EXPERIMENTAL STUDY

Origin of parathyroid hormone (PTH) fragments detected by intact-PTH assays

L Nguyen-Yamamoto, L Rousseau, J-H Brossard, R Lepage¹, P Gao², T Cantor² and P D'Amour

Centre de recherche, Centre hospitalier de l'Université de Montréal-Hôpital Saint-Luc and ¹Departments of Medicine and Biochemistry, Université de Montréal, Montréal, Québec, Canada, and ²Scantibodies Laboratory Inc., Santee, California, USA

(Correspondence should be addressed to P D'Amour, Centre de recherche-CHUM-Hôpital Saint-Luc, 264 René Lévesque Blvd, East, Montréal, Québec H2X 1P1 Canada; Email: rechcalcium.chum@ssss.gouv.qc.ca)

Abstract

Background: Intact parathyroid hormone (I-PTH) assays react with non-(1-84)PTH, large carboxyl-terminal (C) fragments with a partially preserved amino-terminal (N) structure. They account for up to 50% of I-PTH in renal failure and may be implicated in PTH resistance. We wanted to know if they were secreted by the parathyroid glands and generated by peripheral metabolism of PTH(1-84). **Methods:** Anesthetized normal and nephrectomized (NPX) rats were injected i.v. with 1.5 µg human (h) PTH(1-84). Blood was obtained from 8 rats at 2, 4, 6, 8, 12, 24, 48 and 96 min. I-PTH (Allegro I-PTH) was measured in all samples. Pools of serum were fractionated by HPLC at each time point and the fractions assayed to quantitate hPTH(1-84) and non-(1-84)PTH. Secretion studies were performed with dispersed cells from 5 parathyroid adenomas. The serum of 10 patients with primary hyperparathyroidism and cell supernatants were fractionated by HPLC and were analyzed as described.

Results: hPTH(1-84) disappeared from serum biexponentially. The half-life of the first exponential was similar in normal (2.08 min) and NPX (1.94 min) rats, while that of the second was longer in NPX rats (32.4 vs 20.9 min). The residual quantity of hPTH(1-84) under the curve was greater in NPX (6964±2392 pmol) than in normal rats (3229±561 pmol; $P < 0.001$). Non-(1-84)PTH concentration was maximal at 8 min in both groups and was higher in NPX (92.8±13.8 pmol/l) than in normal rats (38.8±7.2 pmol/l; $P < 0.01$). The area under the curve of non-(1-84)PTH was also greater in NPX (1904±405 pmol) than in normal rats (664±168 pmol; $P < 0.001$). All parathyroid adenomas secreted non-(1-84)PTH. It represented 21.1±3.9% of secreted and 32.5±1.3% of circulating I-PTH in primary hyperparathyroidism.

Conclusions: Non-(1-84)PTH, like other C-PTH fragments, originates from both the peripheral metabolism of hPTH(1-84) and from parathyroid gland secretion. Renal failure influences its concentration by increasing the amount of substrate available and by reducing non-(1-84)PTH clearance. Its higher proportion in serum relative to cell supernatants in primary hyperparathyroidism reflects the added role of peripheral metabolism and the longer half-life of fragments.

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Introduction

The existence of circulating carboxyl-terminal (C) fragment(s) of parathyroid hormone (PTH) with a partially preserved amino-terminal (N) structure is a relatively recent finding (1–3). These fragments are of particular interest because, at least theoretically, they could interact with the classic PTH/PTH related peptide receptor to modify some aspects of PTH(1-84) bioactivity. They were first identified by analyzing HPLC-fractionated sera with Nichol's intact PTH (I-PTH) radioimmuno-metric assay (1). This assay and other similar assays do not react with human (h) PTH(1-34) or C-fragments without a partially preserved N-structure (4, 5) but

they do react with hPTH(7-84), a prototype of these circulating C-fragments (1–3). Such is the case because most antisera raised against N-PTH have their epitopes in region 15–34 of the PTH structure (6). These fragments behave like other C-PTH fragments. Their concentration relative to that of hPTH(1-84) increases in hypercalcemia and decreases in hypocalcemia (1, 2). They also accumulate in renal failure to account for 40–50% of I-PTH immunoreactivity while representing only 15–20% in normal subjects (1–3). Recent data suggest that synthetic hPTH(7-84) alone decreases ionized calcium concentration in thyroparathyroidectomized rats (7, 8) and antagonizes the calcemic effect of hPTH(1-84) (8, 9) and hPTH(1-34)

