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Effects of PTH on PTHrP Gene Expression in Human Osteoblasts: Up-Regulation with the Kinetics of an Immediate Early Gene

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Parathyroid hormone-related peptide (PTHrP) is a local regulator of human bone turnover, which shares some sequence homology with the systemic hormone parathyroid hormone (PTH). The two proteins exert cellular effects through interaction with a common G protein-coupled PTH/PTHrP receptor on target cells. Whilst the PTH gene has a relatively simple structure the PTHrP gene is complex, alternative splicing of which can generate multiple mRNA species encoding PTHrP of 139, 141 and 173 amino acids. To date little is known regarding the extent to which PTH and PTHrP interact to modulate bone cell function. In this study we have used the quantitative technique of Real-Time polymerase chain reaction (PCR) to investigate the ability of PTH to induce PTHrP expression in SaOS-2 cells and in primary human osteoblasts. In addition, we have used the semi-quantitative techniques of PCR followed by Southern analysis and scanning densitometry to investigate the effects of PTH(1-34) on expression of mRNA species encoding the three PTHrP isoforms. We report a 50 fold increase in PTHrP mRNA expression 30 min after treatment with 100 ng/ml human recombinant PTH (1-34) in SaOS-2 cells, and a 38 fold rise in human osteoblasts 45-90 min post-PTH treatment. mRNA species encoding for PTHrP 1-139, 1-141 and 1-173 were all induced in human osteoblasts 45 min after exposure to PTH. Whilst the 1-139 mRNA species exhibited a sustained expression, both the 1-141 and 1-173 isoforms showed a biphasic induction with a second peak 6 hr post PTH treatment. These data demonstrate that PTH induces expression of the PTHrP gene in both SaOS-2 and primary human osteoblasts with the kinetics of an immediate early gene. Up-regulation of the PTHrP gene in response to PTH may be an important physiological mechanism by which this systemic factor effects a localised response in bone.

PTHrP was originally isolated from a human tumour cell line and implicated as a mediator of humoral hypercalcemia of malignancy (1). More recently however, the protein has been identified in a wide range of normal adult and foetal tissues including bone (reviewed in 2), whilst in culture, both rodent (3) and human (4–5) osteoblasts produce PTHrP. PTHrP shares limited amino-terminus sequence with parathyroid hormone (PTH); in the human sequence 8 of the first 13 amino acids at the N terminus are identical (1). This common sequence enables the two peptides to interact with a common PTH/PTHrP receptor on target cells. Whilst PTH is a well characterised systemic regulator of bone turnover, it is thought likely that PTHrP produced locally in bone by osteoblasts and other cells may be an autocrine, paracrine or intracrine regulator of bone remodelling.

The human PTHrP gene is complex, consisting of nine exons and three promoters. Alternate splicing of the PTHrP gene gives rise to three mRNA species encoding PTHrP of 139, 141 and 173 amino acids. The three proteins have variable C-termini, the significance of which have yet to be elucidated. The expression of PTHrP by human osteoblasts is regulated *in vitro* by glucocorticoids (5), 1,25 dihydroxy vitamin D_3 (6) and growth factors (7). However, to date, the possibility that PTHrP acts as a local mediator for systemic PTH has not been considered. In these studies we have investigated the effects of human PTH (1-34) on PTHrP gene expression in the human osteosarcoma cell line SaOS-2 and in primary human osteoblasts by quantitative Real-Time PCR. Furthermore, we have used conventional semi-quantitative PCR and Southern analysis to investigate whether PTH stimulation of human osteoblasts induces differential expression of the three PTHrP isoforms.

MATERIALS AND METHODS

Cell culture. Bone samples were obtained from the spine of a 14 yr male subject undergoing corrective surgery. Samples were chopped

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finely into Dulbecco's Modified Minimum Essential Medium supplemented with 10% foetal calf serum and antibiotics as described in detail in other publications (5,8). At confluence cells were passaged into 90mm dishes and treated with 100ng/ml of recombinant human (1-34) PTH for varying time periods in phenol red-free RPMI/1640 in the absence of serum. SaOS-2 cells were maintained in culture, passaged and treated as outlined above for human osteoblasts. Total RNA was extracted from both cell populations and reverse transcribed to generate first strand cDNA as previously described (5).

