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C-type natriuretic peptide does not attenuate the development of pulmonary hypertension caused by hypoxia and VEGF receptor blockade

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Abstract

AIMS—C-type natriuretic peptide (CNP) is a local regulator of vascular tone and remodeling in many vascular beds. However, the role of CNP in modulating pulmonary arterial hypertensive and vascular remodeling responses is unclear. The purpose of this study was to determine if CNP is capable of preventing the development of pulmonary hypertension (PH).

MAIN METHODS—We used animal models of PH caused by chronic hypoxia alone or in combination with the VEGF receptor blocker SU5416. We measured pulmonary hemodynamics, right ventricular hypertrophy and vascular remodeling effects in response to a continuous infusion of low dose or high dose CNP or vehicle placebo.

KEY FINDINGS—Right ventricular hypertrophy and a marked elevation in right ventricular systolic pressure (RVSP) were seen in both models of PH. Rats treated with the combination of SU5416 and chronic hypoxia also developed pulmonary endothelial hyperproliferative lesions. Continuous intravenous infusion of CNP at either dose did not attenuate the development of PH, right ventricular hypertrophy or vascular remodeling in either of the models of PH despite a three-fold increase in serum CNP levels.

SIGNIFICANCE—CNP does not prevent the development of PH in the chronic hypoxia or SU5416 plus hypoxia models of pulmonary hypertension suggesting that CNP may not play an important modulatory role in human PH.

Keywords

C-type Natriuretic Peptide; VEGF Receptor Blockade; Chronic Hypoxia; Pulmonary Hypertension; SU5416

INTRODUCTION

Pulmonary hypertension (PH) is a disease of the small pulmonary arteries that is characterized by vasoconstriction and vascular remodeling (Humbert et al., 2004) including increased medial thickeness, muscularization of non-muscularized vessels (Rich &

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Brundage, 1989; Tuder et al., 2001), and endothelial cell proliferative lesions (plexiform lesions) in distal pulmonary arteries (Tuder et al., 1994; Tuder et al., 2007). In addition, endothelial dysfunction observed in PH decreases the production of vasodilators, whereas it increases that of vasoconstrictors(Budhiraja et al., 2004; Davie et al., 2002). Thus, a therapeutic strategy that can redress the imbalance between endothelial vasodilators and vasoconstrictors or between endothelial mitogens and growth repressors may be effective for the treatment of PH.

Atrial, Brain and C-type natriuretic peptides (ANP, BNP, and CNP, respectively) play a critical role in the coordination of vascular tone and fluid/electrolyte balance. ANP and BNP are predominately produced in the cardiomyocytes. In contrast, CNP has been found in many tissues including lung (Heublein et al., 1992; Stingo et al., 1992; Suga et al., 1992) and is synthesized and secreted primarily by endothelial cells. In addition to vasodilatory properties, CNP has anti-inflammatory (Itoh et al., 2004; Murakami et al., 2004; Scotland et al., 2005) and anti-mitogenic (Doi et al., 2001; Itoh et al., 2004; Murakami et al., 2004) properties and has been shown to induce endothelial regeneration in the injured vasculature (Doi et al., 2001; Ohno et al., 2002; Yamahara et al., 2003).

Though CNP has been shown to be a potent vasodilator in many systemic vascular beds and in multiple animal models (Amin et al., 1996; Garcha & Hughes, 2006; Wei et al., 1993), its role in PH is unclear. While CNP vasodilates the pulmonary vasculature in normoxic settings (Klinger et al., 1998a; Vang et al., 2010), it has an attenuated vasodilatory effect in hypoxia-adapted rats (Klinger et al., 1998a). Chronic infusion of CNP has been shown to attenuate monocrotaline (MCT)-induced PH in rats (Itoh et al., 2004). Although MCT model of PH has been used extensively, it is considered as a more acute toxic model of vascular injury associated with endothelial cell injury and apoptosis (Rosenberg & Rabinovitch, 1988; Thomas et al., 1998), infiltration of monocytes and macrophages in the pulmonary capillary bed (Kimura et al., 1998; Sugita et al., 1983) and impaired fibrinolysis (Schultze & Roth, 1993). Also, intimal proliferative lesions typical in certain human PH are not seen in this model. Moreover, a large number of agents have been demonstrated to be beneficial in this model, including some that are known to cause PH (reviewed in(Stenmark et al., 2009)). Unlike human disease, this model responds well to therapies likely due to the acute/subacute nature of the injury with MCT. Hence, it is imperative to confirm the effect of potential theapeutic agents such as CNP, in other models of PH prior to consideration for human use.