(8) in the same animals, raising the possibility that circulating C-fragments with a partially preserved N-structure could do the same. This could implicate them in the PTH resistance of renal failure (7).

We were thus interested to see how these fragments were generated *in vivo* and how acute renal failure affected their concentration. We wanted to know if, like other C-PTH fragments, they had a dual origin from both peripheral metabolism (9, 10) and secretion by the parathyroid glands (11–14). The data to be presented here suggest a similar origin and behavior to other C-PTH fragments.

Materials and methods

Metabolism of hPTH(1-84) in normal and acutely nephrectomized rats

Experimental protocol This study was performed in male Sprague–Dawley rats weighing 275–300 g, under general anesthesia (Somnotol, 6 mg/100 g). Half the rats were nephrectomized bilaterally via a posterior surgical approach prior to injection (NPX rats) while the other half was sham-operated without removal of the kidneys (normal rats). hPTH(1-84) (Bachem, Torrance, CA, USA) was dissolved in barbital buffer, 0.05 mol/l, pH 8.6, containing 15% normal rat serum, at a concentration of 1.5 µg/500 µl, and 500 µl were injected into a jugular vein. Four groups of 8 normal or NPX rats were studied at the following time points: group 1, 2 and 4 min; group 2, 6 and 8 min; group 3, 12 and 24 min; group 4, 48 and 96 min. Five hundred microliters blood were collected through a jugular vein at the first time point, a volume equal to the injection volume, and exsanguination was performed via the abdominal aorta at the second time point. Serum was stored at –75 °C in aliquots until processed further or analyzed. The protocol was approved by the Animal Care Committee of our center in compliance with the Canadian Council on Animal Care guidelines.

Processing and analysis of samples I-PTH was measured in serum samples from all groups by a commercial I-PTH radioimmunoassay which is non-reactive to endogenous rat PTH (Allegro I-PTH, Nichol's Institute, San Juan Capistrano, CA, USA). This assay has been demonstrated to react in an equimolar fashion with hPTH(1-84) and hPTH(7-84) (2, 3). It also reacts with circulating C-fragments having a partially preserved N-structure, also called non-(1-84)PTH in prior publications (2, 3). A pool of serum from 8 rats of each group at each time point was extracted in Sep-Pak Plus C₁₈µ cartridges (Waters, Chromatographic Division, Wilford, MA, USA) (15) and eluted with 3 ml 80% acetonitrile in 0.1% trifluoroacetic acid (TFA). Acetonitrile was evaporated, and the residual volume freeze-dried. The samples were

reconstituted in 0.6 ml 0.1% TFA for HPLC analysis. Each sample was loaded on the same C₁₈µ Bondapak analytical column of 3.9 × 300 mm (Waters), and eluted with a non-continuous linear gradient of acetonitrile, 15 to 50% in 0.1% TFA, delivered at 1.5 ml/min for 65 min by BioRad Model 2700 HPLC (Richmond, CA, USA). Fractions (1.5 ml) were collected in polypropylene tubes precoated with 0.1% BSA in water. The acetonitrile present in each fraction was evaporated and the residual volume freeze-dried. Each fraction was reconstituted to 1 ml with 0.7% BSA in water, and appropriate volumes assayed for I-PTH. PTH recovery during all these procedures was similar in normal (80 ± 14.26 ; $n = 5$) and in nephrectomized (89.6 ± 9.34 ; $n = 5$) rats and was calculated by dividing the total amount of I-PTH recovered in the HPLC run by the total amount of I-PTH in the processed serum × 100. hPTH(1-84) and hPTH(7-84) (Bachem) processed in rat serum, as described, eluted as single peaks at the expected positions, demonstrating no degradation during the above procedures.

