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PARATHYROID HORMONE-RELATED PEPTIDE AS A LOCALLY PRODUCED VASORELAXANT: REGULATION OF ITS mRNA BY HYPERTENSION IN RATS

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The present study was undertaken to examine the effect of parathyroid hormone-related peptide (PTHrP) on the vascular tone as well as the expression of its mRNA in the cardiovascular system and its regulation in response to systemic hypertension in Sprague-Dawley rats. In aortic rings precontracted with 0.3 μ M norepinephrine PTHrP(1-34) caused a dose-dependent relaxation with the maximal response of 33% being observed at 10^{-6} M. PTHrP was nearly equipotent to PTH or CGRP in the vasodilatory action. Ribonuclease protection assay and Northern blot analysis demonstrated that PTHrP mRNA levels in the heart and aorta were increased as a result of systemic hypertension induced by constant infusion of angiotensin II and salt loading. The results of our in vivo studies suggest an autocrine/paracrine role for PTHrP as a local regulator of the vascular tone.

Parathyroid hormone-related peptide (PTHrP) was originally purified as a PTH-like humoral factor causing cancer-associated hypercalcemia (1). Its cDNA predicted a novel 141 amino acid protein with 8 out of 13 residues at the amino terminus being identical with those of PTH (2). PTHrP produced and secreted by various cancers circulates with elevated concentrations (3), interacts with the PTH/PTHrP receptor in bone and kidney (4), and causes hypercalcemia in cancer patients.

Subsequent studies on the tissue distribution of its mRNA as well as immunoreactivity revealed that PTHrP is expressed in a number of normal

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Abbreviations: PTHrP, parathyroid hormone-related peptide; PTH, parathyroid hormone; CGRP, calcitonin gene-related peptide; A-II, angiotensin II; NE, norepinephrine; DBcAMP, dibutyryl cyclic AMP; SD, Sprague-Dawley; BSA, bovine serum albumin; RNase, ribonuclease; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto rat.

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adult and fetal tissues albeit at very low levels (5), and emerging evidence suggests that PTHrP plays a wide variety of physiologic roles mainly as an autocrine/paracrine factor (6,7). Of particular interest and importance is its role as a smooth muscle relaxant in uterus (8), bladder(9), and stomach (10). It has been reported that PTHrP is produced by rat aortic smooth muscle cells in primary culture (11) and at the same time exerts a potent vasodilatory action in vascular smooth muscle (12,13). In addition, the production of PTHrP in cultured aortic smooth muscle cells seems to be induced by vasoactive agents, such as endothelin, norepinephrine, thrombin, and angiotensin II (11,14), raising the possibility that there may exist a local feedback regulation to maintain the vascular tone. However, it remains to be elucidated whether the expression of PTHrP gene in the vascular system is in fact regulated in vivo by these vasoactive substances and/or resultant changes in blood pressure.

In the present study we have examined the expression of PTHrP mRNA in the cardiovascular system and its regulation by elevated blood pressure in Sprague-Dawley rats. Our results indicate that PTHrP exerts a potent vasorelaxant effect on rat aortic rings in an organ bath and that its mRNA expression in the heart and aorta is substantially increased as a result of hypertension induced by angiotensin II infusion and high salt diet. Taken together, these results are consistent with an autocrine/paracrine role for PTHrP in the local regulation of the vascular tone.

MATERIALS AND METHODS

Materials - Human PTHrP(1-34), human PTH(1-34), and human calcitonin gene-related peptide (CGRP) were kindly provided by Asahi Chemical Industry Co. Ltd. (Tokyo, Japan). Angiotensin II (A-II) and forskolin were purchased from Sigma Co. (St. Louis, MO) and norepinephrine (NE) from Sankyo Co. Ltd. (Tokyo, Japan). Dibutyryl cAMP (DBcAMP) was a kind gift from Yamasa Shoyu Co. Ltd. (Chiba, Japan). [α ^{32}P]UTP and [α ^{32}P]dCTP were purchased from Amersham (Buckinghamshire, UK).

