Selective Uptake of the Synthetic Amino Terminal Fragment of Bovine Parathyroid Hormone by Isolated Perfused Bone

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ABSTRACT Studies from our laboratory have shown that the metabolic clearance rate of carboxy terminal immunoreactive parathyroid hormone (i-PTH) can be accounted for by extraction of i-PTH by liver and kidney. In contrast, there was no demonstrable hepatic uptake of the synthetic amino terminal bovine PTH fragment (syn b-PTH 1-34) and the kidney accounted for only 45% of the metabolic clearance rate of amino terminal i-PTH. This suggested that another major site, presumably bone, played a role in the metabolism of syn b-PTH 1-34. Extraction of i-PTH by isolated perfused bone was studied during infusion of purified bovine PTH (b-PTH) 1-84 or syn b-PTH 1-34. In five studies during infusion of syn b-PTH 1-34 the arteriovenous difference for i-PTH across bone was 36%. In contrast, no significant uptake of carboxy terminal i-PTH was observed in nine studies during infusion of b-PTH 1-84. In addition, when H_2O_2-oxidized (biologically inactive) syn b-PTH 1-34 was used no arteriovenous difference was observed. These findings correlated with the ability of these PTH preparations to stimulate cyclic AMP production by the perfused bone. Syn b-PTH 1-34 increased cyclic AMP production at perfusate PTH concentrations of 1–5 ng/ml, whereas b-PTH 1-84 evoked only a minimal response at concentrations of 10–20 ng/ml. We conclude that bone is a major site of metabolism of the amino terminal PTH fragment, syn b-PTH 1-34. In addition, these data suggest that cleavage of the intact hormone, with the production of amino terminal PTH fragments by peripheral organs (liver and kidney), may play a major role in the regulation of PTH effects on bone.

INTRODUCTION

Circulating immunoreactive parathyroid hormone (PTH)[1] exists as a mixture of the intact hormone and its fragments (1–4). Although there is evidence that PTH fragments may be secreted to some extent directly by the parathyroid glands (5–7), metabolism of intact PTH by peripheral organs undoubtedly contributes to the immunoheterogenous nature of circulating PTH.

Previous studies from our laboratory have shown that after injection of bovine PTH (b-PTH 1-84) to dogs, the kidney accounts for ≅60% of the total metabolic clearance rate of carboxy terminal immunoreactive PTH (8). The remaining 40%, therefore, is a result of PTH uptake at extrarenal sites. In studies designed to investigate the extrarenal sites of PTH metabolism, we have shown that the liver is an important site of PTH degradation (9). Hepatic uptake of immunoreactive PTH (i-PTH) is selective for the intact hormone and this organ does not remove carboxy terminal PTH fragments from the circulation. The hepatic uptake of intact PTH, however, appears to account completely for the extrarenal clearance of carboxy terminal i-PTH.

In studies using the synthetic amino terminal b-PTH fragment (syn b-PTH 1-34) significant differences were apparent between hepatic and renal PTH uptake. The kidney accounted for 45% of the metabolic clearance rate of amino terminal i-PTH, leaving the major portion (55%) to be accounted for by extrarenal sites. How-

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[1] *Abbreviations used in this paper:* A-V, arterio-venous; b-PTH, bovine PTH; cAMP, cyclic AMP; i-PTH, immunoreactive PTH; PTH, parathyroid hormone; syn b-PTH 1-34, synthetic amino terminal bovine PTH fragment.

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ever, our previous studies did not demonstrate any uptake of this amino terminal i-PTH fragment by the liver (9).

Parsons and Robinson (10) provided evidence for mobilization of calcium from bone after intravenous injection of intact b-PTH 1-84 to an anesthetized cat whose tibia was being perfused with its own arterial blood, but no calcium was mobilized when the hormone was added directly to their perfusing blood so that it did not enter the intact animal. These observations suggest that intact b-PTH 1-84 must undergo some alteration in the whole animal before it is capable of exerting its effect on bone. The present studies were designed to examine the possibility that bone might represent the major extrarenal site of metabolism of the syn b-PTH 1-34.

