

Uterine Relaxing Action of Parathyroid Hormone: Effect of Oxidation and Methionine Substitution (41818)

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Abstract. The effects of bPTH-(1-34), oxidized bPTH-(1-34), [Nle⁸,Nle¹⁸,Tyr³⁴]bPTH-(1-34) amide, and oxidized [Nle⁸,Nle¹⁸,Tyr³⁴]bPTH-(1-34)amide were tested in an *in vitro* rat uterine assay. When bPTH-(1-34) was treated with hydrogen peroxide (H₂O₂), the ability of this peptide to reduce oxytocin-stimulated uterine contraction *in vitro* was no longer evident. An analogue of bPTH-(1-34), in which the methionines at positions 8 and 18 were replaced with norleucine ([Nle⁸,Nle¹⁸,Tyr³⁴]bPTH-(1-34)amide), was capable of reducing oxytocin-stimulated uterine contraction. However, when the [Nle⁸,Nle¹⁸,Tyr³⁴]bPTH-(1-34)amide was oxidized, it retained the ability to reduce uterine contraction. Since we have previously shown that H₂O₂ oxidation affected only the methionines, these results suggest that the methionines are not necessary for the uterine activity of bPTH-(1-34). We have previously shown that oxidation of bPTH-(1-34) also destroys its blood vessel relaxing activity but has no effect in the rat or the Japanese quail hypercalcemic assays. These data combined with the results of the present studies suggest that the uterine and vascular smooth muscle relaxing properties of bPTH-(1-34) may require the same structural conformation and that this conformation is different from that required for the hypercalcemic action of the peptide.

The ability of parathyroid hormone (PTH) to regulate serum calcium and phosphate levels is well recognized (1). Target tissues involved in this response include bone and kidney (2, 3). A vasodilatory effect of PTH has been reported by numerous investigators and this property of the hormone has received extensive investigation recently by Pang and colleagues (4, 5). In addition, we have reported that synthetic bovine parathyroid hormone fragment containing the amino terminal 1-34 amino acids [bPTH-(1-34)] inhibited oxytocin, acetylcholine, prostaglandin F_{2α}, and electrically stimulated uterine contraction *in vitro* (6). This action of bPTH-(1-34) on uterine contraction appears to be due to a direct action of the peptide fragment on the uteri *in vitro* (7). Mild oxidation with hydrogen peroxide (H₂O₂) of the intact bovine PTH [bPTH-(1-34)] results in inactivation of the hypercalcemic response of the hormone (8) and inactivation of the hypotensive response in rats (9). It has also been reported that oxidized bPTH-(1-34) is inactive in the dog hypercalcemic and phosphaturic assays (10). In con-

trast, both the avian hypercalcemic response and the renal 1-hydroxylase-stimulating activity of bPTH-(1-34) have been reported to resist inactivation with H₂O₂ treatment (11). Yet the hypotensive property of bPTH-(1-34) is lost upon treatment with H₂O₂ (9). Other properties of bPTH-(1-34) which are reduced or abolished by mild oxidation with H₂O₂ are the hyperphosphaturic response (12) and the renal adenylate cyclase activity (11).

The purpose of the present investigation was to examine the effect of mild oxidation of bPTH-(1-34) with H₂O₂ on the ability of the peptide to inhibit or reduce uterine contraction. Since oxidation of bPTH-(1-34) has been reported to affect only the methionine residues at positions 8 and 18 (9), the action of a synthetic analogue where norleucine is substituted for the methionines [Nle⁸,Nle¹⁸,Tyr³⁴]bPTH-(1-34)amide was examined to determine if replacement of the methionines altered the peptides action on uterine contraction. Also, the effect of oxidation of this analogue was tested to more completely examine the effects of oxidation and the importance of the methionines for bPTH-(1-34) activity.

Material and Methods. Mature female Wistar rats, which weighed 150-200 g were

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injected intraperitoneally with the synthetic estrogen diethylstilbestrol (DES, 50 $\mu\text{g}/\text{rat}$) 14–18 hr before being sacrificed by cervical dislocation. The uterine horns were dissected out and trimmed of surrounding connective tissue and immersed immediately in a tissue chamber containing Van Dyke–Hastings solution (13) equilibrated with 5% carbon dioxide and 95% oxygen (pH 7.4) at a constant temperature (32°C) as previously described (6). A resting tension between 0.5 and 1.0 g was applied to the suspended uterine horns and contractions were measured isometrically with a Grass force-displacement transducer (model FT.03) and recorded with a Grass polygraph (model 7D). The tissue was allowed to equilibrate for 1 hr before beginning the experiment.

