Nitric oxide as a second messenger in parathyroid hormone-related protein signaling

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Abstract

Parathyroid hormone (PTH)-related protein (PTHrP) is produced in smooth muscles and endothelial cells and is believed to participate in the local regulation of vascular tone. No direct evidence for the activation of endothelium-derived nitric oxide (NO) signaling pathway by PTHrP has been found despite attempts to identify it. Based on direct *in situ* measurements, it is reported here for the first time that the human PTH/PTHrP receptor analogs, hPTH(1–34) and hPTHrP(1–34), stimulate NO release from a single endothelial cell. A highly sensitive porphyrinic microsensor with a response time of 0.1 ms and a detection limit of 1 nmol/l was used for the measurement of NO. Both hPTH(1–34) and hPTHrP(1–34) stimulated NO release at nanomolar concentrations. The peak concentration of 0.1 μ mol/l hPTH(1–34)- and 0.1 μ mol/l hPTHrP(1–34)-stimulated NO release was 175 ± 9 and 248 ± 13 nmol/l respectively. This represents about 30%–40% of maximum NO concentration recorded in the presence of (0.1 μ mol/l) calcium ionophore. Two

competitive PTH/PTHrP receptor antagonists, 10 μ mol/l [Leu¹¹,D-Trp¹²]-hPTHrP(7–34)amide and 10 μ mol/l [Nle^{8,18},Tyr³⁴]-bPTH(3–34)amide, were equipotent in antagonizing hPTH(1–34)-stimulated NO release; [Leu¹¹,D-Trp¹²]-hPTHrP(7–34)amide was more potent than [Nle^{8,18},Tyr³⁴]-bPTH(3–34)amide in inhibiting hPTHrP(1–34)-stimulated NO release. The PKC inhibitor, H-7 (50 μ mol/l), did not change hPTH(1–34)- and hPTHrP(1–34)-stimulated NO release, whereas the combined effect of 10 μ mol/l of the cAMP antagonist, Rp-cAMPS, and 50 μ mol/l of the calmodulin inhibitor, W-7, was additive.

The present studies show that both hPTH(1–34) and hPTHrP(1–34) activate NO production in endothelial cells. The activation of NO release is through PTH/PTHrP receptors and is mediated via the calcium/calmodulin pathway.

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Introduction

Apart from being a major physiological regulator of calcium homeostasis, parathyroid hormone (PTH) is known to relax vascular smooth muscle and to acutely lower blood pressure in several species of vertebrates. Accordingly, PTH proved to be a potent vasodilator in dogs (Charbon 1968), rats (Pang *et al.* 1980a), chickens (Pang *et al.* 1980b), frogs (Chiu *et al.* 1983) and snakes (Sham *et al.* 1984). PTH has also been shown to lower blood pressure in hypertensive rats (Nakamura *et al.* 1981). Moreover, responsiveness *in vivo* of small arterioles to PTH fragment 1–34 but not fragment 3–34 indicated that N-terminally located amino acids are important for vascular dilatation (Dowe & Joshua 1987). Despite this evidence, the relative importance of PTH as a physiological regulator of cardiovascular hemodynamics has been debated (Bukoski *et al.* 1995). This is, in part, due to the fact that supra-

physiological concentrations of the hormone were often required to produce vasorelaxant effects.

Parathyroid hormone-related peptide (PTHrP), initially identified as a factor responsible for malignancy-associated hypercalcemia (Philbrick *et al.* 1996), has subsequently been demonstrated not only in tumor tissues but also in a number of normal fetal and adult tissues including vascular endothelial cells (Ishikawa *et al.* 1994, Rian *et al.* 1994, Jiang *et al.* 1996) and smooth muscle cells of non-vascular and vascular origin (Burton *et al.* 1994, Philbrick 1996). The role of PTHrP is increasingly recognized as an important autocrine/paracrine hormone in regulating physiological functions, such as local modulation of microcirculation, whereas PTH only mimics the vascular action of PTHrP. PTHrP has sequence similarity to PTH at its N-terminus and has been shown to share its receptor with PTH in several tissues, including smooth muscle cells (Nickols *et al.* 1990, Urena *et al.* 1993) and endothelial