Secretion of PTH by parathyroid cells from human parathyroid adenomas

Experimental protocol Parathyroid tissue (230–500 mg) was obtained from 5 parathyroid adenomas in patients operated on for primary hyperparathyroidism. Blood was also obtained from 10 similar patients prior to surgery to study I-PTH molecular forms present in the circulation. All these patients were hypercalcemic (mean total calcium of 2.79 ± 0.31 mmol/l; normal value = 2.2–2.5 mmol/l) and had elevated levels of I-PTH (36.2 ± 52.5 pmol/l; range 6.4–136 pmol/l; normal value = 1.2–6.2 pmol/l). The use of serum or tissue for research purposes was approved by the ethics committee of our center and all patients signed an informed consent.

Processing and analysis Parathyroid glands were trimmed of exogenous tissue, sliced and dispersed with collagenase-DNase, according to the method of Brown *et al.* (16, 17), at 37 °C for 45–60 min. The cell suspension was filtered through 200 µm mesh and washed 3 times with culture medium to be described. Cell viability, assessed by the trypan blue exclusion technique, was >95%. Parathyroid cells were incubated in DMEM/F12 medium without NaHCO₃ and buffered with 0.015 mol/l HEPES, pH 7.4, 5% FBS and 0.2% BSA at a concentration of 200 000 to 300 000 cells/ml medium. Calcium concentration was adjusted with CaCl₂ to the original patient serum calcium concentration. Secretion was studied over 1 to 2 h in a humidified incubator at 37 °C. After incubation, the cells were centrifuged in a pellet, and the supernatant was collected for further analysis. Storage and processing of sera and supernatants for HPLC analysis were similar to the preceding

project in rats except that several different columns were used over the time course of these studies. Recovery of I-PTH immunoreactivity during these procedures was $103.4 \pm 17.2\%$ ($n = 10$). The same I-PTH assay described previously was used to analyze all HPLC profiles. A more recent cyclase activating (CA) PTH assay specific for hPTH(1-84) and non-reactive with hPTH(7-84) (18) was also used for the most recent samples.

Statistical analysis

The results are given as means $\pm$ S.D. Comparisons were made on logarithmic concentrations by a two-way repeated measures ANOVA followed by a Tukey test. Planimetric evaluation of HPLC profiles was undertaken with Origin 4.1 software (Microcal Software, Northampton, MA, USA). Percentages of hPTH(1-84) and non-(1-84)PTH were obtained in each case and applied to total I-PTH data for proper quantitation of both molecular forms at each time point and for each rat. The same software was used to study the kinetics of hPTH(1-84) disappearance in both groups, employing a standard formula for half-life and the elimination constant (K_e). hPTH(1-84) and non-(1-84)PTH were also quantitated similarly in the serum of patients with primary hyperparathyroidism and in the supernatant of cells incubation.

Results

Metabolism of hPTH(1-84) in normal and NPX rats

Figure 1 illustrates the disappearance of injected hPTH(1-84) in normal and NPX rats. Each point represents the mean $\pm$ S.D. for 8 rats. In both groups, I-PTH immunoreactivity decayed biexponentially with a rapid first component over the first 10 min and a slower second component over the rest of the study. Mean I-PTH concentrations were significantly higher at 2 min ($P < 0.05$) and from 4 to 96 min ($P < 0.001$) in NPX rats. These decay curves were separated into their components, hPTH(1-84) and non-(1-84)PTH, by HPLC analysis of a pool of sera at each time-point. The fractions were assayed with I-PTH, and peaks were quantitated by planimetry. This is illustrated for both groups at 8, 24 and 48 min in Fig. 2. Only hPTH(1-84) was identified at 2 and 4 min at position 52 min. Large fragments with a partially preserved N-structure were clearly identified as a shoulder to the hPTH(1-84) peak at 6 and 8 min at position 50 min. Their concentration was maximal 8 min after the injection of hPTH(1-84) in both groups and higher in NPX (92.8 ± 13.8 pmol/l) than in normal rats (38.8 ± 7.2 pmol/l; $P < 0.001$). They reached a percentage maximum at 24 min where they represented 40% of I-PTH immunoreactivity in both

