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NPY Revealed as a Critical Modulator of Osteoblast Function In Vitro: New Insights Into the Role of Y1 and Y2 Receptors

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ABSTRACT

Neuropeptide Y (NPY) has recently emerged as a potential regulator of bone homeostasis. However, the relevance of NPY's role in osteoblast activity and the biological functions involving NPY receptors in bone homeostasis remain to be clarified. Here we report that chronically elevated NPY levels leaded to a modulation of the level of Y2 receptor expression marked with a transient down and upregulation according to the stage of osteoblast differentiation. We also show that NPY is a negative regulator of Y1 receptor expression. The pharmalogical activation of Y2 receptor with its agonist resulted in similar effect. Functional analysis also revealed the osteogenic potential of NPY with osteoblast phenotype markers being significantly enhanced in osteoprogenitor cells stimulated by NPY, probably due to the down-regulation of Y1 receptor. In contrasts, these cells exhibit a reduction in calcium deposition in extracellular matrix most likely mediated via Y2 receptor signalling. Furthermore, we show that NPY modulates receptor activator of nuclear factor kB (NF-kB) (RANK) ligand and osteoprotegerin, two key factors regulating bone remodelling. Specifically, NPY inhibits the transcriptional activity of RANKL promoter in osteoprogenitor cells and enhances OPG expression in osteoblasts at early stages of differentiation. However, NPY effect on OPG seemed to be unrelated to Y2 receptor activation. Taken together the present data supported the contribution of NPY pathway in bone homeostasis via a direct action on osteoblasts cells. J. Cell. Biochem. 107: 908–916, 2009.

KEY WORDS: NPY; Y2 RECEPTOR; Y1 RECEPTOR; OSTEOBLASTS; BONE

The concept of a neuro-osteogenic network was first described in the eighties, but only recently major progress has been made in this subject making it one of the most exciting areas of research in skeletogenesis. The idea of a neural regulation of bone homeostasis is based upon morphological studies demonstrating the presence of nerve fibres in bone tissue [Serre et al., 1999; Imai and Matsusue, 2002; Lerner and Lundberg, 2002; Chenu, 2004]; through evidences obtained from clinical observations in patients with nerve injuries [Chenu and Marenzana, 2005; Elefteriou, 2005]; from experimental nerve deletions in animals [Lundberg et al., 1999; Lundberg and Lerner, 2002] and through the demonstration of the presence of several neuropeptides receptors in bone [Lundberg and Lerner, 2002; Chenu and Marenzana, 2005; Elefteriou, 2005]. Furthermore, the demonstration that activation of such receptors leads to changes in the activity of bone cells, similarly to those caused by systemic hormones, cytokines, and growth factors as well as work on animal models either transgenic or knockouts for neuropeptides and neuropeptide receptors, has contributed significantly to our understanding on how neuronal networks influence bone homeostasis [Chenu and Marenzana, 2005; Elefteriou, 2005].

Among different neuropeptides shown to be able to influence bone physiology, NPY has recently emerged as one of the major regulators of bone homeostasis. NPY, first isolated from porcine brain [Tatemoto, 1982], is a 36-amino acid peptide, which belongs to a family of peptides that also includes peptide YY (PYY) and the pancreatic polypeptide (PP). NPY is widely expressed in the central and peripheral nervous system. Among other physiological

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functions, it is one of the most potent regulators of food intake and energy homeostasis [Clark et al., 1985; Herzog, 2003]. NPY mediates its actions via at least five different Y receptors (Y1, Y2, Y4, Y5, and y6). All these receptors are expressed in the hypothalamus, the brain stem, and peripheral tissues [Dumont et al., 1998; Michel et al., 1998] and belong to the large super-family of G-protein-coupled receptors and using similar signal transduction pathways such as inhibiting cAMP accumulation.

Y2 and Y1 receptors have been identified as potential contributors to the regulation of bone homeostasis [Baldock et al., 2002, 2005, 2007; Sainsbury et al., 2003; Allison and Herzog, 2006; Allison et al., 2006; Lundberg et al., 2007]. It has been demonstrated that both hypothalamus-specific and germ line Y2 receptor knockouts resulted in an increase in cancellous bone volume associated with accelerated bone formation [Baldock et al., 2002; Lundberg et al., 2007]. The increased bone volume in Y2 receptor knockouts mice was not associated with changes in the plasma concentrations of hormones that are known to regulate bone physiology, notably, thyroid hormones, corticosterone, testosterone, insulin-like growth factor 1 (IGF-1) or leptin. In addition, it has been demonstrated that in sex-hormone deficient adult mice, despite the increased bone resorption, germline and conditional deletion of hypothalamic Y2-R elicited higher bone formation rate leading to higher bone mass comparing to the control suggesting that Y2 deletion-mediated anabolic pathway is independent of sex hormones [Allison et al., 2006]. Furthermore, recent data have shown that bone marrow cells from Y2 receptor germline knockout mice exhibited increased osteoprogenitor number and altered Y1-receptor expression in osteoblasts [Lundberg et al., 2007]. Taken together these data suggest that osteoblasts are under the control of NPY. However, open questions remain, including those related to the osteoblasts cell responses to direct action of NPY and whether the mechanisms underlying the regulation of bone homeostasis via Y2 receptor are exclusively attributed to the hypothalamic control excluding contribution of the peripheral pathway.

Therefore, in this study, we investigated the direct effect of NPY on osteoblast activity and the possible involvement of the Y2 receptor in this process.