cells (Amizuka *et al.* 1997, Jiang *et al.* 1998). Both PTH and PTHrP have been reported to bind to the receptor with equivalent affinity and to stimulate both adenylate cyclase and phospholipase C activities (Philbrick *et al.* 1996, Schluter & Piper 1998). While the evidence linking cAMP to PTHrP-mediated vasorelaxation in different species and vascular beds is reasonably secure, the question as to whether the vasodilatory action of PTHrP involves an endothelium-derived nitric oxide (NO) signaling pathway is unresolved. It has been reported that the relaxant effect of PTH in isolated rat aorta and mesenteric vasculature did not require an intact endothelium and appeared to result from a direct effect on the vessel medial layer (Nickols *et al.* 1986). On the other hand, in porcine coronary arteries, removal of endothelial cells or pretreatment with N^G-nitro-L-arginine (L-NNA), a NO-synthase inhibitor, impaired PTH-induced relaxations (Schulze *et al.* 1993). In the kidney, it has been shown that the inhibition of NO-synthase by L N^G-nitro-L-arginine-methyl ester (L-NAME) markedly reduced the PTHrP-induced vasorelaxation in rabbit (Simeoni *et al.* 1994). However, endothelial damage in rabbit renovasculature did not alter the inhibitory action of L-NAME on PTHrP-induced vasorelaxation (Massfelder *et al.* 1996). On the contrary, in the more recent studies, it has been reported that PTHrP- and PTH-induced aortic relaxations were largely endothelium dependent in mice (Sutliff *et al.* 1999). In addition, the endothelium-dependent component of PTHrP- and PTH-induced aortic relaxations was unaffected by pretreatment with L-NNA but was inhibited by pretreatment with tetrabutyl ammonium, a potassium channel blocker.

The limitation of all studies reported previously is that the release of NO was suggested based on the comparison of vascular smooth muscle relaxation and not based on direct measurement of NO. The short half-life of NO in biological systems has created several problems in its direct determination. Recently, the design and application of a porphyrinic microsensor for direct *in situ* electrochemical measurement of NO in a single cell have been published (Malinski & Taha 1992, Hill *et al.* 1996). This microsensor, designed for cell culture (Hill *et al.* 1996), allows the direct quantification of NO with a high sensitivity.

The aims of the present study were to explore, by using NO-porphyrinic microsensor, the concentration-related effect of PTHrP on NO release, to compare the NO-stimulating potency of PTHrP to that of PTH, and to evaluate signal transduction systems that are involved in PTHrP- and PTH-stimulated NO release in endothelial cells.

Materials and Methods

Cell culture

Cultured endothelial cells were derived from bovine pulmonary artery, cell line CPAE (CCL-209) from the