Animals - Seven-week-old male Sprague-Dawley (SD) rats were purchased from Charles River Japan Inc. (Atsugi, Japan) and fed a standard rodent chow containing 0.66% sodium chloride (Oriental Yeast, Tokyo, Japan) and water ad libitum. In the experiments to examine the effect of systemic hypertension on PTHrP mRNA expression, animals were randomly divided into four groups and graded degree of hypertension was induced by 1) standard diet (0.66% sodium chloride), 2) high salt diet (8% sodium chloride), 3) administration of angiotensin II, and 4) angiotensin II plus high salt diet. Angiotensin II was dissolved in 0.9% saline and infused for the indicated days at a constant rate of 125 ng/min through an osmotic minipump (Alzet model 2002, Alza, Palo Alto, CA) implanted intraperitoneally. Mean blood pressure was monitored in conscious unrestricted rats through a catheter inserted into the femoral artery using a pressure transducer (model TP-200T, Nihon Kohden, Tokyo, Japan) and recorded on a thermal array recorder (model WS-641G, Nihon Kohden, Tokyo, Japan). The catheterization of the femoral artery was performed under ether anesthesia approximately 24 hours before the measurement of blood pressure.

Organ bath experiments - Aortic rings were prepared as described previously (15). In brief, thoracic aorta was removed and carefully freed of connective tissue without damaging the endothelium. The aorta was cut into segments of 4 mm and placed in an 8-ml organ bath chamber containing oxygenated (95% O_2 and 5% CO_2) Krebs-bicarbonate solution at 37° C. The aortic ring was suspended from a force-displacement transducer (TB-651T, Nihon-Koden, Tokyo, Japan) under 1.0 g tension and allowed to equilibrate for 90 min. For experiments, the ring was precontracted with 0.3 μM norepinephrine, and then test agents (PTHrP, PTH and CGRP) dissolved in 0.1% BSA were directly added to the organ bath.

RNA analysis - Tissues were homogenized in guanidinium thiocyanate solution as described previously (16) and total cellular RNA was prepared by pelleting through cesium chloride cushion and quantitated by A_{260}. Ribonuclease (RNase) protection assay was performed using 50 μg total RNA as described previously (17). The antisense RNA probe was prepared from the linealized rat PTHrP cDNA (18) (courtesy of Dr. Mark Thiede, Pfizer Inc., Groton, CT), using [α ^{32}P]UTP and in vitro transcription system (Promega, Madison, WI). Northern blot analysis was performed using rat PTHrP cDNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes as described previously (16).

Statistical analysis - Data were expressed as the mean ± SEM, and the statistical significance was determined by paired and unpaired Student's t test. A value of p less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Effect of PTHrP on the Vascular Tone

We first examined the vasodilatory effect of PTHrP using rat aortic rings in an organ bath. As shown in a representative tracing from four separate experiments (Figure 1), increasing concentrations of PTHrP(1-34) inhibited the norepinephrine-induced contraction of aortic rings. Figure 2 clearly shows that the vasorelaxant effect of PTHrP was dose-dependent with the maximal relaxation of 33 ± 5 % being observed at 10^{-6} M. PTHrP was nearly equipotent to the same dose of PTH (49 ± 6 %) or CGRP (37 ± 3 %) in relaxing aortic rings, and agents that are known to increase intracellular cAMP levels, such as forskolin and DBcAMP, mimicked the vasodilatory effect of PTHrP (data not shown). Taken together with the findings that PTH/PTHrP receptor is coupled to both adenylate cyclase and phospholipase C pathways (19), it is conceivable that the vasorelaxant effect of PTHrP is at least in part mediated through the stimulation of adenylate cyclase. The vasodilatory effect of PTHrP was observed when the aorta was denuded of the endothelial layer by gentle rubbing with cotton (data not shown), suggesting that PTHrP acts directly on the vascular smooth muscle cells independently of an endothelial-derived vasoactive substance(s).