Oxytocin (Syntocinon, Sandoz, 0.5 mU/ml) was used to stimulate uterine contraction, which was measured as a change in gram tension. Synthetic bovine parathyroid hormone bPTH-(1-34) was obtained from Peninsula Laboratories (San Carlos, Calif.; assigned potency 10 U/ μg). The synthetic analogue of bPTH-(1-34) where norleucine (Nle) is substituted for the methionines at positions 8 and 18, [Nle⁸,Nle¹⁸,Tyr³⁴]bPTH-(1-34)amide, was obtained from Bachem Laboratories (Torrance, Calif.; assigned potency 10 U/ μg). Doses of peptides used were 2.4×10^{-7} M, 7.3×10^{-8} M, 2.4×10^{-8} M, and 2.4×10^{-9} M and were diluted with 0.9% NaCl. Mild oxidation of bPTH-(1-34) and [Nle⁸,Nle¹⁸,Tyr³⁴]bPTH-(1-34)amide with H₂O₂ was performed as previously described (11). Briefly, this procedure consists of dissolving 1 mg of the peptide in acid saline, pH 3, to a concentration of 1000 U/ml and incubated at 25°C with 10 μl of 30% H₂O₂ for 30 min. Control-treated peptide was treated similarly except that water was added in place of the H₂O₂. The samples were then lyophilized overnight and reconstituted in water to the same volume.

The inhibitory effect of bPTH-(1-34) on oxytocin-stimulated uterine contraction has been described previously (6). Briefly this procedure entails adding oxytocin (0.5 mU/ml) and recording the contractile force. After thorough rinsing, the same dose of oxytocin is tested again to determine if a similar response was obtained. If similar contractile responses were obtained for the oxytocin then

varying doses of bPTH-(1-34), H₂O₂-treated bPTH-(1-34), [Nle⁸,Nle¹⁸,Tyr³⁴]bPTH-(1-34)amide, or H₂O₂-treated [Nle⁸,Nle¹⁸,Tyr³⁴]bPTH-(1-34)amide (2.2×10^{-7} to 2.4×10^{-9} M) were added to the bath 1 min prior to addition of 0.5 mU/ml of oxytocin. In this manner log dose–response curves of the peptides were obtained in the presence of one dose of oxytocin.

Statistical analysis included Student's *t* test and paired *t* tests when experimental and control treatments were given to the same tissue. Data are expressed as the mean \pm the standard error. In all experiments, each data point represents between 4 and 14 trials.

Results. Figure 1 shows that both bPTH-(1-34) and the synthetic analogue reduce oxytocin-stimulated uterine contraction in a dose-related manner and do not differ significantly from one another. The data are expressed as the percentage of oxytocin-stimulated contraction in the presence of varying doses of either bPTH-(1-34) or its analogue [Nle⁸,Nle¹⁸,Tyr³⁴]bPTH-(1-34)amide. The control (oxytocin, 0.5 mU/ml.) response is 100%. The data were normalized to percentage of control response due to the variability (1.0–3.0 g) between individual uterine horns.

Data in Figs. 2 and 3 are expressed as the percentage inhibition of oxytocin (0.5 mU/

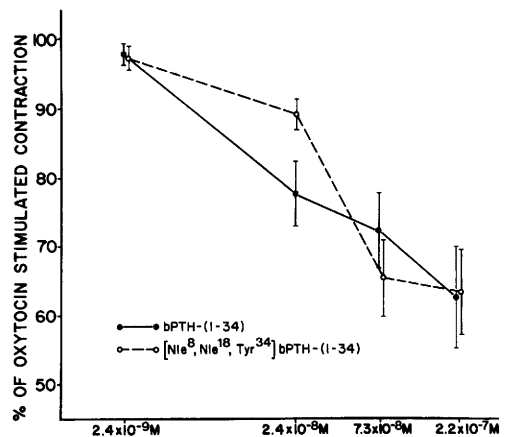


FIG. 1. Dose responses of the inhibitory effect of bPTH-(1-34) and [Nle⁸,Nle¹⁸,Tyr³⁴]bPTH-(1-34)amide on oxytocin (0.5 mU/ml)-stimulated uterine contraction *in vitro*. Concentration of peptides on the abscissas and the percentage oxytocin-stimulated uterine contractions on the ordinate. The two responses do not differ statistically.