PCR analysis. Using cDNA derived from SaOS-2 or human osteoblasts as template, and specific combinations of primers (as detailed below), PTHrP fragments corresponding to the three mRNA species were generated in a 50μl reaction volume (See (6) for detailed description of methods), under cycling conditions of 94°C for 3 min followed by 35 cycles of 94°C for 30 sec, 62°C for 30 sec, 72°C for 30 sec and a final extension period of 5 min at 72°C. Following electrophoresis through a 1% ethidium bromide stained agarose gel, PCR products were transferred to Zeta-bind hybridisation membrane under alkaline conditions, probed with a fluorescently labelled fragment of PTHrP cDNA, and visualised by chemiluminescent detection. Scanning densitometry was used to assign quantitative values to generated PTHrP and glyceraldehyde-3-phosphate (GAPDH) products, enabling PTHrP values to be normalised against GAPDH. Normalised PTHrP absorbance values were expressed as a % of the maximum value.

Primer sequences. The same sense primer was used for all three PTHrP isoforms, with specific antisense primers

PTHrP:		
Sense		5′-ATGCAGCGGAGACTGGTTCAG
Antisense	1-139	5′-AAGGGAGGCAGCTGAGAGCAC
	1-141	5′-GTCCTTGGAAGGTCTCTGCTG
	1-173	5′-TTCTAGTGCCACTGCCCATTG
GAPDH:		
Sense		5′-GGTGAAGGTCGGAGTCAACGG
Antisense		5′-GGTCATGAGTCCTTCCACGAT

Real time quantitative PCR analysis. Quantitative real time PCR was performed using established Tacman chemistry in an ABI Prism™ 1770 sequence detector (9–10). PTHrP (all isoforms) or β-actin specific primers and probes were designed and generated by the manufacturers using 'Primer Express' software.

RESULTS

PTH Induces PTHrP Expression in SaOS-2 Human Osteosarcoma Cells

Using SaOS-2 cDNA as template and PTHrP primers recognising all isoforms, Real-Time PCR analysis was performed. Values obtained for PTHrP all isoforms were normalised for β-actin expression and expressed in Figure 1 as fold increase in PTHrP mRNA. Using this technique we detected a 50 fold increase in PTHrP mRNA expression 30 min after PTH treatment.

PTH Induces PTHrP Expression in Primary Human Bone-Derived Cells

In order to more clearly define a role for PTH in the modulation of PTHrP expression in normal bone we have assessed the effects of 100ng/ml PTH(1-34) on PTHrP mRNA expression by human osteoblasts. cDNA generated from osteoblasts derived from normal human bone was used as template for quantitative Real-Time PCR analysis. Values obtained for PTHrP all isoforms were normalised for β-actin expression and expressed in Figure 2 as fold increase in PTHrP mRNA. In these cells expression of the PTHrP gene was induced, with a 39 fold peak, 90 min after PTH treatment.

PTH-Induced PTHrP Isoform Expression in Primary Human Bone-Derived Cells

The functional role for specific PTHrP isoform expression remains unclear. Using conventional PCR analysis we have assessed isoform specific changes in PTHrP mRNA expression in response to PTH treatment in primary human osteoblasts. Following RT-PCR and Southern analysis quantitative values for generated products were obtained by scanning densitometry. Values were then corrected for corresponding GAPDH levels and expressed as percentage of maximal value. PTH effected an increase in steady state mRNA levels for all three PTHrP isoforms 45 min post-PTH treatment (Figure 3A, B and C). The 1-139 mRNA species displayed a sustained expression only returning to basal levels after 12-24 hr PTH treatment. In contrast, PTH induced a biphasic expression of the 1-141 and 1-173 mRNA species, with expression peaks at 45 min and 6 hr.