Figure 1 Decay curves of injected hPTH(1-84) in normal (●) and nephrectomized (■) rats. Each point represents the mean $\pm$ S.D. of 8 rats. Logarithmic values statistically different from normal rats by a two-way ANOVA for repeated measurements followed by a Tukey test at given time points: * $P < 0.05$; ** $P < 0.001$.

groups. On all HPLC profiles, more fragments and hPTH(1-84) were observed in NPX rats than in normal rats. The HPLC percentages of hPTH(1-84) and fragments at each time point were next applied to individual rat values of Fig. 1 to obtain Fig. 3. The half-life of the first exponential of hPTH(1-84) decay (normal = 2.08 min; NPX = 1.94 min) was similar in both groups. The half-life of the second exponential was shorter in normal (20.9 min) than in NPX (32.4 min) animals. This caused the residual quantity of hPTH(1-84) under the curve to be more than twice as large in NPX (6964 ± 2392 pmol) compared with normal rats (3229 ± 561 pmol; $P < 0.001$). The quantity of non-(1-84)PTH under the curve was three times as large in NPX (1904 ± 405 pmol) than in normal rats (664 ± 168 pmol; $P < 0.001$).

Secretion of PTH by parathyroid cells from human parathyroid adenoma

Figure 4 illustrates the HPLC profiles of immunoreactive PTH in the serum of patients with primary hyperparathyroidism. Ten patients were assessed by I-PTH assay, and 2 peaks were identified in all patients with 3 peaks in some patients. The major peak, at position 52 min, co-migrated with hPTH(1-84) and represented $67.8 \pm 1.4\%$ of the immunoreactivity. The second peak migrated slightly in front of hPTH(1-84) at positions 48–50 min and represented $27.7 \pm 1.8\%$ of I-PTH immunoreactivity. This peak migrated similarly to non-(1-84)PTH, identified during the peripheral metabolism of hPTH(1-84) in rats, and was not recognized by the CA-PTH assay. A third minor peak was

Figure 2 HPLC profiles (left) of circulating PTH in normal (solid line) and nephrectomized (dashed line) rats at 8, 24 and 48 min after the injection of hPTH(1-84). The diagonal lines illustrate the acetonitrile (CH₃CN) gradient. The respective elution positions of hPTH(7-84) and hPTH(1-84) are indicated by arrows from left to right. Planimetric analysis (right) of HPLC peaks at each time point for normal (A) and nephrectomized (B) rats. Data are expressed as percentage of total I-PTH immunoreactivity measured for hPTH(1-84) (●) and recognized fragments (■) in the 2 groups.

also present in front of the last one, at positions 42–46 min, mainly in patients with higher basal PTH levels; it represented $4.5 \pm 1.7\%$ of immunoreactivity and was not recognized by the CA-PTH assay. Figure 5 illustrates similar results for supernatants of parathyroid cells obtained from parathyroid adenomas. Again, with the I-PTH assay 3 peaks were identified. Within one position, these peaks eluted similarly to those described for serum and represented, respectively: position 52 min, $78.9 \pm 4\%$, position 48–50 min, $18.2 \pm 3.8\%$, and position 42–46 min, $2.9 \pm 0.6\%$. The latter two peaks were not recognized by the CA-PTH assay. The amount of fragments identified in cell supernatants was significantly less than the amount observed in serum (21.1 ± 3.9 vs 32.5 ± 1.3 ; $P < 0.001$). When synthetic hPTH(1-84) was processed in hypoparathyroid serum, a single peak, migrating at position 52 min, was identified.