MATERIALS AND METHODS

REAGENTS

NPY_{1-36} and PYY_{3-36} were purchased from Bachem. BIIO246 was purchased from Tocris and 1α,25-dihydroxyvitamin D_3 was purchased from Sigma. α-Modified minimal essential medium (α-MEM) and foetal bovine serum were obtained from Gibco.

CELL LINES AND PRIMARY CULTURES

MC3T3-E1 calvaria pre-osteoblast cell line was purchased from ETCC (European Collection of Cell Cultures). UAMS-32P mouse bone marrow stromal cell line, stably transfected with a reporter containing the entire murine RANKL gene was kindly provided by Dr Charles O'Brain (Center for Osteoporosis and Metabolic Bone Diseases, University of Arkansas for Medical Sciences, Little Rock, AR). This reporter responds to PTH or db-cAMP as well as other hormones and cytokines known to stimulate RANKL [Fu et al., 2006].

MC3T3-E1 and UAMS-32P were cultured in α-MEM supplemented with 10% (v/v) foetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. At pre-confluence, cells were harvested using trypsin (0.25% (w/v) trypsin, 0.1% (w/v) glucose, and 0.05% (w/v) ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline). The medium was refreshed every 2–3 days.

Mouse primary bone marrow stromal cells were obtained from one month old NMRI wild type males. Briefly, after sacrificing the mice by euthanasia, using Eutasil (Safoni Veterinária), the tibias and femurs were excised within few minutes after death. Bone marrow cells were flushed from the bones with α-MEM supplemented with 10% (v/v) foetal bovine serum and 1% (v/v) penicillin/streptomycin. Cells were seeded in 75-cm^2 plastic culture flasks, and incubated in a humidified incubator (37°C and 5% CO_2). The medium was changed after the first 24 h to remove non-adherent cells. Subsequently, the adherent cells were cultured for 10 days and the medium was renewed every 3 days. Cells from the first passage were used.

RNA ISOLATION AND cDNA SYNTHESIS

Total RNA was extracted from cells and purified using RNeasy Mini 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.

First strand cDNA synthesis was performed using Superscript II reverse transcriptase kit (Invitrogen), Random primers (Invitrogen), 10 mM dNTPs (Invitrogen), 5× First Strand synthesis buffer, 0.1 M DTT, and RNaseOUT ribonuclease RNase inhibitor (Invitrogen).

PCR AND REAL-TIME PCR

PCR was performed using cDNA as the template in a 50 μl reaction mixture containing specific primers (Table I). All primers used were located on two different exons to ensure that only properly spliced mRNA and not genomic DNA contaminants was amplified. Ethidium bromide-stained gels were scanned using a Typhoon 8600 (Amersham) and amplified bands were quantified using the ImageQuant software (Amersham). Fluorescence density of each PCR-amplified band was normalized with the corresponding value of β-actin.

Real-time PCR was preformed in an 18 μl reaction volume containing 10 μl master mix of iQ SYBR Green super Mix (Invitrogen), 2 μl template cDNA and 0.5 μM primers (Table I). β-Actin was used for normalization whereas the reserve transcription reaction was omitted for negative controls.

Real-time PCR results were analysed using the Livak method. The melting curve analysis and agarose gel electrophoresis were used to verify the specificity of transcript amplification.

MTT CELL METABOLIC ACTIVITY ASSAY

The MTT assay is based on the reduction of tetrazolium salt to purple formazan crystals, which are solubilised by the addition of a detergent. Cells (4×10^4 cells per well) were seeded and incubated on a 24-well plate in α-MEM supplemented with 10% (v/v) foetal

TABLE I. Primers Sequences and PCR Products Sizes

Primers	Sequence	PCR product (bp)	Annealing temperature (°C)
Y2 receptor–forward	5′-TCCTGGATTCCTCATCTGAG-3′	520	60
Y2 receptor–reverse	5′-GGTCCAGAGCAATGACTGTC-3′		
Y1 receptor–forward	5′CTCGCTGGTTCTCATCGCTGTGGAACGG-3′	323	55
Y1 receptor–reverse	5′-GCGAATGTATATCTTGAAGTAG-3′		
Osteocalcin–forward	5′-CTCTGTCTCTCTGACCTCACAG-3′	262	56
Osteocalcin–reverse	5′-CAGGTCCTAAATAGTGATACCG-3′		
RANKL–forward	5′-TATGATGGAAGGCTCATGGT-3′	512	53
RANKL–reverse	5′TGTCCTGAACTTTGAAAGCC-3′		
OPG–forward	5′-AAAGCACCCTGTAGAAAACA-3′	257	56
OPG–reverse	5′-CCGTTTTATCCTCTCTACACTC-3′		
β-Actin–forward	5′-GTGGGCCGCTCTAGGCACCAA-3′	540	56
β-Actin–reverse	5′-GTCTTTGATGTCACGCACGATTTC-3′		

All cDNA were amplified with 30 cycles.

bovine serum and 1% (v/v) Penicillin/Streptomycin. Cells were exposed to different agents for 24 h. At the end of the incubation time, the culture medium was removed and culture medium without phenol red containing 10% (v/v) MTT solution (sigma) was added to each well and incubated for 4 h at 37°C. At the end of the incubation time, the formazan resulting from the reduction of MTT by mitochondrial enzymes was then extracted using dimethyl sulfoxide (DMSO). The amount of formazan was determined colorimetrically at 540 nm with normalization of interference at 690 nm using a micro-plate spectrophotometer. Results are expressed as a percent of control values.

