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Activation of Avian Medullary Bone Osteoclasts by Oxidized Synthetic Parathyroid Hormone (1-34) (42061)

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Abstract. Synthetic bovine parathyroid hormone (1-34) [bPTH(1-34)] has been treated with hydrogen peroxide and assayed for the effect of such treatment on the ability of bPTH(1-34) to activate medullary bone osteoclasts during their quiescent period in the early phase of the ovulatory cycle in Japanese quail. In addition, the same batches of oxidized and unoxidized bPTH(1-34) were assayed for their hypercalcemic activity in Japanese quail and their capacity to stimulate renal adenylate cyclase activity in the same species. Three groups, each consisting of five 5-month-old egg-laying Japanes quail (*Coturnix coturnix japonica*), were used. Between 4 to 5 hr after oviposition the three groups were injected intraperitoneally with acid saline (control) solution, bPTH(1-34) at 40 μg/bird, or oxidized bPTH(1-34) at 40 μg/bird, respectively. Twenty minutes after injection, the femoral bones were removed, split, fixed, and appropriately processed for examination by electron microscopy. Both oxidized and unoxidized bPTH(1-34) stimulated the development of osteoclast ruffled borders within 20 min after injection of the hormone preparations. As anticipated from previously published work from this laboratory, oxidized bPTH(1-34) retained its hypercalcemic activity and lost its capacity to stimulate renal adenylate cyclase activity in the Japanese quail. These results support, but do not prove, the contention that bPTH(1-34) exhibits its responses in the Japanese quail through the mediation of more than one type of receptor.

Following mild oxidation of synthetic bovine parathyroid hormone (1-34) [bPTH(1-34)] with hydrogen peroxide, the hypotensive (1), hyperphosphaturic (2), uterine-relaxing (3), and renal adenylate cyclase-stimulating (4) activities of bPTH(1-34) are reduced or eliminated. On the other hand, oxidation of bPTH(1-34) fails to inactivate responses classically associated with calcium homeostasis. The latter responses include the hypercalcemic response in the Japanese quail (1, 4), the hypocalciuric response in the rat (5), and the effects on the renal vitamin D endocrine system in the Japanese quail (4).

The purpose of this study was to compare the effects of oxidized and unoxidized bPTH(1-34) on the activation of functionally inactive medullary bone osteoclasts in Japanese quail. The same bPTH(1-34) preparations were also tested for hypercalcemic and renal adenylate cyclase-stimulating activities in Japanese quail.

Materials and Methods. *Hormone preparation.* Synthetic bovine parathyroid hormone tetratricontapeptide, bPTH(1-34), was obtained from Beckman (Beckman Instruments, Inc., Bioproducts Operation, Palo Alto, Calif.). Two lots of bPTH(1-34) were pooled; 1260 μg of lot No. B10845 with a claimed potency of 6800 units/mg was combined with 816 μg of lot No. B01130 with a claimed potency of 7500 units/mg, yielding a pooled preparation with a potency estimated to be 7075 units/mg.

Hydrogen peroxide treatment. One milligram of the pooled bPTH(1-34) preparation was dissolved in 1.0 ml of acid saline (0.154 *M* NaCl adjusted to pH 3.0 with HCl) containing 0.01% of bovine serum albumin. Ten microliters of 30% hydrogen peroxide was added and the mixture was incubated at 25°C for 30 min, lyophilized, and stored below 0°C until required. The lyophilized preparation was reconstituted in 1.0 ml of water and diluted in the appropriate solution for assay. Acid saline was used for the osteoclast activation experiment. Acid saline containing 0.01% bovine serum albumin was used for the renal adenylate cyclase assay. A vehicle consisting of 51 m*M* $CaCl_2$ and 0.01% bovine serum albumin was used for the avian hypercalcemic assay. Chemical characterization of similarly oxidized preparations of bPTH(1-34) has shown that the two methi-

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onine residues were oxidized to methionine sulfoxide; no other amino acid modifications were detected (1).

Medullary bone osteoclast activation in Japanese quail. Medullary bone, which is deposited in female Japanese quail prior to the onset of egglaying, serves as a source of calcium to supplement dietary calcium during the ovulatory cycle. Medullary bone osteoclasts are functionally synchronized during the cycle; they are active in bone resorption during shell formation and inactive during the remainder of the cycle (6, 7). Based on changes in cell ultrastructure, inactive medullary bone osteoclasts are rapidly activated by exogeneous bPTH (8, 9).

