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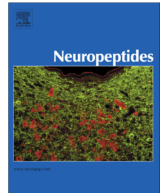

Neuropeptides stimulate human osteoblast activity and promote gap junctional intercellular communication

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A B S T R A C T

Neuropeptides released from the skeletal nerve fibers have neurotransmitter and immunoregulatory roles; they exert paracrine biological effects on bone cells present close to the nerve endings expressing these signaling molecules. The aims of this study were a systematic investigation of the effects of the neuropeptides substance P (SP), calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP), Neuropeptide Y (NPY) and tyrosine hydroxylase (TH) on the cell viability and function of the human osteoblasts, and comparing their difference in the role of regulating bone formation. Cultures of normal human osteoblasts were treated with SP, CGRP, VIP, NPY or TH at three concentrations. We found that each of the five neuropeptides induced increases in cell viability of human osteoblasts. The stimulatory action of NPY was the highest, followed by VIP, SP and TH, while CGRP had the lowest stimulatory effect. The viability index of osteoblasts was inversely associated with the concentration of neuropeptides, and positively with the time of exposure. Moreover, the five neuropeptides increased the ALP activity and osteocalcin to different extents in a dose-dependent manner. The GJIC of osteoblasts was significantly promoted by neuropeptides. The results demonstrated that neuropeptides released from skeletal nerve endings after a stimulus appeared to be able to induce the proliferation and activity of osteoblasts via enhancing GJIC between cells, and further influence the bone formation. These findings may contribute toward a better understanding of the neural influence on bone remodeling and improving treatments related to bone diseases.

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1. Introduction

Bone alters its metabolic and anabolic activities in response to the variety of systemic and local factors such as hormones and growth factors. Classical observations about the abundance of the nerves fibers in bone also predict a paradigm that the nervous system influences bone metabolism. Many of the nerve-derived signaling molecules that may act as efferent agents on the bone cells were fallen into the category of neuropeptides. Five neuropeptides have been known to be involved in the control of bone homeostasis, including calcitonin gene-related peptide (CGRP), Neuropeptide Y (NPY), substance P (SP), vasoactive intestinal peptide (VIP) and tyrosine hydroxylase (TH) (Shi and Bladock, 2012; Togari et al., 1997; Strange-Vognsen et al., 1997). Of them, the most intensive studies have so far been performed on CGRP. It is abundantly distributed in bone via sensory nerves, especially in the epiphyseal trabecular bones, which strongly supports that CGRP influences bone metabolism (Imai and Matsusue, 2002). However, in the current study our focus is to compare the effect of all five neuropeptides on human osteoblast so as to acquire more comprehensive knowledge about neuropeptides regulating bone remodeling.

NPY, a classic neuronal regulator of energy homeostasis, is now also known to be involved in the control of bone homeostasis (Lee and Herzog, 2009). But its role in osteoblast activity and the biological functions involving NPY receptors in bone homeostasis remain to be clarified. Functional analysis made by Teixeira (Teixeira et al., 2009) revealed the osteogenic potential of osteoprogenitor cells significantly stimulated by NPY, probably due to the down-regulation of Y1 receptor. Furthermore, Lundberg (Lundberg et al., 2007) suggested that the greater number of mesenchymal progenitors and the altered Y1 receptor expression within bone cells in the absence of Y2 receptors are a likely mechanism for the greater bone mineralization in vivo and in vitro.

In addition, some observations showed that VIP, a neuropeptide present in peptidergic skeletal nerve fibers, could regulate the activities of osteoblasts and osteoclasts, suggesting the existence

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of a neuro-osteogenic interplay in bone metabolism (Persson and Lerner, 2011; Lundberg et al., 2000). Meanwhile, many articles also demonstrated the distribution of nerve fibers containing SP, another sensory nerve-specific neuropeptide, and TH, the rate-limiting enzyme of catecholamine (Imai and Matsusue, 2002). The distinct effects of SP and catecholamines on the bone cells together with their in vivo influences manifested by experimental denervation studies suggested that the sensory and sympathetic nerves seemed to play important roles in bone metabolism through controlling vascularization and matrix differentiation during skeletal growth (Opolka et al., 2012; Adamus and Dabrowski, 2001; Shih and Bernard, 1997; Goto et al., 2007).

These data support the hypothesis that neuropeptide signaling stimulates bone formation and inhibits bone resorption. But, no information about comparative studies the effect of neuropeptides on bone remodeling is available to date. Hence, to further test this hypothesis we have first fully investigated the role of each of the five neuropeptides. In addition, the ALP activity and osteocalcin were included to exemplify some features of the osteoblast. So, the study examined the effects of neuropeptides on osteoblast in terms of cell viability levels, ALP activity, and osteocalcin determinations in culture supernatants.

