Cultured rat calvarial osteoblast-like cells are provided with orexin type 1 receptors

AGNIESZKA ZIOLKOWSKA[1], MARCIN RUCINSKI[1], CINZIA TORTORELLA[2], MARIANNA TYCZEWSKA[1], GASTONE G. NUSSDORFER[2] and LUDWIK K. MALENDOWICZ[1]

[1]Department of Histology and Embryology, School of Medicine, Karol Marcinkowski University of Medical Sciences, PL-60781 Poznan, Poland; [2]Department of Human Anatomy and Physiology, Section of Anatomy, School of Medicine, University of Padua, Via Gabelli 65, I-35121 Padua, Italy

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Abstract. Orexins A and B are hypothalamic peptides which are derived from the proteolytic cleavage of prepro-orexin and act via two subtypes of receptors, named OX1-R (that almost exclusively binds orexin-A) and OX2-R (nonselective for both orexins). Several lines of evidence show that other neuropeptides, which like orexins are involved in the central control of energy homeostasis (e.g. leptin and ghrelin), may play a role in the regulation of bone metabolism, acting via autocrine-paracrine or endocrine routes. Therefore, we studied by reverse transcription-polymerase chain reaction (RT-PCR) the expression of the orexin system in rat calvarial osteoblast-like (ROB) cells, whose osteoblastic lineage was immunocytochemically demonstrated by their osteonectin and collagen-1α content at day 14 of culture. Conventional PCR detected the mRNA expression of OX1-R, but not OX2-R and prepro-orexin in ROB cells at days 2, 7 and 21 of culture. Semiquantitative real time-PCR evidenced a gradual down-regulation of OX1-R mRNA in relation to the duration of culture. This novel finding suggests that rat osteoblasts could be a target for circulating orexin-A, especially during their early stages of differentiation into mature osteoblasts.

Introduction

Orexins A and B are hypothalamic neuropeptides that stimulate both food intake and energy expenditure, and play a role in sleep-wakefulness regulation and modulation of hypothalamic-pituitary-adrenal axis activity. Orexins originate from the proteolytic cleavage of prepro-orexin and act through two subtypes of receptors, named OX1-R and OX2-R. OX1-R almost exclusively binds orexin-A, while OX2-R is nonselective for both orexins (1-5).

Evidence has been provided that hypothalamic orexinergic neurons are regulated by hormonal signals, including ghrelin and leptin (6-8). Available findings indicate that certain neuropeptides involved in energy homeostasis, such as leptin and ghrelin, may have a role in the regulation of bone metabolism, their receptors being expressed in osteoblasts (see Discussion). However, there are no data available concerning the expression of orexin system components in bone cells. Hence, we investigated the expression of prepro-orexin and OX1-R and OX2-R mRNAs in cultured rat calvarial osteoblast-like (ROB) cells.

Materials and methods

Animals and reagents. Female Wistar rats (final body weight 100-110 g) were kept under a 14-h light:10-h dark cycle (illumination onset at 6.00 a.m.) at 23˚C, and maintained on a standard diet and tap water *ad libitum*. The study protocol was approved by the local Ethics Committee for Animal Studies. Rabbit anti-osteonectin and mouse anti-collagen-1α primary polyclonal antibodies were purchased from Acris Antibodies (Herford, Germany), and goat anti-rabbit MFP488 (emission wavelength, 524 nm) and anti-mouse MFP590 (emission wavelength, 624 nm) secondary antibodies from MoBi Tech (Göttingen, Germany). 4',6'-diamine-2'-phenilindole (DAPI) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), bovine serum albumin (BSA), phosphate-buffered saline (PBS), and all other laboratory reagents were provided by Sigma-Aldrich Corp. (St. Louis, MO).

ROB cell culture. The technique used was described by Boden *et al* (9), with few modifications (10). Briefly, calvarias of 8 rats were placed in DMEM, 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 for 30 min at 37˚C. ROB cells were harvested by centrifugation and re-suspended in DMEM supplemented with $NaHCO_3$, 6% FCS and antibiotic-

Correspondence to: Professor G.G. Nussdorfer, Department of Human Anatomy and Physiology, Section of Anatomy, School of Medicine, University of Padua, Via Gabelli 65, I-35121 Padua, Italy
E-mail: gastone.nusdorfer@unipd.it

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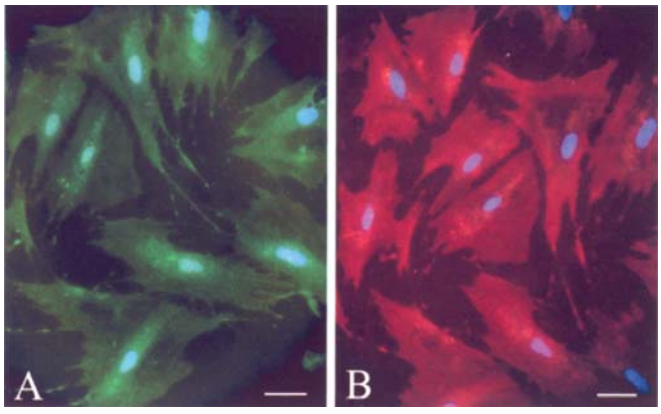

Figure 1. Osteonectin (A) and collagen-1α immunoreactivity (B) in ROB cells cultured for 14 days, as demonstrated by immunofluorescence. Nuclei were stained with DAPI. Magnification as defined by bars, 10 μm.

antimycotic solution. Cells were then plated in culture dishes (10^4 cells/dish), and cultured for up to 21 days at 37˚C in a humidified atmosphere of 95% air-5% CO_2; the medium being changed every 24 h.

