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Neuromedin U directly stimulates growth of cultured rat calvarial osteoblast-like cells acting via the NMU receptor 2 isoform

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Abstract. The neuromedin U (NMU) system is composed of NMU, neuromedin S (NMS) and their receptors NMUR1 and NMUR2. This system is involved in the regulation of energy homeostasis, neuroendocrine functions, immune response, circadian rhythm and spermatogenesis. The present study aimed to investigate the possible role of the NMU system in regulating functions of cultured rat calvarial osteoblast-like (ROB) cells. By using QPCR, high expression of NMU mRNA was found in freshly isolated ROB cells while after 7, 14, and 21 days of culture, expression of the studied gene was very low. In contrast, NMUR2 mRNA expression in freshly isolated ROB cells was negligible and very high in cultured cells. The highest NMUR2 mRNA expression was observed at day 7, and was followed by lower levels at days 14 and 21 of culture. Neither NMS nor NMUR1 mRNA was found in studied cells. Exposure of cultured ROB cells to NMU8 at concentrations 10^{-6} to 10^{-10} M had no effect on expression levels of the genes. During the entire culture period, NMU8 did not affect osteocalcin production, but stimulated proliferative activity of ROB cells at days 14 and 21 of culture. Thus, we demonstrated that cultured rat calvarial osteoblast-like cells are provided with NMUR2, the receptor isoform typical for the central nervous system. Acting via this receptor NMU8 stimulates proliferation of cultured cells and has no effect on their differentiated function (osteocalcin secretion).

Introduction

Recent experimental data suggest that both bone formation and bone remodelling are regulated by numerous factors acting via endocrine, paracrine or autocrine routes and through the nervous system. Among others, peptides involved in regulation of energy homeostasis belong to this group of compounds (1-3), and the best recognised is leptin, an adipocyte-derived anorexigenic hormone, which plays a role in regulating bone formation. Acting directly this pleiotropic cytokine exerts a stimulatory effect on bone formation. While acting through the central nervous system (CNS) leptin suppresses bone formation (4-10). Moreover, OB-Rb mRNA is expressed in osteoblasts, and *in vitro* leptin enhances their proliferation and has no effect on osteocalcin and osteopontin production by cultured cells (6,11-13).

In contrast to leptin, ghrelin, a stomach-derived orexigenic hormone, exerts opposite effects on appetite, energy expenditure and weight control. Both at the mRNA and protein levels, GHS-R1a was found to be present in osteoblast-like cells and ghrelin directly stimulates proliferation and differentiation and has no effect, inhibitory or otherwise, on apoptosis of osteoblasts (14-18).

In search of novel neuropeptides involved in the regulation of energy homeostasis and bone physiology, we recently demonstrated that orexins (OXs), potent orexigenic peptides, might be involved in the regulation of osteoblast function. In rat calvarial osteoblast-like cultured cells (ROB) we identified OX1-R, but not OX2-R and pre/pro-orexin mRNA. This suggests a physiological role of OXs in regulating osteoblast activity (19).

Recently, Sato *et al* (20) reported that neuromedin U (NMU), an anorexigenic neuropeptide, regulates bone mass independently of its regulation of energy metabolism. NMU consists of 23 amino acids, and its C-terminal region, which is essential for the peptide's activity, is highly conserved. Neuromedins U and S (NMS) exert their biological activity through two types of receptors, NMUR1 and NMUR2 (21-28). NMUR1 is a peripheral receptor while NMUR2 is predominantly expressed in the CNS. Both NMU and its receptors are barely detectable in bone, and NMU exerts no effect on proliferation and differentiation of cultured calvarial osteoblasts obtained from WT and $Nmu^{-/-}$ mice (20). Since $Nmu^{-/-}$ mice have a higher bone mass than their WT counterparts, their cultured osteoblasts are not affected by NMU. Sato *et al* (20) suggest that NMU effects on bone formation are mediated via the CNS. Unexpectedly, in search of neuropeptides affecting osteoblast function, we found that cultured ROB cells are provided with neuromedin U receptor,

