Orexin B inhibits proliferation and stimulates specialized function of cultured rat calvarial osteoblast-like cells

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Abstract. Orexin-A (OXA) and orexin-B (OXB) are polypeptides derived from the same 130 amino acid long precursor (prepro-orexin) that bind and activate two closely related orphan G protein-coupled receptors OX1-R and OX2-R. These hypothalamic neuropeptides stimulate food intake and energy expenditure and play a significant role in sleep-wakefulness regulation. Present studies aimed to investigate the effects of orexins on proliferative activity and osteocalcin secretion by cultured rat calvarial osteoblast-like (ROB) cells. Conventional RT-PCR methods detected expression of the OX1-R gene in freshly isolated ROB cells and cells cultured for 7, 14 and 21 days. In contrast, at all time points tested, expression of prepro-OX or OX2-R genes was not demonstrated. QPCR revealed the highest expression of OX1-R gene in freshly isolated bone cells and a notably lower one in cultured ROB cells. Exposure of cultured cells to both OXA and OXB stimulated expression of the OX1-R gene. However, this effect was seen at the lowest tested concentration ($1x10^{-10}$ M). Exposure of cultured ROB cells to OXA for 48 h did not change osteocalcin concentrations in media analyzed at days 7, 14 and 21 of culture. On the contrary, OXB notably stimulated osteocalcin concentrations in media taken at days 14 and 21 of culture. In contrast, OXA exerted a notable inhibitory effect on the proliferative activity of ROB cells at day 7 of culture, while OXB exerted a similar effect at day 14. Thus, the obtained results suggest that: (i)(ROB) cells are provided with functional OX1-R gene; (ii) in ROB cells expression of this gene seems to be up-regulated by low concentrations of both OXA and OXB; (iii) OXB exerts inhibitory effects on proliferative activity and stimulating effects on osteocalcin secretion by cultured ROB cells; (iv) rat calvarial osteoblasts provided with OX receptor may be a target for circulating orexins. Thus, orexins may be included in the expanding group of neuropeptides involved in the physiological regulation of the major bone cell types.

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Introduction

Orexin-A (OXA, hypocretin-1) and orexin-B (OXB, hypocretin-2) are polypeptides derived from the same 130 amino acid long precursor (prepro-orexin) that bind and activate two closely related orphan G protein-coupled receptors OX1-R and OX2-R. OX1-R almost exclusively binds orexin-A, while OX2-R is non-selective for both orexins. Those hypothalamic neuropeptides stimulate both food intake and energy expenditure and play a significant role in sleep-wakefulness regulation. Orexins now are considered critical components for maintaining and regulating the stability of arousal (1-7).

Numerous biologically active peptides are involved in the regulation of biological activity of the major types of bone cells (8,9). The peptides may affect bone cells via endocrine, paracrine or autocrine routes. Peptides involved in energy homeostasis are known to influence functions of bone cells. In search of such peptides, we recently reported expression of OX1-R gene in cultured rat calvarial osteoblast-like (ROB) cells (10). In those cultures expression of prepro-OX and OX2-R genes could not be identified. In the present study we investigated effects of orexins on proliferative activity and osteocalcin secretion by cultured rat calvarial osteoblast-like cells.

Materials and methods

Animals and reagents. Two day-old Wistar rats, born in our animal facilities, were used. The local Ethics Committee for Animal Studies approved the study protocol. Orexins A and B were purchased from Bachem AG, Bubendorf, Switzerland. If not otherwise stated, all other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) or from POCh (Gliwice, Poland).