ALKALINE PHOSPHATASE (ALP) ACTIVITY AND CYTOCHEMICAL STAINING

Cells were seeded into 24-well plates at a density of 4×10^4 cells per well and cultured in medium supplemented with β-glycerophosphate (10 mM) and vitamin C (50 μg/ml). Cells were left undisturbed in the incubator for 24 h and then exposed to the agent to be tested. At defined time points, cells were rinsed twice with phosphate-buffered saline and lysed in 1% (v/v) Triton X-100 in phosphate-buffered saline.

ALP activity was then measured by incubation of cell lysates for 1 h at 37°C in 0.1 M $NaHCO_3$–Na_2CO_3 buffer, pH 10, containing 0.1% Triton X-100, 2 mM MgSO4, and 6 mM PNPP (4-nitrophenyl phosphate). The reaction was stopped by adding 1 M NaOH, and absorbance measured at 405 nm. Enzyme activity was normalized to cell protein content measured using the bicinchoninic acid assay (Pierce, IL).

For cytochemical staining, cells were washed twice with phosphate-buffered saline, fixed in 3.7% (v/v) formaldehyde for 15 min, washed in water, and incubated for 30 min in Naphtol AS-MX phosphate/Fast Violet B salt (Sigma) at room temperature in the dark. Finally, cells were washed in water, air dried, and observed under a stereo microscope (Olympus SZX9).

STAINING FOR MINERALISATION

Von Kossa staining was used. Briefly, cells were washed with phosphate-buffered saline and fixed with 3.7% (v/v) formaldehyde for 15 min. Cells were then rinsed with distilled water and thereafter incubated under UV light in the presence of 5% silver nitrate. After 30 min, cells were washed with distilled water and incubated with 5% sodium thiosulphate.

LUCIFERASE ASSAY

Cells were seeded into 24-well plates at a density of 4×10^4 cells/well, left undisturbed until confluence, and then exposed to various stimulating agents for 24 h. Cells were then washed twice with phosphate-buffered saline and incubated in Reporter Lysis Buffer (25 mM glycyl-glycine, pH 7.8, 1% Triton X-100, 15 mM $MgSO_4$, 4 mM EGTA) for 15 min and then kept at −20°C for 30 min. Cells were then thawed and centrifuged at 500*g*. Luciferase activity and protein concentration of the cleared lysates were determined according to manufactures instructions (Luciferase assay system Kit, Promega).

STATISTICAL ANALYSIS

One-way ANOVA tests were used to evaluate differences between the sample of interest and its respective control, using SPSS software. Two-tailed asymptote parametric values being considered. *P*-value ≤ 0.05 was considered statistically significant.

RESULTS

NPY MODULATES Y2 AND Y1 RECEPTOR EXPRESSION IN OSTEOBLASTS

Primary osteoblast cultures derived from bone marrow stromal cells contain a heterogeneous cell population making it difficult to determine the expression profile of specific cell types. In order to avoid this problem, we analysed the expression of the different Y receptor mRNAs in the mouse pre-osteoblast cell line MC3T3-E1. Real-time RT-PCR was performed on RNA isolated from this cell line using Y2 receptor specific primers [Lundberg et al., 2007] and RNA isolated from wild type mice brain tissue, as a positive control. Y2 receptor expression was detected in MC3T3-E1 cells cultured, under osteoblast differentiation condition, at days 1, 3, and 7 (Fig. 1A). However, real-time PCR analysis showed no significant differences in Y2 mRNA level at these time points. Previous reports provided evidence that NPY up-regulates the expression of the Y2 receptor in endothelial cells and pre-adipocytes of white adipose tissue [Kuo et al., 2007]. Therefore, we analysed the expression of Y2 receptor by MC3T3-E1 in the presence of the universal Y2-receptor

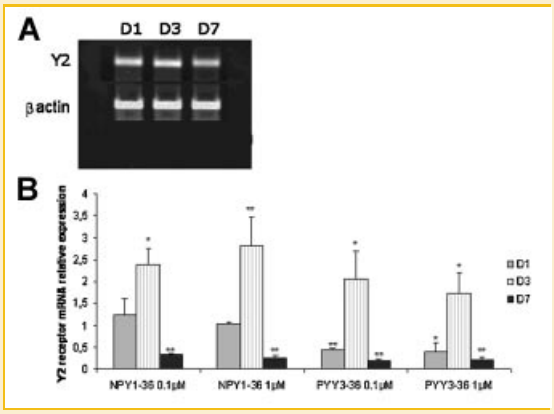

Fig. 1. Y2 receptor expression in MC3T3-E1 pre-osteoblasts under osteoblast differentiation conditions at different time points (A). Quantitative real-time RT-PCR analysis of Y2mRNA level when cells are exposed to NPY_{1-36} (0.1 and 1 μM) or PYY_{3-36} (0.1 and 1 μM) (B). Y2 receptor expression levels were normalized to β actin gene and to the control (untreated cells). The statistical analysis was performed comparing the treatments with the control (*,**). $^{*}P < 0.05$, $^{**}P < 0.01$.

agonist NPY_{1-36} (0.1 and 1 μM) or the Y2 receptor preferring agonist PYY_{3-36} (0.1 and 1 μM). As shown in Figure 1B, while 1 day treatment with NPY_{1-36} (0.1 and 1 μM) did not affect the expression level of Y2 receptor, its activation by PYY_{3-36} leaded to a significant decrease of the mRNA transcript. Nevertheless both NPY_{1-36} and PYY_{3-36} treatments resulted in significant increase of Y2R mRNA transcript following 3 days of cells exposure. The observed up-regulation was then dropped to a low level at day 7.