American Type Culture Collection (Rockville, MD, USA). Cells were grown in T-75 tissue culture flasks (Corning, Greenville, OH, USA) in minimum essential medium (MEM, Cellgro, Herndon, VA, USA) containing 10% fetal bovine serum (FBS, Biocell Laboratories, Inc., Rancho Dominguez, CA, USA) and 0.004% gentamycin. The culture was incubated in an atmosphere of 95% air and 5% CO₂ at 37 °C and passaged every 3–4 days. The cells were detached by exposure for 2–3 min at 24 °C to 0.05% trypsin in 0.15 mol/l NaCl, 0.01 mol/l sodium phosphate and 0.02% EDTA. Once the cells lifted off, 8 ml of the original medium were added to the 2 ml trypsin/cell solution (to inactivate trypsin) and centrifuged at approximately 600 r.p.m. for 10 min. The cell pellet was resuspended in 10 ml fresh culture medium. To maintain the culture, 4 ml of the cell suspension were transferred to a T-75 tissue culture glass containing 11 ml fresh growth medium. Two milliliters of the cell suspension were transferred to a 60 × 15 mm tissue culture dish (Corning). The culture was then incubated (37 °C, in an atmosphere of 95% air and 5% CO₂) for 2–4 days, until the cells were confluent. Cells were rinsed twice with Hank's balanced saline solution (HBSS) containing 137 mmol/l NaCl, 5 mmol/l KCl, 0.8 mmol/l MgSO₄, 0.33 mmol/l Na₂HPO₄, 0.44 mmol/l K₂HPO₄, 1 mmol/l MgCl₂, 1.8 mmol/l CaCl₂, 10 mmol/l Tris-HCl and 1 mmol/l L-arginine (37 °C) prior to assays in order to remove growth media. All PTH and PTHrP relative peptides were dissolved in 10⁻³ mol/l HCl containing 0.1% bovine serum albumin at a final concentration of 2.5 × 10⁻⁴ mol/l and stored at -70 °C in 25 µl aliquots. Before use, peptides were further diluted to the desired concentration in HBSS. To study the effects of PTH and PTHrP receptor blockade, the antagonists, [Nle^{8,18}, Tyr³⁴]-bPTH(3–34)amide and [Leu¹¹, D-Trp¹²]-hPTHrP(7–34)amide, were added to cell incubation buffer at a final concentration of 10 µmol/l 5 min prior to addition of the agonists, hPTH(1–34) and hPTHrP(1–34). For experiments in Ca²⁺-free solution, Ca²⁺ was omitted from HBSS solution and 2 mmol/l ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) were added. In the experiments with NO synthase inhibitors (200 µmol/l each), a membrane-permeable antagonist of cyclic adenosine monophosphate (10 µmol/l), calmodulin and protein kinase C (PKC) inhibitors (50 µmol/l each), the cells were pretreated for 30 min with various blocking agents. Trifluoperazine (TFP) and n-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7), calmodulin inhibitors, and forskolin, an activator of adenylate cyclase, were dissolved in dimethylsulfoxide (DMSO). Calcium ionophore (CaI) A23187 (0.1 µmol/l) was dissolved in absolute ethanol, acetylcholine (Ach; 0.1 µmol/l), 1-(5-isoquinoliny)sulfonyl)-2-methyl piperazine (H-7, a PKC inhibitor), Rp-adenosine cyclic 3',5'-phosphorothioate (Rp-cAMPS, a cAMP antagonist) and the NO synthase inhibitors, L-NAME and

N^G -monomethyl-L-arginine (L-NMMA), were dissolved in HBSS. In a control experiment, an equal volume of DMSO or absolute ethanol replaced the solution of tested agents, where appropriate. The final concentrations of the vehicles in the incubation medium did not exceed 0.1%. The concentration of the endothelial NO synthase (eNOS) agonists, CaI and Ach used in all experiments was selected based on a dose-response curve (maximal response). NO release was measured electrochemically from a single endothelial cell. All chemicals were purchased from Sigma (St Louis, MO, USA), unless otherwise noted.

Nitric oxide measurement

Nitric oxide was prepared according to the procedures published previously (Malinski & Taha 1992, Hill *et al.* 1996). The sensor operated in a three-electrode system, consisting of the sensor working electrode, a platinum wire (0.1 mm) counter electrode and a standard calomel reference electrode. The current proportional to NO concentration was measured by a porphyrinic sensor, which operated in amperometric mode (EG&G PAR Model 283 Potentiostat/Galvanostat was used) at a constant potential of 0.75 V versus the standard calomel electrode. The response time used in these measurements was 0.1 ms and the detection limit was 10^{-9} mol/l. The NO concentration was determined from the measured current by means of a calibration curve (nitric oxide standard – saturated aqueous solution with an NO concentration of 1.76 mmol/l). The working electrode (NO sensor) was placed close to the surface ($20 \pm 5 \mu\text{m}$) of the cell membrane with the help of a computer-controlled micro-manipulator. NO was measured as an increase of the current from its background.

Statistical analysis

Statistical evaluation was carried out using ANOVA followed by Student-Newman-Keul's test. Values are expressed as means $\pm$ S.E.M., with $P < 0.05$ considered statistically significant. In each set of experiments, n is the number of culture dishes studied.

