The Somatostatin Analog Angiopeptin Does Not Reduce Chronic Hypoxic Pulmonary Hypertension in Rats (44034)

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Abstract. **Angiopeptin is an analog of somatostatin-14, which has been found to inhibit cellular proliferation in several models of systemic vascular injury. As proliferation plays a major role in pulmonary hypertension, we examined the hypothesis that angiopeptin would inhibit the development of chronic hypoxic pulmonary hypertension in the rat. Angiopeptin was infused intravenously (90–100 μg/kg/day) by minipumps in 10 rats during a 3-week exposure to hypobaric hypoxia and in six normoxic rats. Normal saline was infused in six hypoxic control rats and in seven normoxic control rats. Angiopeptin produced no significant difference in mean pulmonary arterial pressure and resistance, right ventricular weight, or medial thickness of small pulmonary vessels. Vasoconstrictor responses of isolated lungs to acute hypoxia were not affected by angiopeptin. We conclude that angiopeptin, at the high intravenous dose used, does not significantly reduce the development of chronic hypoxic pulmonary hypertension in rats.** [P.S.E.B.M. 1996, Vol 213]

Patients with chronic obstructive lung disease who have pulmonary hypertension have a worse prognosis than those who do not have pulmonary hypertension (1). The pulmonary hypertension is the result of a combination of vascular remodeling and vasoconstriction. Experimental chronic hypoxia is characterized by pulmonary vasoconstriction (2, 3), pulmonary arterial endothelial, smooth muscle and fibroblast proliferation (4), and increased turnover and amount of connective tissue in pulmonary vessels (5–7). In general, vasodilators have not proved to be effective therapy for the pulmonary hypertension associated with hypoxic lung disease. Inhibition of cellular proliferation in the pulmonary vasculature might be more successful.

Angiopeptin is a synthetic cyclic octapeptide, which is an analog of somatostatin-14. Activation of somatostatin receptors inhibits smooth muscle cell proliferation, probably by dephosphorylating tyrosine residues through the action of a membrane-bound phosphatase (8, 9). Somatostatin can reduce cellular proliferation by a direct effect on cell division (10) and by reduction of the release of both growth hormone (11) and insulin-like growth factor (12). In addition, somatostatin is known to reduce calcium current in neurons (13). Chronic infusion of somatostatin-14 (20 μg/rat/hr) has been reported to reduce pulmonary arterial pressure in chronically hypoxic rats (14). The development of stable, long-lasting analogs of somatostatin (15) provides an opportunity to influence vascular remodeling such as occurs in coronary restenosis and pulmonary hypertension.

Angiopeptin has been demonstrated to reduce cellular proliferation in a variety of vascular injury models (rabbits [16, 17], rats [18], pigs [19], and humans

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[20]). The vascular injury in these reports is clearly of a different severity compared with the more subtle signal for cellular proliferation provided by hypoxia in the pulmonary vasculature. However, the fact that angiopeptin has been effective in inhibiting proliferation in the presence of such powerful stimuli for growth suggests that it might be able to reduce the medial hypertrophy and development of longitudinal smooth muscle bundles in the intima which are observed in chronic hypoxia. To test this possibility, we measured the effects of angiopeptin-infusion on the development of chronic hypoxic pulmonary hypertension.

Materials and Methods

The investigation conformed with the *Guide for the Care and Use of Laboratory Animals,* published by the U.S. National Institutes of Health (NIH Publication #85–23, revised 1985). It was performed in an institution with a Public Health Service Animal Welfare Assurance and accredited by the American Association for Accreditation of Laboratory Animal Care. All experimental procedures involving rats were approved by the Minneapolis VA Medical Center Animal Studies Subcommittee.

Experimental Groups. Male specific pathogen-free Sprague-Dawley rats weighing between 300 and 350 g were divided into four groups: normoxic control (NC; $n = 7$), hypoxic control (HC; $n = 7$), normoxic angiopeptin (NA; $n = 6$), and hypoxic angiopeptin (HA; $n = 10$). Rats of both hypoxic groups (HC and HA) were exposed to hypobaric hypoxia (room air at 0.4 atmosphere; simulated altitude 6700 m) for 3 weeks (21). The hypobaric chamber was opened three times a week for cleaning and replenishing food and water. Three days before the start of the hypoxic exposure, each rat was implanted with an osmotic minipump that continually infused intravenously either sterile normal saline (in NC and HC) or angiopeptin dissolved in sterile water (in NA and HA). Water and food was available *ad libitum* to all rats throughout the study.