We next performed a functional analysis based on the cell metabolic activity known to be modulated by the Y2 receptor in neuronal cells [Thiriet et al., 2005]. MC3T3-E1 cells were treated with NPY_{1-36}, the Y2 receptor antagonist BIIE0246 or NPY_{1-36} plus BIIE0246, under control conditions with no differentiation-inducing supplements in the medium. As shown in Figure 2, cell metabolic activity was significantly stimulated after 24 h treatment with NPY_{1-36}. However, this effect was reversed by BIIE0246 resulting in a significant decrease of cell metabolic activity suggesting a Y2 receptor mediated response.

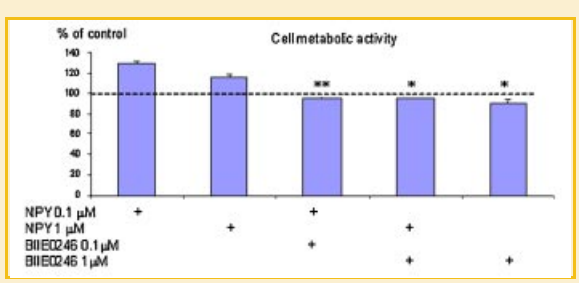

Fig. 2. Cell metabolic activity of MC3T3-E1 pre-osteoblasts exposed to NPY_{1-36}, BIIE0246 or NPY_{1-36}+ BIIE0246 for 24 hours. Data represent in % of the control (untreated cells). The statistical analysis was performed comparing the treatments with the control (*,**). $^{*}P < 0.05$, $^{**}P < 0.01$.

Previous studies have shown that the NPY Y1 receptor mRNA is present in bone marrow stromal cells and osteoblasts and demonstrated that germline Y2 receptor deletion causes Y1 receptor down-regulation in stromal cells and bone tissue, possibly due to the elevated levels of NPY in these mice [Lundberg et al., 2007].

We analysed Y1 receptor expression profile in bone marrow stromal cells and MC3T3-E1 cells osteoblats, at different stages of osteoblast differentiation, in the presence or absence of NPY_{1-36} (0.1 and 1 μM) or PYY_{3-36} (0.1 and 1 μM). As shown in Figure 3A, Y1 receptor transcript was detected in both MC3T3 (3A.I) per-osteoblasts and bone marrow stromal cells (3A.II). The analysis of the expression profile of the Y1 receptor showed an increase of the transcript level throughout bone marrow stromal cells differentiation into osteoblasts. However, in the presence of NPY_{1-36}, a significant decrease of Y1 receptor mRNA level was observed when compared to the control. A Similar effect was detected when cells were treated with the Y2 agonist PYY_{3-36} (Fig. 3B).

NPY IS CRITICAL FOR OSTEOBLAST DIFFERENTIATION

To monitor the effect of NPY on osteoblast differentiation and investigate whether the Y2 R has a functional role in this process, primary bone marrow stromal cell cultures were grown under osteoblast differentiation conditions and were continuously treated with NPY_{1-36} (0.1 and 1 μM), Y2 receptor agonist PYY_{3-36} (0.1 and 1 μM) or NPY_{1-36} (0.1 and 1 μM) plus Y2 receptor antagonist BIIE0246 (0.1 and 1 μM). Parallel untreated cultures were used as control. Osteogenic markers such as alkaline phosphatase activity (ALP), and osteocalcin expression were determined at different stages of osteoblast differentiation.

As shown in Figure 4A, the ALP activity increased in a time dependent manner and peaked on day 7. At this time point, significantly higher values of the enzyme activity were obtained for treatments with NPY_{1-36} (0.1 and 1 μM) and PYY_{3-36} (0.1 and 1 μM) when compared to the control (Fig. 4A,B). On the other hand, NPY_{1-36} stimulatory effect was significantly reduced by the addition of the Y2 receptor antagonist BII0246 (0.1 and 1 μM) leading to a decrease of the ALP activity, at day 7 (Fig. 4C).

To determine whether NPY has an effect in the later stages of osteoblast differentiation, osteocalcin expression was assessed (Fig. 5A,B). We found that in the presence of 0.1 μM NPY_{1-36}, the osteocalcin mRNA level was markedly enhanced at days 1 and 14 (Fig. 5B1) with about a twofold increase relatively to the control level. In presence of PYY_{3-36}, at both concentrations a stimulatory effect was also observed at day 14 (Fig. 5B1). To further confirm that the Y2 receptor may play a functional role in osteogenic differentiation, we assessed whether the co-treatment of cells with Y2 antagonist would reverse the effect induced by NPY. The results show that, while NPY_{1-36} (0.1 μM) induced an increase of osteocalcin gene expression at day 1 and day 14, the combined treatment with BIIE0246 (0.1 μM), reduced osteocalcin gene expression at these time points (Fig. 5B2).

NPY INHIBITS BONE MATRIX MINERALISATION

To study the effect of NPY on bone matrix mineralisation, Von Kossa staining was used to monitor calcium deposition in extracellular