Results

A typical high-resolution amperometric curve (concentration of NO recorded versus time) obtained during CaI A23187-stimulated NO release from a single bovine endothelial cell is depicted in Fig. 1. Immediately after addition of CaI ($0.1 \mu\text{mol/l}$), NO was released resulting in a sharp peak of $583 \pm 30 \text{ nmol/l}$ ($n=5$) about 1 s after stimulation. The rate of concentration increase was 305 nmol/l/s . The peak concentration stimulated by a receptor-independent eNOS agonist represents the maximum NO concentration that can be released by a

Figure 1 Change in NO concentration with time on the surface of a single endothelial cell (pulmonary artery) after stimulation with $0.1 \mu\text{mol/l}$ CaI A 23187.

Figure 2 Change in NO concentration with time on the surface of a single endothelial cell (pulmonary artery) after stimulation with $0.1 \mu\text{mol/l}$ Ach.

particular endothelial cell. After reaching the sharp peak of NO concentration, a rapid decrease of NO concentration with a rate of 134 nmol/l/s was observed.

A different pattern of NO release was observed after stimulation with $0.1 \mu\text{mol/l}$ Ach, a receptor-dependent eNOS agonist (Fig. 2). The peak NO concentration was $388 \pm 20 \text{ nmol/l}$ ($n=5$), which represented about 65% of the maximum concentration achieved in the presence of CaI. Also, the kinetics of Ach-stimulated NO release was slower than those of CaI-stimulated release. An increase in NO concentration was recorded 4 s after the addition of Ach. The rate of NO release amounted to 38 nmol/l/s , which was almost 10 times slower than the rate of CaI-stimulated NO release. A semi-plateau was reached after 8 s and after about 24 s a slow decrease of NO concentration with a rate of 24 nmol/l/s was recorded.

Figure 3 Change in NO concentration with time on the surface of a single endothelial cell (pulmonary artery) after stimulation with 0.1 $\mu\text{mol/l}$ hPTH(1–34).

Figure 4 Change in NO concentration with time on the surface of a single endothelial cell (pulmonary artery) after stimulation with 0.1 $\mu\text{mol/l}$ hPTHrP(1–34).

Figures 3 and 4 show representative amperometric curves for NO release from bovine endothelial cells measured after addition of 0.1 $\mu\text{mol/l}$ hPTH(1–34) and 0.1 $\mu\text{mol/l}$ hPTHrP(1–34) respectively. The kinetics of NO release after stimulation with hPTH(1–34) and hPTHrP(1–34) were very similar. Moreover, the patterns of NO release stimulated by both agents represented mixed features of the patterns of NO release recorded for CaI and Ach. While increases in NO concentration were recorded after the addition of either hPTH(1–34) or hPTHrP(1–34), the patterns resembled the kinetics of NO release after stimulation with CaI. The rate of NO release was 200 nmol/l/s for hPTH(1–34) and 230 nmol/l/s for hPTHrP(1–34). NO concentration peaked at 175 ± 9 nmol/l after stimulation with hPTH(1–34) and at 248 ± 13 nmol/l after stimulation with hPTHrP(1–34) ($n=6$ each). The maximal concentration of NO was significantly smaller after stimulation with 0.1 $\mu\text{mol/l}$ hPTH(1–34) than with 0.1 $\mu\text{mol/l}$ hPTHrP(1–34). It represented about 30% and 40% of the maximum concentration achieved in the presence of CaI, for hPTH(1–34)

Figure 5 Dose–response curve for the peak NO release from an endothelial cell (pulmonary artery) after stimulation with hPTH(1–34) (open circles) and hPTHrP(1–34) (solid circles). * $P<0.05$, ** $P<0.01$ vs hPTHrP.

and hPTHrP(1–34) respectively. After reaching the sharp peak of NO concentration after addition of both analogs, decreases in NO concentrations were more similar to the pattern of NO decay observed for Ach than for CaI. The initial rate of the decrease of NO concentration was slower for hPTH(1–34) than for hPTHrP(1–34) (36 nmol/l/s vs 80 nmol/l/s respectively). However, the rate of NO decay for both peptides was significantly faster (1.5–3 times) after stimulation with Ach. After 2.5 s, a further decrease in the rate of NO decay was observed: 6.7 nmol/l/s for hPTH(1–34) and 9.8 nmol/l/s for hPTHrP(1–34). After 13 s, a further decrease in NO concentration with a rate of 2.6 nmol/l/s for hPTH(1–34) and 1.9 nmol/l/s for hPTHrP(1–34) was observed. After about 20 s, both hPTH(1–34)– and hPTHrP(1–34)–stimulated NO release decreased almost to zero level. It should be noted that the amperograms for NO release depicted in Figs 1–4 were highly reproducible for each of the test substances. Also, the traces of NO production were consistent in relation to maximum concentration as well as to the change in NO concentration with time in all preparations of endothelial cells.