PTHrP mRNA Expression in the Cardiovascular System

Using RNase protection technique with a rat PTHrP antisense RNA probe, we demonstrated that low-abundance PTHrP mRNA was expressed in normal rat tissues including aorta and heart (Figure 3). These results are consistent

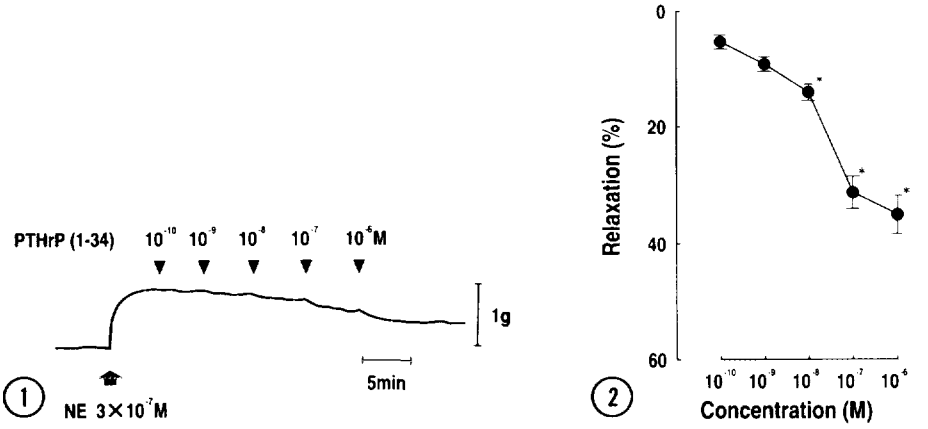

Figure 1. Effect of PTHrP on Norepinephrine-Induced Vascular Contraction.
Rat aortic rings were prepared as described in MATERIALS AND METHODS. After equilibration in an organ bath, the rings were precontracted with 0.3 μM norepinephrine (NE) and then treated with the indicated concentrations of PTHrP(1-34). A representative tracing from four separate experiments is shown.

Figure 2. Dose-response of the Effect of PTHrP on Norepinephrine-Induced Vascular Contraction.
The effect of various concentrations of PTHrP(1-34) on norepinephrine-induced vascular contraction was examined in the same way as in Figure 1, and the results were shown as mean ± SEM of % relaxation. * $p < 0.05$ compared with precontraction level.

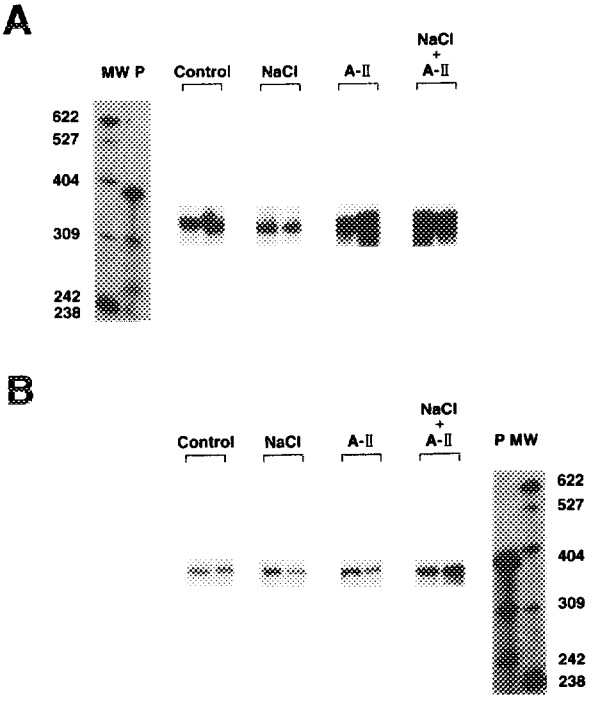

Figure 3. Regulation of PTHrP mRNA Expression in Rat Heart (A) and Aorta (B) by Hypertension on Day 12.
Normal SD rats were given vehicle (control), high salt diet (NaCl), constant infusion of angiotensin II (A-II), or both high salt diet and angiotensin II (NaCl + A-II). Total cellular RNA was extracted from the heart (A) and aorta (B) on day 12, and PTHrP mRNA levels were quantitated by RNase protection assay as described in MATERIALS AND METHODS. MW and P indicate molecular weight markers (pBR322 Msp I fragments) and the undigested probe, respectively.