Five-month-old egg-laying Japanese quail (*Coturnix coturnix japonica*) were used in this study. Several weeks before the start of the experiment the birds were placed in individual suspended cages, which permitted the eggs to roll outside, in an environment with a light/dark cycle (0100 hr on/1500 hr off) anticipated to place oviposition at a time which was convenient for observation during normal working hours. On the day of the experiment, the birds were checked every 30 min for oviposition. Four hours after observing oviposition, the control and bPTH(1-34) solutions were injected intraperitoneally into three groups of five birds each: (a) acid saline control; (b) bPTH(1-34), 40 μg/bird; or (c) oxidized bPTH(1-34), 40 μg/bird. These injections were given at a time when the shell is not being formed and medullary bone osteoclasts are inactive. The birds were sacrificed by halothane euthanasia 20 min after injection; the femora were quickly removed, split open with a razor blade, and immersion fixed in 10% phosphate-buffered Formalin at pH 7.4. After fixation in Formalin the medullary bone was dissected out, minced into pieces about 2 mm^2, and postfixed for 1 hr in sodium cacodylate-buffered 1% osmium tetroxide at pH 7.4. The tissues were hydrated, embedded in Epon, and sectioned for electron microscopy.

The osteoclast ruffled border is a cell specialization found adjacent to bone surfaces that facilitates the resorption of bone (10, 11). The extent of the ruffled border present on osteoclasts was quantified in electron micrographs by measuring the plasma membrane perimeter on that portion of the osteoclast that was apposed to a bone surface, using morphometric methods as previously described (9). For this, measurements were made using grid overlays on micrographs of at least 25 osteoclast profiles from each bird. The osteoclast profiles met the following criteria: (a) cell was apposed to a bone surface; (b) one or more nuclei were present in the cytoplasm; and (c) the entire cell profile was present in the field of view. The data are expressed as the ratio of the perimeter of the osteoclast plasma membrane adjacent to bone to the perimeter of bone covered by the osteoclast. The differences between the treatment groups and the control groups were tested for significance by analysis of variance.

Hypercalcemic activity in Japanese quail. The same batches of oxidized and untreated bPTH(1-34) preparations used for osteoclast activation activity were assayed for hypercalcemic activity in immature male Japanese quail as previously described (12). In this assay, the injections were given iv in a volume of 0.4 ml/bird. The injection vehicle consisted of 51 m*M* $CaCl_2$ and 0.01% bovine serum albumin.

Renal adenylate cyclase activity. The same batches of oxidized and untreated bPTH(1-34) used in the other assays were used to measure adenylate cyclase-stimulating activity in a renal membrane preparation of Japanese quail kidneys (4). This *in vitro* assay is modified from assays using renal membrane preparations of rat kidneys (13–15). The data were analyzed statistically using analysis of

TABLE I. EFFECT OF OXIDIZED SYNTHETIC bPTH(1-34) AND UNTREATED SYNTHETIC bPTH(1-34) ON MEDULLARY BONE OSTEOCLAST RUFFLED BORDER DEVELOPMENT IN EGG-LAYING JAPANESE QUAIL

Treatment	No. birds	Osteoclast plasma membrane per bone surface (mm ± SE)
Control, acid saline	6	2.28 ± 0.21
bPTH(1-34)	6	4.84 ± 0.79[a]
bPTH(1-34) + H_2O_2	6	5.12 ± 0.49[b]

[a] Significantly different from controls, $P < 0.01$.
[b] Significantly different from controls, $P < 0.005$.