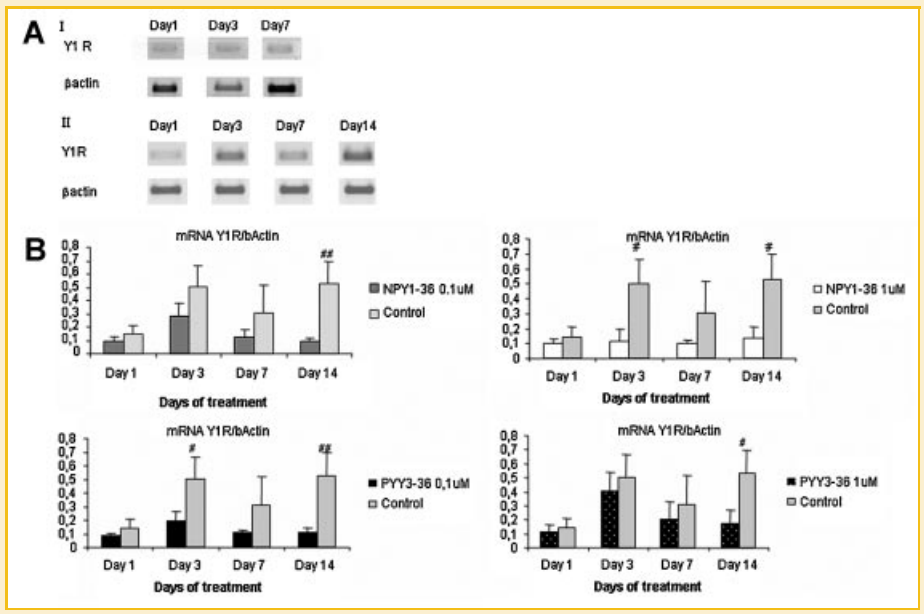

Fig. 3. Expression of Y1 receptor in MC3T3-E1 pre-osteoblasts (A-I) and mouse bone marrow stromal cells (A-II) under osteoblast differentiation conditions at different time points. RNA was extracted, followed by conventional RT-PCR. PCR products were visualized on a 1.4% agarose gel stained with ethidium bromide. B: Graphs showing semi-quantitative RT-PCR of Y1 receptor expression in mouse bone marrow stromal cells exposed to NPY_{3-36} (1 and 1 μM) or PYY_{3-36} (0.1 and 1 μM), under osteoblast differentiation conditions, at different time points. The untreated cells were the control. The statistical analysis was performed comparing the treatments with control ($^{\#,\#\#}$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

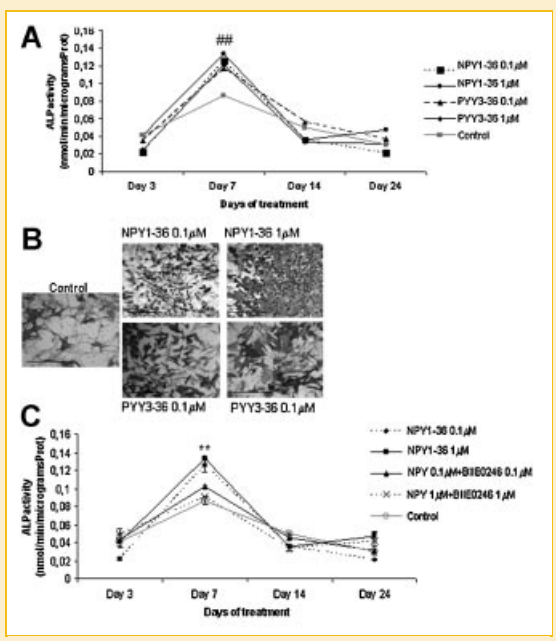

Fig. 4. Alkaline phosphatase activity (A) and Alkaline phosphatase staining (B) of bone marrow stromal cells exposed to NPY_{3-36} (0.1 and 1 μM) or PYY_{3-36} (0.1 and 1 μM), under osteoblast differentiation conditions. C: Alkaline phosphatase activity of bone marrow stromal cells exposed to NPY_{1-36} (0.1 and 1 μM) + BIIE0246 (0.1 and 1 μM) under osteoblast differentiation conditions, at different time points. The untreated cells were the control. Alkaline phosphatase activity was expressed in nanomol per minute per microgram of protein (data normalized to total protein content of each sample). The statistical analysis was performed comparing the treatments (**) and the treatments with the control (##). $^{**,\#\#}P < 0.01$.

matrix of differentiating osteoprogenitor cells to osteoblasts. The results showed that 24 days of treatment with NPY_{1-36} (0.1 and 1 μM) decreased calcium deposition when compared to the control. However, PYY_{3-36} at 1 μM induced an increased reactivity for Von Kossa staining (Fig. 6).

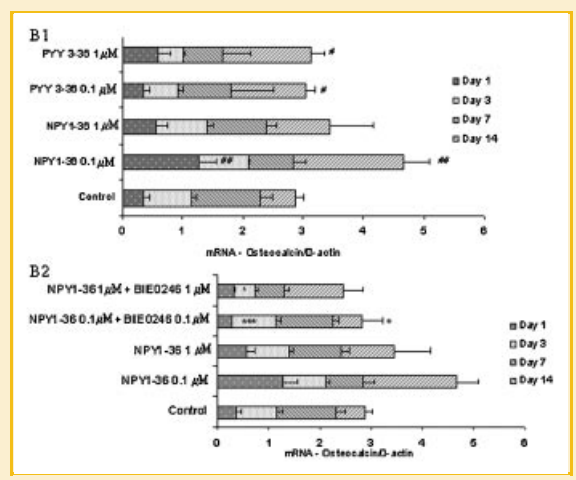

Fig. 5. Graphs showing semi-quantitative RT-PCR of osteocalcin expression in mouse bone marrow stromal cells exposed to NPY_{3-36} (0.1 and 1 μM), PYY_{3-36} (0.1 and 1 μM) or NPY_{1-36} (0.1 and 1 μM) + BIIE0246 (0.1 and 1 μM), under osteoblast differentiation condition at different time points. The untreated cells were the control. The statistical analysis was performed comparing the treatments (*,**,***) and the treatments with the control ($^{\#,\#\#,\#\#\#}$). $^{*,\#}P < 0.05$, $^{**,\#\#}P < 0.01$, $^{***,\#\#\#}P < 0.001$.