DISCUSSION

In these studies we have used the novel quantitative technique of Real-Time PCR to describe an up-regulation of PTHrP gene expression in human osteoblasts treated with 100ng/ml of recombinant human PTH(1-34). This induction occurred with kinetics normally associated with early response genes, providing the first specific demonstration, to our knowledge, of the rapid and transient effect of PTH (1-34) on PTHrP expression in human bone cells.

Other factors have been shown to rapidly up-regulate PTHrP mRNA expression in a number of cell types (11). Consistent with a rapid up-regulation of PTHrP mRNA expression, the PTHrP gene displays properties similar to other members of the immediate early gene family including *c-fos* and *c-myc*. These properties include a rapid and transient induction at the level of transcription following treatment with serum, growth factors and cycloheximide, and the presence of multiple AUUUA motifs in the 3′ untranslated region of the gene influencing stability of the mRNA. Whilst functional roles for other members of the early gene family have been characterised, an intracrine role for PTHrP remains to be firmly elucidated.

The process of skeletal turnover is controlled ultimately by circulating calcium concentration, the effects

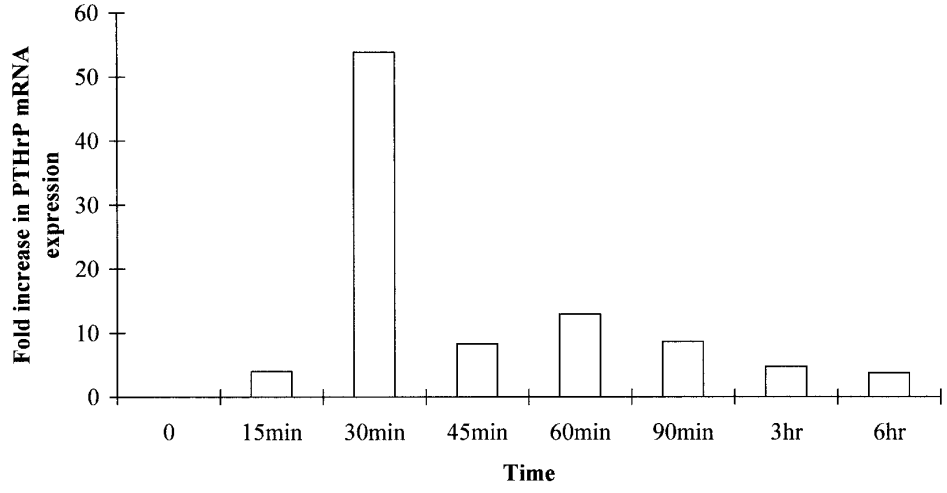

FIG. 1. The fold increase in PTHrP mRNA expression in SaOS-2 cells following exposure to human (1-34) PTH (100ng/ml) for times indicated. Quantitative Real Time PCR was carried out in an ABI Prism Sequence Detector using established Tacman chemistry, and oligonucleotide primers amplifying SaOS-2 cDNA corresponding to all PTHrP isoforms or β-actin. Data were normalised for the expression of β-actin and expressed as fold increase in mRNA expression.

of which are mediated through systemic hormones including PTH. However, bone remodelling is essentially a focal phenomenon requiring mechanisms to localise systemic hormonal responses. Rapid transcriptional up-regulation of the PTHrP gene in response to PTH may provide cells with a means of generating a labile local effector with autocrine, paracrine and intracrine capability.

In order to explain the induction of the PTHrP gene seen in our experiments we have considered the activated signalling cascades which may follow binding of PTH to the PTH/PTHrP receptor. Receptor activation results in an accumulation of cAMP and phosphorylation of the cAMP responsive element binding protein (CREB). The PTHrP gene contains 3 promoter regions, 2 of which have been reported to have cAMP responsive elements (CRE). The activation of the CRE by phosphorylated CREB will initiate gene transcription. This cAMP-mediated pathway would occur in the absence of *de novo* protein synthesis and could result in an induction of transcription of the PTHrP gene with the kinetics of an immediate early gene seen in our experiments.

