

## Inhibition of hypoxic pulmonary vasoconstriction in isolated rat resistance arteries by atrial natriuretic peptide

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*Inhibition of hypoxic pulmonary vasoconstriction in isolated rat resistance arteries by atrial natriuretic peptide. T.K. Rogers, J.S. Thompson, A.H. Morice. ©ERS Journals Ltd 1997.*

**ABSTRACT:** Atrial natriuretic peptide (ANP) is a vasodilator secreted by the heart in response to right atrial stretch. We have hypothesized that ANP may be released to attenuate pulmonary hypertension due to hypoxia. We have examined whether ANP inhibits hypoxic pulmonary vasoconstriction (HPV) in isolated pulmonary resistance vessels (PRV) from chronically hypoxic (CH) rats, compared to air-breathing, control (C) rats.

After at least 17 days of chronic hypoxia, vessels (n=29) were dissected from CH and C littermates and mounted in an automated myograph. The inhibitory effect of ANP on the rapid first contractile phase of HPV, and the relaxant effect of ANP on vessels tonically contracted in the second phase of HPV, were studied.

ANP caused concentration-dependent inhibition of HPV in both C and CH vessels ( $p < 0.001$ ), whilst vehicle had no effect (mean maximum inhibition was 88 and 101%, respectively, at 17 nM ANP). ANP also caused significant concentration-dependent relaxation of the second contractile phase of HPV, which was similar in C and CH vessels (mean maximum relaxation of 89 and 94%, respectively; median effective concentrations were 2.4 and 2.0 nM, respectively).

We conclude that atrial natriuretic peptide is a potent antagonist of both contractile phases of hypoxic pulmonary vasoconstriction in isolated rat pulmonary resistance vessels at concentrations similar to those observed in hypoxic pulmonary hypertension in life. There was no difference between vessels from chronically hypoxic and control animals.

*Eur Respir J 1997; 10: 2061–2065.*

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Keywords: Atrial natriuretic peptide, chronic hypoxia, hypoxic pulmonary vasoconstriction, myograph, natriuretic peptides, pulmonary hypertension

Received: December 4 1996  
Accepted: July 4 1997

This work was supported by a fellowship from Bristol-Myers Squibb Pharmaceuticals Ltd (TKR). JST was funded by The Special Trustees for the Former United Sheffield Hospitals.

We have hypothesized that the cardiac hormone, atrial natriuretic peptide (ANP), may be released to attenuate pulmonary hypertension due to hypoxia. We have examined whether ANP inhibits hypoxic pulmonary vasoconstriction (HPV) in isolated pulmonary resistance vessels.

ANP is a vasodilator of the pulmonary vasculature hypothesized to have a physiological role in modulating pulmonary vascular resistance. Release of ANP is governed by right atrial stretch and because of its short half-life, intravascular concentrations are highest in the pulmonary circulation. ANP is elevated in conditions characterized by pulmonary hypertension. Levels are modestly increased in chronic obstructive pulmonary disease (COPD) and more markedly in severe pulmonary hypertension [1–4]. In patients with COPD we have demonstrated that infusion of ANP leads to a fall in mean pulmonary artery pressure, pulmonary artery wedge pressure and total pulmonary vascular resistance, without significant effects on the systemic vasculature [4].

The normal pulmonary vasculature has low intrinsic tone and to demonstrate the vasodilator properties of ANP precontraction is required. In the isolated lung and in isolated vessels, ANP has been shown to cause vasodilatation when tone is increased by pharmacologi-

cal means [5–10]. The most important physiological vasoconstrictor of the pulmonary circulation is hypoxia and the effect of ANP on hypoxic pulmonary vasoconstriction (HPV) has been studied in anaesthetized animals [6, 11, 12] and using isolated lung preparations [10]. Until recently, it had proved difficult to demonstrate consistent HPV in isolated pulmonary arteries. We and others have developed a technique which allows the study of HPV in isolated resistance arteries by priming with prostaglandin  $F_{2\alpha}$  [13, 14]. In this system, HPV is biphasic, consisting of a rapid, endothelially independent first phase and a slowly developing second phase, which is endothelially dependent. We have now examined the effect of ANP on both phases of HPV in isolated vessels. Chronic hypoxia causes pulmonary hypertension in both humans and animal models, which is associated with muscular remodelling of pulmonary arteries and arterioles. In a previous study, we examined the effect of chronic hypoxia on the relaxant response to ANP in prostaglandin  $F_{2\alpha}$ -precontracted pulmonary resistance vessels [9]. ANP caused a greater relaxation in vessels from chronically hypoxic animals, and in this present study we have used similar experimental conditions to determine whether this is also true of HPV.