For pump implantation, the rats were anesthetized intraperitoneally with sodium pentobarbital (50 mg/kg). Using aseptic technique, the right internal jugular vein was exposed and cannulated with a PE-60 polyethylene tubing attached to an Alzet osmotic minipump (Model 2ML4; Alza Corp., Palo Alto, CA). The pump was then placed subcutaneously in the midscapular region. With the pump infusion rate of 2.5 μl/hr, the dose of angiopeptin delivered was 90–100 μg/kg/day. The surgical wound was checked visually on a daily basis and no healing complications were encountered. At the end of the study, each rat was used for *in vivo* pulmonary hemodynamics measurements, isolated lung preparation, pulmonary vascular morphology evaluation, and plasma angiopeptin assay.

***In Vivo* Pulmonary Hemodynamics.** After 24 days of infusion, the rats were removed from the chamber, anesthetized intraperitoneally with sodium pentobarbital (50 mg/kg), and mechanically ventilated with room air through a tracheostomy. The chest was opened in the midline, while care was taken to prevent bleeding. Cardiac output was measured by an ultrasonic flow probe placed on the ascending aorta (The flow probe [3S] was calibrated by the company Transonic Systems [Ithaca, NY]; accuracy: ±15% over the range of flow used). Although the reported values of cardiac output in intact rats (22) are higher than values in this study, this method was sufficient for our goal to compare the groups. Pulmonary arterial pressure was measured by direct puncture of the pulmonary artery with a 25-gauge needle connected to a pressure transducer. The same needle was then used to puncture the ascending aorta to measure the systemic arterial pressure. Because of the differences in rat size between the groups at the end of the study, cardiac index was calculated as cardiac output divided by body weight, and total pulmonary resistance index was obtained by dividing mean pulmonary arterial pressure by cardiac index. After the cardiac output and pressure measurements, blood was withdrawn from the left ventricle for hematological evaluation and angiopeptin concentration measurement. The lungs were then isolated and perfused *ex vivo* as detailed below.

Isolated Lungs. After heparinization (200 IU), the lungs were isolated and perfused as described previously (23). The pulmonary artery was cannulated through a right ventriculotomy with a double lumen perfusion cannula allowing continuous measurement of perfusion pressure. The left atrium was drained through a large outflow cannula. Lungs were perfused at 0.04 ml/g/min with Krebs' solution containing 4% albumin and 17 μ*M* meclofenamate. The lungs were removed from the chest and suspended from a tracheal cannula in a thermostat-regulated (38°C) humid chamber. The respiratory rate was 65 breaths/min, a positive end-expiratory pressure of 2.5 cm H_2O was applied, and FI_{CO_2} was 5%.

Once perfused, the preparation was allowed to equilibrate for 15 min before a bolus injection of angiotensin II (Ang II; 0.15 μg) was given in the inflow line to prime the lung prior to the hypoxic challenge (24). It caused a transient increase in pulmonary arterial pressure for about 2 min. The Ang II injection was followed after 8 min by an acute hypoxic challenge of 6 min (2.5% O_2 + 5% CO_2 + balance N_2 for 6 min; effluent perfusate $PO_2 = 42 \pm 2$ mm Hg, $PCO_2 = 25 \pm 1$ mm Hg, pH = 7.40 ± 0.01) and then a control 10-min period of normoxic ventilation (effluent perfusate $PO_2 = 128 \pm 1$ mm Hg, $PCO_2 = 28 \pm 1$ mm Hg, pH = 7.36 ± 0.01). The Ang II/hypoxia/control cycle was repeated twice.