ROB cell culture. The technique was that described earlier (11-14) with few modifications. Briefly, 8 calvarias of 2-day-old rats were placed in DMEM (Gibco, UK), and the connective tissue was removed. Calvarias were then cut into small fragments, which were dissociated to cell suspensions by enzymatic digestion with 0.1% collagenase-I (Sigma-Aldrich)

Table I. Real time-PCR analysis of prepro-orexin (OX) and orexin receptor (OX1-R, OX2-R) mRNAs in cultured rat calvarial osteoblast-like cells.

cDNA	Genbank accession number	Primer or probe	Primer or probe sequence (5'-3') (Universal probe library number)	Position	PCR product size (bp)
OX	NM 013179	S	CAGACACCATGAACCTTCCTT	83-103	126
		A	GACAGCAGTCGGGCAGAG	191-208	
		probe	CTGCTGCC (74)	148-155	
OX1-R	NM 013064	S	TGCTGGTTTTTGCACTCTGT	1476-1495	74
		A	GCGAAACATCCCAAACACTC	1530-1549	
		probe	TCTGCCCA (113)	1498-1505	
OX2-R	NM 013074	S	GGCTTATCTCCAAATATTCCGTAAA	782-806	69
		A	CTCTGAACCACAGAAGAAGTTCC	828-850	
		probe	GCAGACAG (112)	814-821	
HPRT	NM 012583	S	CAGTCAACGGGGGACATAAAAG	391-412	146
		A	ATTTTGGGGCTGTACTGCTTGA	515-536	
		probe	TGGTGGAG (22)	417-426	

Oligonucleotide sequences for sense (S) and antisense (A) primers and the sequence of the probes are shown. HPRT, hypoxanthine-guanine phosphoribosyl transferase.

for 30 min at 37˚C. ROB cells were harvested by centrifugation and resuspended in DMEM supplemented with $NaHCO_3$, 6% FCS (fetal calf serum) and antibiotic-antimycotic solution. Cells were then plated in culture dishes (Nalge Nunc International, Denmark, $1x10^4$ cells/dish), and cultured for 21 days at 37˚C in an humidified atmosphere of 95% air-5% CO_2, medium being changed every other day (14). The experiments were performed on freshly isolated cells (RT-PCR) and cells harvested at days 7, 14 and 21 of culture. Before sampling cells were maintained in medium for 48 h without FCS. In such medium osteoblasts were exposed for 48 h to various concentrations of orexin A and B (Bachem, Switzerland).

Characterization of ROB cell culture by double immuno-fluorescence microscopy. Isolated osteoblasts were placed into LabTek™ Slide Chamber™ (Nalge Nunc International, Denmark), and incubated as described above. Immunofluorescence studies were performed on ROB cells cultured for 7, 14 and 21 days. Cells were fixed in 4% paraformaldehyde in PBS, pH 7.4, for 10 min, at room temperature. Then, slides were washed 3 times for 5 min in PBS and subsequently preincubated in PBS with 5% bovine albumin for 30 min. They were incubated for 18 h at 4˚C with primary antibodies against osteonectin and collagen 1α (Acris Antibodies GmbH, Germany) in concentrations of 1:400, in a humidified chamber. After incubation, slides were washed in PBS 3 times for 5 min. As secondary antibodies goat anti-rabbit MFP488 (absorption wavelength, 503 nm, emission wavelength, 524 nm) and goat anti-mouse MFP590 (absorption wavelength 597 nm, emission wavelength: 624 nm) (MoBiTech, Göttingen, Germany), respectively, in concentrations of 1:1000 (60 min, room temperature), were used. Slides were then washed in PBS 3 times for 5 min, coverslips were mounted with a drop of mounting medium for fluorescence with DAPI (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Reactions were documented using Axiolmager Z1 microscope with ApoTome system (Carl Zeiss AG, Germany).

Osteocalcin determination. Osteocalcin concentrations in the culture medium were estimated using a rat osteocalcin EIA kit (Biomedical Technologies Inc., Stoughton, MA, USA). The sensitivity of the assay was 0.5 ng/ml, and inter- and intra-assay CVs were 7% and 4%, respectively.

Cell proliferation. Proliferation rate of ROB cells was measured by the EZ4U Non-radioactive Cell Proliferation and Cytotoxic Assay kit (Biomedica, Vienna, Austria) (15). Cultured cells were incubated for 90 min with EZ4U, and formazan derivative production, which is linearly related to the cell number, was measured at 490 nm wavelength in a microplate autoreader EL-13 (BIO-TEK Instruments, Winooski, VT, USA). It is worthy to emphasize that EZ4U assay system is highly compatible with the standard ^{3}H-thymidine incorporation assay.