METHODS

Experimental model. The experimental model used in these studies was an isolated perfused canine tibia preparation. Mongrel dogs were anesthetized with pentobarbital (30 mg/kg). A longitudinal incision was made along the anterolateral aspect of the hind limbs to expose the muscle groups. The peroneal muscles were retracted superiorly and the anterior tibial artery exposed by sharp dissection to localize the nutrient artery to the tibia. The origin of the nutrient artery is somewhat inconstant and in some animals had its origin from the popliteal artery. After a similar procedure on the opposite limb the animal was heparinized and the tibiae removed. Adhering muscles were stripped from the bone and the nutrient artery cannulated via the main anterior tibial artery. The bones were then placed in a specially designed apparatus and perfused at 37°C as shown diagrammatically in Fig. 1. The perfusate—a Krebs-Henseleit buffer containing 10% canine plasma, Ca^{++} 4.5 mg/100 ml, pH 7.4, when continuously gassed with 95% oxygen, 5% carbon dioxide—was pumped into the bone at a pressure of 90–100 mm Hg. The venous return seeped from the bone surface and from the nutrient vein (when present) and was collected for analysis. Flow rates ranged from 1–2.5 ml/min. In some experiments, the venous return was returned to the perfusate reservoir and allowed to recirculate through the bone.

Adequacy of the perfusion was checked by either of two means. In the initial studies, a mixture of ^{3}H inulin and ^{45}Ca in 250–500 μl of perfusate was injected into the arterial line close to the cannula in the nutrient artery. Serial samples of venous effluent were collected and counted in a liquid scintillation counter (Packard Tri-Carb model 3390, Packard Instruments Co., Inc., Downers Grove, Ill.) for ^{3}H and ^{45}Ca. Recovery of inulin was 90% in 5 min whereas recovery of ^{45}Ca was 45–55%. The retention of ^{45}Ca, presumably by chemical exchange with bone mineral, indicated that bone was being perfused. The alternate method used in later studies was to inject a dye (Indocyanine Green, Hynson Westcott & Dunning, Inc., Baltimore, Md.) and simply to observe the bone surface turn green. This procedure ensured that there were no untied branches of the nutrient artery and that the correct artery was cannulated.

Study protocol. Once perfusion was established, the bone was allowed to equilibrate for 20–30 min. Then, purified b-PTH 1-84 or syn b-PTH 1-34 was added to the perfusate. 5 min after PTH reached the bubble chamber, serial simultaneous perfusate and venous samples were collected into chilled tubes for determination of arterio-venous (A-V) differences for i-PTH across the bone. In studies designed to assess the effects of PTH on cyclic AMP (cAMP) production by the perfused bone the above procedure was modified in that 4-6 arterial and venous samples were collected before addition of PTH to determine basal cAMP output by the perfused bone.

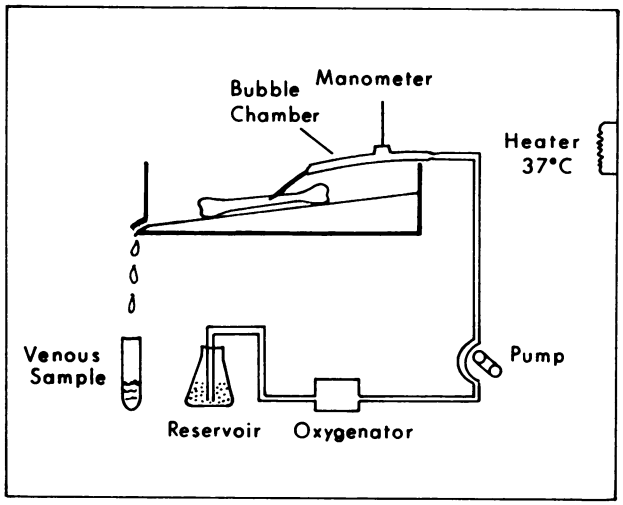

FIGURE 1 Diagrammatic representation of bone perfusion system.

