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Parathyroid Hormone-Related Protein Overexpression Decreases Blood Pressure in Spontaneously Hypertensive Rats

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We have recently demonstrated that arterial PTHrP expression and cardiovascular responses to this protein are altered in SHR compared with normotensive animals, Wistar Kyoto (WKY) and Sprague-Dawley (SD) rats. To investigate whether the slightly, but significantly decreased, aortic PTHrP gene expression observed in SHR, compared to that of normotensive animals, may play a causative role in the maintenance of the elevated arterial blood pressure (ABP) of the SHR, we transfected a hepatic lobe with a PTHrP expression vector in a sense and antisense orientation. At 24 and 48 hours, sense pSV2neo-ECE induced a significant five-fold increase in PTHrP mRNA abundance with respect to antisense pSV2neo-ECE and vehicle. This increment in the PTHrP mRNA induced by the sense PTHrP expression vector was totally inhibited by the co-administration of the antisense PTHrP expression vector. At the same time, we observed a significant decrease of mean ABP (MABP) in SHR transfected with the sense pSV2neo-ECE to similar values as those obtained in the normotensive strain. Neither antisense PTHrP expression vector nor vehicle had any significant effect in any strain. Again, the effect of the sense PTHrP expression vector on MABP was blocked by the simultaneous treatment with the antisense PTHrP expression vector. At 48 hours, the hypotensive effect of the sense pSV2neo-ECE in SHR was reverted by the IV bolus injection of a specific competitive PTHrP receptor antagonist such as Nle8,18,Tyr34-bPTH(3–34)amide. We propose that a defect of this potent local vasodilator may contribute to the development and/or maintenance of arterial hypertension in SHR. This defect can be ameliorated by transfecting tissues with protein-exporting capabilities, such as the liver. Finally, our work adds additional data to a cumulative body of evidence suggesting that it might be possible to design an effective gene therapy to treat the common polygenic and multifactorial form of hypertension by increasing the activity of potent and physiological vasodilators.

Keywords PTHrP, parathyroid hormone-related protein, hypertension, gene therapy, SHR, rats

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Introduction

Parathyroid hormone-related protein (PTHrP) first identified as the tumor-derived protein responsible for the humoral hypercalcemia of malignancy syndrome (1–3) is produced in many normal tissues (4). However, the protein is present at very low levels in the circulation in normal conditions (5) suggesting that it might act in an autocrine or paracrine fashion. Although the physiological function of PTHrP remains unclear, evidence accumulated to date suggests that among other actions, the protein may function as a local relaxant or compliance factor in smooth muscle (6–9). We have showed that PTHrP is also expressed in vascular smooth muscle where it is markedly induced by serum addition in quiescent cells (10). Furthermore, vasoconstrictor agents such as angiotensin II (Ang II) induce PTHrP gene expression in rat aortic smooth muscle cells (SMC) by increasing both gene transcription and mRNA stabilization and, in turn, PTHrP inhibits Ang II stimulation of SMC proliferation (11). Additional studies from our group have also shown that vascular PTHrP gene expression can be activated by mechanical stimuli both in vitro and in vivo (12). As both PTH and PTHrP are powerful vasodilators when infused systemically into rats and rabbits (13), it seemed reasonable to propose that one of the functions of PTHrP within the arterial wall is to serve as a local vasorelaxant activated by vasoconstrictors and mechanical stimuli and to oppose to both the contractile and mitogenic effects of these agents.

On the other hand, abnormalities of blood vessel smooth muscle is a hallmark of hypertension in both man and at least one experimental rat model, the spontaneously hypertensive rat (SHR) (14). The vascular changes are generally considered to be a product of high blood pressure, but it is possible that they precede hypertension (15) and reflect an intrinsic abnormality or the effect of humoral influences, since SHR may have among other biochemical disturbances, decreased serum-ionized calcium concentration and elevated plasma PTH levels (16). Thus, we recently demonstrated that arterial PTHrP expression and cardiovascular responses to this protein are altered in SHR compared with normotensive animals—Wistar Kyoto (WKY) and Sprague-Dawley (SD) rats (17). Here we show for the first time that increased PTHrP overexpression by means of an eukaryotic expression vector injected in the liver induced a long-lasting depressor effect in SHR that can be reversed by a specific PTHrP receptor antagonist.