Discussion

This study was designed to examine the origin of non-(1-84)PTH, large C-fragments of PTH with a partially preserved N-structure (1–3). These fragments could theoretically activate the protein kinase-C domain of the classical PTH/PTH related peptide receptor (19–23), modulate hPTH(1-84) biological effects (7, 8, 23) or react with the less well-defined C-PTH receptor (24–27).

To study the origin of these fragments, we used different experimental models. Rats, either normal or NPX, have been used extensively to understand the metabolism of various PTH(1-84) preparations (9, 10, 28, 29), and they still appear to be the best model to examine the generation of fragments from injected hPTH(1-84). Rats were injected intravenously with $1.5 \mu\text{g}$ hPTH(1-84) and we have demonstrated previously that this dose does not saturate the hepatic extraction

Figure 3 Application of the planimetric analysis result at each time point (from Fig. 2) to circulating I-PTH levels at the same time point (from Fig. 1) in normal (●) and nephrectomized (■) rats. Results are means $\pm$ S.D. The fate of hPTH(1-84) and of non-(1-84)PTH is shown. Values of normal and NPX rats were analyzed using a logarithmic transformation of the data and a two-way ANOVA for repeated measurements followed by a Tukey test: * $P < 0.05$; ** $P < 0.001$.

of PTH(1-84) (30). Parathyroid adenomas obtained at surgery have also been used to study the secretion of C-PTH fragments by the parathyroid glands (11, 12). To ensure that the glandular secretory products were relevant to those found in the circulation, we also looked at circulating molecular forms of PTH in patients with primary hyperparathyroidism.

Intravenously (i.v.) injected hPTH(1-84), like other PTH preparations injected i.v. into rats (9, 10, 28, 29), disappeared from serum biexponentially, first with a rapid and then with a second slower component. As expected, more I-PTH immunoreactivity was present in serum at each time point in NPX animals, reflecting decreased clearance in the absence of the kidneys (28, 29). This has been observed in prior studies dealing with the peripheral metabolism of ^{125}I -bPTH(1-84) or ^{125}I -bPTH(39-84) (9, 10, 28, 29). HPLC fractionation of total I-PTH, at each time point, disclosed exclusively hPTH(1-84) at the 2 earliest time points, and a second peak in front of hPTH(1-84) at all other time points. This second peak first appeared

as a shoulder to hPTH(1-84) at 6 and 8 min, and as a distinct peak later on, with maximum percentage expression at 24 min where it represented 40% of total I-PTH immunoreactivity in both groups of rats. This is similar to what has been described for ^{125}I C-fragments of ^{125}I -bPTH(1-84) in normal and NPX rats (9, 10, 28, 29). The elution positions of this fragment peak and of hPTH(1-84) in rats were also similar to our findings in humans (1-3). The application of HPLC planimetric data to total I-PTH values permitted us to construct a true disappearance curve for hPTH(1-84) and a mixed curve (generation + clearance) for non-(1-84)PTH. Kinetic analysis of hPTH(1-84) disclosed a similar half-life for early exponential decay in both groups, but a longer half-life for the second component in NPX animals. Again, this is similar to prior reports (28, 29), with the exception of a shorter half-life for the second component in both groups in our study. The difference from prior results is probably best explained by the fact that the hPTH(1-84) preparation used here was biologically active while the ^{125}I -bPTH(1-84) used previously was not (9, 10, 29, 30) because of chloramine-T iodination. Prior studies have demonstrated a slightly different tissue distribution (more in the liver, less in the kidneys) of bioactive ^{125}I -bPTH(1-84) (31) and of bioactive internally labeled bPTH(1-84) with a somehow shorter half-life (32). Finally, more PTH fragments were observed in NPX rats at all time points, reflecting the absence of kidney clearance and increased production in the presence of more available substrate. Again, this is similar to what has been described for other C-fragments of PTH (28, 29) which are produced by the liver. In the end, one may wonder why non-(1-84)PTH was not identified previously in experiments dealing with the metabolism of ^{125}I -bPTH(1-84) or internally labeled bPTH(1-84). In the first case, gel chromatography was used and was probably unable to separate these large ^{125}I -PTH fragments from ^{125}I -bPTH(1-84), and/or ^{125}I -PTH fragments were not sequenced long enough to identify ^{125}I -non(1-84) PTH (9, 10, 29). In the second case (32), HPLC was performed on gel chromatography fractions and not on untouched samples, leading to the same conclusion.