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Table I. PCR and real-time PCR analyses of mRNA of neuromedin U (NMU), neuromedin S (NMS) and their receptors (NMUR1 and NMUR2) and of the reference gene (HPRT).[a]

cDNA	Genbank accession no.	Primer	Primer sequence (5'-3')	Position	PCR product size (bp)
NMU	NM 022239	S	GCTTTAACACCCGCACAACA	50-69	200
		A	TGAGGCGATATTGGCGTACC	230-249	
NMS	NM 001012233	S	AGTTTGCTCCCGTCCATC	276-293	190
		A	TTGTCAGTGTATCTTCCATTCC	444-465	
NMUR1	NM 023100	S	ATGCTCTCCCCAAATGCTTC	1-20	190
		A	CCAGAGTGCCCACTACGAA	172-190	
NMUR2	NM 022275	S	GAATCCCTTGAGGCGAACA	841-859	137
		A	CTGAAGAAGAGCCGGTCCAC	958-977	
HPRT	NM 012583	S	CAGTCAACGGGGGACATAAAAG	391-412	146
		A	ATTTTGGGGCTGTACTGCTTGA	515-536	

[a]Oligonucleotide sequences for sense (S) and antisense (A) primers are shown.

and we revealed a direct effect of NMU on their proliferative activity.

Materials and methods

Chemicals. Neuromedin U8 (NMU8) was purchased from Bachem Feinchemikalien AG. Bubbendorf, Switzerland. If not otherwise stated, the remaining reagents were obtained from Sigma-Aldrich or POCh (Gliwice, Poland).

Primary rat calvarial osteoblast-like (ROB) cell cultures. The technique used, was that previously described (29-33) with a few modifications. Briefly, calvarials of eight 2-day-old rats were placed in DMEM (Gibco, UK), and the connective tissue was removed. Calvarials were then cut into small fragments, which were dissociated to cell suspensions by enzymatic digestion with 0.1% collagenase-I (Sigma-Aldrich) 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 (10^4 cells/dish), and cultured for 21 days at 37˚C in a humidified atmosphere of 95% air-5% CO_2, with the medium being changed every other day (34). The cells in culture were confirmed to be osteoblasts both by morphology and osteonectin and collagen-1α expression (19). The local Ethics Commitee for Animal Studies approved the study protocol.

The experiments were performed on freshly isolated cells. Cells were harvested at days 7, 14 and 21 of culture, 48 h before sampling, cells were maintained in medium without FCS. In such medium osteoblasts were exposed for 48 h to various concentrations of neuromedin U8.

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 using the EZ4U Nonradioactive Cell Proliferation and Cytotoxic Assay (Biomedica, Vienna, Austria) (35). 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 important to emphasise that the EZ4U assay system is highly compatible with the standard ^{3}H-thymidine incorporation assay.

Conventional RT-PCR and QPCR. ROB cells were harvested, and total RNA was extracted, as previously detailed (32,36-39). Contaminating DNA was eliminated by DNase-I treatment (RNase-Free DNase Set; Promega, Madison, WI, USA), and the amount of total RNA was determined by measuring optical density at 260 nm. Purity was estimated using the 260/280 nm absorption ratio, which was consistently higher than 1.8. RT and was performed using AMV Reverse Transcriptase (Promega, USA) with Oligo dT (PE Biosystems, Warrington, UK) as primers. Conventional and semi-quantitative real-time PCR were carried out in a Roche Light-Cycler 2.0 (Roche, Mannheim, Germany), as previously described, (38,39). Primers were designed using Primer 3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) (Table I). They were purchased from the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw.

For conventional PCR, the amplification program included a denaturation step (94˚C for 10 min), and 35 cycles of three-step amplification (denaturation, 94˚C for 30 sec; annealing, 57˚C for 60 sec; and extension, 68˚C for 120 sec). A final extension step at 68˚C for 7 min was then carried out. Detection of the PCR amplicons was performed by size fractionation on 2% agarose gel electrophoresis. To eliminate the possibility of amplifying genomic DNA, in some experiments PCR was carried out without prior RT of the