## Materials and methods

### Animals

Male Wistar rats ( $n=29$ ), supplied by the University of Sheffield Field Laboratories, were used. Animals were 5 weeks old at the start of the experiments.

### Study design

Littermate rats were divided at 5 weeks of age, when half were placed in a normobaric hypoxic chamber and half were designated as normoxic control (C) animals and were housed in the same room, breathing room air. Animals were placed in the hypoxic chamber in order to model the development of pulmonary hypertension. Chronic hypoxia (CH) was achieved by maintaining the internal environment at 10%  $O_2$  and all rats were allowed food and water *ad libitum*. After at least 17 days (mean 30 days) littermate C and CH pairs were studied.

### Methods

**Vessel dissection and mounting.** Animals were anaesthetized with intraperitoneal pentobarbitone ( $60 \text{ mg}\cdot\text{kg}^{-1}$ ) and heart and lungs were removed *en bloc*. Further dissection and mounting of small pulmonary arteries onto an automated microvascular myograph (Cambustion Ltd, Cambridge, UK) was performed using an operating microscope as described previously [9]. Vessels were studied as ring preparations at a resting tension equivalent to a transmural pressure of 4.7 kPa (35 mmHg). The organ bath contained physiological salt solution (PSS) maintained at  $37^\circ\text{C}$  and bubbled with 95%  $O_2$ /5%  $CO_2$ . C and CH vessel pairs were mounted in each of the two organ baths of the multichannel myograph. After an initial equilibration period of approximately 1 h, vessel viability and contractile reproducibility were assessed by triplicate contractions to a maximal depolarizing concentration of KCl (100 mM). Any vessel that did not actively contract by greater than  $0.5 \text{ mN}\cdot\text{mm}^{-1}$  was assumed to be damaged and was discarded.

**Hypoxic contractions.** Vessels were precontracted with prostaglandin F ( $\text{PGF}_{2\alpha}$ ) ( $10 \mu\text{M}$ ), and the response to hypoxia measured after steady-state tension was achieved. The induction of basal tone with vasoactive agents has previously been demonstrated to greatly enhance HPV [13, 14] in rat isolated pulmonary arteries.  $\text{PGF}_{2\alpha}$  was used because it produces a sustained and more reproducible precontraction than other agents (*e.g.* KCl, angiotensin II). To produce hypoxic conditions, the PSS in the organ bath was perfused with 5%  $CO_2$ /95%  $N_2$ . With the bath uncovered and exposed to room air, this produced a resting partial pressure of oxygen in arterial blood ( $P_{a,O_2}$ ) within the PSS of 10–12 kPa. When the organ bath was covered, the  $P_{a,O_2}$  decreased to 2–3 kPa (Corning blood gas analyser, Corning Halstead, Essex, UK; and Strathkelvin Instruments oxygen meter model 781 Bearsdon, Glasgow, UK). Perfusion was maintain-