Conventional RT-PCR and QPCR. The applied methods were described earlier (10,16-20). The applied primers for studies of prepro-orexin, OX1-R and OX2-R gene expression (Table I) were purchased from the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw. HPRT (hypoxanthine-guanine phosphoribosyl transferase) gene was used as reference to normalize the data.

Statistics. Data were expressed as the means ± SEM and their statistical comparison was performed using the unpaired Student's t-test.

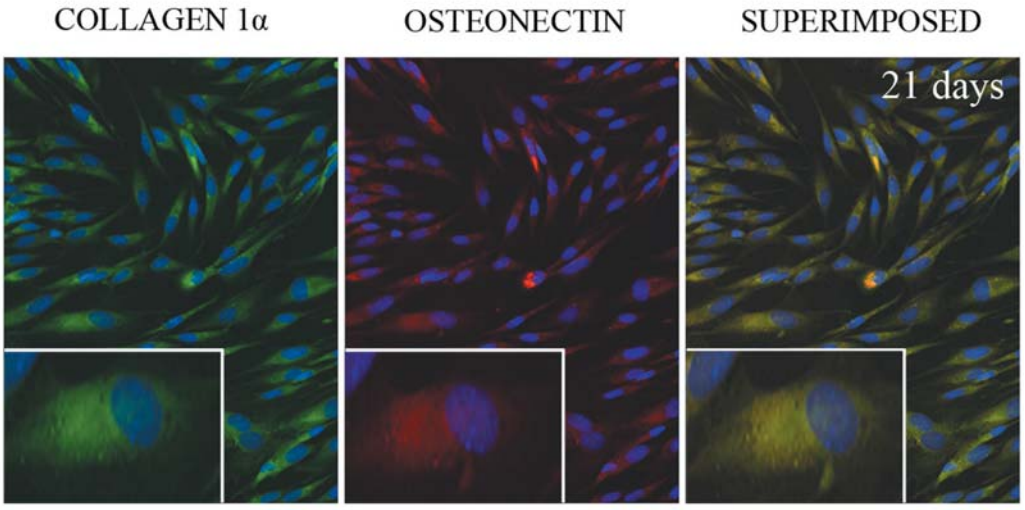

Figure 1. Double immunofluorescence microscopy illustrating the colocalization of collagen 1α and osteonectin in cultured calvaria rat osteoblast-like cells during mineralization of the extracellular matrix stage (day 21). Cultures were fixed in 4% paraformaldehyde in PBS, pH 7.4, for 10 min at room temperature and double labeled with primary antibodies against osteonectin and collagen 1α (Acris Antibodies GmbH, Germany), and followed by goat anti-rabbit MFP488 and goat anti-mouse MFP590 (MoBiTech, Göttingen, Germany) secondary antibodies. Coverslips were mounted in medium for fluorescence with DAPI (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Reactions were documented using Axiolmager Z1 microscope with ApoTome system (Carl Zeiss AG, Germany). Collagen 1α, green; osteonectin, red and nuclei, blue. Colocalization was determined by merging green and red pictures to give yellow/orange stained cells. Magnification ~x400, insert ~x1000.

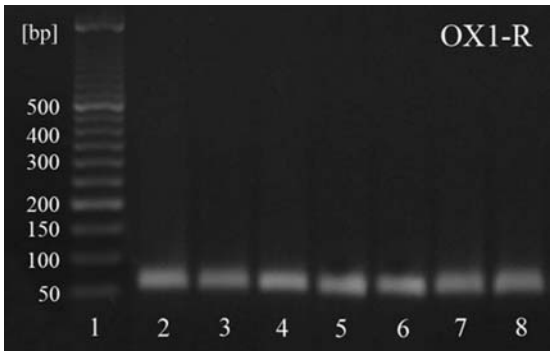

Figure 2. Ethidium bromide-stained 2% agarose gel showing cDNA amplified with rat specific primer for OX1-R from RNA of freshly isolated bone cells and the cultured (7, 14 and 21 days) ROB cells. Lane 1, size marker (O'Range Ruler 50 bp DNA Ladder; MBI Fermentas, Lithuania); lane 2, freshly isolated bone cells; lanes 3 and 4, day 7; lanes 5 and 6, day 14; lanes 7 and 8, day 21. Reaction products with expected size of 74 bp are seen.