The role of the three isoforms of PTHrP has not been elucidated. It has been suggested that these different isoforms represent tissue specific forms of the PTHrP

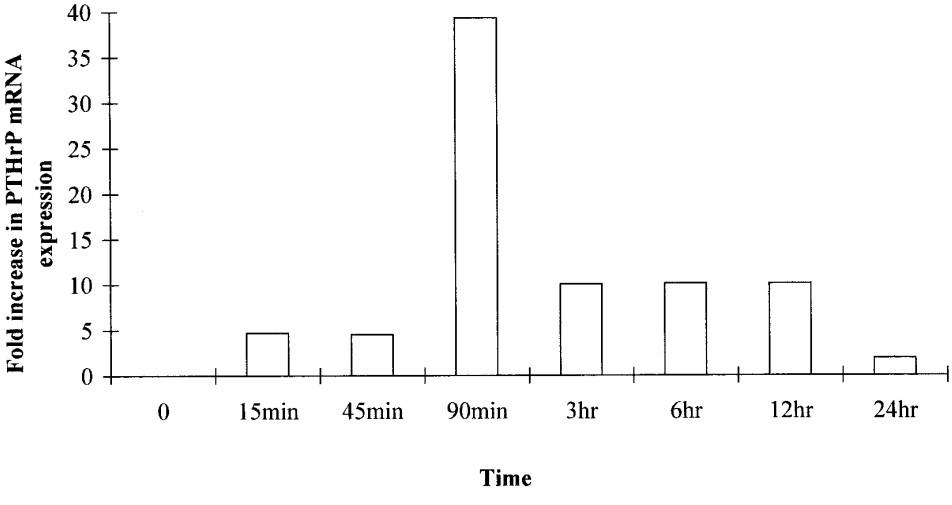

FIG. 2. Human osteoblasts, derived in culture from the bone of a 14 yr male subject, were treated with 100 ng/ml human (1-34) PTH for time periods indicated and total RNA extracted. cDNA generated from this cell population was used as template for quantitative Real-Time PCR. Again values obtained for PTHrP all isoforms were normalised for β-actin expression and expressed here as fold increase in PTHrP mRNA expression.