The second part of our study dealt with the secretion of non-(1-84)PTH by parathyroid adenomas. The HPLC profiles of PTH in sera from 10 patients with primary hyperparathyroidism were first investigated to make sure that parathyroid gland secretory products were compatible with the circulating PTH molecular forms. hPTH(1-84) and a fragment peak corresponding to circulating non(1-84) PTH were identified in all patients and a third fragment peak, not identified before, was also seen in some patient sera. These 3 peaks were also identified as secretory products of parathyroid cell incubations. The smaller third peak was more apparent when high PTH levels were used for HPLC. These non-(1-84)PTH peaks represented 32.5% of the

Figure 4 HPLC profiles of I-PTH and of CA-PTH present in serum of patients with primary hyperparathyroidism. Arrows indicate the elution positions of hPTH(7-84) and hPTH(1-84) from left to right respectively. The diagonal lines illustrate the acetonitrile (CH_3CN) gradient. Results are expressed as absolute values on the left (A, C, E) and as a percentage of total immunoreactivity on the right (B, D, F). Ten profiles were analyzed with the I-PTH assay (A, B) and the mean of these results is illustrated (C, D). Three of these profiles were also analyzed with the CA-PTH assay (E, F). hPTH(1-84) and 2 more fragment peaks were recognized by the I-PTH assay in serum (A, B, C, D) but only hPTH(1-84) was recognized by the CA-PTH assay (E, F).

immunoreactivity in serum, slightly more than in normal individuals (1–3), but less than what is seen in advanced renal failure (2, 3). They also represented 21.1% of parathyroid gland secretory products. These

peaks were not recognized by the CA-PTH assay indicating loss of the first amino acids of the PTH structure. Thus, like other C-fragments, non-(1-84)PTH is also secreted by the parathyroid glands (11–14). The

Figure 5 HPLC profiles of I-PTH present in the supernatant of parathyroid cells from parathyroid adenomas studied over 2 h. Arrows indicate the elution positions of hPTH(7-84) and hPTH(1-84) from left to right respectively. The diagonal lines illustrate the acetonitrile (CH_3CN) gradient. Results are expressed as absolute values on the left (A, C, E) and as a percentage of total immunoreactivity on the right (B, D, F). Five profiles were analyzed with the I-PTH assay (A, B) and the mean of these results is illustrated (C, D). Two of these profiles were also analyzed with the CA-PTH assay (E, F). hPTH(1-84) and 2 more fragment peaks were recognized by the I-PTH assay but only hPTH(1-84) was recognized with the CA-PTH assay.

higher proportion of non-(1-84)PTH in sera than in cell supernatants reflects both the longer half-life of C-fragments in serum relative to hPTH(1-84) (28) and the contribution of peripheral metabolism to circulating non-(1-84)PTH. Earlier studies (14, 33, 34), performed with porcine, bovine and human parathyroid cells, indicated that apart from fragments starting at positions 34, 37 and 43, these glands also secreted fragments starting at positions 24 and 28. These two secreted products could contribute to what we describe as non-(1-84)PTH HPLC peaks.

In conclusion, we can say that non-(1-84)PTH, like other C-fragments of PTH, has a dual origin from both the peripheral metabolism of hPTH(1-84) and the secretory process of parathyroid cells.

In renal failure, more non-(1-84)PTH is observed in relation to the decreased renal clearance of these and other C-fragments. These findings in renal failure may be important to explain PTH resistance in that situation (7, 8). Our results, as far as metabolism is concerned, were obtained in rats and we must assume that similar metabolism occurs in humans. The precise identity of non-(1-84)PTH remains uncertain, and more studies will be required to elucidate non-(1-84)PTH structure.

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