Concentration–response curves of the peak NO release for hPTH(1–34) and hPTHrP(1–34) are shown in Fig. 5 ($n=6$ each point). There is a linear response for both analogs in the range of concentrations between 10^{-12} mol/l and 10^{-9} mol/l. At concentrations of hPTH(1–34) or hPTHrP(1–34) higher than 10^{-9} mol/l, a significant deviation from the linear relationship was observed. A semi-plateau was reached at concentrations of both hPTH(1–34) and hPTHrP(1–34) higher than 0.1 $\mu\text{mol/l}$. hPTHrP(1–34) showed a higher NO release than hPTH(1–34) at concentrations above 10^{-9} mol/l ($P<0.05$).

As expected, both 0.1 $\mu\text{mol/l}$ CaI– and 0.1 $\mu\text{mol/l}$ Ach–stimulated NO release was inhibited by 100 $\mu\text{mol/l}$

Figure 6 NO release stimulated by 0.1 μmol/l Cal A 23187 and 0.1 μmol/l Ach (Fig. 6a), and 0.1 μmol/l hPTH(1–34) and 0.1 μmol/l hPTHrP(1–34) (Fig. 6b) in the presence and absence of extracellular Ca^{2+} , in the presence of 100 μmol/l L-arginine analogues: L-NAME or L-NMMA and in the presence of 10 μmol/l competitive PTH/PTHrP receptor antagonists: [Nle^{8,18},Tyr³⁴]-bPTH(3–34)amide and [Leu¹¹,D-Trp¹²]-hPTHrP(7–34)amide. * $P < 0.01$ vs control (+ Ca^{2+}); + $P < 0.01$ vs [Nle^{8,18},Tyr³⁴]-bPTH(3–34)amide.

L-NAME and 100 μmol/l L-NMMA (Fig. 6; $n=6$ each bar). The average inhibition was about 60% for both NOS agonists. Similarly, both 0.1 μmol/l hPTH(1–34)- and 0.1 μmol/l hPTHrP(1–34)-stimulated NO production was inhibited: by $55 \pm 3\%$ and $63 \pm 4\%$ respectively in the presence of L-NAME and by $60 \pm 4\%$ and $65 \pm 4\%$ respectively in the presence of L-NMMA ($P < 0.01$). In the Ca^{2+} -free buffer (2 mmol/l EGTA was also added to chelate residual Ca^{2+}) the peak concentration of NO stimulated by all tested agents was significantly diminished by $88\% \pm 5$ (Cal), $67\% \pm 5$ (Ach), $35\% \pm 2$ (hPTH(1–34)) and $39\% \pm 2$ (hPTHrP(1–34)). Two competitive PTH/PTHrP receptor antagonists at concentrations of 10 μmol/l, [Nle^{8,18}, Tyr³⁴]-bPTH(3–34)amide and [Leu¹¹,D-Trp¹²]-hPTHrP(7–34)amide were devoid of agonist activity, but markedly inhibited the NO increase elicited by both hPTH(1–34) and hPTHrP(1–34). The inhibition of NO release was $67\% \pm 3$ and $69\% \pm 3$ in the presence of [Nle^{8,18}, Tyr³⁴]-bPTH(3–34)amide and $70\% \pm 4$ and $82\% \pm 4$ in the presence of [Leu¹¹,D-Trp¹²]-hPTHrP(7–34)amide after stimulation with hPTH(1–34) and hPTHrP(1–34) respectively ($P < 0.01$). The PTH/PTHrP receptor antagonists were equipotent in antagonizing hPTH(1–34)-stimulated NO release, whereas the inhibitory effect of [Leu¹¹,D-Trp¹²]-hPTHrP(7–34)amide on hPTHrP(1–34)-stimulated NO release was significantly higher compared with that of the same dose of [Nle^{8,18}, Tyr³⁴]-bPTH(3–34)amide ($P < 0.05$). The antagonists had no measurable effect on NO response after addition of either Cal or Ach.