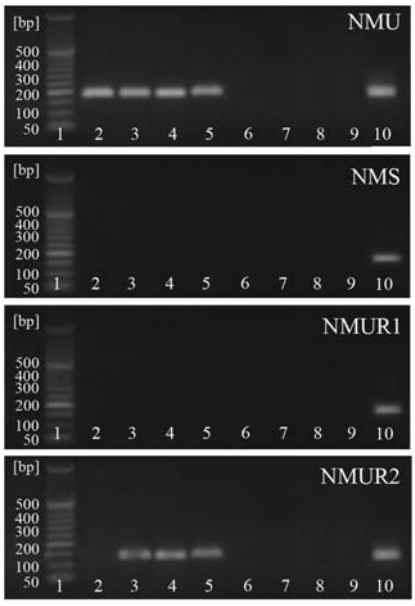

Figure 1. Ethidium bromide-stained 2% agarose gel showing cDNA amplified with rat NMU-, NMS-, NMUR1- and NMUR2-specific primers from RNA of ROB cells cultured for 7, 14 and 21 days and of freshly isolated cells. Lane 1, 200 ng of a DNA size marker (50 bp DNA Ladder; MBI Fermentas, Vilnius, Lithuania); lane 2, freshly isolated cells; lanes 3-5, days 7, 14 and 21 of culture. Respective negative controls (no RT of the RNA) are shown in lanes 6-9. Lane 10, positive control organs (hypothalamus for NMS and adrenal cortex for NMUR1) Specific primers amplified a single band of the expected length: NMU, 200 bp; NMS, 190 bp; NMUR1, 190 bp, and NMUR2, 137 bp.

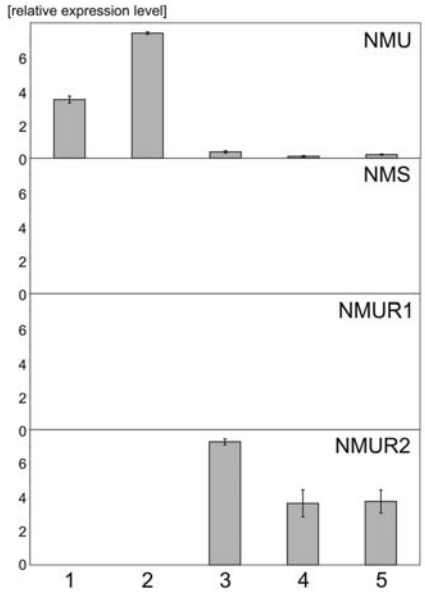

Figure 2. QPCR analyses of NMU, NMS, NMUR1 and NMUR2 gene expression in the ROB cells at days 7, 14 and 21 of culture and in freshly isolated cells (day 0). All samples were amplified in triplicates, and HPRT gene expression was used as reference to normalize data. In each group n=3. Bars represent means and SE. Lines: 1, rat calvaria bone; 2, freshly isolated bone cells; 3, day 7 of culture; 4, day 14 of culture; 5, day 21 of culture.

RNA. For QPCR, the following program involved, a denaturation step (95˚C for 10 min), and 45 cycles of three-step amplification (denaturation, 95˚C for 10 sec; annealing, 58˚C; and extension, 72˚C for 4 sec). Subsequently, melting curve (60-90˚C with heating rate of 0.1˚C/sec) was performed to check the specificity of amplification and the presence of by-products. All samples were amplified in duplicate, and the HPRT (hypoxanthine-guanine phosphoribosyl transferase) gene was used as reference to normalize data.

Statistics. Data were expressed as the mean ± SEM and the statistical comparison was conducted by the unpaired Student's t-test.

Results

NMU, NMS and their receptor expression in ROB cells. Standard RT-PCR revealed expression of specific mRNA for NMU in freshly isolated ROB cells which were cultured for 7, 14 and 21 days (Fig. 1). Significantly, NMUR2 mRNA was found only in cultured, but not in freshly isolated cells. In all assays reaction products were of the expected length. In contrast, neither NMS nor NMUR1 mRNA were found in studied cells, while in positive control organs (hypothalamus and adrenal cortex, respectively) expression of the genes was apparent.

By means of QPCR, high expression of NMU mRNA was found in freshly isolated ROB cells while after 7, 14, and 21 days of culture, expression of the studied gene was very low (Fig. 2). In contrast, NMUR2 mRNA expression in

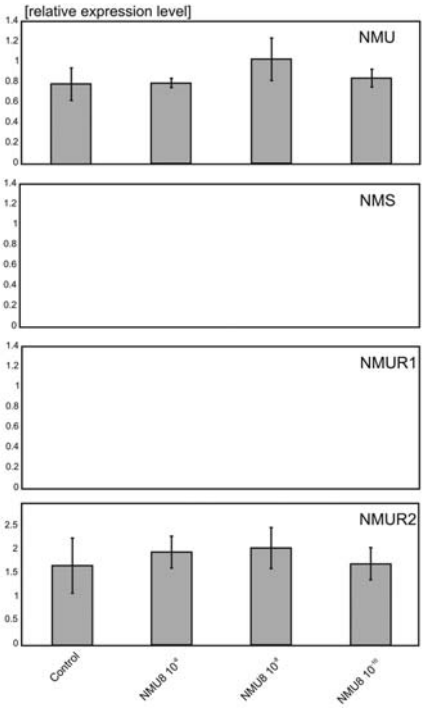

Figure 3. QPCR analysis of NMUR2 gene expression in ROB cells cultured in the presence of different concentrations of NMU8.

freshly isolated ROB cells was negligible and very high in cultured cells. The highest NMUR2 mRNA expression was observed at day 7, and was followed by lower levels at days 14 and 21 of culture. Similar to standard RT-PCR, neither NMS nor NMUR1 mRNA were detected in QPCR.

