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Response of the Renal Vitamin D Endocrine System to Oxidized Parathyroid Hormone (1-34) (41497)

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Abstract. Two preparations of bovine parathyroid hormone (bPTH), the natural bPTH(1-84) and the synthetic bPTH(1-34) fragment, have been treated with hydrogen peroxide and assayed for the effect of such treatment on the renal vitamin D endocrine system in Japanese quail. The oxidized and untreated preparations were injected intramuscularly into 4-week-old male Japanese quail, 12 hr after which the kidneys were removed and homogenized. The kidney homogenates were incubated with tritiated 25-hydroxyvitamin D_3[25-(OH)D_3] and the production rates of 1,25-$(OH)_2D_3$ and of 24,25-$(OH)_2D_3$ were determined as indices of 25-(OH)D_3-1-hydroxylase and 25-(OH)D_3-24-hydroxylase activities, respectively. Both untreated bPTH(1-34) and untreated bPTH(1-84) stimulated 1-hydroxylase and suppressed 24-hydroxylase activities. Oxidation of either bPTH(1-34) or bPTH(1-84) did not eliminate these responses, whereas the effects of oxidation on other responses to bPTH(1-34), namely inactivation of the hypotensive and renal adenylate cyclase stimulating responses, were observed as anticipated from our earlier observations. The importance of these findings is heightened when viewed in the context of our previous reports that oxidation of bPTH(1-34) leaves the hypercalcemic and hypocalciuric responses intact while partially or possibly totally inactivating all other major responses studied to date. It may be concluded that the mechanisms involved in effecting the hypercalcemic, hypocalciuric, and renal 25-(OH)D_3-1-hydroxylase responses to bPTH(1-34) demand structural requirements in the peptide molecule which are different from those needed to effect the hyperphosphaturic, hypophosphatemic, hypotensive, smooth muscle relaxing, and renal adenylate cyclase responses.

It is well known that the hypercalcemic activity of intact bovine parathyroid hormone [bPTH(1-84)] is largely inactivated by mild oxidation with hydrogen peroxide (1, 2). Recently, using conditions known to substantially inactivate the hypercalcemic activity of bPTH(1-84) in Japanese quail, we have shown that the synthetic fragment, bPTH(1-34), resists such inactivation with hydrogen peroxide (3, 4). Further work from our laboratory has revealed that, although the avian hypercalcemic activity of the synthetic bPTH(1-34) is unaffected, the hypotensive (4), hyperphophaturic (5), and uterine relaxing (6) activities of bPTH(1-34) are reduced by oxidation with hydrogen peroxide.

We now wish to report the effect of mild oxidation on the ability of intact bPTH(1-84) and of synthetic bPTH(1-34) to modify the renal 25-hydroxyvitamin D_3-1-hydroxylase and 25-hydroxyvitamin D_3-24-hydroxylase activities in Japanese quail. The untreated and oxidized preparations were also monitored for hypercalcemic, hypotensive, and renal adenylate cyclase stimulating activities.

Materials and Methods. *Hormone preparations.* Synthetic bovine parathyroid hormone tetratricontapeptide, bPTH(1-34), was obtained either from Beckman (Beckman Instruments, Inc., Bioproducts Operation, Palo Alto, Calif.; lot No. B01130) or from Peninsula (Peninsula Laboratories, Inc., San Carlos, Calif.; lot No. 001752). The bPTH(1-34) preparations had assigned potencies of 6000 and 10,000 IU/mg, respectively, when assayed *in vitro* using activation of rat renal cortical adenylate cyclase as the response. The intact bovine parathyroid hormone, bPTH(1-84), was purified material (N1BSC 77/533) kindly supplied by Dr. Joan Zanelli of the National

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Institute for Biological Standards and Control, London. It had an assigned potency of 2300 IU/mg.