Table I. Hemodynamic and Related Data

	Group			
	NC	NA	HC	HA
Number of rats studied	7	6	6	10
Body weight (g)	460 ± 12	443 ± 5	328 ± 3[a]	337 ± 5[a]
Cardiac output (ml/ min)	74 ± 7	83 ± 5	42 ± 1[a]	49 ± 6[a]
Cardiac index (ml/min/kg)	163 ± 20	187 ± 11	127 ± 5	145 ± 17
Mean SAP (mm Hg)	118 ± 7	121 ± 6	98 ± 10	112 ± 5
Mean PAP (mm Hg)	18.9 ± 0.9	17.0 ± 1.0	27.0 ± 1.5[a]	27.2 ± 1.5[a]
BLPP (mm Hg)	7.5 ± 0.6	7.7 ± 0.8	10.7 ± 1.8[a]	9.1 ± 0.5

Note. SAP, systemic arterial pressure; PAP, pulmonary artery pressure in open chest rats; BLPP, baseline pulmonary artery pressure in the isolated, perfused lung.
[a] The value differs from the respective normoxic value ($P < 0.05$). No differences were detected between the groups in other variables.

Angiopeptin Measurement. The blood samples for angiopeptin analysis were treated with EDTA and used to prepare plasma which was stored at −20°C. Plasma levels of angiopeptin in rat were measured by radioimmunoassay (RIA), developed by one of us (S. S. C.). The immunoassay buffer used was 100 m*M* phosphate buffer (pH 7.4) containing 0.1% Triton X-100, 0.2% bovine serum albumin, 0.9% NaCl, and 1% sodium azide. Standard curves were established in the peptide-free plasma to compensate for the nonspecific interference from plasma. Standard or plasma samples in triplicates (100 μl) were preincubated with 100 μl (initial dilution of 1:800) of rabbit antiserum (gift from Plessey, Robinson, France) for 24 hr at 4°C, then angiopeptin labeled with Iodine 125 (150 Bq in 100 μl) prepared using a slight modification of the procedure of Hunter and Greenwood (25, 26) was added and incubation was continued further for 24 hr at 4°C. The mixture was precipitated with cold ethanol at 4°C for 30 min and spun in a cold centrifuge at 3000 rpm for 30 min. Supernatant was discarded and the pellets were counted in a gamma counter (LKB Wallac 1272 clinigamma). The minimal detectable dose (95% confidence limit) was 50 pg/ml. The cross-reactivity of endogenous peptides such as somatostatin-14, tyrosine releasing hormone, or luteinizing hormone releasing hormone was less than 0.005%. The interassay coefficient of variation ($n = 20$) was 12%, 11%, and 15% for 0.2, 1, and 10 ng/ml, respectively. The assay allows direct measurement, without extraction of the peptide, in plasma or serum from the rat, dog, monkey, rabbit, and human.

Morphologic Analysis. At the end of each experiment, the heart was dissected, and the right ventricle to left ventricle plus septum weight ratio (RV/LV + S), as well as the right ventricle to body weight ratio (RV/BW, expressed as a percentage), was determined. The lungs were perfused with 4% formaldehyde (12 to 15 ml/min), and the same solution was infused through the trachea at 13 cm H_2O. Lungs were then placed in 4% formaldehyde for several days before transverse sections at the level of the hilum were prepared for light microscopy and stained with Van Gieson's stain. The thickness of opposing walls (WT_1 and WT_2) and the smallest external diameter (D) of pulmonary vessels were measured using light microscopy at a magnification of ×450 (27). The percentage of medial thickness was calculated as $\%MT = (WT_1 + WT_2)/D \times 100$. Vessels were defined by their relationship to airways: B, muscular vessels (D: 100–200 μm) associated with large bronchioles; AD, alveolar duct vessels (D: 50–100 μm) associated with alveolar ducts; and P, parenchymal vessels (D: 30–60 μm) associated with alveoli. The number of parenchymal vessels measured from each slide varied between 55 and 84, of alveolar duct vessels between 78 and 130, and of bronchiolar vessels between 11 and 15.

Drugs. All drugs were from Sigma Chemical Co. (St. Louis, MO) except for angiopeptin, which was a gift from the Henri Beaufour Institute-USA, Inc. (Washington, DC). For all drugs, normal saline was used as the solvent, except for angiopeptin, for which sterile water was used.