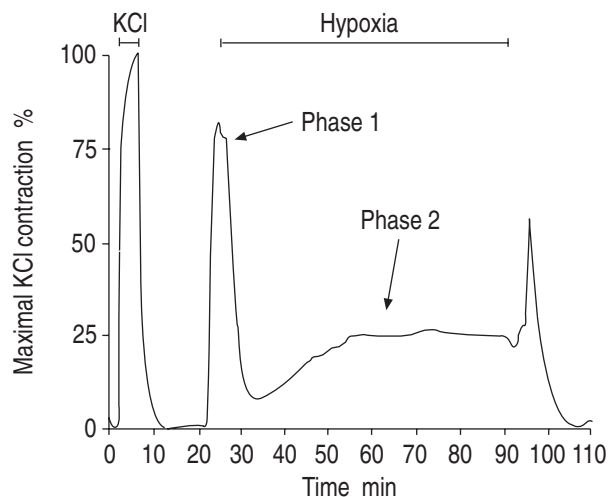


Fig. 1. – A typical contraction of a control pulmonary resistance vessel (diameter  $232 \mu\text{m}$ ) in response to hypoxia, expressed as percentage of a maximum KCl contraction. The organ bath was initially open to room air, being bubbled with 95%  $N_2$ /5%  $CO_2$ . A contraction in response to KCl (100 mM) was performed and the bath washed four times with physiological salt solution (PSS). Tone was produced in the vessel with  $\text{PDG}_{2\alpha}$  ( $10 \mu\text{M}$ ), and when this contraction had stabilized, the bath was covered, causing the partial pressure of oxygen of the PSS to fall. The biphasic nature of the hypoxic contraction is demonstrated. The lid was removed after 95 min.

ed until a maximum contractile response was seen, generally within 2–5 min (first contractile phase). After 30 min, a second contractile peak was observed (fig. 1). Hypoxic responses were reported as maximum active tension generated above baseline during the first and second contractile phases of hypoxia.

### Experiment 1: inhibition of first phase of hypoxic vasoconstriction by ANP

After stabilization of the precontraction to  $\text{PGF}_{2\alpha}$  ( $10 \mu\text{M}$ ), C and CH vessel pairs were exposed to hypoxia. When the first contractile phase of the hypoxic response reached a plateau, the baths were opened, resulting in rapid relaxation to the  $\text{PGF}_{2\alpha}$  baseline, and the bath was washed four times with oxygenated PSS. Four such challenges were performed to establish a baseline, before subsequent hypoxic challenges in the presence of incremental doses of ANP (17 pM to 60 nM) or ANP vehicle (as negative control).

### Experiment 2: the effect of ANP on the second phase of HPV

Different C and CH vessels were used in this study. Cumulative doses of ANP (0.017–17 nM) were added when the second contractile phase of HPV had plateaued, after about 1 h of continuous bubbling with the hypoxic gas mixture.

### Solutions and drugs

PSS consisted of NaCl (120 mM), KCl (4.7 mM),  $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$  (2.5 mM),  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$  (1.17 mM),  $\text{NaHCO}_3$

(25 mM),  $\text{KH}_2\text{PO}_4$  (1.18 mM), ethylenediamine tetraacetic acid (EDTA) (26.9  $\mu\text{M}$ ), and glucose (5.5 mM) dissolved in de-ionized water. This was continuously bubbled with 95%  $\text{O}_2$ , 5%  $\text{CO}_2$  mixture, maintaining a pH of approximately 7.4.  $\text{PGF}_{2\alpha}$  (Sigma, Poole, Dorset, UK) was dissolved in normal saline. Human ANP (Sigma) was dissolved in a solvent of 50% (w/v) Haemacel (Hoechst, Hanslow, Middlesex, UK) and 50% normal saline (150 mM), at pH 4, by adding drops of 1% acetic acid, to make a stock solution of 100  $\mu\text{g}\cdot\text{mL}^{-1}$ . Subsequent dilutions were made with normal saline. This procedure was followed to minimize adsorption of ANP onto plastic containers and pipette tips.

### Statistical analysis

Parametric data were analysed using analysis of variance (ANOVA) or student's t-tests, as appropriate. Median effective concentrations ( $\text{EC}_{50}$ s) did not follow a normal distribution and the Wilcoxon signed rank test was used. A p-value of less than 0.05 was considered significant. Values were reported as mean  $\pm$  SEM, except where stated. The tension data for individual vessels was recorded digitally. The data for hypoxic responses of the various groups of vessels were averaged, after ensuring that the peak of the first phase of contraction coincided for all the vessels in the group.

## Results

CH animals weighed significantly ( $p < 0.001$ ) less than their littermate controls ( $247 \pm 10$  and  $287 \pm 12$  g, respectively), and the vessels from both groups were of similar diameter ( $278 \pm 58$  and  $245 \pm 54$   $\mu\text{m}$ , respectively, table 1).