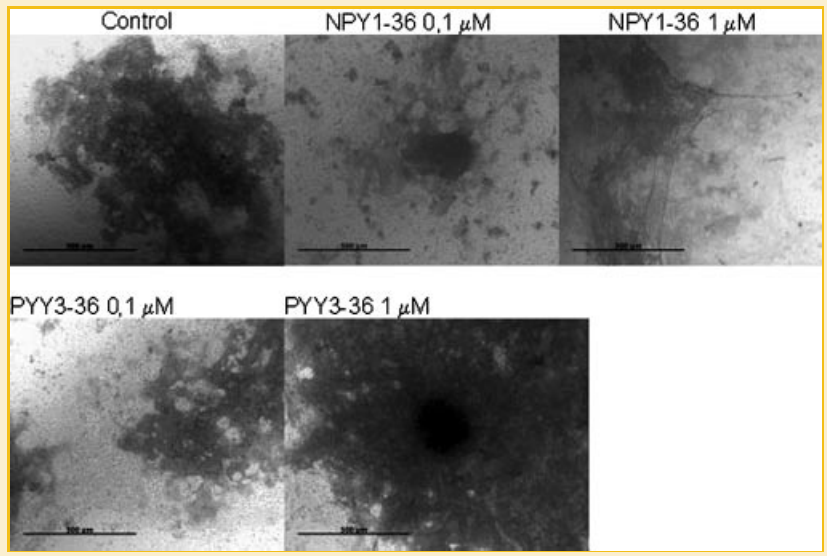

Fig. 6. Representative digital histographs of Von Kossa staining for mouse bone marrow stromal cells exposed to NPY_{3-36} (0.1 and 1 μM), PYY_{3-36} (0.1 and 1 μM) and control (untreated cells), under osteoblast differentiation condition, after 24 days of treatment. The histographs were taken with a digital camera coupled to a stereo zoom microscope (Olympus SZX9).

NPY INHIBITS RANKL EXPRESSION ON OSTEOBLAST

It is well established that the osteoblast lineage plays a fundamental role in bone resorption through receptor activator of nuclear factor kB (NF-kB) (RANK) ligand (RANKL) that stimulates osteoclast differentiation and fusion. Recent reports showed that NPY elicits an inhibition of osteoclastogenesis by reducing isoprenaline-induced production of RANKL by bone marrow cells [Amano et al., 2007].

We performed a short time course analysis to test whether NPY could modulate RANKL expression by acting directly on the osteoblast. MCT3T3-E1 pre-osteoblasts were treated with NPY_{1-36} (0.1 and 1 μM) for 2, 6, and 8 h. The RANKL expression level was analysed by real-time PCR and as shown in Figure 7A, NPY_{1-36} strongly reduced RANKL mRNA expression when compared to the control. In the presence of NPY_{1-36} either concentrations 0.1 or 1 μM this effect was observed after 2 h of exposure and was maintained until 8 h.

To further confirm the inhibitory effect of NPY on RANKL expression, we analysed the transcriptional activity of the RANKL promoter using the UAMS-32P- bone marrow stromal cell line stably transfected with a construct containing the entire murine RANKL gene coupled to luciferase as a reporter gene. As expected, 1α,25-dihydroxyvitamin D_3 known to promote RANKL expression, induced a significant increase of promoter activity when compared to the control. However, when cells were challenged with NPY_{1-36} (0.1 and 1 μM) the promoter activity was decreased significantly to levels much lower than the untreated control. Similar effect was observed when cells were treated with the Y2 receptor agonist PYY_{3-36} (0.1 and 1 μM) (Fig. 7B).

NPY ENHANCES OPG EXPRESSION ON OSTEOBLASTS

It has been reported that osteoblast differentiation is associated with an increase of osteoprotegerin (OPG); a decoy receptor that neutralizes the effect of RANKL in bone [Gori et al., 2000].

Therefore, we investigated the effect of NPY on OPG expression at different stages of osteoblast differentiation. Primary bone marrow stromal cells were grown under osteoblast differentiation conditions and were continuously treated with NPY_{1-36} (0.1 and 1 μM) for 1, 3,

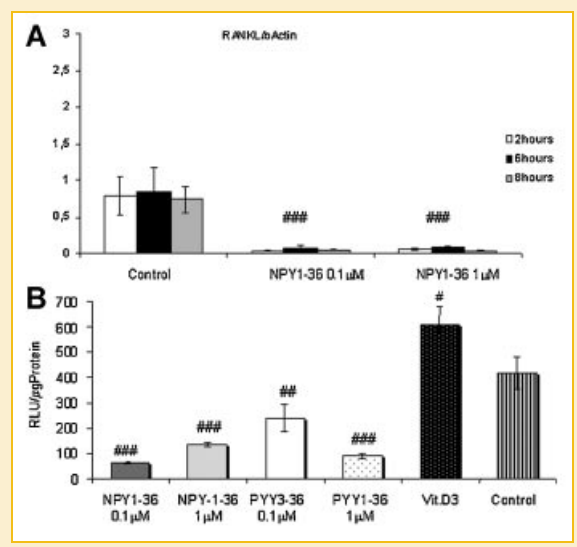

Fig. 7. A: Quantitative real-time RT-PCR analysis of RANKL expression in MC3T3-E1 pre-osteoblasts exposed to NPY_{3-36} (0.1 and 1 μM) or PYY_{3-36} (0.1 and 1 μM). The untreated cells were the control. The values shown are quantity of RANKL mRNA normalized to that of β-actin. B: Luciferase activity of UAMS-32P bone marrow stromal cell line after 24 h of treatment, normalized with total protein content. The following treatments were assessed: NPY_{3-36} (0.1 and 1 μM); PYY_{3-36} (0.1 and 1 μM); 1,25-dihydroxyvitamin D3 (Vit.D3) as the positive control; untreated cells as the control. The statistical analysis was performed comparing the treatments with the control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.