Methods

Unless indicated, all reagents were from Sigma Company (St. Louis, MO, USA).

Animals

Hypertensive male rats between 18–21 weeks of age of the Okamoto-Aoaki Strain (SHR) and age-matched normotensive Wistar Kyoto (WKY) rats (founders were obtained from Charles River Laboratories, Portage, MI, USA) rats were housed in a room with controlled temperature (24 ± 1°C) under a schedule of 12 h light :12 h darkness. Food and water were ad-libitum. The Institutional Animal Care and Use Committee approved the experimental protocol.

Four animals per experiment were anesthetized with ip pentobarbital (33–45 mg/kg) and they were chronically instrumented for direct mean arterial blood pressure (MABP) and heart rate (HR) recorded throughout the experiment by a polyethylene cannula, inserted into the left carotid artery and connected to a Statham transducer, coupled to a Grass polygraph. The jugular vein was cannulated for peptide injections. Mean arterial

blood pressure and HR were monitored before (basal) and every minute during a 30-min period post-injection of a bolus of 0.3–30 μg/kg body weight hPTHrP(1–34)NH_2 (Bachem Fine Chemicals, Torrance, CA, USA) in saline supplemented with 0.1% BSA (until the maximum cardiovascular effect was reached). To analyze PTHrP agonist responsiveness, a complete dose-response curve was performed in the same animal allowing MABP and HR to return to baseline before administration of the next dose of peptide. Administration of vehicle produced no changes either in MABP or HR. Results reflecting maximal changes for each dose are expressed as mean ± SE from four separated experiments.

After these procedures for intrahepatic injections, the superior lobe of the liver was exposed through a small incision on the abdominal wall and a total volume of 100 μl was injected using a microsyringe. One hundred μg of sense pSV2neo-ECE and antisense pSV2neo-ECE alone or in combination were dissolved in phosphate-buffered saline (PBS). The control group received vector without insert (V).

As vectors, we used the two plasmids based on pSV2neo-ECE (generously donated by Dr. Thomas Clemens, University of Cincinnatti, OH, USA) which contained the 1.1 Kb PTHrP cDNA inserted into the Eco RI site (18) in the sense and antisense orientation with regards to the position of the early promoter of SV40. The pSV2neo vector without PTHrP insert was used as a control DNA (V).

After 24 h, MABP and HR were obtained and every 24 h thereafter from the carotid artery cannula up to 1 week. In an independent experiment, a subgroup of five SHR that was instrumented and treated with sense pSV2neo-ECE (n = 4) as described, 30 μg of the specific and competitive PTHrP receptor antagonist Nle8,18,Tyr34-bPTH(3–34)amide (Sigma Co.), St Louis, MO, USA) was IV injected as a bolus 48 h after transfection to evaluate whether the hypotensive effect of the sense pSV2neo-ECE was due to PTHrP receptor activation. In preliminary experiments, we found that 30 μg of Nle8,18,Tyr34-bPTH(3–34)amide is able to completely inhibit the hypotensive effect of up to 10 ug of the PTHrP agonist, hPTHrP(1–34)NH_2.

In another series of experiments, anesthetized 16 SHR and 16 WKY were divided into four groups and treated with intrahepatic injections of V, sense pSV2neo-ECE, antisense pSV2neo-ECE or sense pSV2neo-ECE plus antisense pSV2neo-ECE as described. After 48 h, for total RNA extraction, an individual piece of liver around the injection site from each animal was immediately frozen on dry ice.