Table II. Hematologic Data

	Group			
	NC	NA	HC	HA
Platelets (thousands/mm^3)	106 ± 83	101 ± 70	51 ± 63[a]	62 ± 34[a]
Hematocrit (%)	39 ± 0.8	38 ± 1.0	65 ± 0.5[a]	64 ± 0.8[a]
Hemoglobin (g/dl)	13.8 ± 0.3	13.9 ± 0.3	22.4 ± 0.2[a]	22.6 ± 0.3[a]

[a] The value differs from the respective normoxic group ($P < 0.05$). No other significant differences were detected.

Statistics. Results are given as mean ± SEM. The differences between the groups were evaluated with factorial analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test. Due to the negative findings of this study, the relatively liberal Fisher's LSD test was preferred over the more rigorous Sheffé test in order to guard primarily against type II error. For %MT, the values used in ANOVA were the averages for each individual and each vessel category (i.e., B, AD, and P). To compare the reactivity to Ang II and acute hypoxia between the groups, the repeated measures ANOVA was used. Differences were considered significant at $P < 0.05$.

Results

During the 3 weeks after pump implantation one hypoxic control rat died. At the end of the experiment, the body weights of the normoxic rats were significantly higher than those of both hypoxic groups. There was no significant difference in body weight between the control and angiopeptin groups (Table I).

Angiopeptin Levels. In angiopeptin-infused groups, plasma levels of angiopeptin-like immunoreactivity were 476 ± 20 (SEM) pg/ml in the normoxic rats and 298 ± 27 pg/ml in the hypoxic rats ($P < 0.0005$). Angiopeptin was undetectable in the control groups.

***In Vivo* Hemodynamics.** Both groups of chronically hypoxic rats had higher mean pulmonary arterial pressure and total pulmonary resistance index than did normoxic rats (Fig. 1). Mean pulmonary arterial pressure was not reduced in the groups with angiopeptin in comparison with their control groups (Fig. 1A and Table I). Similarly, total pulmonary resistance index was not significantly reduced in the groups with angiopeptin (NA: 94 ± 10 mm Hg/ml/min/g; HA: 200 ± 15 mm Hg/ml/min/g) compared with the groups who did not receive angiopeptin treatment (NC: 124 ± 13 mm Hg/ml/min/g; HC: 214 ± 14 mm Hg/ml/min/g) (Fig. 1B). Cardiac index and mean systemic arterial pressure were not significantly different between normoxic and hypoxic rats (Table I).

Hematology. In agreement with previous reports (21, 28), the hypoxic rats had lower platelet counts than normoxic rats (Table II). The platelet count did not differ significantly between control and angiopeptin groups. Hemoglobin concentration and hematocrit were higher in chronically hypoxic rats than in normoxic rats but were not affected by angiopeptin infusion (Table II).

Morphologic Data. The right ventricular weight, which was significantly higher in the chronic hypoxic than in normoxic groups, was not reduced by angiopeptin (Fig. 2). In fact, the RV/BW ratio was larger ($P < 0.05$) in the HA group (0.09% ± 0.002%) as compared to HC rats (0.08% ± 0.003%) (Fig. 2A). Such a difference did not exist in normoxic rats (0.05% ± 0.002% in NC; 0.05% ± 0.002% in NA; $P > 0.05$). The results were similar for the RV/LV + S ratio (Figure 2B): it was higher ($P < 0.05$) in the HA group (0.45 ± 0.01) than in the HC group (0.38 ± 0.01), while there was no difference between the normoxic groups (0.27 ± 0.01 in NC; 0.24 ± 0.01 in NA; $P > 0.05$).

Medial thickness of parenchymal vessels was increased by chronic hypoxia (from 8.8% ± 0.5% in NC to 15.4% ± 2.0% in HC and from 9.1% ± 0.7% in NA to 16.3% ± 1.3% in HA). Similarly, %MT was increased by chronic hypoxia in the alveolar duct vessels (from 6.7 ± 0.4 in NC to 10.3 ± 1.0 in HC and from 7.2 ± 0.7 in NA to 10.6 ± 0.9 in HA). Medial thickness of the bronchiolar vessels was not significantly altered by chronic hypoxia (12.5% ± 1.4% in NC; 11.2% ± 1.1% in NA; 12.4% ± 1.7% in HC; and 12.6% ± 0.8% in HA). Angiopeptin treatment had no significant effect on %MT in any of the vessel levels (Fig. 3).