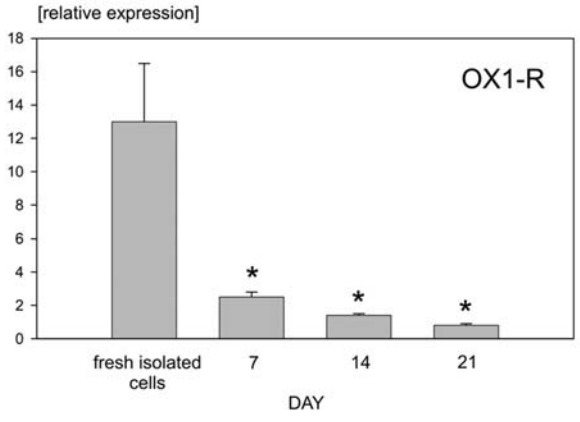

Figure 3. QPCR analysis of OX1-R gene expression in the ROB cells at days 7, 14 and 21 of culture and in freshly isolated bone cells. All samples were amplified in triplicate, and HPRT gene expression was used as a reference to normalize data. In each group n=3. Bars represent the mean and ± SEM. Statistical comparisons (in relation to control) were performed by the unpaired Student's t-test, $^{*}p<0.01$.

Results

As revealed by double immunofluorescence microscopy, collagen 1α and osteonectin were demonstrated in all cultured ROB cells during cell proliferation (day 7), differentiation (day 14) and mineralisation (or bone nodule formation, day 21) stages (Fig. 1). Collagen 1α (green) and osteonectin (red) exhibited high degrees of colocalization in ROB cells. That colocalization was determined by merging green and red pictures to obtain yellow/orange stained cells. The obtained results proved high purity of culture.

Classic RT-PCR methods demonstrated expression of OX1-R gene in freshly isolated bone cells and the cultured (7, 14 and 21 days) ROB cells (Fig. 2). With specific primer RNA amplification by means of RT-PCR revealed presence of reaction products with expected size of 74 bp. In contrast, at all time points tested, expression of neither prepro-OX nor OX2-R genes was demonstrated (data not shown). QPCR revealed the highest expression of OX1-R gene in freshly isolated bone cells (Fig. 3). Expression of that gene was notably lower in cultured ROB cells. In order to test the possibility of local OX influence on expression of orexin and orexin receptors in osteoblast-like cells, ROB cells were cultured for 48 h in the presence of different ($1x10^{-10}$-$1x10^{-6}$ M) OXA and OXB concentrations and the RNAs were isolated at day 7 of culture (Fig. 4). Under these conditions both OXA and OXB stimulated expression of OX1-R gene but this effect was seen after exposure of cells to the lowest tested

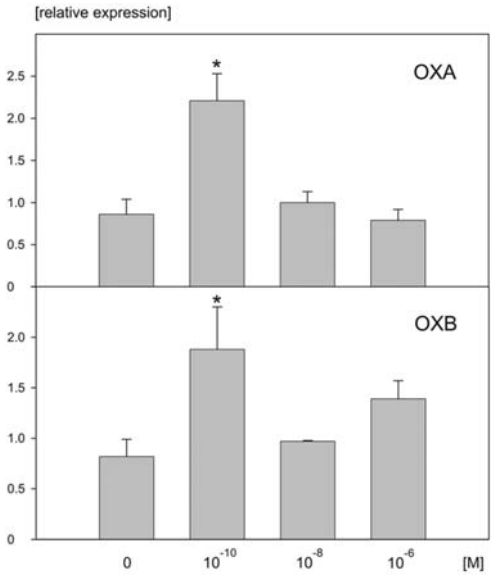

Figure 4. QPCR analysis of OX1-R gene expression in ROB cells cultured in the presence of different concentrations of orexin A (OXA) and B (OXB). Neuropeptides were added to culture 48 h before medium collection at day 7. All samples were amplified in triplicate, and HPRT gene expression was used as reference to normalize data. In each group n=3. Bars represent the mean and ± SEM. Statistical comparisons (in relation to control) were performed using the unpaired Student's t-test, $^{*}p<0.01$.