Hypoxic PH may be a more relevant model in which to examine the pulmonary vascular effects of CNP as it occurs commonly in patients with obstructive pulmonary disease (COPD), sleep disordered breathing, and lung parenchymal diseases (Stenmark et al., 2009). Recently, the VEGF receptor antagonist SU5416 has been evaluated as an experimental model of PH that may more closely resemble clinical PH (Taraseviciene-Stewart et al., 2001). The combination of SU5416 plus hypoxia has been shown to induce severe PH and obstructive intravascular lesions in distal pulmonary arterioles that resemble the plexiform lesions observed in idiopathic PH (Abe et al., 2010). The purpose of this study was to determine if CNP is capable of preventing the development of hypoxic PH or PH caused by SU5416 plus chronic hypoxia.

METHODS

Animals

All animal protocols were approved by the Institutional Animal Use and Care Committee of the Providence VA Medical Center.

Experimental Protocol for Hemodynamic studies

Adult male Sprague Dawley rats (Strain: CD, 200-250g) were anesthetized with an injection of intraperitoneal ketamine (75 mg/kg) and medetomidine (0.5 mg/kg), and buprenorphine was used for analgesia. Subsequently Alzet osmotic pumps were implanted subcutaneously between the scapulae of the animals to deliver intravenous CNP (0.75μg/hr or 2.25μg/hr) or vehicle (5% Dextrose) for three weeks. After recovering for 48h, the animals received a single subcutaneous (s.c.) dose of SU5416 (20mg/kg) or diluent and were placed in either normoxic or normobaric hypoxic (10% FiO2) environment for 3 weeks. A total of 85 animals were divided into four groups: i) SU5416 rats exposed to hypoxia, ii) diluent rats exposed to hypoxia, iii) SU5416 rats exposed to normoxia, and iv) diluent rats exposed to normoxia. The animals were maintained on standard rat chow. After 3 weeks of normoxia or hypoxia, rats were weighed and anesthetized.

Transthoracic echocardiograms and hemodynamic studies were performed on Day 21of hypoxic or normoxic exposure. Transthoracic echocardiogram was used to measure right ventricular outflow tract (RVOT) diameter, pulse-wave Doppler across RVOT, and heart rate. Rats were anesthetized with ketamine (75 mg/kg ip) and medetomidine (0.5 mg/kg ip) and parasternal short axis views were obtained using a 5-10 MHz linear transducer (ATL CL 10-5) and HDI 3000 ultrasound machine. The pulse-wave Doppler recording at the RVOT was used to measure pulmonary acceleration time (PAT) and the velocity time integral (VTI). Stroke volume was calculated by multiplying the VTI with the area of RVOT. Cardiac output was calculated by multiplying the stroke volume with the heart rate.

For hemodynamic measurements, rats were anesthetized with ketamine (75 mg/kg ip) and medetomidine (0.5 mg/kg ip) and a polyethylene catheter was inserted into the right carotid artery to measure mean arterial pressure and heart rate. A polyethylene catheter was inserted into the RV to measure RV pressure. After hemodynamic measurements, blood was collected and the animals were euthanized. The ventricles and lungs were excised, dissected free, and weighed. The ratio of RV weight to body weight, the ratio of RV weight to left ventricular plus septal weight, and the ratio of left ventricular plus septal weight to body weight were calculated as indices of ventricular hypertrophy.