Figure 7 NO release stimulated by 0.1 μmol/l hPTH(1–34) and 0.1 μmol/l hPTHrP(1–34) in the presence of 10 μmol/l Rp-cAMPS (a cAMP antagonist), 50 μmol/l TFP or W-7 (calmodulin inhibitors), 10 μmol/l Rp-cAMPS + 50 μmol/l W-7, or 50 μmol/l H-7 (a PKC inhibitor). * $P < 0.01$ vs without an inhibitor (solid bars) (0.1 μmol/l hPTH(1–34) or 0.1 μmol/l hPTHrP(1–34) alone).

The addition of 10 μmol/l Rp-cAMPS (a cAMP antagonist) significantly reduced, but did not completely prevent, the hPTH(1–34)- and hPTHrP(1–34)-stimulated NO release, by $22 \pm 4\%$ and $25 \pm 3\%$ respectively ($P < 0.01$) (Fig. 7; $n=6$ each bar). As expected, 0.1 μmol/l Ach (cAMP independent NO agonist)-stimulated NO release was not significantly affected by Rp-cAMPS (data not shown). The addition of 50 μmol/l of the calmodulin inhibitors, TFP or W-7, reduced but did not completely prevent either hPTH(1–34)- and hPTHrP(1–34)-stimulated NO release. Both hPTH(1–34)- and hPTHrP(1–34)-stimulated NO release was inhibited by $37 \pm 2\%$ and $38 \pm 3\%$ respectively in the presence of TFP and by $43 \pm 3\%$ and $41 \pm 4\%$ respectively in the presence of W-7 ($P < 0.01$). By contrast, in the presence of 50 μmol/l H-7 (a PKC inhibitor), both hPTH(1–34)- and hPTHrP(1–34)-stimulated NO release was unaltered. The combined effect of Rp-cAMPS and W-7 on either hPTH(1–34)- or hPTHrP(1–34)-stimulated NO release was additive.

With a view to confirming the involvement of adenylate cyclase activation in the peptide-mediated NO production, the effect of different concentrations of forskolin on NO release in endothelial cells was also studied. Forskolin produced a concentration-dependent linear increase of the peak NO release with 1 μmol/l being the threshold concentration of the compound. Rp-cAMPS (10 μmol/l) significantly inhibited the NO release induced by forskolin (Fig. 8; $n=6$ each point).

Discussion

While the evidence linking the adenylyl cyclase transduction pathway to PTHrP-mediated vasorelaxation is well

Figure 8 The peak concentration of NO released from an endothelial cell (pulmonary artery) after stimulation with different concentrations of forskolin in the presence (solid circles) or absence (open circles) of 10 µmol/l Rp-cAMPS. * $P < 0.01$ vs without Rp-cAMPS.

proven (Schluter & Piper 1998), the involvement of an endothelium-derived NO signaling pathway has not been indicated despite attempts to identify it. These studies provide, for the first time, direct *in situ* measurements of NO after stimulation by PTH (hPTH(1–34)) and PTHrP (hPTHrP(1–34)) close to the membrane of endothelial cells. The cell membrane does not present a barrier to the diffusion of NO and is not a rate-determining factor in its propagation between cells. On the membrane of endothelial cells, the concentration of NO is three to four times higher than that in the cytoplasm (Kiechle & Malinski 1993). From an analytical point of view, the detection of NO in the location with the highest concentration, the surface of the cell membrane, is the most efficient and accurate method of measuring endogenous NO levels. The short half-life of NO and its loss due to reaction with transition metals or free radicals make accurate quantitative measurements of NO difficult. Most current methods for NO detection are indirect, relying on measurements of secondary species such as nitrite removed from biological systems, or on bioassays that rely on secondary effects. The hPTHrP(1–34) concentrations that stimulate NO release in endothelium may be considered physiologically relevant, as PTHrP has a potent vasorelaxant activity with EC_{50} values in the nanomolar range. hPTHrP(1–34) at concentrations higher than 100 nM produced a greater increase in NO concentrations as compared with the same dose of hPTH(1–34). It has previously been found independently of the experimental model used (Schluter & Piper 1998), that PTHrP(1–34) is more potent than PTH(1–34) in vasodilation. It can be assumed that the discrepancy of both peptides in vasorelaxant potency is due, in part, to the different ability for stimulation of NO in endothelial cells.