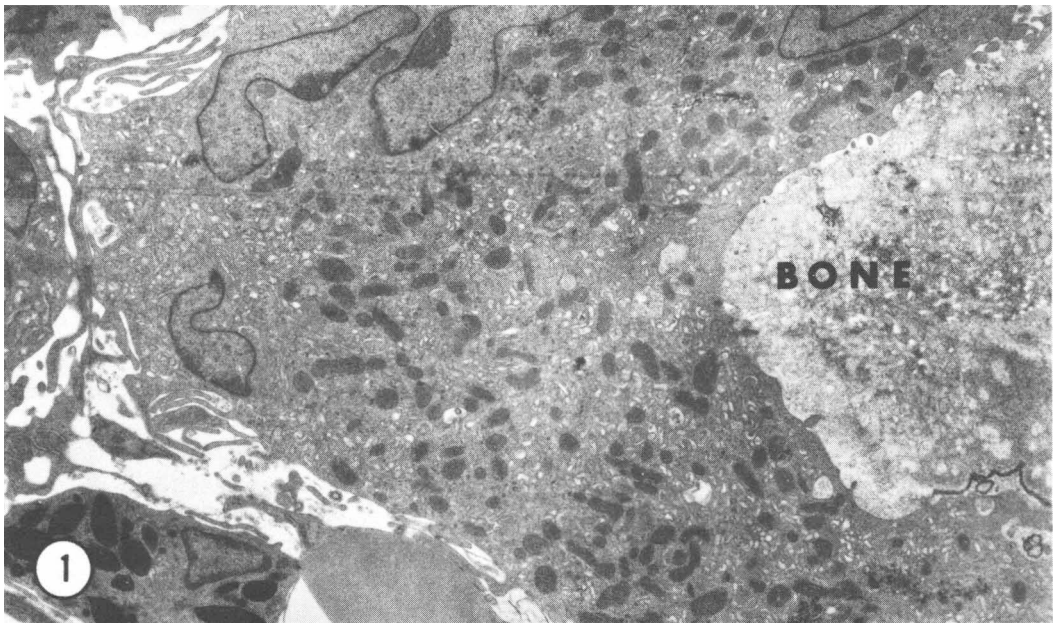

FIG. 1. Electron micrograph of a medullary bone osteoclast taken at 4 hr after oviposition and 20 min after the administration of acid saline (control). At this time in the egg-cycle, medullary bone is not being resorbed and ruffled borders on osteoclasts are rarely seen (×5450).

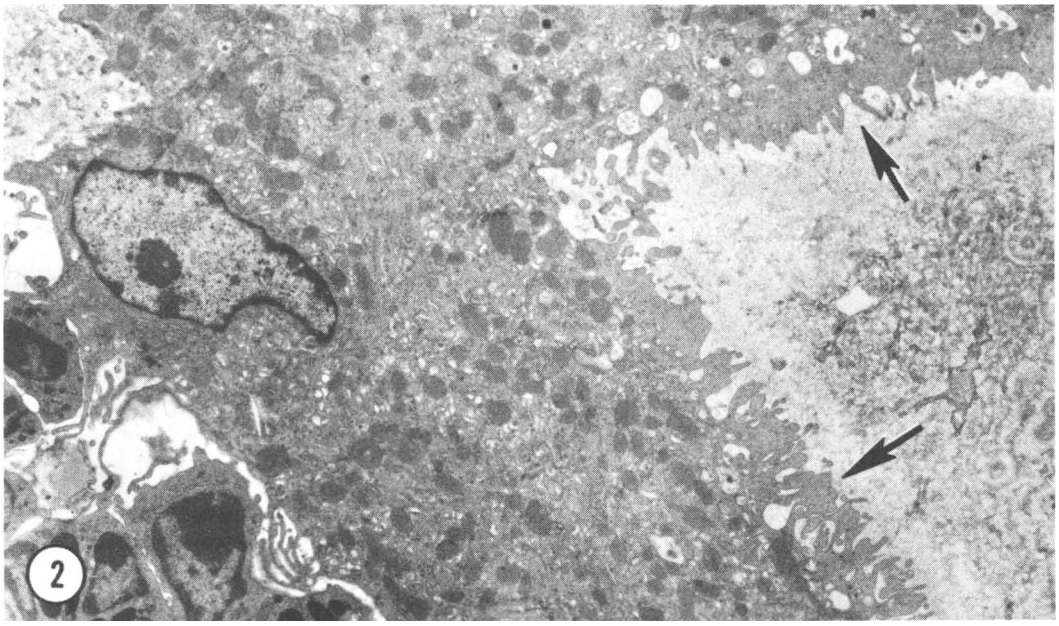

FIG. 2. Electron micrograph of a medullary bone osteoclast taken at 4 hr after oviposition and 20 min after the administration of bPTH(1-34). Ruffled borders (arrows) adjacent to bone surfaces have appeared on the osteoclasts (×4850).

variance, least-significant difference, and linear regression.

Results. Both oxidized and unoxidized bPTH(1-34) stimulated the development of osteoclast ruffled borders within 20 min after injection of the hormone in Japanese quail (Table I, Figs. 1 and 2). The hypercalcemic activity of bPTH(1-34) was also unaffected by oxidation (Table II). In contrast, mild oxidation of bPTH(1-34) significantly reduced the renal adenylate cyclase activity (Table III). Although slight residual activity was present at lower doses (90 and 270 ng/ml in assay I-102), the higher doses (0.91, 2.73, and 9.09 μg/ml in assay I-84) exhibited no activity.