Immunocytochemistry. ROB cells cultured for 14 days were fixed in 4% paraformaldehyde for 10 min at room temperature, washed in PBS and then preincubated with 5% BSA for 30 min. Cells were incubated with the primary anti-osteonectin and anti-collagen-1α antibodies (1:400 dilution) for 18 h at 4˚C, and then, after washing in PBS, with the secondary fluorescent antibodies (1:1,000 dilution) for 60 min at room temperature. Control specimens were obtained using primary antibodies preabsorbed with antigen excess (11). Slides were washed in PBS, nuclei were stained with DAPI, and specimens were observed and photographed using a Nikon Eclipse E600 microscope.

Reverse transcription (RT)-polymerase chain reaction (PCR). Total RNA was extracted from ROB cells cultured for 2, 7 and 21 days, as well as from the hypothalamus and anterior pituitary of two rats, and reverse transcribed to cDNA (12-14). Conventional PCR was carried out in a Perkin-Elmer 480 DNA Thermal Cycler (Perkin-Elmer Life Sciences, Milan, Italy), using the amplification program previously described (15) and the primers indicated in the legend of Fig. 2. The specificity of the PCR was verified by sequencing analysis (16). Semiquantitative real time-PCR of OX1-R mRNA was performed in a Roche Light Cycler 2.0 with software version 4.0, as detailed previously (17-19). The following program was used: a predenaturation step to activate Taq DNA polymerase (95˚C for 10 min), followed by a three-step amplification (denaturation at 95˚C for 10 sec; annealing at 56˚C for 5 sec; and extension at 72˚C for 2 sec). Subsequently, a melting curve (60-90˚C with a heating rate of 0.1˚C/sec) was carried out to check the specificity of amplification and the presence of by-products. All samples (n=6) were amplified in duplicate, and data were normalized in relation to the housekeeping gene hypoxanthine phosphoribosyl-transferase 1 (HPRT) and expressed as the means ± SEM.