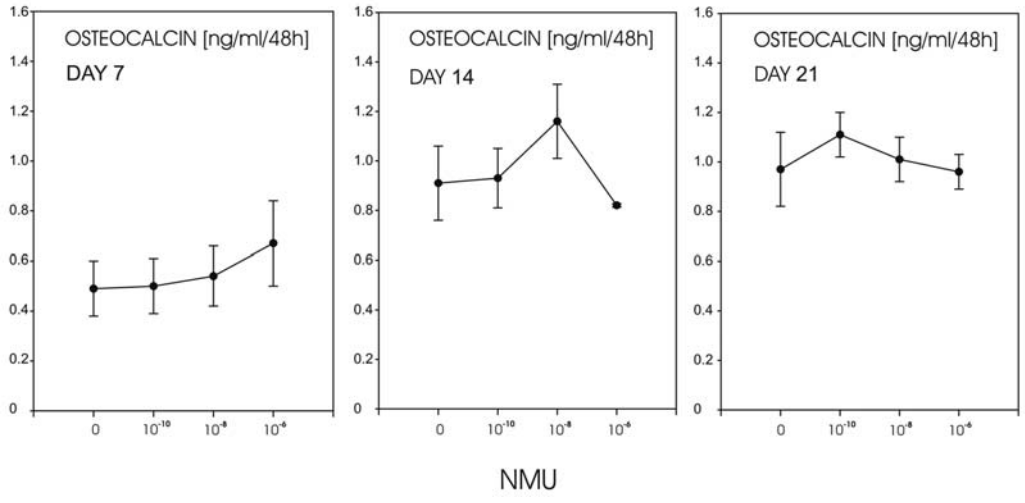

Figure 4. Effect of NMU on osteocalcin secretion by cultured ROB cells. Cells were exposed to different concentrations of NMU8 ($1x10^{-10}$ to $1x10^{-6}$ M) for 48 h. Neuropeptide was added to the culture 48 h before medium collecting at days 7, 14 and 21. Data are expressed as means ± SEM, n=8. Statistical comparisons (in relation to control) were performed by the unpaired Student's t-test: $^{*}p<0.05$, $^{**}p<0.02$, $^{***}p<0.01$, $^{****}p<0.001$.

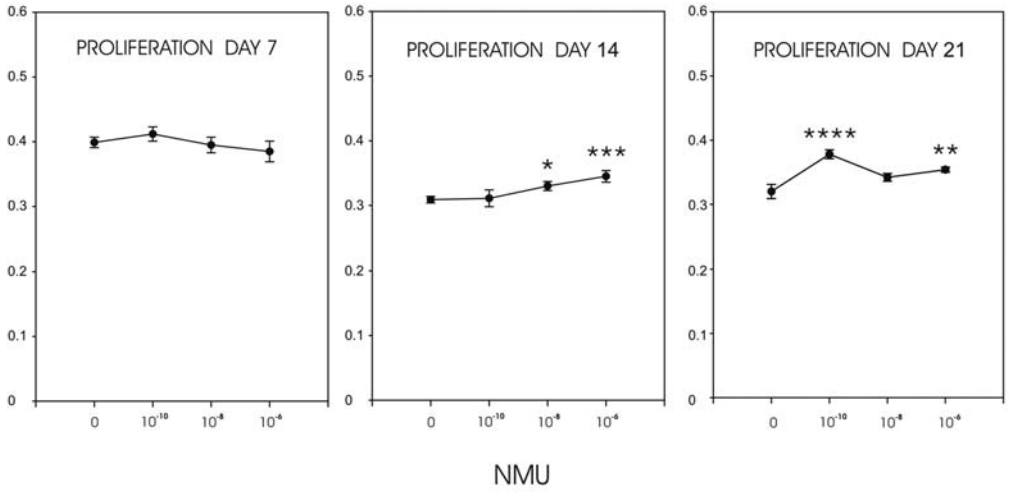

Figure 5. Effect of NMU on proliferative activity of cultured ROB cells. Cells were exposed to different concentrations of NMU8 ($1x10^{-10}$ to $1x10^{-6}$ M) for 48 h. Neuropeptide was added to the culture 48 h before medium collecting at days 7, 14 and 21. Data are expressed as means ± SEM, n=8. Statistical comparisons of means in relation to control groups were performed by the unpaired Student's t-test: $^{*}p<0.05$, $^{**}p<0.02$, $^{***}p<0.01$, $^{****}p<0.001$.

