Effects of parathyroid hormone-related protein on human mesangial cells in culture

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Bosch, Ricardo J., Pilar Rojo-Linares, Guadalupe Torrecillas-Casamayor, M. Carmen Iglesias-Cruz, Diego Rodriguez-Puyol, and Manuel Rodriguez-Puyol. Effects of parathyroid hormone-related protein on human mesangial cells in culture. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E990–E995, 1999.—Parathyroid hormone (PTH) and PTH-related protein (PTHrP) produce similar biological effects through the PTH/PTHrP receptor. Because PTHrP exhibits vasodilatory properties, we evaluated the hypothesis that this hormone interacts with human mesangial cells (HMC). The PTHrP prevented both the expected reduction in the planar cell surface area and the increase in myosin light-chain phosphorylation induced by platelet-activating factor (PAF) on HMC, in a dose-dependent manner. This effect was completely blocked by pertussis toxin and dideoxyadenosine, suggesting that a G protein-coupled receptor and cAMP are important in the PTHrP transduction mechanism. Moreover, PTHrP increased cAMP synthesis and thymidine incorporation in HMC. However, whereas RT-PCR and Southern and Northern blot analyses demonstrated the expression of human PTH/PTHrP receptor in human kidney cortex, no expression could be demonstrated in HMC. These results show that PTH and PTHrP directly interact with mesangial cells. These effects might be mediated by a receptor different from the PTH/PTHrP receptor.

planar cell surface area; platelet-activating factor; 2',3'-dideoxyadenosine

PARATHYROID HORMONE (PTH) is a well-known hormone that plays a central role in calcium and phosphate metabolism. Less is known about the physiological role of PTH-related protein (PTHrP), which was first identified in 1982 as the agent of the humoral hypercalcemia of malignancy (see review in Ref 16). The PTHrP consists of 141 amino acid residues, and the first 13 amino acids exhibit 70% amino acid homology with PTH. Although PTHrP and PTH are expressed by different genes, the NH2-terminal fragment of PTHrP and PTH interact with a common PTH/PTHrP receptor (also called “type 1” PTH/PTHrP receptor) in bone and kidney, which accounts for the clinical similarity of hypercalcemia of malignancy and primary hyperparathyroidism (16). Despite the similarities in actions of these peptides in bone and kidney, there are some differences in their biological activities. In this regard, a novel G protein-coupled receptor, the PTH-2 receptor, has recently been cloned and shown to selectively bind PTH (24).

During the last decade, a great amount of information clearly showed that the PTHrP gene is expressed not only in cancers but also in the vast majority of normal tissues. Despite the widespread production of PTHrP in healthy individuals, the concentration of the protein is below the detectable limit of current assays. Thus, under normal circumstances, PTHrP seems to play predominantly a paracrine and/or autocrine role in contrast to the situation of humoral hypercalcemia of malignancy, in which PTHrP plays the role of the classical “endocrine” hormone (1, 14, 22). These apparent physiological functions are also complex and appear to include regulation of 1) smooth muscle (vascular, intestinal, uterine, bladder) tone; 2) transepithelial (renal, placental, oviduct, mammary gland) calcium transport; and 3) tissue and organ development, differentiation, and proliferation (14, 16).

It is well known that mesangial cells modulate glomerular function and are closely related to the vascular smooth muscle cells (4, 5, 7), in which PTHrP (see recent review in Ref. 1) exerts a relaxant effect (14, 16). We therefore propose the hypothesis that PTHrP could modulate glomerular function by interacting with the contracting-relaxing capabilities of human mesangial cells. In the present study, we tested the effect of PTHrP on the ability of platelet-activating factor (PAF) to influence human mesangial cells in culture.

MATERIALS AND METHODS

Human mesangial cell culture. Human glomerular mesangial cells (HMC) were cultured according to a previously described procedure (4). Portions of macroscopically normal cortical tissue were obtained from human kidneys immediately after nephrectomy for renal cell carcinoma. The cortex was cut into slices and washed (with Hanks’ balanced salt solution); then, the material was pushed through 180- and 105-µm stainless steel sieves and washed to obtain isolated glomeruli free from tubular contamination. The glomeruli were then treated with collagenase, cultured with RPMI 1640 supplemented with 10% fetal calf serum, and buffered with bicarbonate. The experiments were performed in passages 3-5. The identity of the cells was confirmed by morphological and functional criteria, as previously described (4, 5) including a positive staining for myosin and anti-Thy-1 (thymus) antibody. No cells with morphological features of epithelial or endothelial cells were detected in the cultures. The ability of cells to exclude trypan blue dye was tested in all the different experimental conditions.