Hydrogen peroxide treatment. One milliliter of a solution of either bPTH(1-84) or bPTH(1-34), each dissolved in acid saline (0.154 *M* NaCl adjusted to pH 3.0 with HCl) at a concentration of 1000 IU/ml, was treated with 0.3% hydrogen peroxide by adding 10 μl of 30% hydrogen peroxide (Fisher Scientific Co., Pittsburgh, Pa.; catalog No. H-325) to the 1.0-ml sample. Following addition of hydrogen peroxide, the mixture was incubated for 30 min at 25°. Control incubations were performed using 10 μl of water instead of the 30% hydrogen peroxide. The mixture was immediately lyophilized following incubation. The lyophilized preparations were sealed and stored below 0° until required. They were reconstituted in 1.0 ml of water and diluted in acid saline for injection. Total enzymatic digestion of the bPTH(1-34) preparation, subjected to oxidation under these conditions, has revealed that such treatment is 100% effective by reducing the methionine content from 1.74 moles per mole of peptide to zero (4). The evidence also indicated that the methionine is oxidized mole for mole to methionine sulfoxide. No other amino acid modifications were detected.

Renal 1- and 24-hydroxylase assays in Japanese quail. Four-week-old immature male Japanese quail (*Coturnix coturnix japonica*), weighing approximately 100 g and bred and maintained in the vivarium of the Texas Tech University Health Sciences Center as described elsewhere (7), were used. The control or bPTH solutions were injected intramuscularly in a volume of 0.4 ml/bird to five groups of five birds each: (a) control, acid saline; (b) bPTH(1-34); (c) oxidized bPTH(1-34); (d) bPTH(1-84); and (e) oxidized bPTH(1-84). Twelve hours after injection, the birds were sacrificed at which time the kidneys were removed for assessment of 1- and 24-hydroxylase activities *in vitro* as described elsewhere (8, 9). Each kidney homogenate was incubated for 10 min in the presence of 50 pmole of 25-[26,27-^{3}H]hydroxyvitamin D_3 with a specific activity of 9.6 Ci/mmole. The incubation mixture was extracted with appropriate solvents and the extract was dried, dissolved in 300 μl of chloroform/hexane (65:35), and applied to a 0.7 × 18-cm Sephadex LH-20 column. The column was eluted with the same solvent system and 50 fractions (125 drops or 0.92 ml each) were collected. The tritium content of each fraction was determined by liquid scintillation counting. Total disintegrations per minute (dpm) under each peak was calculated and corrected for extraction losses and the data were expressed as picomoles min^{-1} g^{-1} kidney. The 1,25-$(OH)_2D_3$ peak generated by Japanese quail kidney homogenates under these circumstances has failed to reveal any cochromatographic contaminants when subjected to more rigorous analysis (10).

Hypercalcemic activity in Japanese quail. The bPTH preparations were assayed for hypercalcemic activity in Japanese quail by the method described by Dacke and Kenny (2). All injections were given intravenously in a volume of 0.4 ml/bird using an injection vehicle consisting of 51 m*M* $CaCl_2$ and 0.01% bovine serum albumin.

Renal adenylate cyclase activity. A renal membrane preparation of adenylate cyclase was made from Japanese quail kidneys and used for the *in vitro* assay of the bPTH peptides. Details of the protocol, which was modified from assays using rat renal cortical membrane preparations (11–13), will be supplied on request. The assay is sensitive to about 0.01 μg/tube (2×10^{-8} *M*) of synthetic bPTH(1-34).

Hypotensive activity in the rat. The hypotensive effect of the bPTH peptides was assayed in the rat following intravenous injection as described elsewhere (14). A significant hypotensive response may be seen with 1 μg/kg. A typical response to 7 μg/kg is presented in Fig. 1.

Plasma calcium. Heparinized blood was obtained from the Japanese quail by heart puncture following light anesthesia with halothane (Fluothane, Ayerst Laboratories, Inc., New York, N.Y.) and prior to removal of the kidneys. Plasma calcium concentration was determined by atomic absorption spectrophotometry (Perkin–Elmer

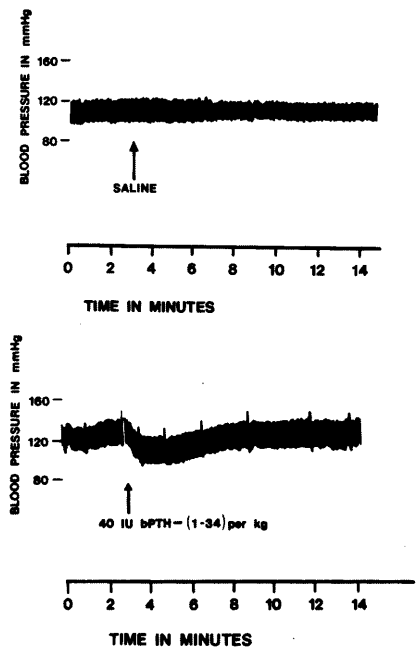

FIG. 1. Typical hypotensive response to bPTH(1-34) in the anesthetized rat. Upper panel: control injection of saline. Lower panel: response to 40 IU/kg of bPTH(1-34).