Total RNA Extraction

In an additional experiment, five SHR and five WKY rats were killed by decapitation. A section of the aorta, from the aortic arch to the level of the renal arteries, and 1 cm^2 of liver around the injection site were excised. In the case of aortae, adhering fat and connective tissue from the adventitia were stripped off by blunt dissection. For total RNA extraction, the aortae was immediately frozen on dry ice.

Northern Blot Analysis

RNA was extracted from hepatic tissue according to the method of Chomczynski et al. (19) with slight modifications. Briefly, tissue samples (100 mg) were homogenized in 1.2 ml of a reagent made by mixing one volume of solution "D" (4 mol/L sodium isothiocyanate, 25 mmol/L sodium citrate, 15 mmol/L sarcosyl, and 0.1 mol/L β-mercaptoethanol) plus one volume of water-saturated phenol and 0.1 volume of 2 mol/L sodium acetate pH: 4.0 using a glass vessel-glass pestle homogenizer. Then, 240 μl of

chloroform:isoamyl alcohol (24:1) were added to each tube that was vortexed by 30 sec, and kept on ice. After 15 min, tubes were centrifuged at 15,000 rpm during 15 min at 4°C. Isopropyl alcohol (600 µl) was added to the separated aqueous phase and vortexed by 30 sec. After 1 h at −20°C, samples were centrifuged at 15,000 rpm during 15 min at 4°C. Pellets were washed with 400 µl of 75% ethanol, dried at room temperature, and resuspended in autoclaved diethylpyrocarbonate-treated water. UV spectrophotometry at 260 nm was used for quantitation of total RNA. The integrity and accuracy of the RNA quantitation were confirmed by running 5 µg aliquots of each sample by 1% agarose-formaldehyde gel electrophoresis and ethidium bromide staining. Only undegraded samples with intact 28S/18S ribosomal RNA and ratios between optical densities (OD) at 260 and 280 nm above 1.8 were processed. For Northern blots, 40 µg of total RNA were electrophoresed on 1.5 % agarose gels containing formaldehyde and ethidium bromide. RNA samples were transferred onto nylon membranes and then hybridized to a rat PTHrP cDNA probe (rPLPm10, G. Hendy and D. Goltzman, McGill University, Toronto, Canada) labelled with α32P-dCTP by a random primer method (20). The cDNA probe encodes most of the coding region and the 3' untranslated region of rat PTHrP (21). In some cases, the blots were reprobed with a rat cyclophilin cDNA [BamHI restriction fragment of pCD15.8.1 (22)] since, as cyclophilin is a housekeeping gene, it is not altered by experimental conditions (11), its gene expression was used as a control for loading. Filters were washed three times (30 min each) in 0.1 × SSC (1 × SSC = 0.15 mol/L NaCl, 15 mmol/L sodium citrate), 0.1% sodium dodecyl sulphate (SDS) at 49 and 55°C, respectively, and exposed to Kodak X-Omat films (Eastman Kodak, Rochester, NY, USA) with

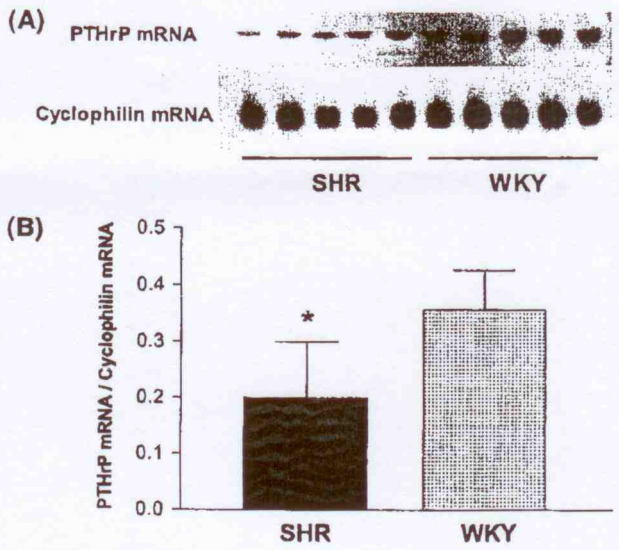


Figure 1. Aortic PTHrP gene expression in 18-week-old SHR and age-matched normotensive WKY. Northern blot of 40 µg of total RNA extracted from aortic walls hybridized with rat PTHrP cDNA probe and rat cyclophilin cDNA probe as a control for loading (A). Each lane corresponds to one independent experiment. A single PTHrP mRNA transcript of approximately 1.4 kb was expressed. Densitometry data expressed as a ratio between PTHrP and cyclophilin mRNA band intensities (mean ± SE, n = 5) (B). *: $p < 0.02$ with respect to WKY rats.