Morphometric Analysis of Pulmonary Arteries

Lung sections were prepared for histological analysis by placing a cannula in the trachea and infusing formalin under 12cm H_2O pressure. Lungs were removed from the chest and placed in normal buffered formalin. Transverse sections were cut, paraffin embedded and stained with hematoxylin and eosin (H&E) stain. Three animals in each treatment arm and three sections per animal were evaluated to calculate medial wall thickness. The data was averaged from all sections of the same animal for n=1 value. The external diameter and the medial thickness were measured in all arteries with external diameter from 25-150μm. The medial wall thickness was calculated as follows: percentage of wall thickness = ([medial thickness × 2]/external diameter) × 100 and compared between groups. Histological analysis was performed in a blinded fashion by two observers. In order to assess for muscularization, forty random vessels (<100 μm) from each slide were evaluated and scored as non muscularized (no smooth muscle cell layer), partially muscularized (smooth muscle cell layer < ¾ of the circumference), and fully muscularized (smooth muscle layer > ¾ of circumference) (Klinger et al., 1998b).

Immunohistochemical Analysis

Histological sections were used to perform immunoperoxidase staining using antibodies directed against von-Willebrand Factor (vWF). Endothelial proliferation was calculated as the percent occluded vessels relative to the total vessels on the slide. Criteria for inclusion of

a vessel in the quantitation was that the vessel was cut *en face* and had a diameter between 50-150μM. Histological analysis was performed in a blinded fashion by two observers. Data was obtained from 18 animals treated with SU5416 and chronic hypoxia (9 CNP, 9 vehicle) and 8 animals with chronic hypoxia (4 CNP, 4 vehicle), examining 2-4 sections per animal with each animal n=1.

Immunoblot analyses

Protein was isolated from whole lung lysate harvested from animals treated with either CNP or vehicle infusion. Equivalent amounts of lysate (200 μg protein) were resolved by SDS-PAGE and immunoblotted as previously described (Simon et al., 2009). Briefly, the proteinw as transferred to a poly(vinylidene difluoride) membrane.

Membranes were blocked in TBS Tween (0.2%) containing 5% nonfat dry milk and incubated with NPR-B antibody (C-15, Catalog # SC-34421) obtained from Santa Cruz Biotechnology in 1:250 dilution overnight at 4°C. Bound antibodies were visualized by using secondary antibodies with conjugated horseradish peroxidase and ECL reagent (Amersham Pharmacia).

CNP Assay

CNP concentration from serum obtained at the end of the 21 day exposure was determined using ELISA assay (Bachem Americas,Inc., Torrance, CA) following the manufacturers protocol.

Statistical Analysis

All data were expressed as mean ± SEM unless otherwise indicated. Comparisons of parameters among the three groups were made by one-way analysis of variance followed by Fisher-Hayter analysis for pairwise comparisons. A value of p less than 0.05 was considered statistically significant.

RESULTS

Effect of CNP infusion on development of pulmonary arterial hypertension and right ventricular hypertrophy

The RV systolic pressure was significantly increased in both the chronic hypoxia and SU5416 plus hypoxia models compared to normoxic controls (Figure 1). Both groups had mean RV systolic pressure more than double the control group. Representative RV pressure tracings of animals exposed to 3 weeks of Normoxia/ Vehicle (D5W) **(a)** and SU5416 + Hypoxia/ Vehicle **(b)** are shown in Figure 1A. There was a trend toward increased RVSP in rats given SU5416 under normoxic conditions, versus normoxic controls, but the difference was not statistically significant (Figure 1B). Increases in RV systolic pressure (RVSP) were associated with a marked RV hypertrophic response as evidenced by the increase in RV/LV +S (Figure 2) or RV/ body weight (Supplementary Figure 1). There was also an increase in RV/LV+S or RV/ body weight in rats given SU5416 under normoxic conditions although the increase was not nearly as great as that seen in the chronic hypoxia and SU5416 + hypoxia groups (Figure 2, Supplementary Figure 1). CNP infusion at either 0.75μg/hr or 2.25μg/hr or did not attenuate the increases in RVSP or RV mass as compared with vehicle alone in any of the experimental groups (Figures 1B and 2, Supplementary Figure 1). In addition, there was no significant difference in mean carotid arterial pressure or heart rate among the four groups (data not shown). We further noted that CNP had no significant affect on the carotid arterial pressures nor heart rates of the infused animals (data not shown). We assayed the level of circulating CNP in the animals which received the chronic

infusion of CNP and noted a 3-fold higher serum CNP level in animals in the SU5416 + hypoxia group implanted with CNP pumps compared to animals implanted with vehicle pumps (Vehicle pumps: 0.13 ± 0.01 ng/ml, CNP pumps: 0.38 ± 0.01 ng/ml; $p<0.05$, n=8).