with those of in vitro studies that PTHrP is produced and secreted by rat aortic smooth muscle cells in primary culture (11) and cardiac myocytes (20).

It has been reported that PTHrP mRNA levels in the aorta of the spontaneously hypertensive rat (SHR) are increased compared with those in the normotensive Wistar-Kyoto rat (WKY), raising the possibility that PTHrP mRNA expression in the aorta changes as a function of blood pressure (21). However, the possibility that differences in the genetic background between SHR and WKY affected the expression of PTHrP gene cannot be ruled out. In order to determine whether PTHrP gene expression in the cardiovascular system was regulated by changes in blood pressure in normal SD rats, graded degree of hypertension was induced by giving high salt diet and/or continuous infusion of angiotensin II. As shown in Table 1, high salt diet alone had no effect on blood pressure while constant infusion of angiotensin II for 12 days caused a significant elevation in blood pressure. As reported previously (22), angiotensin II plus salt loading further raised the blood pressure in SD rats (Table 1).

Tissues were removed from the four groups of rats, and PTHrP mRNA levels were determined by a quantitative RNase protection assay. As shown in

Table 1. Mean blood pressure in the four experimental groups

Treatment	Mean blood pressure
	mm Hg
Control	113 ± 3
NaCl	115 ± 3
A-II	143 ± 4[a]
A-II + NaCl	182 ± 12[a,b]

In nine-week-old male Sprague-Dawley rats graded degree of hypertension was induced by 1) standard diet containing 0.66% sodium chloride (Control), 2) high salt diet containing 8% sodium chloride (NaCl), 3) constant infusion of angiotensin II (A-II), and 4) angiotensin II plus high salt diet (A-II + NaCl). After each treatment for 12 days, mean blood pressure was measured through a catheter inserted into the femoral artery as described in MATERIALS AND METHODS. Data are expressed as mean ± SEM (n = 6). [a] and [b] indicate significant difference ($p<0.05$) from the control and the A-II group, respectively.

Figure 3A, salt loading alone had no effect on PTHrP mRNA expression in the heart whereas a marked increase in PTHrP mRNA was observed in the hypertensive rats treated with angiotensin II or angiotensin II plus high salt diet for 12 days. Repeated experiments revealed no apparent difference in PTHrP mRNA levels between the angiotensin II-infused group and the angiotensin II plus high salt diet group, suggesting either that PTHrP gene expression is regulated primarily through the action of angiotensin II on the heart, and not as a result of hypertension itself, or that the response of PTHrP mRNA is saturated at a certain degree of hypertension.

PTHrP mRNA expression in the aorta was also increased when marked hypertension was induced by angiotensin II plus high salt diet for 12 days (Figure 3B). In contrast to its regulation in the heart, moderate hypertension induced by angiotensin II infusion alone caused no apparent increase in PTHrP mRNA levels in the aorta (Figure 3B), suggesting either that the aorta does not respond to angiotensin II itself but to the marked elevation in blood pressure or that the aorta is less sentitive to changes in blood pressure than the heart. Since plasma norepinephrine concentrations are increased by angiotensin II plus sodium chloride loading, but not by angiotensin II infusion alone, and since norepinephrine has been reported to increase PTHrP mRNA expression in aortic smooth muscle cells (11), it is also possible that norepinephrine plays a role in the induction of PTHrP mRNA in the aorta.