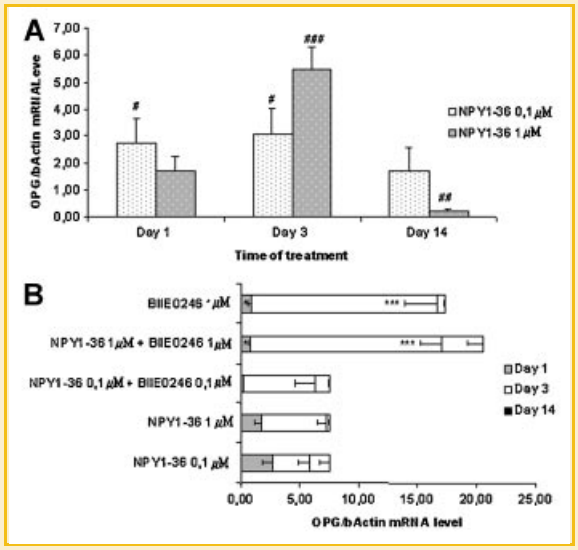

Fig. 8. Quantitative real-time RT-PCR analysis of OPG expression in mouse bone marrow stromal cells exposed to (A): NPY_{3-36} (0.1 and 1 μM), (B) NPY_{1-36} (0.1 and 1 μM) plus BIIE0246 (0.1 and 1 μM) or BIIE0246 (0.1 and 1 μM), under osteoblast differentiation condition, at different time points. OPG expression levels were normalized to β-actin gene and to the control (untreated cells). The statistical analysis was performed comparing the treatments (*,**,***) and the treatments with the control ($^{\#,\#\#,\#\#\#}$). $^{*,\#}P < 0.05$, $^{**,\#\#}P < 0.01$, $^{***,\#\#\#}P < 0.001$.

and 14 days. Real-time PCR showed an enhancement of OPG mRNA level at day 3 followed by a decrease at day 14. This profile was more evident in the presence of 1 μM NPY_{1-36} (Fig. 8A).

To further address whether the NPY effect on OPG expression could be related to Y2 receptor, we assessed whether the co-treatment of cells with Y2 receptor antagonist would reverse the effect obtained with NPY_{1-36} treatments. At day 1, the combined treatment with BIIE0246 inhibited the stimulatory effect of NPY resulting in a reduction of the OPG mRNA level. Surprisingly, at days 3 and 14, cell response in presence of BIIE0246 was significantly enhanced resulting in an increase of OPG mRNA level when compared to NPY's effect at these time points. This enhancement was more pronounced at day 3 when cells were treated with 1 μM NPY_{1-36} + 1 μM BIIE0246. To further assess whether the observed increase of OPG expression was exclusively attributed to Y2 receptor antagonist, primary bone marrow stromal cells were grown under osteoblast differentiation conditions and were continuously treated with 1 μM BIIE0246. As shown in Figure 8B, a large stimulation of OPG expression was obtained at day 3 of culture.

DISCUSSION

In the present report, we demonstrated that NPY modulates the expression of Y2 and Y1 receptors in osteoblasts cells. We also show the osteogenic potential of NPY mediated through a direct action on osteoblasts and the contribution of the Y2 receptor in osteoblast cell responses.

There are divergent findings among studies utilizing osteoprogenitor cell lines or primary culture systems on whether osteoblasts express Y2 receptor [Fu et al., 2006; Amano et al., 2007]. In an attempt to clarify some of these conflicting observations, and to avoid cell heterogeneity of the primary culture, we addressed the expression of Y2 receptor in MC3T3-E1 perosteoblasts. These cells are derived from mouse calvaria bone, are nontumorigenic and undergo a typical program of osteoblast differentiation in vitro [Sudo et al., 1983]. The data presented demonstrated Y2 mRNA expression in MC3T3-E1 cells under osteoblast differentiation condition. This finding are in agreement with those reported by Hosaka et al. [2008] showing the expression of all types of Y receptor mRNA, including Y2 in these cells.

NPY induced Y2 receptor up-regulation was demonstrated in different cells types; neuronal and non-neuronal cells, and has been associated with some NPY mediated physiological processes [Kuo et al., 2007]. In our study, we observed a modulation marked with a transient down and upregulation of the level of Y2 receptor expression according to the stage of osteoblast differentiation. This effect was triggered by both NPY and Y2 receptor agonist, PYY_{3-36}, suggesting a transcriptional regulation that might be mediated by the activation of Y2 signalling.

In light of these reports and considering our findings, it is reasonable to assume that a possible direct effect of NPY through Y2 receptor on osteoblast cells may occur in physiological conditions, when NPY levels are high within bone tissue as occurs when sympathetic activity is increased. It is noteworthy to point out that bone tissue, as well as bone marrow, receive a rich supply of nerve fibres namely NPY fibres [Hill et al., 1991; Ahmed et al., 1994]. Furthermore, NPY is also expressed by non-neuronal cells present in the vicinity of the bone compartment, namely megakaryocytes in the bone marrow and endothelial cells of blood vessels [Ericsson et al., 1987; Movafagh et al., 2006]. Thus, it is therefore not unreasonable to speculate that during the process of bone regeneration throughout fracture healing, where angiogenesis and tissue innervation [Li et al., 2001; Szczesny, 2002] are stimulated, NPY levels might be increased and subsequently leading to a modulation of Y2 receptor level in osteoblast cells.