Amino terminally truncated PTH and PTHrP peptides are potent PTH/PTHrP receptor antagonists. In the present study, two competitive PTH/PTHrP receptor antagonists, [Leu¹¹,D-Trp¹²]-hPTHrP(7–34)amide and [Nle^{8,18}, Tyr³⁴]-bPTH(3–34)amide, markedly antagonized both hPTH(1–34)- and hPTHrP(1–34)-stimulated NO release in endothelial cells. This clearly suggests that stimulation of NO release occurred via the PTH/PTHrP receptor mechanism. On the other hand, the analogs were equipotent in antagonizing hPTH(1–34)-stimulated NO release, whereas [Leu¹¹,D-Trp¹²]-hPTHrP(7–34)amide was more potent than [Nle^{8,18}, Tyr³⁴]-bPTH(3–34)amide in inhibiting hPTHrP(1–34)-stimulated NO release. These findings indicate that part of hPTHrP(1–34) other than the first two amino acids contribute to its activation of NO production. hPTHrP(1–34) was more potent than hPTH(1–34) in the stimulation of NO production, a fact which may be of importance in the speculation on the possible physiological functions of PTH and PTHrP. In contrast to PTHrP, PTH has so far been localized only in the parathyroid glands, hypothalamus and pituitary in all vertebrates (Pang *et al.* 1988, Philbrick *et al.* 1996). The amount of PTH required to produce a vasorelaxant effect is still much higher than is found in the circulation; the circulating levels of PTH in humans and animals are in the order of 0.025 nM (Bukoski *et al.* 1995). Thus it seems that under normal physiological conditions PTHrP is the endogenous ligand and PTH mimics the vascular action of PTHrP pharmacologically.

NO produced by vascular endothelial cells is synthesized by Ca^{2+} -dependent constitutive NO synthase (eNOS) (Bredt & Snyder 1990). Both hPTH(1–34) and hPTHrP(1–34) activated eNOS to release NO. The release of NO was inhibited by the analogs of L-arginine, L-NAME and L-NMMA. The Ca^{2+} that activates the eNOS may originate from intracellular stores or from the extracellular space. It has been demonstrated that the release of NO strongly depends on the entry of extracellular Ca^{2+} (Bredt & Snyder 1990). Data presented here indicate that the removal of extracellular Ca^{2+} significantly affected, to an equal extent, the release of NO stimulated by both hPTH(1–34) and hPTHrP(1–34). Numerous experimental data suggest that either PTH or PTHrP exert their vasorelaxant action via cAMP-dependent inhibition of L-type Ca^{2+} channel currents in vascular smooth muscle cells (Philbrick *et al.* 1996, Schluter & Piper 1998). Little data exist with regard to the effect of the peptides on endothelial Ca^{2+} content. It has been reported that intracellular Ca^{2+} of human vascular endothelial cells was raised by intact PTH(1–84), but fragment 1–34 had no effect in human vascular and bovine bone-derived endothelial cells (Fujita *et al.* 1992, Ida *et al.* 1994). The present study revealed that pharmacological blockage of the cAMP/protein kinase A-dependent process partially reduced both hPTH(1–34)- and hPTHrP(1–34)-stimulated NO release. However, additional second messenger pathways must be