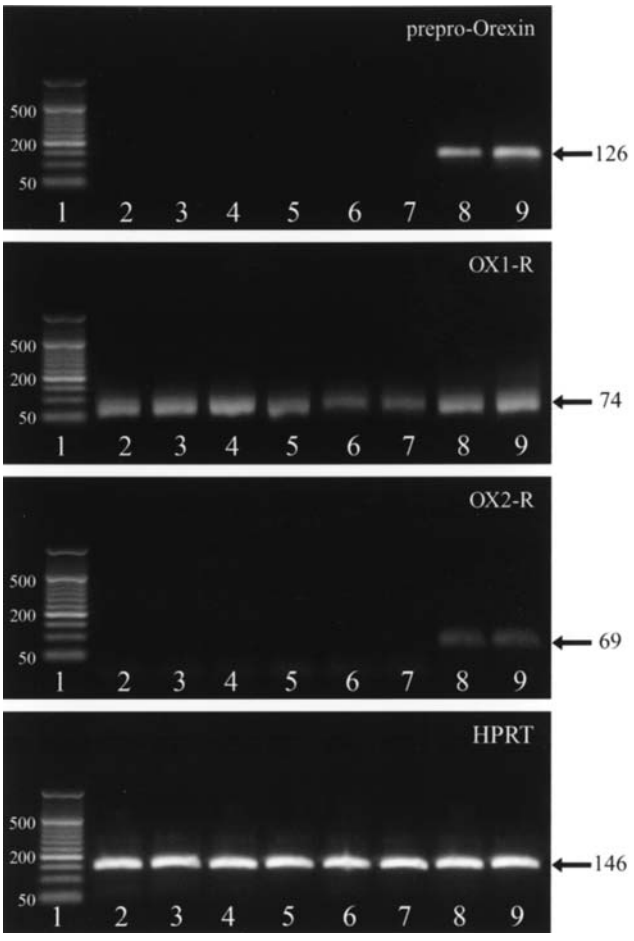

Figure 2. Ethidium bromide-stained 2% agarose gel showing cDNA amplified with rat specific primers from RNA of exemplary ROB cells cultured for 2 days (lanes 2 and 3), 7 days (lanes 4 and 5) and 21 days (lanes 6 and 7). Positive controls for prepro-orexin (hypothalamus) and other genes (anterior pituitary) are shown in lanes 8 and 9. Primer sequences were: prepro-orexin sense (83-103) CAGACACCATGAACCTTCCTT, antisense (191-208) CAGAGCAGTCGGGCAGAG and probe (148-155) CTGCTG CC (amplicon 126 bp; accession no. NM013179); OX1-R sense (1476-1495) TGCTGGTTTTTGCACTCTGT, antisense (1530-1549) GCGAAAC ATCCCAAACACTC and probe (1498-1505) TCTGCCCA (amplicon, 74 bp; accession no. NM013064; OX2-R sense (782-806) GGCTTATCTCCAAA TATTCCGTAAA, antisense (828-850) CTCTGAACCACAGAAGAAGT TCC and probe (814-821) GCAGACAG (amplicon, 69 bp; accession no. NM013074; and HPRT sense (391-412) CAGTCAACGGGGGACATAAA AG, antisense (515-536) ATTTTGGGGCTGTACTGCTTGA and probe (417-424) TGGTGGAG (amplicon, 146 bp; accession no. NM012583). Lane 1 was loaded with 200 ng of a DNA size marker (O'Range Ruler 50 bp DNA Ladder, MBI Fermentas, Vilnius, Lithuania).

Results

Immunocytochemistry revealed the expression of both osteonectin and collagen-1α in all ROB cells at 14 days of culture (Fig. 1).

Conventional PCR did not detect prepro-orexin and OX2-R mRNA expression in ROB cells, but detected it in the rat hypothalamus and anterior pituitary, respectively. Conversely, OX1-R mRNA was observed in ROB cells at days 2, 7 and 21 of culture (Fig. 2). Real time-PCR evidenced a gradual decrease in OX1-R mRNA expression in ROB cells in relation to the duration of culture (Fig. 3).

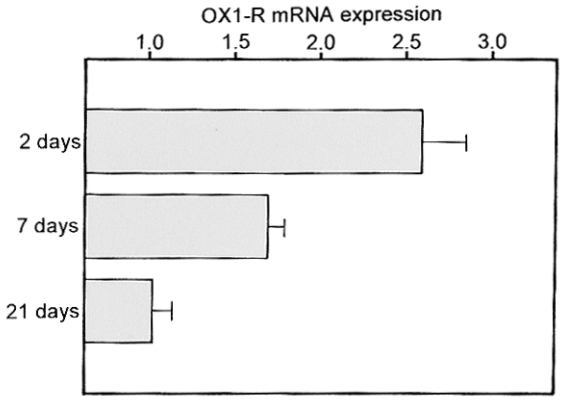

Figure 3. Relative OX1-R mRNA expression (normalized to HPRT) in ROB cells cultured for 2, 7 and 21 days. Bars are the means ± SEM (n=6).

Discussion

ROB cell primary culture is one of the most common *in vitro* models used to investigate specific gene events associated with osteoblast proliferation, differentiation and mineralization of extracellular matrix. Cultured ROB cells spontaneously differentiate into osteoblasts, and this process is coupled to the increased expression of both collagen and non-collagenous bone proteins, including osteonectin, osteocalcin and osteopontin (9,10,14,20-24). Our immunofluorescence findings showed that all cultured ROB cells expressed both osteonectin and collagen-1α, suggesting their osteoblastic lineage.

Consistent findings indicate that both bone formation and remodeling are regulated by several biologically active peptides, that act via endocrine, paracrine and autocrine mechanisms. Such peptides include substance-P, VIP, CGRP and neuropeptide-Y (25,26), as well as leptin and the endogenous ligand of growth hormone secretagogue (GHS) receptor ghrelin, which, like orexins, play a major role in the central control of energy homeostasis. Evidence has been provided that leptin is expressed in and secreted from human osteoblasts in primary culture (27-29), and that functionally active leptin-receptor isoforms are present in human osteoblast-derived cell lines and rat osteoblasts (30,31). Leptin was found to enhance human marrow stromal cell differentiation into osteoblasts (32), to stimulate proliferation of cultured osteoblasts, and to promote matrix production and mineralization (27-29). In contrast, *in vivo* studies evidenced an inhibitory effect of leptin on bone formation (33). Ghrelin and its GHS-R1a have been shown to be expressed in human and rat osteoblasts, and ghrelin has been reported to stimulate *in vitro* their proliferation and differentiation (34-36).

Our study demonstrated that ROB cells express OX1-R mRNA, but not prepro-orexin and OX2-R mRNAs. This novel finding may suggest that rat osteoblasts could be a target for circulating orexin A, for which OX1-R is selective. Moreover, real time-PCR reveals a time-dependent down-regulation of OX1-R expression in cultured ROB cells, which could be related to the stage of their differentiation into mature osteoblasts. Further studies are underway to elucidate the physiological relevance of these findings and to ascertain whether orexins may be included in that group of neuropeptides regulating bone metabolism.

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