The regulation of Y2-receptor expression has been associated to Y1 receptor in several physiological processes such as anxiety and aggression [Wittmann et al., 2005]. In bone, due to the lack of additive effects in Y1Y2−/− mice, Y2 and Y1 receptors were thought to interact or share a common pathway by which they regulate osteoblast activity [Baldock et al., 2007]. Moreover, it has been suggested that germline Y2 receptor deletion causes Y1 receptor down-regulation in stromal cells and bone tissue because of elevated levels of NPY in these mice [Lundberg et al., 2007]. This is consistent with our results that demonstrate that treatment with NPY resulted in a significant decrease of Y1 receptor transcript in differentiating osteoblasts. This effect on the Y1 receptor mRNA synthesis was mimicked by the Y2 receptor agonist, thus confirming that the Y2 receptor is involved in the regulation of Y1 receptors in osteoblasts.

Despite the merits of the Y1 and Y2 receptor knockouts, little is known about the direct effects of NPY signalling in osteoblasts.

Therefore, as a next step, we addressed the relevance of NPY role in osteoblast differentiation and activity and focused on the functional connection to the Y2 receptor.

Osteoblast differentiation is accompanied by the differential expression of several osteoblastic markers such as alkaline phosphatase and osteocalcin. We show that NPY stimulates the differentiation of murine bone marrow stromal cells into osteoblasts. This was based upon the observation that NPY treatments leads to an enhancement of alkaline phosphase activity and osteocalcin expression. Similar effects were achieved by Y2 agonist whereas Y2 receptor antagonist reversed the NPY stimulatory effects to nearly control levels. Taking into account that Y1 receptor expression is subject to downregulation in these cells and in view of previous report [Lundberg et al., 2007] suggesting that Y1 receptor reduction in marrow stromal cells cultures from *Y2−/−* mice might be important for the anabolic bone phenotype of the *Y2−/−* model, our data might also be related to an impairment of Y1 receptor. However, before drawing out a final conclusion, it is necessary to examine in detail whether the blockade of Y1 receptor mechanism by mean of selective antagonist would be associated to an enhancement of osteoblast differentiation.

Matrix mineralisation was examined as an in vitro end-point indicator of the osteoblastic phenotype. Under our experimental conditions, NPY and Y2 receptor agonist PYY_{3-36} had an opposite effect on calcium deposition in the extracellular matrix of differentiating osteoprogenitor cells. While NPY acted as an inhibitor, PYY_{3-36} stimulated matrix mineralisation. This observed effect of PYY_{3-36} is consistent with a recent study showing reduction of bone mineral density in PYY−/− mice [Wortley et al., 2007]. Nevertheless, only further studies focusing on genes known to regulates bone extracellular matrix mineralisation such as tissue-*nonspecific alkaline phosphatase* (Tnap) and Type I collagen coexpression in osteoblasts will provide clear understanding on the effect of NPY on this process.

RANKL and OPG, both produced by osteoblasts and stromal cells are known key factors regulating bone remodelling and thereby coordinating bone resorption and formation. We examined the effect of NPY on RANKL and OPG expression by the osteoblast lineage and demonstrated that a short time course NPY treatment inhibited RANKL expression in MC3T3-E1 osteoblasts. UAMS-32P stromal cells, stably transfected with a construct containing a reporter gene under the control of the RANKL promoter, were used to analyse the effect of NPY on RANKL at the molecular level. Both, NPY and the Y2 agonist, significantly inhibited RANKL promoter activity. This can be related to the well known inhibition of cAMP accumulation associated to the activation of NPY receptors [Holliday et al., 2004]. Indeed, the construct used in the present study contains conserved consensus sequences which can bind the cAMP- associated (CRE)-binding protein (CREB) [Fu et al., 2006]. Previous reports had demonstrated that in stromal/osteoblastic cells the CREB pathway is a central regulator of RANKL gene expression in response to a variety of stimuli such as parathyroid hormone and 1,25 $(OH)_2D_3$ [Kondo et al., 2002; Lee and Lornezo, 2002]. The inhibitory effect of NPY on RANKL production by bone marrow stromal cells was recently addressed by Amano et al. [2007]. They suggest that the inhibitory effect of NPY on osteoclastogenesis was caused by the suppression of isoprenaline-induced RANKL production by stromal cells at a site upstream of RANKL mRNA expression.

OPG acts as a decoy receptor that inhibits RANKL activation of osteoclastogenesis, thereby decreasing bone resorption [Simonet et al., 1997]. Our experiments showed that NPY exerted a transient stimulatory effect on OPG expression by osteoblasts. The enhancement of OPG mRNA levels was observed during the early stages of osteoblast differentiation. However, this effect seemed to be unrelated to Y2 receptor activation. In fact, within the same time period, Y2 receptor antagonist treatment up-regulated basal levels of OPG mRNA. These data supports the hypothesis that pharmacological blockade of the Y2 receptor might be in favour of bone formation.

Overall, our study indicates that NPY induces Y2 receptor upregulation and Y1 receptor down-regulation in osteoblast cells. In addition, Y2 receptor may play a pivotal role in NPY-modulated osteoblast activity. However, although these data suggest a direct mechanism of Y2 receptor signalling in osteoblasts the question whether these effects might also be a consequence of NPY induced down-regulation of Y1 receptor remains open. Further investigations are necessary to explore in more detail the relation among Y1 receptor, Y2 receptor signalling and osteoblast activity.

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