Discussion. This study provides evidence of a dissociation in the ability of bPTH(1-34) to stimulate osteoclast activity and hypercalcemia from the stimulation of renal adenylate cyclase activity. The same batch of oxidized hormone was used in all assays; hence variations in hormone preparations cannot explain our results. These data provide additional evidence for the concept that bPTH(1-34) exhibits its responses through the mediation of more than one type of receptor (1–5). This concept assumes that bPTH(1-34) effects its responses as an agonist by binding with a receptor on the outside of the cell membrane. It is further assumed that modified forms of bPTH(1-34), such as the oxidized form, which exhibit a reduction in a specific response must have a reduced affinity for the receptor (or receptors) associated with the reduced response. However, the receptor or receptors mediating a response, which is unaffected by oxidation of the agonist, must retain the same affinity for the modified agonist as that associated with the untreated bPTH(1-34). Such differences in affinities are best explained by assuming different receptor types.

Because the *in vivo* osteoclast and hypercalcemic activities were unaffected by oxidation of bPTH(1-34), while the *in vitro* adenylate cyclase activation response was substantially altered, it is possible that the oxidized hormone was reduced back to an active form *in vivo.* However, the fact that the hypercalcemic activity of intact bPTH(1-84) in Japanese quail is largely inactivated by mild oxidation with hydrogen peroxide, unlike the response to oxidized bPTH(1-34) (1, 4), militates against this possibility. Mild oxidation is assumed to affect only the methionine residues at positions 8 and 18 of both the bPTH(1-34) and bPTH(1-84) molecules (1). The difference in hypercalcemic activity between the oxidized bPTH(1-34) and bPTH(1-84) is probably due to a conformational change in the intact peptide, hindering interactions with its receptor (4). The dissociation of the hypercalcemic and adenylate cyclase activities of the bPTH(1-34) molecule by oxidation is not the only known such dissociation of these two activities; the bPTH(2-34) fragment does not stimulate renal adenylate activity but remains active in the chick hypercalcemia assay (16).

TABLE II. EFFECT OF HYDROGEN PEROXIDE TREATMENT ON THE HYPERCALCEMIC RESPONSE TO bPTH(1-34) IN JAPANESE QUAIL

Treatment	Dose, iv (per bird)	No. birds	Plasma Ca[a] (mg/dl, mean ± SD)
Assay P248			
Control, vehicle alone[b]	0.4 ml	6	11.9 ± 0.37
bPTH(1-34)	0.4 μg	6	11.7 ± 0.36
bPTH(1-34)	1.2 μg	6	12.3 ± 0.20
bPTH(1-34)	3.6 μg	6	14.1 ± 0.34[c]
H_2O_2-treated bPTH(1-34)	0.4 μg	6	12.5 ± 0.27
H_2O_2-treated bPTH(1-34)	1.2 μg	6	12.9 ± 0.48
H_2O_2-treated bPTH(1-34)	3.6 μg	5	13.7 ± 0.15[c]

[a] 1 hr after injection.
[b] 51 m*M* $CaCl_2$ + 0.01% bovine serum albumin.
[c] Significant at $P < 0.01$ when compared with control group.

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

Treatment	Dose (μg/ml)	Adenylate cyclase activity (pmole cAMP/mg protein)	
		Assay I-102	Assay I-84
Control (vehicle only[a])	—	99 ± 17.7	90 ± 5.2
bPTH(1-34)	27	154 ± 22.4[b]	—
	90	227 ± 22.0[b]	221 ± 24.6[b]
	270	520 ± 6.6[b]	384 ± 25.3[b]
	910	—	461 ± 19.7[b]
H_2O_2-treated bPTH(1-34)	27	139 ± 9.6	—
	90	147 ± 13.6[b]	—
	270	152 ± 10.6[b]	—
	910	—	131 ± 28.5
	2730	—	121 ± 18.2
	9090	—	113 ± 7.4
NaF, 10 m*M*	—	1415 ± 291.5[b]	1247 ± 72.3[b]

Note. Treatments were done in triplicate; data are means ± SE. The standard curves for the untreated bPTH(1-34) in assays I-102 and I-84 exhibited significant ($P < 0.001$) slopes and did not depart from linearity.

[a] Acid saline containing 0.01% bovine serum albumin, pH 3.0.

[b] Significantly different ($P < 0.05$) from control.

The dissociation of osteoclast and renal adenylate cyclase activities, as indicated in the present study, is particularly interesting in view of data showing that bone resorption, but not bone adenylate cyclase, is stimulated with several bPTH fragments (17). These data support the suggestion that cyclic AMP does not necessarily play a major role in PTH-stimulated bone resorption (18).

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