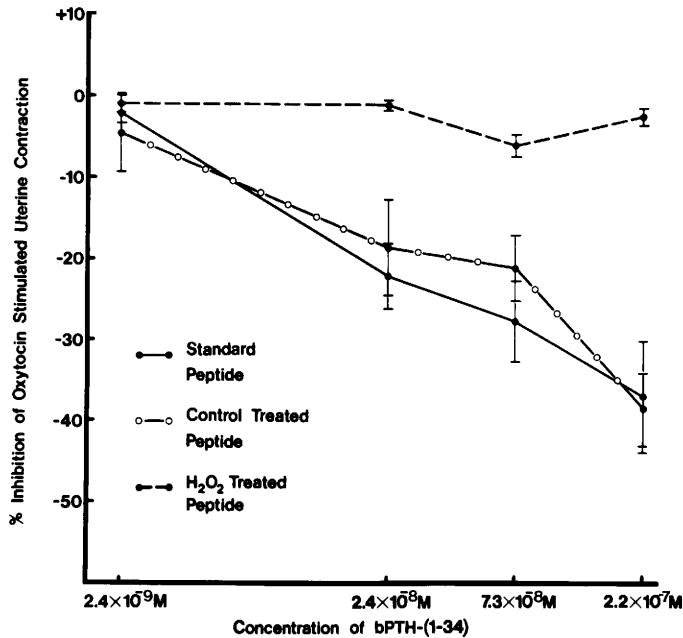


FIG. 2. Typical dose-response curves of the inhibitory effect of bPTH-(1-34) and control-treated bPTH-(1-34). The H₂O₂-treated bPTH-(1-34) has lost its ability to inhibit oxytocin (0.5 mU/ml)-stimulated contractions. The H₂O₂-treated peptide differs significantly ($P < 0.05$) from the other peptides at the concentrations of 2.4×10^{-8} M and greater.

ml)-stimulated uterine contraction. Figure 2 demonstrates that when bPTH-(1-34) is treated by mild oxidation with H₂O₂ (H₂O₂-treated peptide) the peptide fragment's ability

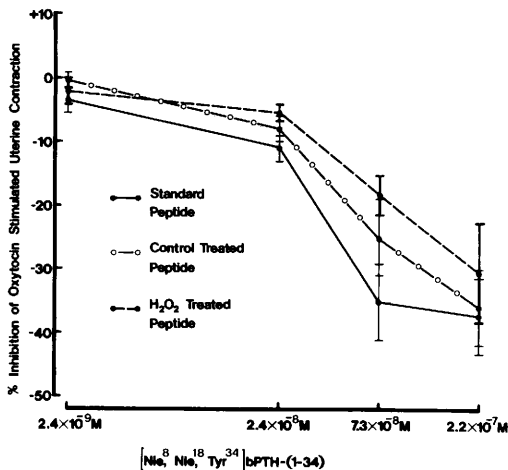


FIG. 3. Dose-response curves of the inhibitory effect of standard, control, and H₂O₂-treated [Nle⁸, Nle¹⁸, Tyr³⁴]bPTH-(1-34)amide. The dose responses are not statistically significantly different from one another.

to reduce oxytocin-stimulated uterine contraction was abolished. When bPTH-(1-34) was exposed to the same conditions used for oxidation except water replaced H₂O₂ (control-treated peptide), its response was similar to that of untreated bPTH-(1-34) (standard peptide). These results suggest that the inactivation following H₂O₂ treatment of the peptide is due to the mild oxidation by H₂O₂ rather than some other factor in the oxidation procedure method.

Figure 3 shows the response of oxytocin-stimulated contraction after pretreatment with [Nle⁸, Nle¹⁸, Tyr³⁴]bPTH-(1-34)amide. The norleucine analogue reduced oxytocin-stimulated uterine contraction in a dose-related manner. Treatment with H₂O₂ failed to abolish the uterine relaxing effect of the norleucine analogue. The control treated norleucine analogue reduced uterine contraction in a similar manner as the standard norleucine analogue.