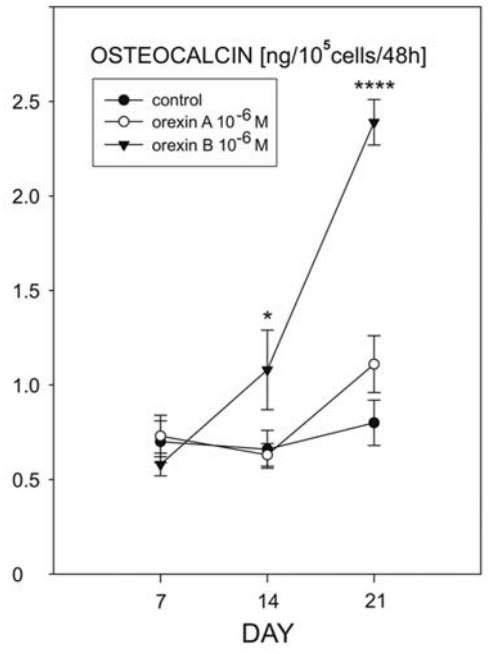

Figure 5. Effects of orexin A and B on osteocalcin secretion by cultured ROB cells (ng/10^5 cells/48 h). Cells were exposed to orexins ($1x10^{-6}$ M) for 48 h. Neuropeptides were added to culture 48 h before medium collection at days 7, 14 and 21. Data are expressed as a mean ± SEM, n=6. Statistical comparisons (in relation to control at given day) were performed using the unpaired Student's t-test, $^{*}p<0.05$, $^{****}p<0.001$.

concentration ($1x10^{-10}$ M) of polypeptides while the higher concentrations were ineffective. Again, in these cells expression of neither OX nor OX2-R was demonstrated.

Exposure of cultured ROB cells to OXA for 48 h did not change osteocalcin concentrations in media analyzed at days 7, 14 and 21 of culture (Fig. 5). On the contrary, OXB notably stimulated osteocalcin concentrations in media sampled at days 14 and 21 of culture. In contrast, OXA exerted a notable inhibitory effect on proliferative activity of ROB cells at day 7 of culture, while OXB exerted a similar effect at day 14 (Fig. 6).

Discussion

Primary culture of the rat calvarial osteoblast-like cells (ROB cells) is one of the most frequent *in vitro* models of investigation on specific gene events associated with osteoblast proliferation, differentiation, and mineralization of extracellular matrix (11,21-26). In primary culture these cells spontaneously differentiate into osteoblasts and the entire process is divided into proliferation (days 0-12), differentiation (or maturation, days 12-20) and mineralization (days 16-32) stages (27,28). The process of differentiation is associated with increased expression of both collagen and non-collagenous bone proteins, including osteocalcin, osteopontin, and osteonectin (14,22,29-31).

Recent experimental data indicate that numerous neuropeptides are involved in physiological regulation of the major bone cell types. In this regard, arginine-vasopressin (AVP), acting via V1 receptor, stimulates proliferation of human and mouse osteoblast-like cells (32,33). Substance P (SP), acting via NK1R, stimulates osteocalcin mRNA expression in ROB cells and the effect is observed at the late-stage of culture (34,35). Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) receptors are also expressed in osteoblasts and VIP-1R expression is induced during osteoblastic differentiation (36-39). Moreover, VIP stimulates ROB alkaline phosphatase biosynthesis and bone noduli formation without affecting osteoblast proliferation. Glucose-dependent insulinotropic peptide (GIP) receptor mRNA and protein are expressed in osteoblasts and GIP stimulates expression of collagen type I messenger RNA and alkaline phosphatase synthesis (40).