Planar cell surface area measurements. Planar cell surface area (PCSA) measurements were performed as we previously...
described (4, 5). HMC were grown in 35-mm tissue culture dishes (Nunk, Denmark). In every experiment, cells were washed twice (with the culture media discarded), placed in fresh buffer A (containing in mM: 20 Tris, 139 NaCl, 5 KCl, 10 sodium acetate, 5 glucose, and 2.5 calcium, pH 7.45), and maintained at room temperature. After 15 min, the experiments were started.

In the first set of experiments, cells were preincubated for 10 min with buffer or 10 nM human synthetic PTH fragment 1–34 [PTH(1–34)] or 10 nM human synthetic hypercalcemia of malignancy factor 1–34 [PTHrP(1–34); Sigma, St. Louis, MO]. Then 1 µM PAF, a known “contractor” of HMC (5), was added to the incubation media. Microphotographs were taken just before PAF addition and after 30 min of incubation. Control cells incubated only with buffer were used in each case. Thereafter, in a second set of experiments, cells were preincubated with buffer or different final concentrations of PTHrP (from 100 pM to 1 µM), and then 1 µM PAF was added to the incubation media.

Photomicrographs were also taken in the presence of some pharmacological modulators. After 15 min of incubation with 2’,3’-dideoxyadenosine (DDA, 0.1 mM), 10 nM PTHrP were added to the incubation media for 10 min, and then 1 µM PAF was also added. Photomicrographs were taken just before PAF addition and 30 min later. Pertussis toxin was also used in some experiments (0.5 µg/ml); if this occurred, the preincubation time was extended to 18 h.

While incubations were performed at room temperature (22 ± 2°C), cells were observed under phase contrast with an inverted Olympus IMT 2 photomicroscope (Shibuya-Hu, Tokyo, Japan) with ×150 magnification. Serial photographs were taken under the experimental conditions we have cited. Ten to eighteen cells were analyzed per photograph. The PCNA measurement was determined by computer-aided planimetric techniques. Actual areas were calculated after correction for microscope and photographic magnification. The measurements were performed blindly by two different researchers. The intraobserver and interobserver variations were 2 and 5%, respectively (4, 5).

Measurements of myosin light-chain phosphorylation. To confirm the contractile nature of PCSA changes, a biochemical parameter of contraction was analyzed. Thus myosin light-chain phosphorylation was determined in confluent monolayers of HMC after immunoprecipitation and SDS-PAGE protein separation by use of a human anti-platelet myosin antibody (generously given by Christine A. Kelley, M.D., National Institutes of Health (NIH), Bethesda, MD), as we previously reported (4, 5). The phosphorylated myosin light chains were identified on the autoradiographs (20 kDa), and the optical density of the 20-kDa band was measured by an Image-store Colour Onescanner and analyzed using the NIH Image 1.55 Software USA (obtained from the NIH internet home page at http://www.nih.gov) following manufacturer instructions. Results are expressed in arbitrary density units. In all cases of protein measurement, a correction for the protein content of the sample was performed (2).

Measurement of cAMP synthesis. To measure cAMP synthesis by cultured HMC, cells were washed twice with 2 ml of fresh buffer A and then preincubated in the same buffer containing 0.1 mM IBMX at 37°C for 5 min. PTH, PTHrP, or buffer was added to the incubation media, and cells were incubated for 5 min under these conditions. The medium was siphoned off, and 1 ml of cold ethanol was added to the flask, which was maintained in ice for 30 min. The cellular extracts were centrifuged for 20 min at 2,000 g, and the supernatant fraction was evaporated to dryness. The concentration of cAMP was determined by RIA (Du Pont, Boston, MA), as previously described (4).

Proliferation experiments. HMC proliferation was assessed by measuring [3H]thymidine uptake according to previously described techniques (17). Briefly, cells were incubated in standard culture medium with or without 15% fetal calf serum for 24 h and then were pulsed with [3H]thymidine (1 µCi/well) 16 h after the start of the experiment. PTHrP was added to the incubation media at different concentrations (from 0.1 nM to 0.1 µM) and for different times (6–48 h) before cell collection. Then the media were siphoned off, and the cells were washed (3 times) with PBS and precipitated directly by addition of 0.5 ml (3 times) of 10% trichloroacetic acid. Cell suspensions were collected, and the trichloroacetic-precipitable material was pelleted by centrifugation at 2,000 g, redissolved in 0.2 ml of 1 M NaOH, and neutralized with 1 M HCl. The radioactivity was measured in a scintillation counter.

