OPG mRNA expression was evident in control cells as well as in mrleptin treated cells. However, the exposure of MC3T3-E1 osteoblasts to mrleptin had no significant effect on OPG mRNA. This result is in apparent conflict with others showing an increase of OPG expression following leptin

treatment. Indeed, the expression of OPG by bone marrow stromal cells/osteoblasts in vitro was reported to be increased by leptin [Burguera et al., 2001; Gordeladze et al., 2002]. Nevertheless, it is important to emphasize that the duration of the treatment, the concentrations and the cell culture system used (cell line or primary culture) were different between studies. Burguera et al. described that 24 h of leptin treatment (2.4 μg/ml) stimulates OPG mRNA level by conditionally immortalized human marrow stromal cells [Burguera et al., 2001]. Gordeladze and coworkers reported that continuous leptin exposure (14–35 days) of iliac crest osteoblasts increase the mRNA level of OPG [Gordeladze et al., 2002]. In our study, the duration of leptin treatment was short (24 h) and the concentrations were lower than 2.4 μg/ml. Therefore, it is possible that the OPG mRNA level depends on the exposure time to leptin hormone and dose. It is known that leptin serum levels in humans are associated with food intake, periods of growth, and reproduction. Therefore, this observation raises the hypothesis that within physiological context, leptin effect on bone remodeling, through RANKL/OPG pathway, may depends on leptin concentration.

In summary, we find that Ob-Rb and Ob-Ra leptin receptor isoforms are expressed in MC3T3-E1 osteoblasts cells. When exposed to leptin hormone at high concentrations the expression of RANKL in these cells was down regulated whereas the OPG expression was not affected.

These results support the hypothesis that leptin is in favor of positive OPG/RANKL balance and therefore may directly modulate bone remodeling. This effect could be mediated by targeting RANKL gene.

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