intensifying screens at −70°C. Intensity of the bands was estimated by scanning, using a dedicate computer program (SigmaScan, Jandel Co., San Rafael, CA, USA).

Statistical Analysis

Statistical studies were performed using two-way analysis of variance with repeated measurements and Tukey's test for individual mean differences.

Results

Northern blots using total RNA extracted from aortae showed a significant reduction of PTHrP mRNA abundance in SHR to almost half of the levels observed in WKY animals (Figure 1). To certify the appropriate cardiovascular responsiveness to PTHrP agonists in SHR compared to WKY rats, we injected the synthetic N-terminal fragment of the rat PTHrP molecule. In pentobarbital-anesthetized animals, PTHrP(1–34)NH_2 induced a long-lasting (10–15 min), dose-dependent absolute hypotensive response that reached a maximum with 10 μg of PTHrP(1–34)NH_2/Kg body weight and was greater in SHR (65 ± 4 mmHg) than that in WKY rats (36 ± 5 mmHg) (Figure 2). These findings indicate that SHR retain responsiveness to the vasodilator. Conversely, the dose-dependent tachycardia elicited by PTHrP(1–34)NH_2 was significantly higher in WKY rats than in SHR at all doses of the peptide tested (at 10 μg of PTHrP(1–34)NH_2/Kg body weight, WKY: 105 ± 6 versus SHR: 62 ± 4 beats/min).

To investigate whether the slightly, but significantly decreased, aortic PTHrP gene expression observed in SHR compared to that of normotensive animals may play a causative role in the maintenance of the elevated ABP of the SHR, we transfected a hepatic lobe with a PTHrP expression vector in a sense and antisense orientation. At 24 and 48 h, only sense pSV2neo-ECE induced a significant five-fold increase in PTHrP mRNA abundance with respect to antisense pSV2neo-ECE and vehicle (Figure 3). This increment in the PTHrP mRNA induced by the sense PTHrP