On transthoracic echocardiogram, the PAT (milliseconds) was significantly increased in both hypoxic groups, as compared to the normoxic groups. This correlated with increased pulmonary pressures in the hypoxic groups. All other variables recorded, including heart rate (bpm), RVOT diameter (cm), RVOT velocity time integral (cm), stoke volume (cc) and cardiac index (cc/gm), were not significantly different between any of the experimental groups (Table 1). Furthermore, the high dose of infused CNP had no inhibitory effect on PA acceleration time as assessed by echocardiogram (Table 1).

Effect of CNP infusion on microvascular remodeling in severe PH

The medial wall thickness increased in vessels sized below 150μm in rats exposed to SU5416, hypoxia or SU5416 plus hypoxia, as compared to control animals (Figure 3A). CNP infusion did not significantly inhibit the increase in medial wall thickness, as compared with vehicle placebo (Figure 3B). In order to confirm these results, we performed additional analyses to evaluate the muscularization of arteries. CNP infusion (2.25 μg/hr) failed to alter the distribution of non-muscularized, partially muscularized, and fully muscularized arteries in both hypoxic and SU5416 plus hypoxia groups.

Endothelial proliferative lesions occluding the vascular lumen in vessels <150 μm were noted in the animals exposed to SU5416 plus hypoxia treated with vehicle or CNP and in animals exposed to hypoxia alone treated with CNP (Figure 4, Table 2). In vehicle treated animals, there were an increased number of endothelial proliferative lesions, as assessed by immunoperoxidase staining of lung sections in animals exposed to SU5416 plus hypoxia, compared to hypoxia alone (Table 2). Also, there was a trend towards increased number of endothelial proliferative lesions in the lungs of animals exposed to hypoxia or SU5416 plus hypoxia and treated with 2.25 μg/hr infusion of CNP compared to animals treated with vehicle (Table 2). The percent of vessels with endothelial proliferative lesions in Normoxia + SU5416 animals that received CNP was 2.6 ± 1.3% compared to 5.0 ± 3.8% in those that received D5W (p=ns).

Effect of CNP on NPR-B expression in severe pulmonary hypertension

To determine if chronic infusion of CNP affected the expression of its particulate guanylyl cyclase receptor, NPR-B, we performed immunoblot analysis in the whole lung lysates. Compared to rats treated with vehicle alone, CNP infusion resulted in a marked down-regulation of pulmonary NPR-B receptor expression in rats exposed to SU5416 plus hypoxia (Figure 5).

DISCUSSION

Natriuretic peptides are endogenous vasodilators that have potential therapeutic application in pulmonary vascular diseases such as PH. In an earlier study, Itoh *et al.* (Itoh et al., 2004) found that continuous intravenous infusion of CNP was effective in blunting the development of MCT-induced pulmonary hypertension and partial reversing established PH in this model. In their study, CNP infusion was associated with regeneration of pulmonary endothelium, inhibition of endothelial cell apoptosis and blunting of monocyte/macrophage infiltration of the pulmonary capillary bed. Considering the important roles that endothelial apoptosis and pulmonary vascular inflammation play in the pathogenesis of PH, these results suggested a potential therapeutic role for CNP infusion in the management of PH.