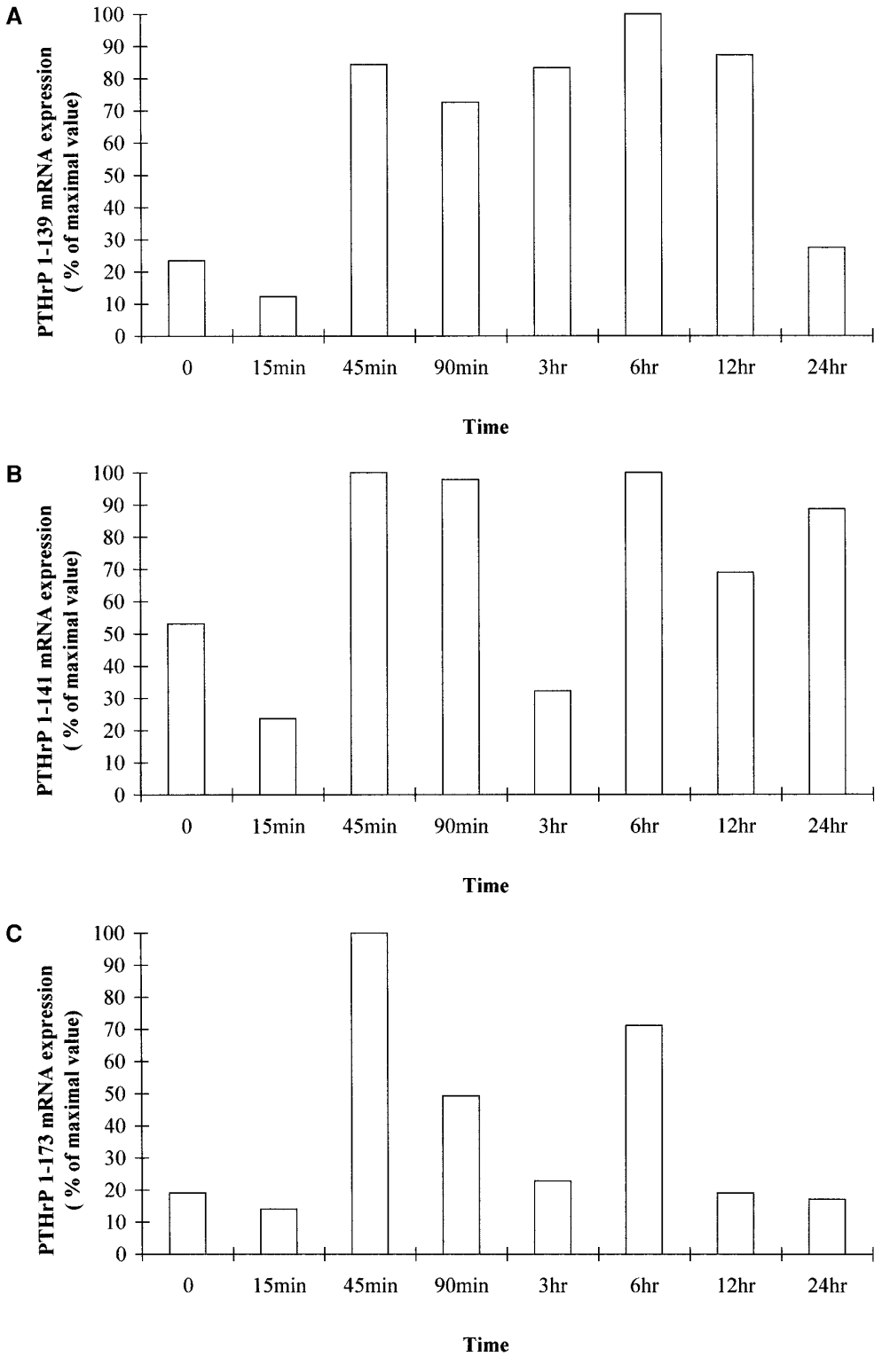

FIG. 3. Conventional RT-PCR analysis using PTHrP isoform specific or GAPDH primers and primary human bone derived cell cDNA as template was used to amplify products corresponding to all three PTHrP or GAPDH mRNA species. Generated products were analysed by agarose gel electrophoresis and visualised following ethidium bromide staining and exposure to U.V light. Southern analysis and subsequent chemiluminescent detection using a random primed fluorescein labelled PTHrP probe and Amersham detection kit confirmed the specificity of PCR fragments. Scanning densitometry was used to assign quantitative values to generated PTHrP and GAPDH products, enabling PTHrP values to be normalised against GAPDH. Normalised PTHrP absorbance values were expressed as a % of the maximum value.

molecule, but this proposal remains to be validated. mRNAs encoding the three PTHrP isoforms were all induced with early gene kinetics in response to PTH stimulation in these studies. Interestingly, mRNAs encoding PTHrP 1-141 and 1-173 exhibited a biphasic expression, with a second induction peak 6 hr after PTH treatment. The significance of these findings are unclear, but further demonstrate the complex nature of the PTH/PTHrP response.

If rapidly up-regulated PTHrP is translocated to the nucleus, as has been suggested (12), a cascade of events could be triggered that would influence cellular growth or differentiation as well as promoting a positive feedback to maintain or initiate further PTHrP transcription. Alternatively, the secondary induction seen here may result from activation of the PTHrP gene by the protein products of other immediate early genes including *c-fos*. This could be achieved using the AP1 binding site which has been identified in the PTHrP gene promoter region. Any secondary or sustained induction resulting from these processes may be responsible for the generation of protein products for secretion.

In conclusion, data presented here demonstrates that PTH modulates PTHrP production by SaOS-2 osteosarcoma cells and human osteoblasts *in vitro*. The production of PTHrP by osteoblasts, in response to PTH, may be an important physiological mechanism *in vivo* by which this systemic hormone exerts local effects in bone. The PTH-induced up-regulation of all PTHrP isoforms occurs rapidly and transiently suggesting that PTHrP may, in line with other members of the early gene family, be an intracrine regulator of cellular function.

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