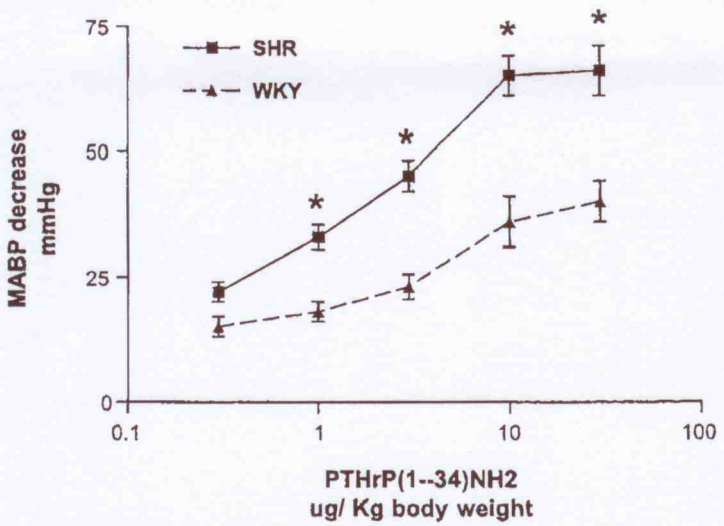
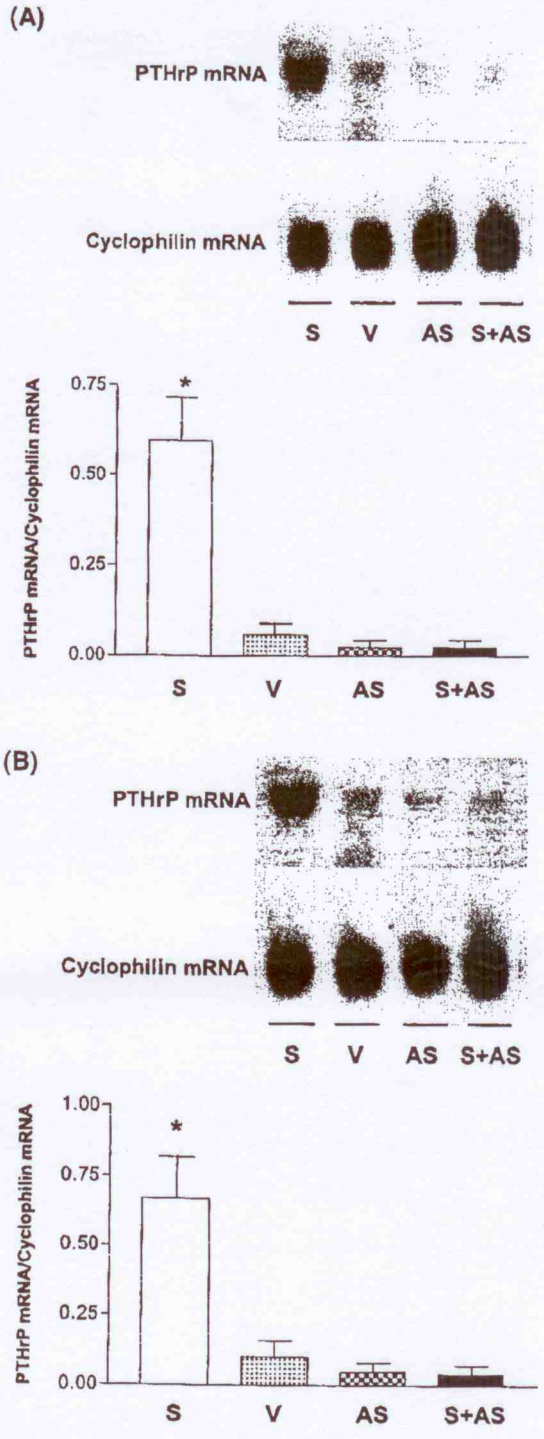


Figure 2. Dose-response curve for the maximum mean arterial blood pressure (MABP) decrease induced by the intravenous injection of PTHrP (1–34)NH2 in SHR and WKY rats.