In the present study, we aimed to extend this observation to other models of pulmonary hypertension that have been used to explore the therapeutic potential of anti-pulmonary hypertensive agents. Chronic hypoxia is a well-established model of experimental pulmonary hypertension and has clinical relevance to PH associated with a variety of chronic lung diseases such as emphysema, pulmonary fibrosis and sleep disordered breathing (Stenmark et al., 2009). However, most of the pulmonary vascular changes associated with this model involve increased medial wall thickness and muscularization of small normally non-muscularized vessels without the development of the endothelial proliferative changes and intravascular obliterative lesions seen in human PH. Recently, the combination of the VEGF receptor antagonist SU5416 and chronic hypoxia has been developed as an experimental model of PH that more closely resembles PH (Abe et al., 2010; Taraseviciene-Stewart et al., 2001). In addition to increased pulmonary artery pressure, this combination consistently results in the appearance of intravascular changes that resemble plexiform lesions in human disease (Abe et al., 2010).

We found that rats exposed to chronic hypoxia or SU5416 plus hypoxia, over a period of three weeks, developed severe pulmonary hypertension, right ventricular hypertrophy and pulmonary vascular remodeling and rats given SU5416 under normoxic conditions developed a milder degree of pulmonary hypertension. Continuous infusion of CNP failed to attenuate the rise in RVSP, increase in RV mass or increase in medial wall thickness in any of the 3 models of pulmonary hypertension. Moreover, the dose of CNP used in the current study was up to 3-fold greater than that found to be effective in blunting pulmonary hypertension in MCT-treated rats (Itoh et al., 2004). The lack of an effect by CNP administration in the present study despite a 3-fold elevation in circulating CNP levels suggests that CNP has little ability to attenuate pathogenic mechanisms promoting pulmonary vascular remodeling in chronic hypoxia and SU5416/hypoxia models of pulmonary hypertension.

Other members of the natriuretic peptide family, ANP and BNP have been shown to blunt hypoxic pulmonary hypertension in rats using similar methods of continuous intravenous infusion (Jin et al., 1990; Klinger et al., 1998b). In these studies, infusion of ANP and BNP increased circulating levels by 2.3 fold and 2-fold, respectively, and significantly attenuated hypoxic pulmonary hypertension and decreased pulmonary vascular remodeling (Jin et al., 1990; Klinger et al., 1998b). The efficacy of these agents in mitigating pulmonary hypertension has been attributed to their ability to elevate intracellular cGMP levels. Considering that the primary effect of CNP is also the elevation of intracellular cGMP, it is unclear why similar results were not obtained in the present study in the rats given CNP during exposure to chronic hypoxia. Interestingly, CNP infusion resulted in a marked downregulation of the CNP specific particulate guanylate cyclase-linked receptor, NPR-B in the lungs. It has previously been demonstrated that CNP down-regulates expression of its cognate receptor in rat aortic smooth muscle cells (Rahmutula & Gardner, 2005). Thus, it is possible that any initial augmentation of intracellular cGMP production by CNP infusion was offset by an eventual down regulation in NPR-B. In support of this hypothesis, Klinger *et al.* previously demonstrated a marked attenuation of the vasodilatory effects of CNP in isolated perfused lungs and in isolated pulmonary arteries obtained from hypoxia-adapted rats (Klinger et al., 1998a). An increase in cyclic GMP is also a result of treatment with phosphodiesterase inhibitors that are approved for treatment of pulmonary hypertension. It has been noted that the activity/expression of phosphodiesterase is increased in pulmonary circulation in the setting of pulmonary hypertension (Murray et al., 2002; Wharton et al., 2005). While synergistic effects of ANP/ BNP and phosphodiesterase inhibitors have been reported (Klinger et al., 2006; Preston et al., 2004), it remains to be seen whether such an approach would be beneficial with CNP.

In addition to guanylate cyclase receptors NPR-A and NPR-B, the natriuretic peptides can bind to NPR-C receptor. In addition to clearance functions, NPR-C has been shown to be important in modulating cellular functions such as membrane potential regulation, proliferation, permeability, and secretion of various mediators (Anand-Srivastava, 2005). Activation of NPR-C signaling by CNP has been shown to inhibit smooth muscle proliferation and enhance endothelial cell proliferation (Khambata et al., 2011). Klinger at. al. have demonstrated that the expression of NPR-C is reduced in animals exposed to hypoxia (Klinger et al., 1994). Thus, alteration in NPR-C expression and signaling may be another alternative or complementary potential mechanism to explain the lack of attenuation of pulmonary hypertension seen in animals treated with CNP in our study and requires further investigation.