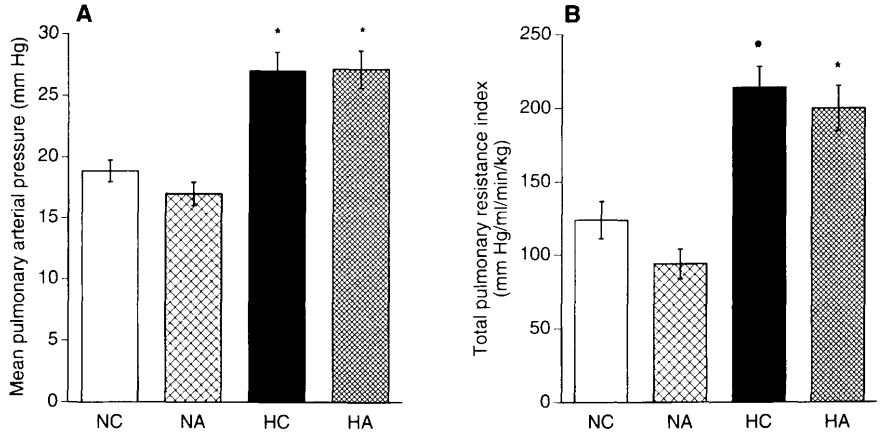

Figure 1. Angiopeptin does not alter the pulmonary arterial pressure (A) and total pulmonary resistance index (B) in normoxic rats or reduce the rise in pressure and resistance caused by chronic hypoxia. NC, normoxic control; NA normoxic angiopeptin; HC, hypoxic control; HA, hypoxic angiopeptin. *Different from respective normoxic value ($P < 0.05$).

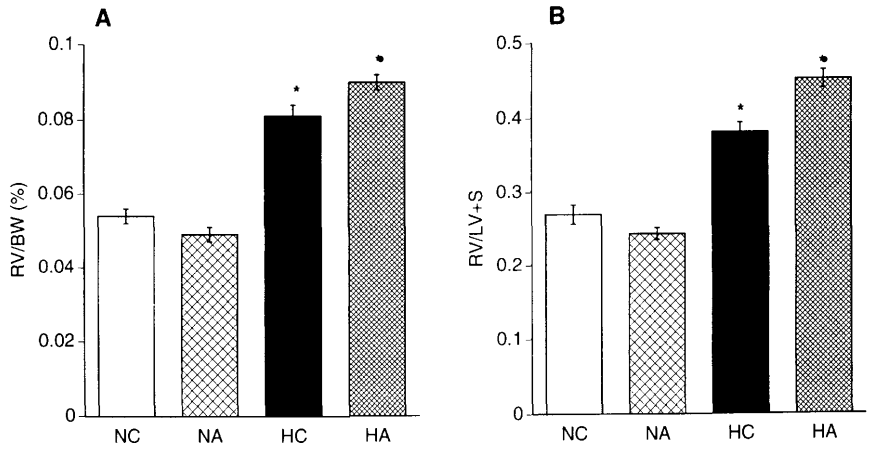

Figure 2. Angiopeptin does not reduce the right ventricular hypertrophy caused by chronic hypoxia. Right ventricular weight is expressed in relation to body weight (A) and to left ventricle plus septum weight (B). NC, normoxic control; NA, normoxic angiopeptin; HC, hypoxic control; HA, hypoxic angiopeptin. *Different from respective normoxic value ($P < 0.05$).

Isolated Perfused Lungs. The baseline perfusion pressure in isolated lungs did not differ significantly between the angiopeptin-treated and -untreated groups (Table I). There was a reduction in reactivity to acute hypoxic challenges in chronically hypoxic rats (Fig. 4B). Angiopeptin did not affect significantly the acute hypoxic pulmonary vasoconstriction in either control rats (change in perfusion pressure at constant flow: 3.8 ± 1.7 mm Hg in NC; 2.4 ± 1.1 mm Hg in NA) or chronic hypoxic rats (HC: 0.2 ± 0.1 mm Hg; HA: 0.1 ± 0.1 mm Hg). Unexpectedly, the lungs of the chronically hypoxic rats treated with angiopeptin did not respond to Ang II injection (change in perfusion pressure: 0.0 ± 0.0 mm Hg), whereas the lungs from chronic hypoxic controls did (5.3 ± 1.6 mm Hg) (Fig. 4A). A similar trend was observed in the normoxic groups, although it was not statistically significant (NC: 3.0 ± 1.1 mm Hg, NA: 1.0 ± 0.35 mm Hg).