In five studies the perfusate (50 ml) was allowed to recirculate after addition of b-PTH 1-84 and the reservoir sampled at intervals to assess if degradation of intact b-PTH 1-84 was occurring.

Assay methods. Perfusate PTH concentrations during infusion of b-PTH 1-84 or syn b-PTH 1-34 were measured by radioimmunoassay as described in detail (8, 9) and remained stable throughout the duration of the experiments. Antiserum CH9 with antigenic determinants for the carboxy terminal portion of the PTH molecule was used in studies with b-PTH 1-84. Antiserum CH9N which is specific for the amino terminal portion of PTH was used in studies with syn b-PTH 1-34. cAMP was measured by radioimmunoassay according to the method of Steiner et al. (11). The antiserum was a generous gift of Dr. Charles Parker (Washington University, St. Louis, Mo.). ^{125}I-succinyl cAMP was purchased from New England Nuclear, Boston, Mass. 1-ml samples for cAMP determination were drawn into tubes containing trichloroacetic acid at a final concentration of 5%. After centrifugation the supernate was extracted with 20 vol of water-saturated ether and evaporated to dryness. The dried samples were reconstituted in 0.5 ml sodium acetate (200 mM, pH 6.2), and assayed in duplicate. Results are expressed as cAMP produced in pmol/min (venous cAMP minus arterial cAMP multipled by perfusate flow rate).

Source of PTH. Highly purified b-PTH 1-84 (2,000 U/mg by bioassay) used in these studies was either a gift from Dr. James Hamilton and Dr. David Cohn, University of Kansas, Veterans Administration Hospital, Kansas City, Mo., or prepared in our laboratory according to the method of Hamilton et al. (12). Both preparations were identical in biological activity and in their migration profile on polyacrylamide gel electrophoresis (*vide infra*). syn b-PTH 1-34 (3,850 U/mg in a renal adenylate cyclase system) was obtained from Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif. Both b-PTH 1-84 and syn b-PTH 1-34 were equipotent on a molar

basis in stimulating cAMP production in fetal rat calvaria. Oxidized syn b-PTH 1-34 was prepared by dissolving lyophilized 10–20-μg aliquots of syn b-PTH 1-34 in 60 μl of 150 mM acetic acid and adding 40 μl of 30% vol/vol hydrogen peroxide. After incubation at 37°C for 45 min the reaction mixture was lyophilized. The H_2O_2-oxidized syn b-PTH 1-34 was identical to the biologically active PTH fragment in the radioimmunoassay.

Assessment of degradation of b-PTH 1-84. In studies in which the perfusate was allowed to recirculate for prolonged periods (2 h), aliquots of perfusate were analyzed by polyacrylamide gel electrophoresis in 10% (W/V) acrylamide, 1% (W/V) bis-acrylamide gels according to the method of Reisfield et al. (13) with the following modifications. All gel solutions contained 8 M urea. The running buffer (five times) contained 78 g β-alanine and 20 ml acetic acid/liter and was diluted 1/5 with deionized water before use. The pH of the separating gel was 5.2 and the pH of the stacking gel was 6.3. Electrophoresis was performed at 6 mA/gel for 3.5 h. 2-mm slices of the separating gel were shaken overnight at 4°C in 0.5 ml of 0.1 M barbital buffer, pH 8.6, containing 10% plasma and Trasylol (FBA Pharmaceuticals, Inc., New York) 500 U/ml before radioimmunoassay for PTH in carboxy and amino terminal assays. This system is capable of excellent resolution of intact hormone and carboxy and amino terminal fragments.

RESULTS

Studies with syn b-PTH 1-34. The results of five experiments during constant infusion of syn b-PTH 1-34 are shown in Table I. These results are compared with four experiments during infusion of H_2O_2-oxidized syn b-PTH 1-34 in Fig. 2. Extraction of amino terminal i-PTH, i.e. the A-V difference for i-PTH divided by the arterial i-PTH concentration, remained relatively constant at 36% for the biologically active PTH. Extraction of i-PTH by the perfused bone appeared to remain unchanged at perfusate PTH concentrations as high as 20 ng/ml. The specificity of the extraction of this PTH fragment by bone is shown by the results of studies using H_2O_2-oxidized syn b-PTH 1-34. No significant A-V difference was demonstrable when the oxidized hormone fragment was used.