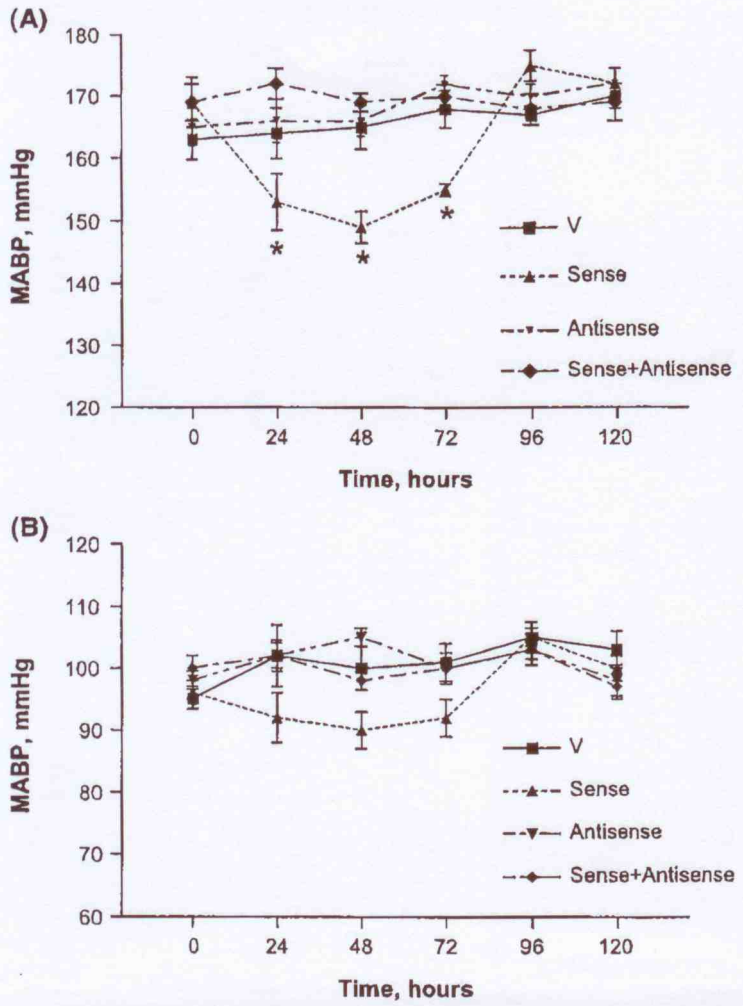


Figure 4. Time course of mean arterial blood pressure (MABP) of spontaneously hypertensive rat [SHR, (A)] and normotensive rats [WKY, (B)] transfected with sense and antisense PTHrP expression vector and vehicle (V). * $p < 0.02$ with respect to V and AS-treated SHR.

Figure 3. Liver PTHrP gene expression in SHR (A) and age-matched normotensive WKY (B) 48 h after liver transfection with vehicle (V), sense pSV2neo-ECE (S), antisense pSV2neo-ECE (AS), and both S and AS simultaneously. Inserts show northern blots of 40 μg of total RNA extracted from hepatic tissue hybridized with rat PTHrP cDNA probe and rat cyclophilin cDNA probe as a control for loading in a representative experiment. A single PTHrP mRNA transcript of approximately 1.4 kb was expressed. Densitometry data expressed as a ratio between PTHrP and cyclophilin mRNA band intensities (mean ± SE, n = 4 independent experiments) are shown as bars. *: $p < 0.01$ with respect to the other treatments.

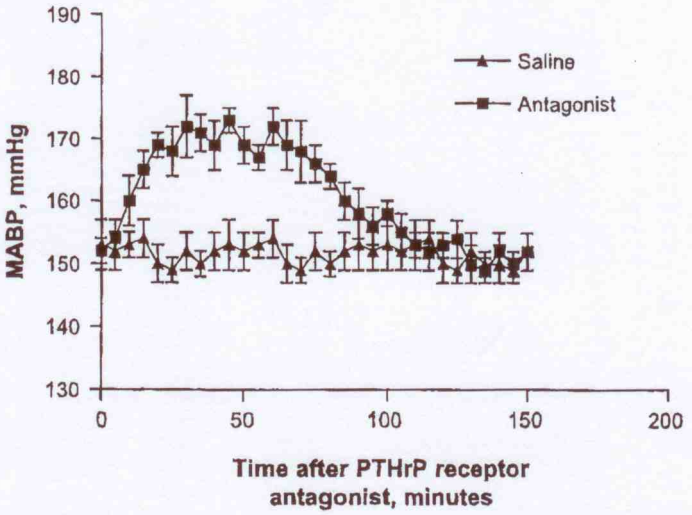


Figure 5. Time-response curve for the effect of the PTH/PTHrP receptor antagonist (Nle8,18,Tyr34-bPTH(3–34)amide) or saline on MABP of SHR treated 48 h before with pSV2neo-ECE.

expression vector was totally inhibited by the co-administration of the antisense PTHrP expression vector.