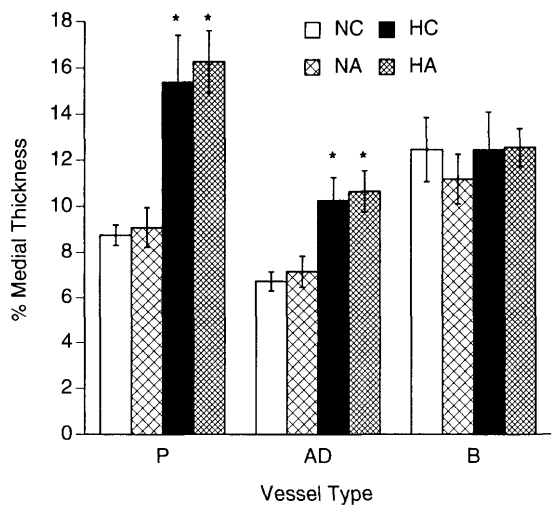

Figure 3. Medial thickness in three types of pulmonary vessels (P, parenchymal; AD, alveolar duct; B, bronchiolar) in the four groups: NC, normoxic control; NA, normoxic angiopeptin; HC, hypoxic control; HA, hypoxic angiopeptin. *Chronically hypoxic groups differ from respective normoxic groups ($P < 0.05$). There were no other significant differences.

Discussion

Chronic hypoxia has been used to induce pulmonary hypertension and to study the ability of agents to decrease the long-term development of pulmonary hypertension. In this study, hypobaric hypoxia for 3 weeks increased pulmonary arterial pressure and resistance in the rat compared with normoxic controls. In addition, chronic hypoxia increased the ratio of right ventricle to body weight and the medial thickness of small pulmonary vessels. None of these measures of hypoxic pulmonary hypertension were significantly changed by chronic angiopeptin infusion.

Previous studies have demonstrated that angiopeptin reduces cellular proliferation in a number of models. In one experiment on neointimal hyperplasia following balloon injury of rabbit aorta, angiopeptin was shown to significantly reduce the expression of c-*fos* and c-*jun* protooncogenes, which promote proliferation (16). Angiopeptin also reduces the proliferation of human coronary smooth muscle cells grown in culture (12). In a recent clinical trial, angiopeptin given subcutaneously for 5 days at the time of coronary angioplasty markedly reduced restenosis at follow-up angiography performed at 6 months (20). Balloon injury of systemic vessels gives rise to myointimal hyperplasia; the migration of smooth muscle cells through the internal elastic lamina and proliferation in the intima. Chronic treatment with angiopeptin decreases intimal thickening in response to balloon injury in the rabbit (17) and pig (19). Similarly, chronic angiopeptin reduces intimal thickening when aortic allografts are studied in the rat (18).

The dose of angiopeptin used, 90–100 μg/kg/day iv, was greater than that found to be effective in reducing myointimal hyperplasia in models of intimal damage, 20 and 50 μg/kg/day sc in rats (29) and 20 μg/kg/day sc or ip in rabbits (16, 17, 30), and in chronic rejection of aortic allografts, 80 μg/kg/day sc in rats