When the organ baths were exposed to atmospheric air, the mean (SD) partial pressure of oxygen ( $P_{\text{O}_2}$ ) was  $10.9 \pm 2.3$  kPa, partial pressure of carbon dioxide ( $P_{\text{CO}_2}$ ) was  $3.7 \pm 0.5$  kPa, pH  $7.45 \pm 0.07$  ( $n=6$ ). Under these conditions, the maximum KCl responses were significantly lower in CH vessels;  $2.3 \pm 0.2$  compared with  $3.8 \pm 0.9$   $\text{mN}\cdot\text{mm}^{-1}$  in C vessels.

Approximately 5 min after the organ baths were sealed, the vessels generated a rapid "phase 1" vasoconstriction (fig. 1). The threshold gas tensions for first-phase HPV were  $P_{\text{O}_2}$   $5.5 \pm 1.7$  kPa,  $P_{\text{CO}_2}$   $4.4 \pm 0.7$  kPa, pH  $7.42 \pm 0.10$  ( $n=20$ ). Vasoconstriction was significantly greater in C

Table 1. – Mass of rats, and internal diameter and contractility of pulmonary resistance vessels from control rats of chronically hypoxic rats

	Control vessel (n=10)	Chronically hypoxic vessels (n=10)	ANP vehicle control (n=9)
Animal mass g	287 (12)	247 (10)	282 (10)
Vessel diameter $\mu\text{m}$	252 (55)	278 (58)	245 (54)
$\text{KCl}_{\text{max}}$ $\text{mN}\cdot\text{mm}^{-1}$	3.8 (0.9)	2.3 (0.2)	4.1 (0.9)
Mean HPV $\text{mN}\cdot\text{mm}^{-1}$	3.5 (1.2)	1.8 (0.2)	3.4 (0.9)

Results are mean ( $\pm$ SEM).  $\text{KCl}_{\text{max}}$ : maximum mean contractile response to KCl (100 mM); HPV: maximum first contractile phase of hypoxic pulmonary vasoconstriction.

than CH vessels. Active tension was  $3.55 \pm 1.24$   $\text{mN}\cdot\text{mm}^{-1}$  in C vessels and  $1.94 \pm 0.19$   $\text{mN}\cdot\text{mm}^{-1}$  in CH vessels ( $p < 0.05$ ). If expressed as a percentage of maximum KCl contractions, however, these responses were similar at 82 and 88%, respectively.

The first contractile phase spontaneously reduced after about 5 min and was succeeded by a sustained, second phase of vasoconstriction ("phase 2"), which reached a plateau approximately 40 min after the peak of phase 1 (fig. 1). The gas tensions during peak second phase contraction were  $P_{\text{O}_2}$   $2.1 \pm 0.9$  kPa,  $P_{\text{CO}_2}$   $6.7 \pm 0.6$  kPa and pH  $7.17 \pm 0.04$ . This second phase of vasoconstriction produced similar increases in active tension in C and CH vessels,  $1.1 \pm 0.22$  and  $1.16 \pm 0.21$   $\text{mN}\cdot\text{mm}^{-1}$ , respectively. Both phases of contraction were rapidly and completely reversible by re-oxygenation.

### Experiment 1: effect of ANP on phase 1 of the hypoxic response

The first phase of the hypoxic response was significantly (ANOVA,  $p < 0.001$ ) inhibited by ANP (17 nM) in both C and CH vessels, whilst ANP vehicle alone