involved in hPTH(1–34)/hPTHrP(1–34) formation since the combined effect of both Rp-cAMPS and W-7 inhibitors was additive. It suggested that the phospholipase C/phosphatidylinositol 4,5-bisphosphate (PIP₂) turnover pathway is involved in mediating this effect. On the other hand, the PKC inhibitor, H-7, was unable to inhibit NO formation response to hPTH(1–34) or hPTHrP(1–34). Moreover, the combined effect of the inhibitors on hPTH(1–34)- and hPTHrP(1–34)-stimulated NO release was higher than the effect after the removal of extracellular Ca²⁺ and was comparable with the effect of L-NAME or L-NMMA. It suggests also a possible role for the release of Ca²⁺ from intracellular stores after stimulation of NO formation with hPTH(1–34) or hPTHrP(1–34). It is known that hydrolysis of PIP₂ by phospholipase C yields the other inositol 1,4,5-trisphosphate (IP₃) and subsequently may result in the influx of extracellular Ca²⁺ and Ca²⁺ release from the intracellular stores in endothelial cells (Busse *et al.* 1994). Since H-7 had no effect on the peptides-stimulated NO production, it does not follow that PKC via the PIP₂/diacylglycerol transduction pathway is involved in mediating this effect. In turn, the experiments with forskolin, which does not provoke phosphatidylinositol 4,5-bisphosphate breakdown, additionally confirm that activation of adenylate cyclase is associated with NO production in endothelial cells. The exact cAMP/protein kinase A-dependent mechanism which leads to stimulation of NO release with hPTH(1–34) or hPTHrP(1–34) in endothelial cells will need further investigation. At present, it is possible to speculate that, as in smooth muscle cells (Wang *et al.* 1991), the effect of PTH(1–34) or PTHrP(1–34) on L-type Ca²⁺ channel currents may be mediated by cAMP.

It can be considered that PTHrP (or PTH) induces relaxation by stimulation (or inhibition) of signaling pathways that differ among different vascular beds and possibly also between different species. For instance, in experiments with mice, PTHrP- and PTH-induced aortic relaxations were largely endothelium dependent, whereas an intact endothelium was not necessary for maximal portal vein relaxation (Sutliff *et al.* 1999). In the same studies, the endothelium-dependent component of PTHrP- and PTH-induced aortic relaxation was prevented by pretreatment with tetrabutyl ammonium (TBA), a potassium channel blocker. In this regard, it has been suggested that incomplete inhibition of NO production may enable low levels of NO that can activate the TBA-sensitive potassium channel (Cohen *et al.* 1997). On the other hand, in vessels of rabbit kidney, activation of both adenyl cyclase/protein kinase A and NO-synthase/guanylyl cyclase pathways are directly linked to the renodilatory action of PTHrP (Massfelder *et al.* 1996). Along with this assumption, both a marked increase in cAMP and a small increase in cGMP were seen in rabbit aortic strips during PTH-induced relaxation (Nickols & Cline 1987). Moreover, the vasodilating property which

hPTHrP(1–34) has shown may be mediated, in part, through its inhibitory effect on endothelin-1 production, which is probably mediated through NO and cGMP in pulmonary arterial endothelial cells (Jiang *et al.* 1996).

Apart from being a crucial vasorelaxant agent, NO given locally exerts potent antiatherosclerotic effects, such as inhibition of smooth muscle proliferation, inhibition of platelet aggregation, inhibition of adhesion leukocytes and the expression of leukocyte adhesion molecules (Munzel *et al.* 1997). It has recently been reported that both hPTHrP(1–34) and hPTH(1–34) inhibit the migration and proliferation of cultured vascular smooth muscle cells through PTH/PTHrP receptors (Ishikawa *et al.* 1998). It prompts the suggestion that the antiatherosclerotic effect of PTHrP could be, at least partially, influenced by a release of NO from the endothelium.

In conclusion, the present studies clearly indicate that the N-terminal fragments of PTHrP and PTH, as well as exhibiting the well known vasorelaxing action via cAMP-dependent inhibition of L-type Ca²⁺ channel currents in smooth muscle cells, are also potent activators of endothelium-derived NO production. The NO release is presumably mediated through PTH/PTHrP receptors and dependent on a combination of pathways (cAMP, PIP₂) converged on Ca²⁺/calmodulin effector.

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