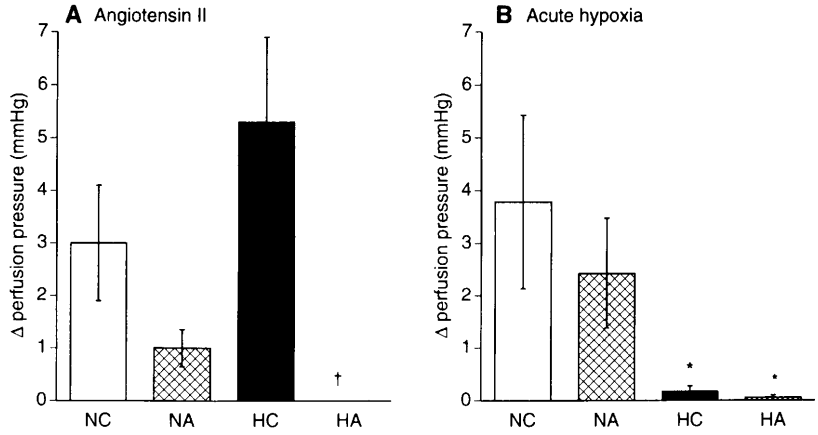

Figure 4. The effects of chronic hypoxia and angiopeptin treatment on the pressor responses to Ang II injection (A) and acute hypoxic challenge (B) in isolated lungs perfused at constant flow rate. Angiopeptin treatment abolishes the reactivity to Ang II in chronically hypoxic rats. Chronic hypoxia reduces pressor response to acute hypoxia. NC, normoxic control; NA, normoxic angiopeptin; HC, hypoxic control; HA, hypoxic angiopeptin. *Different from respective normoxic value ($P < 0.05$); †Different from hypoxic control ($P < 0.01$).

(18). A comparable dose, 100 μg/kg/day sc, reduced intimal hyperplasia following balloon injury in swine (19). In the present study, the angiopeptin was infused directly into the blood and measurable plasma levels were demonstrated by radioimmunoassay. The abolished reactivity to Ang II in the HA group confirms that angiopeptin was effective in the dose delivered. However, we cannot exclude the possibility that angiopeptin was degraded into fragments recognized by the RIA but with physiological effects different from angiopeptin. Angiopeptin levels in the plasma were lower in the hypoxic rats than in the normoxic, possibly because of binding to the greater mass of erythrocytes. Alternatively, angiopeptin degradation, clearance, or uptake (receptor binding) could be increased in chronic hypoxia. It is possible that hypobaria affects pump function and might result in a lower rate of angiopeptin administration. Presently, it is not known if chronic hypoxia alters the angiopeptin half-life. In normoxia, the elimination half-life of intravenously administered angiopeptin is 2–2.5 hr (26).

Angiopeptin is a longer-lasting analog of somatostatin-14. Somatostatin-14 has been reported to reduce pulmonary arterial pressure in chronically hypoxic rats (14). Consequently angiopeptin would be expected to have similar or greater effects. Unlike somatostatin-14, somatostatin-28 exacerbates chronic hypoxic pulmonary hypertension (14). Somatostatin-14 and -28 have different receptors and different physiologic effects in other systems (31). It is possible that angiopeptin had some overlap-reactivity with the somatostatin-28 receptor. Stimulation of the somatostatin-28 receptor might counterbalance any beneficial effect of angiopeptin stimulation of the somatostatin-14 receptor. Alternatively, methodological differences between our study and that of Tjien-A-Looi *et al.* (14) may explain the apparently disparate results on the effect of angiopeptin and somatostatin-14 on pulmonary hypertension. The duration of hypoxia was longer in our study (21 days) than in that of Tjien-A-Looi *et al.* (4–16 days). They studied four rats infused with somatostatin-14 during 16 days of hypoxia. Although they found pulmonary arterial pressure reduced by the somatostatin-14 infusion, they did not measure cardiac output, so the effect of somatostatin-14 on pulmonary vascular resistance is unknown. The right ventricular hypertrophy, which is known to correlate with chronic hypoxic pulmonary hypertension in rats (32), was unchanged by the somatostatin-14 infusion (14), suggesting that pulmonary hypertension might not have been significantly reduced. The effect of somatostatin-14 treatment of pulmonary vascular morphology was not studied by Tjien-A-Looi *et al.* (14).

As we expected angiopeptin to affect mainly the pulmonary vascular remodeling rather than reactivity, we used Ang II primarily to prime the lungs for the subsequent hypoxic response (24). Surprisingly, angiopeptin eliminated the Ang II reactivity in chronic hypoxic pulmonary hypertension. The mechanism of this effect is unknown.

The chronic hypoxic lungs showed a marked decrease in the rise in pulmonary arterial pressure caused by acute hypoxia compared with that observed in the normoxic lungs. This is consistent with previous observations (21, 33). Angiopeptin exerted no effect on the responses to acute hypoxia in either group.

We conclude that angiopeptin at the high intravenous dose used in this study does not significantly reduce chronic hypoxic pulmonary hypertension in the rat.

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