Model 303) following dilution with 0.5% lanthanum chloride and deproteinization with 5% trichloroacetic acid.

Results. Both unoxidized bPTH(1-84) and unoxidized bPTH(1-34) significantly enhanced renal 1-hydroxylase activity and suppressed 24-hydroxylase activity. Mild oxidation with hydrogen peroxide did not significantly modify these responses (Table I). The hypercalcemic activity of bPTH(1-34) was unaffected by oxidation, whereas that of bPTH(1-84) was markedly reduced (Table II). In contrast, oxidation essentially eliminated the hypotensive (Fig. 2), and significantly reduced the renal adenylate cyclase (Table III), activities of bPTH(1-34). The plasma calcium concentrations were normal 12 hr following injection in all birds as anticipated; the hypercalcemic response to bPTH is very rapid in onset and short in duration in Japanese quail (15).

Discussion. The significance of this work becomes more apparent by placing it within the context of our other structure–activity studies associated with hydrogen peroxide treatment of bPTH(1-34). Whereas the hypercalcemic activity of intact bPTH(1-84) is largely inactivated by mild oxidation with hydrogen peroxide, that of the synthetic fragment, bPTH(1-34), is unaffected by such treatment (3, 4). Prior to our report, apparently no laboratory had investigated the *hypercalcemic* activity of oxidized synthetic bPTH(1-34). Mild oxidation is assumed to affect only the methionine residues at positions 8 and 18 which occur in both bPTH(1-34) and bPTH(1-84). Why, then, does such treatment affect the activity of one molecule and not the other? Perhaps

TABLE I. EFFECT OF H_2O_2 ON THE RESPONSE OF THE RENAL 1- AND 24-HYDROXYLASES TO bPTH(1-34) AND bPTH(1-84) IN 4-WEEK-OLD MALE JAPANESE QUAIL

Treatment	Dose, im (per bird)	No. birds	Plasma Ca (mg/dl)	Metabolite production (pmole·min^{-1}·g^{-1} kidney)	
				1,25-$(OH)_2D_3$	24,25-$(OH)_2D_3$
Control, acid saline	0.4 ml	5	9.8 ± 0.24	2.4 ± 2.35	27.6 ± 3.44
bPTH(1-34)	20 μg	5	9.6 ± 0.16	17.2 ± 1.82**	12.7 ± 3.37*
bPTH(1-34) + H_2O_2	20 μg	5	9.4 ± 0.21	22.5 ± 3.57**	6.4 ± 4.03**
bPTH(1-84)	17 μg	5	9.5 ± 0.25	8.3 ± 3.24***	12.4 ± 4.25*
bPTH(1-84) + H_2O_2	17 μg	5	9.5 ± 0.26	10.9 ± 1.98*	8.0 ± 3.30**

Note. Results are means ± SE. bPTH(1-34): synthetic bovine PTH(1-34) from Beckman (6000 IU/mg claimed potency). bPTH(1-84): purified bPTH(1-84) from National Institute for Biological Standards and Control, London (NIBSC 77/533) with an assigned potency of 2300 IU/mg.

* Significant at $P < 0.05$.