At the same time points, we observed a significant decrease of MABP in SHR transfected with the sense pSV2neo-ECE to similar values as those obtained in the normotensive strain. Although there was a trend toward a decrease in MABP of WKY treated with sense pSV2neo-ECE, this effect did not reach statistical significance and neither antisense PTHrP expression vector nor vehicle had any significant effect in any strain (Figure 4). Again, the effect of the sense PTHrP expression vector on MABP was blocked by the simultaneous treatment with the antisense PTHrP expression vector. At 48 h, the hypotensive effect of the sense pSV2neo-ECE in SHR was reversed by the IV bolus injection of a specific competitive PTHrP receptor antagonist such as Nle8,18, Tyr34-bPTH(3–34)amide (sense pSV2neo-ECE plus saline: 152 ± 4 mmHg versus sense pSV2neo-ECE plus antagonist: 169 ± 5 mmHg, $n = 4$, $p < 0.002$). In Figure 5, the time-response curve for the antagonist effect can be seen showing that it was apparent after 10 min and lasted up to 125 min.

Discussion

PTHrP is expressed abundantly in vascular smooth muscle cells both in vivo and in vitro (10–12). Thus, we recently reported that mechanical stimuli such as sheer stress and vasoconstrictors such as Ang II, endothelin, norepinephrine, serotonin, and thrombin, increased PTHrP mRNA abundance in SMC by transcriptional and post-transcriptional mechanisms both in vitro and in vivo (11, 12). Therefore, in order to determine whether PTHrP gene expression in the vascular bed is regulated by blood pressure rise, we studied the expression of PTHrP mRNA in aortae of genetic hypertensive animals compared to normotensive controls. We found that SHR did not show any increase in aortae PTHrP mRNA abundance. On the contrary, we observed a slightly albeit significant decrease in PTHrP gene expression. Although these results are

in conflict with another study performed on the aorta (23), they are consistent with what we have reported (17). These results combined suggest that in adult SHR the aortic wall fails to respond to hypertensive stimuli with an increase of PTHrP. Since hypertension induced by constant infusion of Ang II or salt-loading produced an increase in aortic PTHrP mRNA abundance of normal rats (24, 25), it could be suggested that differences in the genetic background between SHR and normotensive rats may explain our results. Furthermore, in order to analyze the SMC response in the absence of in vivo hemodynamic masking factors, we have studied the effect of Ang II on PTHrP gene expression in cultured smooth muscle cells (SMC) and found that also in vitro SHR SMC lack a normal response to Ang II when compared to that derived from 4- and 18-week-old WKY or adult SD normotensive rats. The abnormal angiotensin II response of SHR SMC was not due to altered type-1 angiotensin II receptor (AT1aR) expression, since SHR SMC had a higher basal abundance of AT1aR mRNA than that of SD rats. AT1aR activation by Ang II appeared to be normal since angiotensin II induction of c-myc mRNA was even higher in SHR SMC (17).

Seeing that smooth muscle PTHrP gene expression may be enhanced in the prehypertensive state and becomes defective with established hypertension, it is tempting to speculate that a late onset and permanent deficiency in this compensatory mechanism may be required for the development of hypertension. Since in fura 2-loaded SMC, PTH, and PTHrP attenuated Ang II-induced calcium mobilization (26), the lack of a physiological antagonism of PTHrP on vasoconstrictor-induced intracellular calcium rise may explain the hypersensitivity seen in vessels of adult SHR.

Since in a hypertensive milieu characterized by a relative deficiency in PTHrP, it might be expected that PTHrP injected intravenously would produce higher cardiovascular effects, we injected SHR and WKY with a N-terminal rat PTHrP fragment with agonist activity. In this way, when injected in anesthetized animals, PTHrP(1–34)NH_2 induced a higher absolute hypotensive response in SHR than in WKY rats, indicating that spontaneously hypertensive animals, at least, retain responsiveness to the vasodilator. Similar results were also shown by us as well as DiPette et al. (17, 27).