Discussion. Parathyroid hormone is recognized as one of the mineral (calcium) regulating hormones. It is generally believed that PTH is acting on mineralized tissue and the kidneys to maintain serum calcium concen-

trations. The hypercalcemic activity of bPTH-(1-34) may be determined in a number of different biological assays, including the avian (14), rat (15), and dog (16) hypercalcemic assays and various *in vitro* bone calcium mobilization assays (2). Synthetic bPTH-(1-34) is also hyperphosphaturic (17), stimulates renal adenylate cyclase activity (11, 18) and the hormone is also capable of acting as a hypotensive agent (4, 5, 19). Pang and colleagues have investigated in depth the hypotensive properties of bPTH-(1-34) (4, 5). We have also reported that bPTH-(1-34) inhibits stimulated uterine contraction *in vitro* (6) and *in vivo* (20). This uterine relaxing action of bPTH-(1-34) appears to be a direct action of the peptide fragment (7).

When the intact hormone, bPTH-(1-34) is treated with H_2O_2 its hypercalcemic activity is abolished (8, 14). By comparison, mild oxidation does not inactivate the avian hypercalcemic activity of the synthetic fragment, bPTH-(1-34) (9). Yet the hyperphosphaturic and the hypotensive responses to bPTH-(1-34) are abolished when treated with H_2O_2 (11, 12). In the present study we have demonstrated that H_2O_2 -treated bPTH-(1-34) also inactivates the peptide fragments ability to inhibit oxytocin stimulated uterine contraction. In a previous study (9) the amino acid compositions of oxidized and untreated bPTH-(1-34) were reported and the methionines were oxidized in H_2O_2 -treated peptide. The oxidation is believed to affect only the methionine residues at positions 8 and 18. The control-treated bPTH-(1-34) was treated identically to the H_2O_2 -treated peptide and it retained the ability to reduce oxytocin-stimulated uterine contraction. These results suggest that the replacement of the methionines by methionine sulfoxide was probably the cause of the inactivation of the uterine effect. There have been other reports that treatment of bPTH-(1-34) with H_2O_2 results in partial oxidation to produce different forms of the fragment (21). In bone calcium flux studies *in vitro* and bone cell adenosine 3',5'-cyclic phosphate (cyclic AMP) assays, H_2O_2 -treated bPTH-(1-34) was approximately 100 times less potent (22). At the concentrations of H_2O_2 -treated bPTH-(1-34) tested, it was ineffective in inhibiting oxytocin stimulated uterine contraction *in vitro*.

We examined the role of the methionines further by testing the synthetic analogue where the methionines at positions 8 and 18 are replaced by isosteric norleucine, [Nle⁸,Nle¹⁸,Tyr³⁴]bPTH-(1-34)amide. The norleucine analogue is biologically active in the renal adenylate cyclase assay (23) and is equipotent with bPTH-(1-34) in bone cyclic AMP-stimulating activities (24) and hypotensive assays (9). Similarly, when the norleucine analogue was tested in the uterine assay it did not differ significantly from bPTH-(1-34) activity. Oxidation of the norleucine analogue failed to inactivate its ability to reduce uterine contraction and H_2O_2 -treated peptide did not differ from control or standard norleucine analogues. Recently, it has been suggested that the amino acid tryptophan (Trp) is affected by the oxidation procedure and this may be the cause of the inactivation of the peptide (21). However, the H_2O_2 -treated norleucine analogue retained the ability to inhibit oxytocin-stimulated uterine contraction. These results suggest that if Trp is affected during our oxidation procedure it is not inactivating the peptide. These results further substantiate the effect of H_2O_2 on the methionines and further indicate that the methionines are not absolutely necessary for any of the uterine activities associated with bPTH-(1-34). The effects of other PTH analogue fragments [(3-34) and (7-34)] on uterine tissue are of interest and this data will be reported elsewhere (Shew and Pang, submitted for publication).

In summary, bPTH-(1-34) and the analogue [Nle⁸,Nle¹⁸,Tyr³⁴]bPTH-(1-34)amide both reduce oxytocin-stimulated uterine contraction *in vitro* in a similar dose-related manner. Mild oxidation of bPTH-(1-34) with H_2O_2 inactivates this property of the peptide and this oxidative procedure appears to affect only the methionines at positions 8 and 18. When [Nle⁸,Nle¹⁸,Tyr³⁴]bPTH-(1-34)amide was treated with H_2O_2 it retained the ability to reduce oxytocin-stimulated uterine contractions. These results suggest that the methionines are not absolutely necessary for the peptide's uterine activity. Also, it has been reported previously that oxidized bPTH-(1-34) lost its blood vessel relaxing activity (9) but was fully active in the Japanese quail hypercalcemic assay. These results suggest that there may be a structural conformation common

to the vascular and uterine action which is different from that for the hypercalcemic action of the peptide. Such conformational change has been reported recently (25).

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