An increasing body of evidence indicates also that some neuropeptides involved in energy homeostasis may have a role in the regulation of bone metabolism in animals and humans. In this regard, Lee *et al* (41) suggest that bone and energy metabolisms exert reciprocal regulations. Leptin and neuropeptide Y (NPY) are major players in the control of energy homeostasis and both affect bone metabolism. Experimental data suggest that both leptin and NPY inhibit bone formation via activation of the sympathetic nervous system, both neuropeptides, however, also exert direct effects on osteoblasts. Intracerebroventricular infusion of leptin results in bone loss in leptin-deficient and wild-type mice (42-44). Leptin enhances human marrow stromal cell differentiation to osteoblasts (45). The cytokine is expressed in and secreted from primary cultures of human osteoblasts, stimulates their proliferation, regulates apoptosis and promotes bone mineralization (46-48). In contrast, leptin mRNA was not identified in commercially available human

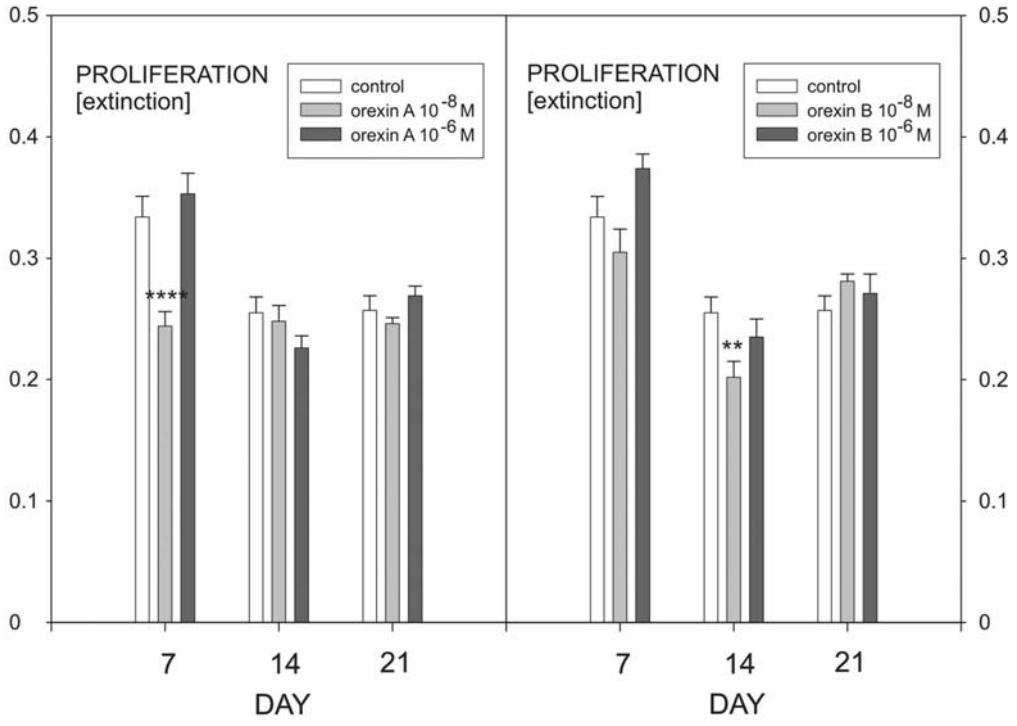

Figure 6. Effects of orexin A and B on proliferative activity of cultured ROB cells (extinction). Cells were exposed to orexins ($1x10^{-8}$ M and $1x10^{-6}$ M) for 48 h. Neuropeptides were added to culture 48 h before medium collection at days 7, 14 and 21. Data are expressed as a mean ± SEM, n = 6. Statistical comparisons (in relation to control at given day) were performed using the unpaired Student's t-test, $^{**}p<0.02$, $^{****}p<0.001$.