TABLE I
Uptake of syn b-PTH 1-34 by Isolated Perfused Bone

Exp.	Flow rate	Extraction*	Perfusate i-PTH‡
	ml/min	*%*	*ng/ml*
1	2.6	45	3.2
2	2.5	27	1.5
3	2.0	43	1.0
4	1.8	33	2.5
5	2.0	31	7.2

Mean PTH extraction for the five experiments = 36%.
* Percent extraction of i-PTH: perfusate PTH concentration minus venous PTH concentration divided by perfusate PTH concentration.
‡ Perfusate amino terminal PTH concentration.

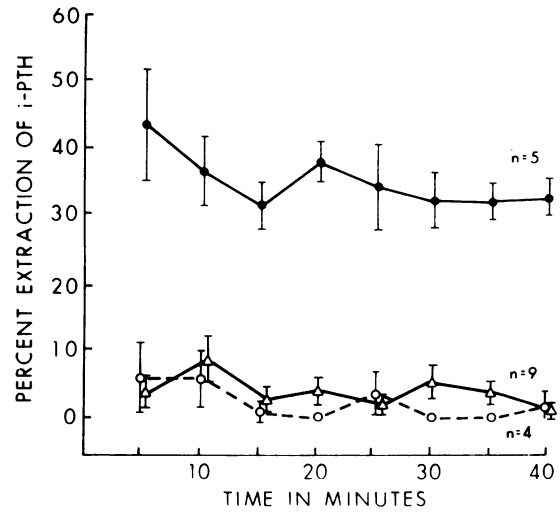

FIGURE 2 Mean percent extraction (±SEM) of amino terminal i-PTH by isolated perfused bone during constant infusion of syn b-PTH 1-34 in five studies (●), H_2O_2-oxidized syn b-PTH 1-34 in four studies (○) and of carboxy terminal i-PTH during infusion of b-PTH 1-84 in nine studies (△).

Studies with intact b-PTH 1-84. Also in Fig. 2 the results of nine experiments during constant infusion of highly purified intact b-PTH 1-84 are compared with the results of studies using syn b-PTH 1-34 ($n = 5$) and H_2O_2-oxidized syn-b-PTH 1-34 ($n = 4$). No significant A-V difference for carboxy terminal i-PTH could be demonstrated during infusion of the intact hormone over a wide range (0.5–30 ng/ml) of PTH concentrations in the perfusate. Similarly, no significant A-V difference was evident when three of those nine studies were assayed in the amino terminal assay system.

Studies of PTH-stimulated cAMP production. To correlate the results of A-V differences found in the above studies with a biological effect, additional studies were performed to assess the ability of the two PTH preparations to stimulate cAMP production by the perfused bone. Representative studies are portrayed in Fig. 3. cAMP production by the perfused bone increased markedly after the infusion of 5 ng/ml of syn b-PTH 1-34 was begun. Significant increases in cAMP production could readily be seen at perfusate PTH concentrations as low as 1 ng/ml. H_2O_2-oxidized syn b-PTH 1-34 at a concentration of 20 ng/ml did not result in any increase in cAMP production.

In contrast, the lower panel of Fig. 3 illustrates the results of a typical study during constant infusion of intact b-PTH 1-84 at a concentration of 20 ng/ml of perfusate. Although cAMP production increased somewhat in response to b-PTH 1-84, the increase was markedly less than that seen with syn b-PTH 1-34 in spite of the high concentration, even on a molar basis, of b-PTH 1-84. At perfusate concentrations of b-PTH 1-84 < 20 ng/ml no significant response was seen.