Similar increase in PTHrP mRNA was observed in the heart of angiotensin II or angiotensin II plus high salt diet group on day 4 (Figure 4A), but no change in PTHrP mRNA was seen in the aorta of either experimental group on day 4 (Figure 4B). PTHrP mRNA expression in other tissues, such as lung and brain, was not influenced by the changes in blood pressure (data not shown).

The mechanism by which high blood pressure causes an induction of PTHrP mRNA in the smooth muscle cells of the cardiovascular system is not clear. PTHrP mRNA expression in the myometrium of the uterus at a late stage of pregnancy is shown to depend on uterine occupancy based on the observation that its expression is confined to the pregnant horn when unilateral pregnancy is induced (8). In fact, it has been demonstrated that PTHrP gene expression in uterus (24), bladder (9), and aortic smooth muscle cells (25) is induced by mechanical stretch. In light of these observations, it is tempting to speculate that PTHrP gene expression in the cardiovascular system is regulated by mechanical stretch increased by systemic hypertension in the current model. It is also intriguing to note that mechanical stretch and angiotensin II synergistically stimulate PTHrP mRNA expression in rat aortic smooth muscle cells in vitro (25). Further studies will be required to elucidate the detailed molecular mechanism by which mechanical stimulus is transduced to a nuclear signal(s) leading to the activation of PTHrP gene in smooth muscle cells.

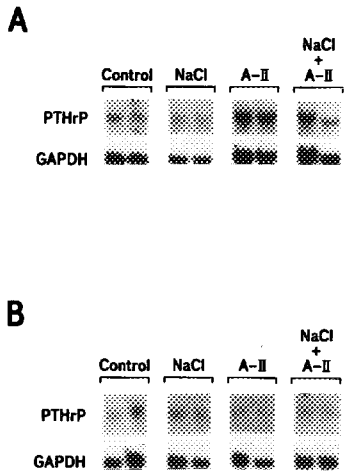

Figure 4. Regulation of PTHrP mRNA Expression in Rat Heart (A) and Aorta (B) by Hypertension on Day 4.
Total cellular RNA was extracted from the heart (A) and aorta (B) of the four groups of rats on day 4, and PTHrP and GAPDH mRNA levels were determined by Northern blot analysis as described in MATERIALS AND METHODS.

PTH and PTHrP genes share many structural and functional features and are thought to have arisen through duplication from a common ancestor gene. While PTH has evolved as a major calcium-regulating hormone produced exclusively in the parathyroid glands, PTHrP is a product of almost all normal tissues and exerts diverse physiologic functions in an autocrine/paracrine fashion (7). Although it is widely accepted that besides its classic actions in calcium homeostasis, PTH exerts a variety of nonclassic actions, such as a potent vasodilatory effect (26), its physiologic significance remains unclear. It is now evident that PTH and PTHrP binds with almost identical affinity to the molecularly cloned PTH receptor, thus referred to as the PTH/PTHrP receptor (4). Binding of PTH and PTHrP has been demonstrated in the vascular smooth muscle (27) and RNA analysis using the cloned PTH/PTHrP receptor cDNA probe has shown that its mRNA is expressed in a number of normal rat tissues including aorta and heart (28). Taken together, it appears likely that PTHrP, rather than PTH, is the natural ligand for the PTH/PTHrP receptor expressed in the cardiovascular system and plays an important physiologic role in maintaining the vascular tone.

In conclusion, the present results suggest the existence of a local feedback regulation in vivo between PTHrP gene expression and its action in the vascular system: when blood pressure is elevated by vasoconstrictors, such as angiotensin II, norepinephrine and endothelin, PTHrP gene expression is induced either through the direct action of the vasoactive substances on the vascular smooth muscle cells or in response to the mechanical stretch

stimulated by resultant hypertension, and PTHrP thus produced and secreted acts on nearby smooth muscle cells to cause vasorelaxation, thereby playing a physiologic role in the local regulation of the vascular tone.

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