To ascertain whether there was an interrelationship between NMU concentration and NMUR2 gene expression, ROB cells were cultured at days 6 and 7 in the presence of different concentrations of NMU8. Two days exposure of cultured ROB cells to NMU8 at concentrations 10^{-6} to 10^{-10} M had no effect on expression levels of studied genes (Fig. 3).

Osteocalcin production. To investigate whether NMU8 affects osteocalcin production, cultured ROB cells were exposed for 48 h to various concentrations of the neuropeptide. When compared with day 7, osteocalcin concentration in the medium was higher at days 14 and 21 of culture (Fig. 4). During the entire culture period NMU8 did not affect osteocalcin production by the studied cells.

Cell proliferation. At days 14 and 21 of culture, proliferation rates of ROB cells were lower than at day 7 (Fig. 5). NMU8 exposure for 48 h significantly stimulated ROB cell proliferation at days 14 and 21 but was ineffective at day 7.

Discussion

In the rat both NMU and NMS exert potent anorexigenic effects, which in part are mediated via CRH neurons (26-28,40). It appears that NMU is also involved in bone remodelling. In search of neuropeptides involved in both regulation of energy homeostasis and bone physiology Sato *et al* (20) demonstrated that NMU controls bone remodelling in the mouse via CNS. Furthermore, they suggest that NMU may be a central mediator of leptin-dependent regulation of bone mass. By means of *in situ* hybridization they found that NMU and its receptors were barely detectable in the bone of WT and $Nmu^{-/-}$ mice and 'both WT and $Nmu^{-/-}$ osteoblasts proliferated normally *in vitro* in response to NMU treatment'.

trast with the above cited data we found that cultured rat calvarial osteoblast-like cells express NMUR2, a central nervous type of NMU receptor, but not NMUR1, a peripheral one. In this context it is worth noting that in freshly isolated cells we could not identify NMUR2 mRNA.

This finding confirms observations of Sato *et al* (20) on negligible expression of NMU receptors in *in situ* osteoblasts. Furthermore, this intriguing observation leads to further investigation as to why NMUR2 receptor is expressed in cultured but not in freshly isolated osteoblasts. It could be speculated that NMUR2 expression is a marker of osteoblast differentiation.

This assumption, however, is restricted by the following facts: i) osteoblasts *in situ* are not provided with that receptor (20) and ii) the highest expression of the NMUR2 gene is observed in the proliferative stage of osteoblast culture (day 7). An alternative explanation may be that in the bone, local factors of the microenvironment (haematopoietic stem cell niche or the osteoblastic niche) suppress expression of NMUR2 in osteoblasts (41-43). This possibility implies that such factors would be removed during isolation of rat calvarial osteoblasts.

One of the environmental factors involved in regulation of NMUR2 gene expression in osteoblasts could be the NMU. In our experiments expression of the NMU gene was very high in freshly isolated ROB cells and absent in cultured cells. This finding suggests that isolated from calvarials of newborn rats NMU mRNA expressing cells undergo elimination in the course of long-term culture. It is still unclear whether NMU suppresses NMUR2 expression in osteoblasts. To investigate such a possibility we added various concentrations of NMU into medium of cultured ROB cells and such exposure did not affect the expression levels of the studied genes.

To assess whether NMU affects ROB cell differentiation, we measured osteocalcin [a late osteoblastic differentiation marker (44)] concentrations in culture medium. Exposure of cultured cells to various NMU concentrations had no effect on osteocalcin secretion. This finding confirms observations of Sato *et al* (20) regarding the lack of NMU effect on osteocalcin secretion by cultured osteoblasts isolated from either WT or $Nmu^{-/-}$ mice. However, in contrast with their data we found that NMU stimulates proliferation of cultured ROB cells and this effect was observed at days 14 and 21 of culture but not in the proliferative stage of culture (day 7). Whether this finding is of physiological relevance remains unclarified.

Thus, results of the present study are the first demonstration that cultured rat calvarial osteoblast-like cells express NMUR2. Moreover, we found that NMU8, acting probably through NMUR2, stimulates proliferation of cultured cells and has no effect on their differentiating functions.

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