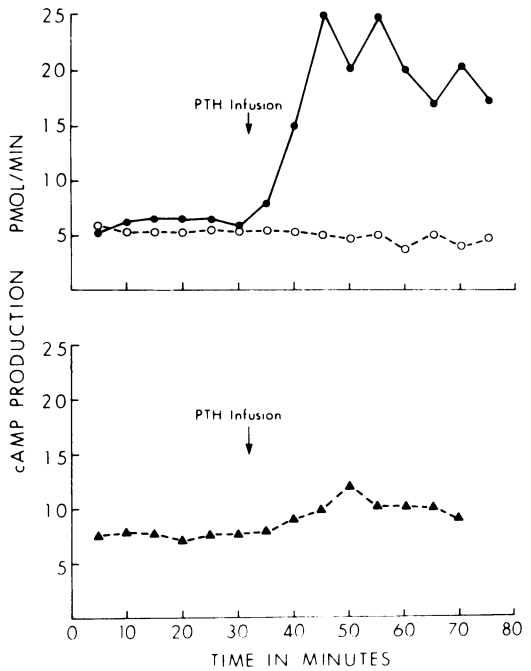

FIGURE 3 cAMP production by isolated perfused bone in representative studies during infusion of syn b-PTH 1-34 at perfusate concentration of 5 ng/ml (●), H_2O_2-oxidized syn b-PTH 1-34 at a concentration of 20 ng/ml (○), and b-PTH 1-84 at a concentration of 20 ng/ml (▲). Similar results were obtained in three additional studies in each group.

Recirculating studies. The possibility that the minimal increase in cAMP seen with b-PTH 1-84 was a result of cleavage of the intact hormone resulting in the production of an amino terminal fragment was assessed by analysis of perfusate which was allowed to recirculate for periods as long as 2 h. Portions of perfusate were analyzed by polyacrylamide gel electrophoresis and eluates of the gel slices assayed using carboxy and amino terminal specific antisera. No signifiant degradation of b-PTH 1-84 to carboxy and(or) amino terminal fragments was apparent.

DISCUSSION

The immunoheterogenous nature of circulating parathyroid hormone is well recognized (1–4). However, controversy continues as to the major source of circulating PTH fragments. Although there is evidence that the parathyroid glands are a source at least of carboxy terminal PTH fragments, there is considerable evidence for a role of peripheral organs in the generation of these PTH fragments. Previous studies from our laboratory have shown that after injection of intact b-PTH 1-84 to dogs, the pattern of circulating i-PTH changes from intact hormone to a mixture of carboxy and amino terminal fragments and finally to carboxy terminal fragments alone (8, 9). Studies using the isolated perfused liver of the rat (14) and the isolated perfused canine kidney (15) have shown extensive degradation of intact b-PTH 1-84 by these organs. Furthermore, these studies demonstrated that the rate of PTH degradation in liver and kidney was modulated by perfusate calcium concentration. Elevated perfusate calcium concentration impaired the metabolism of the intact hormone to carboxy and amino terminal fragments, whereas the production of PTH fragments was accelerated in the presence of low perfusate calcium concentrations. However, the overall physiological significance of the peripheral metabolism of intact PTH remains obscure. Is the peripheral degradation of intact PTH necessary for the expression of its biological effect or is the formation of fragments a consequence of the biological action of the intact hormone? Studies in vitro with bovine and canine renal cortical membranes have shown that the intact hormone can exert a biological effect per se in the absence of degradation (16), although other investigators have shown extensive degradation of intact PTH by renal cortical membranes of the rat (17). Previous studies from our laboratory have shown interesting differences in the uptake of PTH by liver and kidney (9). The canine liver demonstrates a selective uptake of the intact hormone but does not remove carboxy or amino terminal PTH fragments from the circulation, whereas the kidney removes both the intact hormone and its fragments from the circulation. The renal uptake of biologically active forms of PTH, namely, the intact hormone and the syn b-PTH 1-34, occurs mainly at peritubular sites, whereas the renal removal of biologically inactive PTH fragments depends exclusively on the process of glomerular filtration and tubular reabsorption (18).