Study of the human PTH/PTHrP receptor mRNA expression by RT-PCR. To study the expression of the human PTH/PTHrP receptor, procedures reported in detail by Li et al. (12) were used. Messenger RNA (mRNA) expression in human kidney cortex (HKC) and HMC was analyzed by a reverse-transcription polymerase chain reaction (RT-PCR) kit from Promega (Madison, WI) by following the manufacturer’s procedure. Briefly, after total RNA and mRNA extraction by standard procedures (3), one tube containing the two-enzyme system (0.1 µg/ml avian myeloblastosis virus RT and 0.1 µg/ml thermus flavus DNA polymerase), 0.2 mM dNTP mix, 1 mM MgSO4, and 0.2 µl oligonucleotide specific primers was set up in a total volume reaction of 50 µl.

To amplify the human PTH/PTHrP receptor mRNA, synthetic primers for the human receptor sequence (sense, 5’-GATGCT AGATG AGCTC ATGAC-3’; and antisense, 5’-CAGGGGCTCA AACAC ATCCTGG-3’) were used to amplify a 483-bp fragment corresponding to position 107–590 in the human PTH/PTHrP receptor cDNA (12, 13, 20, 21). In all samples, glyceraldehyde-3-phosphate dehydrogenase (GADPH) mRNA expression was also measured by RT-PCR as a housekeeping gene. The GAPDH primer sequences were sense, 5’-GTA AAG GGT CGG TGT CAA CGG ATT T-3’; and antisense, 5’-CAC AGT CTT CTG AGT GGC AGT GAT-3’, respectively, which yielded a single band corresponding to a 558-bp cDNA fragment (18).

For RT-PCR amplification, a thermal cycler (MJ Research, Watertown, MA) was programmed for an initial incubation at 94°C for 4 min, followed by 45 cycles consisting of the following steps: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The resulting ethidium-bromide staining gel was blotted into a nylon membrane to perform a Southern blot analysis of the RT-PCR products (15), as we will describe in the next section. Contamination was ruled out both by using mRNA as a template and by the fact that RT-PCR was negative when the reaction was performed without AMV reverse transcriptase.

Southern and Northern blot analysis. Twenty micrograms of total RNA were denatured and separated by electrophoresis, transferred to a Nylon membrane (Amersham International, Buckinghamshire, UK), cross-linked by ultraviolet irradiation for 1 min, and baked (2 h, 70°C). To identify the human PTH/PTHrP receptor mRNA, the filters from Southern and Northern analysis were hybridized with the (32P-labeled) full-length human PTH/PTHrP receptor cDNA (generously given by H. J. Uppner and E. Schipani, Boston, MA) at 42°C overnight. In the case of the Northern blot experiments, the membranes were reprobed with a 32P-labeled GADPH cDNA (25).
Statistical methods. Results are expressed as means ± SE, and the number of experiments is shown in every case. Comparisons were performed by the paired and unpaired Student's t-test, one- or two-way analysis of variance, Scheffé's multiple comparison test, and Friedman's test, as needed. A P < 0.05 was considered significant.

RESULTS

As shown in Fig. 1A, PTH and PTHrP exerted a preventive effect on the PAF-dependent decrease of PCSA in HMC. When cells were incubated with either PTH or PTHrP alone, no changes in PCSA were observed; however, when PTH or PTHrP was added before PAF, there was a complete blockade of the reduction of PCSA. This inhibitory effect of PTHrP occurred in a dose-dependent fashion, appearing at concentrations of ~0.1 nM and over (Fig. 1B). It is important to mention that our previous studies suggest that cells maintained in plastic flasks can reduce their PCSA, but they cannot increase it (see DISCUSSION).

In an attempt to study the possible role of pharmacological modulators on PCSA, we investigated the effects of G proteins and cAMP as mediators of the PTHrP effects on HMC. Cells were preincubated with pertussis toxin (Fig. 2A) or DDA (Fig. 2B), and PTHrP-dependent cell relaxation was analyzed. Both pertussis toxin and DDA completely blocked the effect of PTHrP on the PAF-induced reduction in PCSA. Moreover, to confirm the importance of cAMP as a second messenger of the PTHrP effects, HMC cAMP synthesis was measured by RIA. PTHrP induced a significant increase in the synthesis of the cAMP when compared with control cells (from 16.7 ± 0.3 to 33.2 ± 0.2 nmol/mg protein, P < 0.005).