Having proved that SHR are responsive to the vasodilator effect of PTHrP agonist, we hypothesized that an increased production of circulating PTHrP may decrease arterial blood pressure in SHR. Then we injected expression vectors in the liver. This has been a successful maneuver to induce the expression of other vasodilator such as kallistatin in SHR (28). Multiple injections of 100 ug of the sense PTHrP expression vector (pSV2neo-ECE with the cDNA of PTHrP in the sense orientation) effectively increases PTHrP mRNA abundance and decreases arterial blood pressure in the SHR. A trend toward a similar effect in WKY did not reach statistical significance. Although we did not measure circulating PTHrP levels, our findings indicate that circulating protein levels may reach a particular level to show an increased sensitivity of SHR vascular bed to the vasodilating effect of PTHrP. The effectiveness of transfection with expression vectors for PTHrP and type-1 PTHrP receptor in decreasing vascular tone in the renal circulation of SHR was also shown by Massfelder et al. (29). The ability of an increased production of PTHrP to decrease blood pressure was also demonstrated in a series of elegant studies using transgenic mice. Maeda et al. (30) have shown that mice with targeted PTHrP overexpression to vascular smooth muscle cells have a significant decrease in blood pressure. Similarly, the same group also reported that PTHrP receptor overexpression produces a similar effect (31). The authors were not able to study double transgenic animals since fetuses died from heart malformations. Altogether these data suggest that PTHrP overexpression is an effective way to decrease blood

pressure in SHR indicating that the reduction in PTHrP expression may be a causative factor in this genetic form of hypertension. A note of caution should be added. The expression of PTHrP using naked expression plasmids is very transient, lasting for up to 72 h at best. This probably reflects the fact that the transfection is of low efficiency and transitory because plasmids are epigenomic elements. More experiments are necessary to develop more efficient strategies.

The effect of the sense PTHrP expression vector seems to be specific for two reasons. First, the effect was abolished by co-transfection with the antisense PTHrP expression vector that arguably should inhibit PTHrP mRNA synthesis and translation. Second, the vasodilator effect of sense PTHrP expression vector was almost completely blocked by the specific PTHrP receptor antagonist—Nle8,18,Tyr34–bPTH(3–34)amide. This synthetic fragment of PTH has been extensively used for PTHrP/PTH receptor blockade since 1977 (32) and the dose we used was able to block the hypotensive effect of up to 10 ug of the PTHrP receptor agonist hPTHrP(1—34)NH_2 (data not shown).

In addition, the lack of effects of the antisense PTHrP expression vector is consistent with the very low expression of PTHrP in the liver.

In summary, aortic PTHrP gene expression of SHR is similar or lower than that of normotensive rats and may be abnormally low considering the high arterial blood pressure of SHR. We propose that a defect of this potent local vasodilator may contribute to the development and/or maintenance of arterial hypertension in this model. This defect can be compensated by transfecting tissues with protein-exporting capabilities, such as the liver or smooth muscle cells themselves although the generalized transfection of smooth muscle cells may be technically more complicated. PTHrP may also serve as a locally produced antiproliferative agent in many cells (6, 33). As this protein inhibites angiotensin II-induced DNA synthesis in SMC (11), this novel vasorelaxant may be a physiological antagonist of the proliferative actions of angiotensin II. Therefore, its remains to be explored whether a diminished local PTHrP production may be a contributory factor to the remodeling process that takes place in the arterial walls of spontaneously hypertensive animals. Unfortunately, because of the short-lasting PTHrP overexpression, our experimental design did not allow us to test this intriguing possibility and more experiments are warranted to analyze it. But since PTHrP is highly expressed in atherosclerotic lesions (34) and in vascular walls suffering the restenosis process, after balloon injury and local application of PTHrP agonist directly to the vessel wall has been shown to impede restenosis (12, 35), that presumption seems to be the case.

To summarize, it has been shown for the first time that increased PTHrP overexpression by means of an eukaryotic expression vector injected in the liver induced a long-lasting depressor effect in SHR that can be reversed by a specific PTHrP receptor antagonist. Finally, our work adds additional data to a cumulative body of evidence that suggests that it might be possible to design an effective gene therapy to treat the common polygenic and multifactorial form of hypertension in the near future by increasing the activity of potent and physiological vasodilators such as PTHrP.

Acknowledgments

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