The present studies demonstrate that uptake of PTH by the other major target organ for PTH, i.e. bone, differs from the uptake observed in both liver and kidney. In bone, a marked A-V difference for the syn b-PTH 1-34 is apparent, whereas no significant uptake of b-PTH 1-84 could be demonstrated. These uptake data correlate well with the biological effect of PTH in stimulating cAMP production. syn-b-PTH 1-34 clearly induced a marked increase in cAMP production by the perfused bone at concentrations as low as 1-ng/ml of perfusate. Intact b-PTH 1-84 produced only a minimal increase in cAMP production even at concentrations as high as 20 ng/ml.

The present findings are consistent with the observations of Parsons and Robinson (10) who demonstrated mobilization of calcium from bone after intravenous injection of intact b-PTH 1-84 to an anesthetized cat whose isolated tibia was being perfused with its own arterial blood. However, this effect was not seen when the hormone was added directly to the perfusing blood so that it did not enter the intact animal.

Because of the small though significant effect of large doses of b-PTH 1-84 on cAMP release by the perfused bone in the present experiments, studies were performed to evaluate whether degradation of the intact hormone occurred. We have been unable to demonstrate significant metabolism of b-PTH 1-84 to carboxy or amino terminal fragments in this system. Although it may be argued that metabolism of b-PTH 1-84 by bone yields fragments that are not immunoreactive in our assays, this possibility is unlikely because the intact hormone peak did not change significantly. These data are in contrast to previous findings in fetal rat calvaria in which intact b-PTH 1-84 readily stimulates cAMP production (19, 20). However, b-PTH 1-84 is extensively degraded to PTH fragments by fetal bone cells whereas oxidized b-PTH 1-84, devoid of biological activity is not metabolized (20). Thus, degradation of b-PTH 1-84 appears to be related to expression of biological activity in fetal rat calvarial preparations. Our failure to demonstrate degradation of intact b-PTH 1-84 in the present studies may represent a difference between adult and fetal bone, a species difference, or differences in experimental design.

Fig. 4 illustrates our proposed scheme for the peripheral metabolism of PTH. PTH is secreted from the parathyroid glands predominantly in the intact form and undergoes metabolism by the liver and kidney resulting in the production of carboxy and amino terminal fragments. The carboxy terminal fragments are further metabolized by the kidney by glomerular filtration and tubular reabsorption. The amino terminal fragments, in addition to intact PTH, may act on the peritubular side of the renal tubular cells and mediate the biological effect of PTH on the kidney. The present studies suggest that peripheral metabolism of intact PTH may be necessary for the expression of the biological effect of PTH on bone and emphasize the major role and possible physiological control that the peripheral metabolism of PTH by kidney and liver may play on the effects of the hormone in bone. Regulation of PTH metabolism, and hence the formation of fragments at the level of kidney and liver, may significantly affect the circulating levels of amino terminal (biologically active) PTH fragments and hence the effects of PTH at the level of bone.

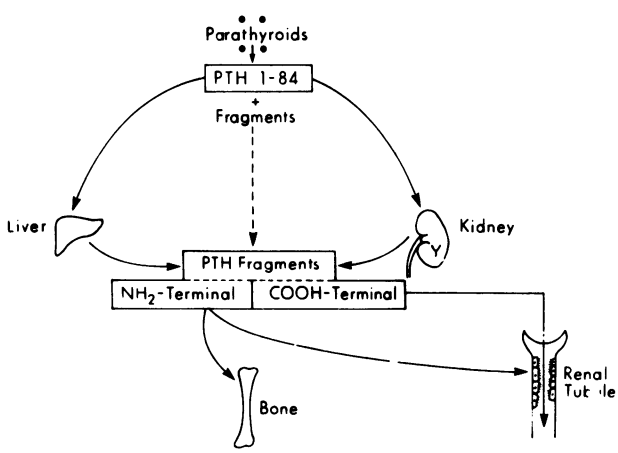

FIGURE 4 Current scheme of the peripheral metabolism of parathyroid hormone. For a complete description see text. NH_2-terminal, amino terminal; COOH-terminal, carboxy terminal.

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