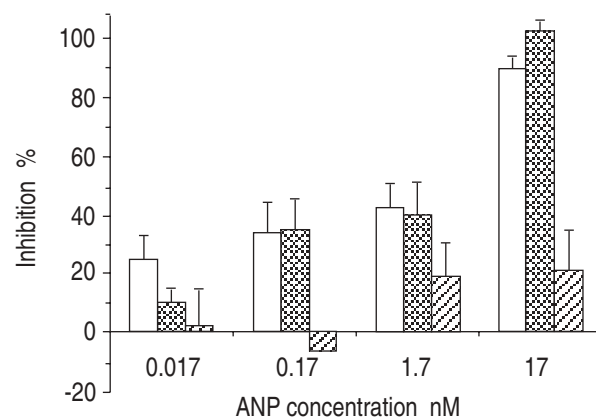


Fig. 2. – The mean  $\pm$  SEM percentage inhibition of phase 1 of the hypoxic contractile responses to control and chronically hypoxic pulmonary resistance vessels by atrial natriuretic peptide (ANP) (17 pM–17 nM). □: control vessels ( $n=10$ ); ▨: chronically hypoxic vessels ( $n=10$ ); ■: ANP vehicle control ( $n=9$ ).

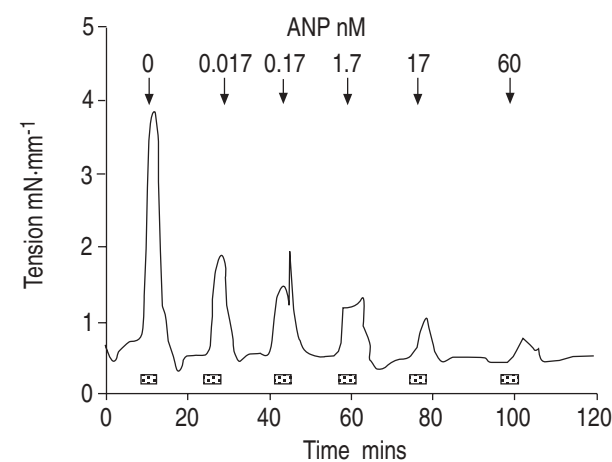


Fig. 3. – Typical trace showing phase 1 contractile hypoxic responses of a control pulmonary resistance vessel, internal diameter 266  $\mu\text{m}$ , in the presence of increasing concentrations of atrial natriuretic peptide (ANP) (17 pM–60 nM). Shaded areas represent periods of hypoxia.

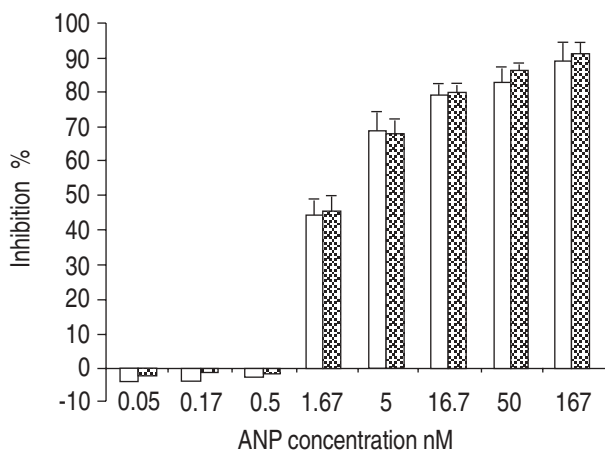


Fig. 4. — The spasmolytic effect of atrial natriuretic peptide (ANP) (0.17–288 nM) on stable, phase 2 contractile hypoxic response of control (□) and chronically hypoxic (▨) pulmonary resistance vessels.  $n=10$  vessels in each group.

caused no significant change in contractility (ANOVA,  $p=0.4$ ) (fig. 2). A typical trace is shown for a C vessel (internal diameter 266  $\mu\text{m}$ ) in figure 3. There were no significant differences between the effects of ANP at any concentration on the C and CH vessels.

#### Experiment 2: effect of ANP on phase 2 of the hypoxic response

There was dose-dependent relaxation of the vessels tonically contracted by hypoxia. Peak relaxation was similar at 89 and 94% in C and CH vessels, respectively (fig. 4). The  $EC_{50}$  was also similar, with values of 2.4 and 2.0 nM in C and CH vessels, respectively ( $p=0.87$ ).

### Discussion

In this series of experiments we looked at the effect of a putative homeostatic hormone, ANP, on the main physiological mediator of pulmonary vascular resistance, namely HPV. HPV arose early in evolution and is present in amphibians and reptiles. In mammals it has two roles, one at birth to facilitate the change from foetal to adult circulation and secondly to prevent systemic hypoxaemia by ensuring correct ventilation perfusion matching in the presence of lung disease [15]. Excessive HPV such as occurs in high altitude pulmonary oedema is harmful and a counter regulatory mechanism which protects the right heart has been hypothesized by several authors [16].