osteoblast cell lines or in different human monoclonal osteosarcoma cell lines. These cells, however, are provided with the long form of the leptin receptor. Leptin expression is also observed in osteoblasts in the mineralization and/or the osteocyte transition period but not during the matrix maturation period. Likewise, rat osteoblasts express various functionally active leptin receptor isoforms (49,50). Intracerebroventricular administration of NPY also causes bone loss (51,52). Furthermore, mouse osteoblasts are provided with Y1 and Y2 receptors and it has been suggested that Y1 and Y2 receptor pathways may also directly inhibit bone formation (53). Recently it has been demonstrated that neuromedin U (NMU), an anorexigenic neuropeptide, also regulates bone mass independently of its regulation of energy metabolism, and that NMU-induced bone alterations are mediated via the central nervous system (54). Furthermore, NMU directly stimulates the growth of cultured ROB cells acting via NMU receptor 2 isoform (55).

Orexins (OXA and OXB) belong to the group of potent orexigenic neuropeptides and recently we described expression of OX1-R at the mRNA level in cultured ROB cells while expression of prepro-OX and OX2-R genes could not be demonstrated (10). In present studies we searched for a possible role of orexins in regulating functions of cultured rat calvaria osteoblast-like cells. As proven by double immunofluorescence microscopy, such culture is composed exclusively of cells expressing collagen 1α and osteonectin, typical markers of osteoblasts (56,57). In agreement with earlier observations, we confirmed that cultured ROB cells were provided with functional OX1-R gene while in all performed experiments expression of neither prepro-OX nor OX2-R genes was demonstrated.

Of note was the higher expression of the OX1-R gene in cells freshly isolated from the rat calvaria than in cultured ROB cells. At days 7, 14 and 21 of culture expression of the gene was notably lower and similar in all groups. With great probability we can assume that the high expression of the OX1-R gene in freshly isolated cells from the rat calvaria was connected with expression of that gene in cells other than osteoblasts. Furthermore, the obtained results suggest that cells with high expression of the OX1-R gene underwent elimination in the course of culture.

The pattern of expression of prepro-OX, OX1-R and OX2-R genes in culture was not influenced by 48 h exposure of cells to either OXA or OXB. This finding suggests that in cultured ROB cells neither OXA nor OXB induced expression of prepro-OX and the OX2-R gene. In contrast, OXA and OXB stimulated expression of the OX1-R gene, an effect seen after exposure of cells to the lowest tested concentration ($1x10^{-10}$ M) of polypeptides while the higher concentrations were ineffective. Thus, in very low concentrations OXA and OXB up-regulated expression of their OX1-R receptor isoform gene.

We also demonstrated that OXB but not OXA stimulated osteocalcin secretion by cultured rat calvaria osteoblast-like cells, an effect observed at differentiation and mineralization stages of culture. In contrast, OXA exerted a notable inhibitory effect on proliferative activity of ROB cells at day 7 of culture, while OXB exerted a similar effect at day 14. To our knowledge, this is the first report on possible involvement of orexins in the regulation of proliferation and a specialized function of rat osteoblasts. Furthermore, since orexins are present in the blood, our finding suggests that rat osteoblasts may be their target cells (58-61).

It was generally accepted that OX1-R almost exclusively binds OXA, while OX2-R is non-selective for both orexins (1,6). However, in cultured ROB cells provided with OX1-R, OXB but not OXA stimulated osteocalcin secretion. This is a rather unexpected finding and deserves further studies. We cannot exclude the possibility that this difference may depend on faster inactivation of OXA than OXB in the applied culture medium. In this regard it should be emphasized that in other cells orexins may induce similar effects. For example, in the guinea-pig individual myenteric neurons both OXA and OXB caused membrane depolarizations via a direct action (62). In these studies OXB was sometimes more effective than OXA and vice versa. The cited authors also suggested that OXB increased neuronal activity via mechanisms similar to OXA.

Our studies demonstrated a role for OX1-R in regulation of proliferation and osteocalcin secretion by cultured calvarial ROB cells. A possible physiological relevance of this finding remains to be elucidated.

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