![Fig. 1](https://example.com/fig1.png)

Fig. 1. Parathyroid hormone-related protein (PTHrP) prevents the changes induced by platelet-activating factor (PAF) in planar cell surface area (PCSA) of cultured human mesangial cells. Results are expressed as percentage of PCSA at time 0 and are means ± SE of 6 experiments. A: C, cells with buffer; PTHrP, cells with 10 nM PTHrP; PTH, cells with 10 nM PTH; PAF, cells with 1 µM PAF; PTHrP/PAF, cells preincubated for 10 min with 10 nM PTHrP and then 1 µM PAF was added for 30 min; PTH/PAF, cells preincubated for 10 min with 10 nM PTH and then 1 µM PAF was added for 30 min. *P < 0.05 vs. C. B: cells were preincubated for 10 min with different PTHrP concentrations, and then 1 µM PAF was added for 30 min. *P < 0.05 vs. 0 dose.

![Fig. 2](https://example.com/fig2.png)

Fig. 2. Effects of pharmacological modulators on PCSA. Results are expressed as percentage of PCSA at time 0 and are means ± SE of 6 experiments. A: effect of G proteins on PTHrP prevention of PAF-induced reduction in PCSA in human mesangial cells in culture. Open bars, cells incubated with pertussis toxin; filled bars, cells incubated without pertussis toxin. *P < 0.05 vs. C. B: effect of cAMP on PTHrP prevention of PAF-induced reduction in PCSA in human mesangial cells in culture. DDA, cells with 0.1 mM 2',3'-dideoxyadenosine; DDA/PTHrP/PAF, after 15 min of cell incubation with DDA (0.1 mM), 10 nM PTHrP was added for 10 min and then 1 µM PAF was also added. *P < 0.05 vs. C.
As it can be observed in the top panel of Fig. 3, PAF induced a significant increase in labeled phosphate incorporation in the myosin light-chain molecule; this effect was blocked by preincubating the cells with PTHrP. Interestingly, the PAF-induced myosin light-chain phosphorylation reappeared after the cells were retreated with DDA. The numerical analysis of the different experiments is shown in Fig. 3, bottom, where the densitometric analysis of X-ray films was corrected by protein content. The results confirm the morphological appearance: PTHrP blocked the ability of PAF to induce labeled phosphate incorporation in myosin light chain, and this effect disappeared in the presence of DDA. Moreover, PTHrP alone induced a statistically significant decrease in the myosin light-chain phosphorylation with respect to control cells.

Figure 4 shows the effect of PTHrP on HMC thymidine incorporation. An increased thymidine incorporation was detected at PTHrP concentrations as low as 100 pM. The expression of the human PTH/PTHrP receptor was first analyzed by RT-PCR. Figure 5 (top) shows the Southern blot analysis of the RT-PCR products. Amplification from HKC shows the expected 483-bp fragment, whereas this amplification was not detected in HMC. In all cases, the viability of the RNA was demonstrated by both the presence of the corresponding 28S ribosomal RNA band, as visualized with ethidium bromide, and the expression of GADPH mRNA by RT-PCR (data not shown).

Northern blot analysis (with the full-length human PTH/PTHrP receptor cDNA) shows the expected 2.4-kb transcript in total RNA from HKC, whereas no transcript was found in RNA from HMC (Fig. 5, middle). Assays were performed 12 times using both different samples and different astringency conditions. In all cases, the viability of the RNA was demonstrated both by presence of the corresponding 28S ribosomal RNA band, as visualized with ethidium bromide (data not shown), and by reprobing the membrane with a 32P-labeled GADPH cDNA showing the expected 1.2-kb transcript in total RNA from HKC and HMC (Fig. 5, bottom).

DISCUSSION

The present results clearly demonstrate that PTHrP exerts a direct action on HCM in vitro. Thus PTHrP inhibits both the expected reduction in PCSA and the increase in myosin light-chain phosphorylation induced by PAF in cultured HMC. This parallel experimental approach, analyzing both morphological and biochemical criteria of contraction, is very important in assessing the vasoactive properties of any potential modulator on mesangial cell contraction, because the presence of only morphological changes does not always provide a proper balance of information (4, 5, 7). PTHrP reduced 32P incorporation in myosin light chain of mesangial cells, supporting the hypothesis that the peptide exerts a direct relaxing effect on HMC. Nevertheless, PTH and PTHrP were unable to induce any significant change in PCSA in the cells maintained in plastic flasks, probably because under these culture conditions cells are unable to increase their PCSA (7).