The cellular events leading to HPV are still poorly understood. The effector mechanism is likely to be a depolarization caused by a closure of an outward potassium channel thus leading to influx of calcium through voltage gated calcium channels. This hypothesis is supported by direct patch clamping studies [17, 18] and by the potency of drugs acting at these channels to inhibit HPV [13]. However, the intracellular events which lead to this change in ion flux are still a mystery.

Whilst ANP has been shown to be a vasodilator of the pulmonary vascular bed *in vivo* and *in vitro*, we felt it important to study the effect of ANP in isolated ves-

sels in response to hypoxia particularly since in this system HPV has recently been shown to be characteristically biphasic [13, 14]. Our results indicate that ANP is a highly effective vasorelaxant of HPV in pulmonary artery resistance vessels with  $EC_{50}$  values within the range that occur *in vivo*, thus supporting a physiological role. This contrasts with a study performed under identical conditions using  $PGF_{2\alpha}$ -contracted vessels [9]. Here, ANP was much less potent: the  $EC_{50}$  was 62 mM in control and 28 nM in CH vessels, in comparison to 2.4 and 2.0 nM, respectively, when the agonist was hypoxia. The magnitude of the relaxation achieved was also much less: 37 and 63% of the  $PGF_{2\alpha}$  contraction in C and CH vessels, respectively, in comparison with the virtually complete inhibition and relaxation of hypoxic contractile responses. This apparent selectivity for HPV as a contractile agonist may be linked with the intracellular activity of ANP causing release of cyclic guanosine monophosphate (cGMP) *via* particulate guanylate cyclase. In pulmonary vascular smooth muscle, cGMP may act directly on a calcium-sensitive potassium channel, decreasing the tendency for membrane depolarization and thus the ability of the cell to react to hypoxia with vasoconstriction [19].

Which of the two phases of HPV seen in isolated vessels represents the true HPV seen *in vivo* is currently a matter of much debate. The first phase has the right time course of onset, but rapidly diminishes. In this study, the second phase generated much less wall tension, but this was more than adequate to close the vessel lumen. Calculated equivalent intramural pressures of the second phase were 9.0 kPa (68 mmHg) and 9.6 kPa (72 mmHg) in C and CH vessels, respectively [12]. Unfortunately, the response to ANP was not helpful in separating these two phases since it was highly potent against both. It is likely that HPV *in vivo* represents a summation of both phases in whole lung.

Chronic hypoxia leads to pulmonary hypertension and is known to cause pulmonary vascular remodelling with extension of identifiable muscle distally into previously thin-walled arterioles of diameter less than 80  $\mu\text{m}$ . We made an effort to consistently select vessels of a similar size, and the diameters of the C and CH vessels in both experiments were comparable. The contractility of the vessels from CH animals both to hypoxia and KCl was less than that of the vessels from their normoxic littermates. This is likely to be due to morphological changes within the vessel wall secondary to pulmonary vascular remodelling. In the rat, characteristic features are the development of a double elastic lamina and medial hyperplasia. Both phases of hypoxic pulmonary vasoconstriction were demonstrable in normal and remodelled rat pulmonary resistance vessels and were potently inhibited by ANP. The responses of the C and CH vessels to ANP were similar. We have previously found that the potency of ANP against HPV was greater in isolated perfused lungs from CH rats compared to controls [10]. It may be that the neomuscularized vessels, which are rather smaller than those examined in this study, contribute substantially to the pulmonary vascular resistance of CH animals. Thus, the explanation for the increased potency of ANP in the intact remodelled vascular bed may be the extended site of action available to ANP.

These findings are further evidence for an important action of atrial natriuretic peptide on the pulmonary circulation, showing for the first time powerful antagonism of both phases of hypoxic contraction of isolated pulmonary resistance vessels, and with no attenuation of the response in remodelled vessels from chronically hypoxic rats.

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