** Significant at $P < 0.01$.

*** Significant at $P < 0.10$ (one-tail test).

TABLE II. EFFECT OF H_2O_2 ON THE HYPERCALCEMIC RESPONSE TO bPTH(1-34) AND bPTH(1-84) IN JAPANESE QUAIL

Treatment	Dose, iv (per bird)	No. birds	Plasma Ca (mg/dl, mean ± SE)
Assay P120			
Control, vehicle alone	0.4 ml	7	10.1 ± 0.28
bPTH(1-34)	7 μg	7	12.2 ± 0.34***
H_2O_2-treated bPTH(1-34)	7 μg	7	13.0 ± 0.16***
Assay P162[a]			
Control, vehicle alone	0.4 ml	6	9.4 ± 0.21
bPTH(1-84)	44 μg	5	12.4 ± 0.31***
H_2O_2-treated bPTH(1-84)	44 μg	6	10.2 ± 0.52

Note. See footnotes to Table I. Vehicle: 51 m*M* $CaCl_2$ + 0.01% bovine serum albumin.
[a] These data (assay P162) are reproduced from Pang *et al.* (4).

oxidation of the two methionines at positions 8 and 18, respectively, results in conformational changes such that the intact bPTH(1-84) molecule, with its large biologically inactive C-terminal tail, cannot adequately interact with its receptor. The synthetic N-terminal fragment, bPTH(1-34), on the other hand, may not experience any steric hindrance resulting from such a conformational change.

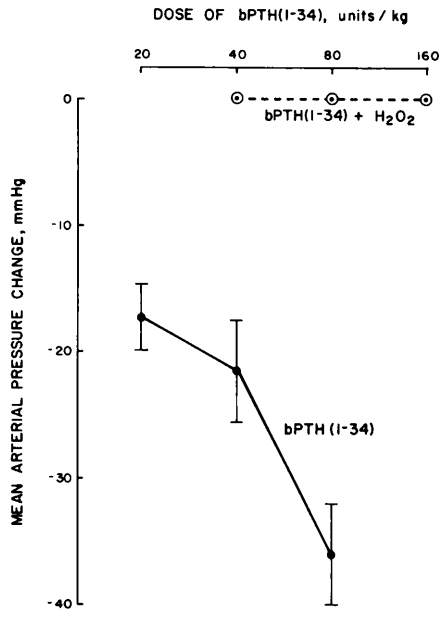

FIG. 2. Effect of different doses of untreated and oxidized bPTH(1-34) on mean arterial pressure in the anesthetized rat.

Equally important is our finding that mild oxidation partially or possibly totally inactivates some but not all of the responses associated with the synthetic bPTH(1-34) fragment. Of the responses studied to date, three resist inactivation. These are: (i) the hypercalcemic response in the Japanese quail (3, 4); (ii) the hypocalciuric response in the rat (16); and now, from the present study, (iii) the ability to activate the renal 25-hydroxyvitamin D_3-1-hydroxylase and suppress the 25-hydroxyvitamin D_3-24-hydroxylase enzymes in the Japanese quail. All three responses are classically associated with the mobilization of calcium into extracellular fluid from bone, kidney, and gut. Other responses studied are partially or totally eliminated by oxidation of bPTH(1-34). These include: (i) the hyperphosphaturic response in the rat (5); (ii) renal adenylate cyclase activation in the Japanese quail (Table III); (iii) relaxation of induced contraction of smooth muscles (6); and (iv) the hypotensive response in the rat ((4) and Fig. 2).

One other interesting point emerges from these findings. It is obvious that the mechanisms involved in the hypercalcemic, hypocalciuric, and renal 1-hydroxylase responses demand structural requirements in the bPTH(1-34) molecule which are differ-

TABLE III. EFFECT OF H_2O_2 ON THE AVIAN RENAL ADENYLATE CYCLASE RESPONSE TO bPTH(1-34)

Treatment	Dose (per tube)	Adenylate cyclase activity (pmole cAMP/mg protein)
Control	—	101 (87–115)
bPTH(1-34)	0.1 μg	408 (361–454)
bPTH(1-34) + H_2O_2	0.1 μg	131 (121–141)

Note. Results are means; range of duplicates in parentheses. bPTH(1-34): synthetic bPTH(1-34) from Peninsula (10,000 IU/mg).

ent from those needed to effect the responses, such as the hyperphosphaturic, renal adenylate cyclase, hypotensive, and smooth muscle responses, which are not elicited by the oxidized form of bPTH(1-34). Is it possible that the different structural requirements revealed in our functional studies using the oxidation technique approach are related to the existence of different types of receptors? Further work is needed before this question can be answered with assurance.

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