One important characteristic of the PTHrP-dependent contractile inhibition must be stressed; this effect was dose dependent, with an effective blocking concentration of ~0.1 nM and over, supporting the possibility
that the effect of this hormone might not be only pharmacological but also physiological. To the best of our knowledge, this is the first report to demonstrate that PTHrP directly antagonizes the effect of PAF on HMC contraction. Thus some experimental protocols were designed to analyze the mechanisms responsible for the observed effects in HMC. Because convincing evidence indicates that the action of PTH and PTHrP is receptor mediated (16), the first question to consider would be whether specific membrane receptors are present in HMC. Although radio-ligand studies with labeled PTHrP were not performed, the dose-dependent relaxant and proliferative effects of this peptide, as well as the induction of cAMP synthesis by HMC, support the notion of the presence of such a receptor. It is interesting to note that Soifer et al. (23) also found a proliferative effect of PTHrP on rat mesangial cells in culture, supporting the existence of a PTHrP receptor on these cells.

The second question to answer was the possible dependence of the observed effect of PTHrP on the presence of a specific G protein in the cell membrane, as well as its intracellular mechanism of action. The ability of pertussis toxin to block the PTHrP-dependent cell relaxation suggests the presence of a membrane G protein that specifically mediates the cellular response. On the other hand, the ability of the peptide to stimulate cAMP synthesis, as well as the observed inhibition of the PTHrP effects in presence of DDA, points to cAMP as a possible intracellular messenger of the relaxing effects of PTHrP. However, these results must be considered carefully, because classical signals mediated by adenylate cyclase are enhanced by pertussis toxin, as this substance blocks an adenylate cyclase-coupled inhibitory G protein with a subsequent permissive effect of the system (24). In this context, opposite results were expected with DDA and pertussis toxin. Nevertheless, the biological response to PTHrP by mesangial cells does not appear to be a standard response, as in this cell type the relaxing agonist usually acts as an antiproliferative (4, 6, 7, 19), and this is not the case for PTHrP. Moreover, it has been previously described that, in some cell types, pertussis toxin may have inhibitory effects on the cAMP-generating system, although the exact nature of this inhibition has not been adequately explained (8). Consequently, even though our results point to a PTHrP receptor coupled to a G protein and with adenylate cyclase activity, additional investigation is needed to adequately clarify these mechanisms. Finally, this unexpected effect of pertussis toxin might be taken as an additional clue for the existence of a novel receptor (see later remarks).

Although receptors for PTH are known to be present in both mesangial and epithelial cells (23), a clear functional characterization of the receptor has not been performed. In this regard, Lee et al. (11), although demonstrating the expression of the PTH/PTHrP receptor in glomeruli podocytes, failed to demonstrate this receptor in mesangial cells in culture. Our results are in agreement with those of Lee et al., because we found the expression of the PTH/PTHrP receptor mRNA in HMC, whereas no expression of the receptor was found in HKC. Moreover, we also found that PTHrP is able to increase thymidine incorporation in HMC in a dose-dependent fashion, thereby suggesting a role for this hormone in the process of HMC proliferation. This result is in agreement with the previous studies reported by Soifer et al. (23). These authors also suggested the presence of a subtype of PTH receptor with PTHrP as the primary ligand in the mesangium, and our results would strongly support the suggestion that a novel receptor may, in fact, be present in HMC.

The renal expression of alternative splicing products of the PTH/PTHrP receptor gene has been reported (9), yet the failure to hybridize by Northern analysis with the full-length cDNA of the PTH/PTHrP receptor as a probe strongly argues against that possibility, at least in HMC.

One important question to consider is the physiological meaning of the present findings. Several studies propose that mediators capable of relaxing mesangial cells could be involved in the maintenance of the renal...
plasma flow and glomerular filtration rate in pathological situations in a similar way, because they act as vasodilatory agents in the circulation [14, 16]. In this regard, Sofer et al. (23) found that PTHrP mRNA increases after acute renal ischemia, stimulating DNA synthesis in mesangial cells and thus suggesting a role for PTHrP in the normal glomerulus and in the injured kidney. More recent data from Largo et al. (10) support this concept, because they found an overexpression of both PTHrP mRNA and protein in a rat model of tubulointerstitial damage.

In summary, we found that PTHrP is able to counteract the contracting effects of PAF on HMC, and the induction of proliferation of these cells. Moreover, these effects seem to be mediated by a G protein-coupled receptor, and cAMP acts as a second messenger. Finally, we confirm the presence of the PTH/PTHrP receptor mRNA in the human kidney cortex, which does not seem to be responsible for the effects of PTHrP in the human mesangium.

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REFERENCES