Gap junctions formed by connexins (Cx) play an important role in transmitting signals between bone cells such as osteoblasts and osteoclasts, cells responsible for bone formation and bone remodeling, respectively. GJIC has been demonstrated to mediate the process of osteoblast differentiation and bone formation. Furthermore, GJIC propagates Ca^{2+} signaling, conveys anabolic effects of hormones and growth factors, and regulates gene transcription of osteoblast differentiation markers (Stains and Civitelli, 2005; Wróbel et al., 2011). To date, no available information on the effects of neuropeptides on GJIC is available. Therefore, we also first investigated the effects of neuropeptides on GJIC in osteoblasts. It seemed to allow some initial extrapolations to the possible mechanism of neuropeptides regulating bone remodeling.

2. Materials and methods

2.1. Cell culture

The normal human osteoblast frozen in liquid nitrogen in our lab was thawed. After the cells were subcultured, the thawed cells showed more than 85% of vitality and the same characteristics as before in terms of growth, morphology, and genetic characteristics (Ma et al., 2010). The cells were cultured in Dulbecco Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. Incubation was conducted at 5% CO_2 at 37 °C. The medium was changed three times during one week. Cells of the second or third passage were used for the experiment. Cells growing in medium without any of neuropeptides were used as controls. The neuropeptides used in the study were human neuropeptide Y (NPY) (Sigma, USA), substance P (SP) (Sigma, USA), calcitonin gene-related peptide (CGRP) (Sigma, USA), and vasoactive intestinal peptide (VIP) (Sigma, USA), tyrosine hydroxylase (TH) (Sigma, USA). All neuropeptides were diluted with serum-free DMEM to corresponding conceration.

2.2. Cell viability

Cell viability was measured by the MTT (3–4, 5-dimethylthiazol2, 5-diphenyltetrazolium bromide) assay. Cells were seeded into 96-well plate (clear bottom), at a density of 10,000 cells per well in 100 μl medium, incubated for 24 h, and treated with five neuropeptides at final concentrations of 1 μg/ml, 0.1 μg/ml and 0.01 μg/ml, using 6 replicates per concentration per treatment. After 24, 48 and 72 h, respectively, 20 μl of 5 mg/ml MTT solution were added to each well and incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 5 h. After incubation, the cells were washed with PBS solution. Subsequently, 100 μl of isopropanol acid 4% and hydrochloric acid were added to each well, and the cells were incubated at room temperature for 10 min. The absorbance was measured by Elisa Reader (Organon Teknika Reader 230S, Austria) at 492 nm with 620 nm as reference.

2.3. ALP activity

The cells (2×10^4 cell in 100 μl) from the second passage were seeded in four 24-well plates and incubated for 3–5 days near confluence and were treated with the NPY, SP, CGRP, VIP, TH at final concentrations of 0.1 μg/ml, respectively. After 24 h, the cells in two plates were measured for ALP activity. For simulating the clinical mode of administration, the cells in the two remaining plates continued to be cultured for 3 days, and then treated with five neuropeptides again. After incubation for 24 h, the cells were assayed. The determinations were performed using 6 replicates each treatment. To assay the ALP activity, the remaining medium was removed, and the cells were washed with PBS, digested with 0.25% trypsin 1 min. Then, the suspension of cells were collected in Eppendorf tubes, respectively, added distilled water in each tube, freezed with liquid nitrogen and thawed repeatedly for 3–4 times to destroy cell membrane. After centrifugation, the supernatants were collected to measure with Automatic Biochemistry Analyzer (Hitachi 7150, Japan) at 405 nm. All treatments were compared against control wells (cell culture with ordinary DMEM without neuropeptides and BMP).

2.4. Osteocalcin assay

This assay for the osteocalcin was performed with radioimmunoassay (RIA). Cells were cultured and treated in the way described above in ALP assay. The NPY, SP, CGRP, VIP, TH were added at final concentrations of 0.1 μg/ml, respectively. The osteocalcin kit used in the study was purchased from China Institute of Atomic Energy. For measuring the osteocalcin, the supernatants were collected in Eppendorf tubes, respectively, to perform the determinations. A total of 6 replicates were used per treatment.

2.5. Gap junctional intercellular communication (GJIC) in human osteoblasts

The effects of the neuropeptides on GJIC were determined by fluorescence recovery after photobleaching (FRAP) technique using a laser scanning confocal microscope (LSCM). Cells were seeded into 96-well plate, at a density of 5000 cells per well, incubated to confluence. The cells were rinsed by D-Hank's for two times and treated with 10 μmol/L CFDA (5, 6-carboxy fluorescein diacetate) (Sigma, USA), 37 °C for 90 min, rinsed by D-Hank's again.

Table 1
The ALP activity and osteocalcin contained in osteoblasts and fibroblasts.

Group	ALP (U/L)	Osteocalcin (ng/ml)
Osteoblast	34.61 ± 2.94	0.189 ± 0.006
Fibroblast	20.85 ± 0.69	0.049 ± 0.004
Unpaired t-test	**<0.0001**	**<0.0001**
P values	(t = 10.6)	(t = 44.5)

Values of the ALP activity and osteocalcin are expressed as mean ± SD. The results of unpaired *t-test* demonstrate that the levels of alkaline phosphatase and osteocalcin are significantly higher in osteoblasts than fibroblasts. Statistical significance is noted by bold text.

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