In the systemic circulation, CNP has been shown to suppress the growth of vascular smooth muscle cells through an elevation of cGMP *in vitro* and inhibit the development of vascular remodeling of injured arteries *in vivo* (Doi et al., 2001). In the present study, CNP infusion did not attenuate the increase in medial wall thickness of peripheral pulmonary arteries suggesting that the effect of CNP on vascular smooth muscle cells may be dependent upon the vessel type (systemic vs. pulmonary) or the nature of injury.

In the MCT model of pulmonary hypertension, the protective effects of CNP on vascular injury were attributed to regeneration of pulmonary endothelial cells and inhibition of pulmonary endothelial cell apoptosis (Itoh et al., 2004). Endothelial cell injury activates platelets and vasoconstrictor factors, resulting in pulmonary hypertension and vascular remodeling (Rosenberg & Rabinovitch, 1988). Recent studies have demonstrated that transplantation of endothelial progenitor cells or whole bone marrow cells attenuates MCT-induced pulmonary hypertension (Aliotta et al., 2009; Nagaya et al., 2003; Zhao et al., 2005), suggesting that endothelial regeneration may have beneficial effects on pulmonary hemodynamics. Interestingly, in the present study, we found a trend toward increased endothelial proliferation resulting in occluded microvessels in CNP- treated rats compared to vehicle treated rats in both hypoxia-induced and SU5416 plus hypoxia-induced pulmonary hypertension. Recent in vitro studies evaluating the effect of CNP on human umbilical vein endothelial cells and rat aortic smooth muscle cells noted that while CNP inhibited the proliferation of smooth muscle cells, it augmented the proliferation of endothelial cells (Khambata et al., 2011). The increase in endothelial cell proliferation associated with CNP exposure would be consistent with the increase in endothelial cell proliferative lesions in rats treated with CNP as observed in our study. We speculate that the type of endothelial injury that occurs with MCT results in both apoptosis and proliferation, with CNP restoring the balance towards endothelial regeneration and repair (Doi et al., 2001; Ohno et al., 2002). However, in endothelial injury resulting from hypoxia alone or the combination of SU5416 plus hypoxia (Sakao et al., 2005; Taraseviciene-Stewart et al., 2001) where endothelial proliferation predominates, the protective effects of CNP are less evident. In the in vitro study with SU5416, it was noted that high shear stress was required to increase the proliferation of apoptosis resistant cells (Sakao et al., 2005). Hypoxia is associated with an increase in shear stress through the pulmonary circulation and could explain the observation that the increase in endothelial proliferative lesions with CNP is more marked in the hypoxia groups. In addition to the effects on endothelium, CNP blunted the monocyte/macrophage infiltration of the pulmonary capillary bed in the MCT model. In contrast, the lungs of animals with SU5416 plus hypoxia-induced pulmonary hypertension do not have macrophage infiltration (Taraseviciene-Stewart et al., 2001) and may partially explain the lack of effect of CNP in this model.

It is possible that the lack of efficacy of CNP infusion in the present study was due to the severe degree of pulmonary hypertension that developed in the hypoxia and SU5416 plus

hypoxia models. The pulmonary hypertensive response to hypoxia has been shown to vary considerably between rat strains (Colice et al., 1997) and rats exposed to chronic hypoxia in the present study developed marked elevation of pulmonary artery pressures (PAP) (between 60-80mmHg) compared to the pressures reported by others using different strain of Sprague-Dawley rats. However, rats given SU5416 under normoxic conditions developed only mild increases in RV/LV+S and medial wall thickness, but also showed no attenuation in response to CNP infusion, making it unlikely that CNP would have been effective in a more moderate model of pulmonary hypertension. It is possible that longer-term treatment with CNP (> 3 weeks) could attenuate some of the PH findings as observed in our study. However, 2 weeks of treatment with BNP in hypoxic pulmonary hypertension and 3 weeks of treatment with CNP in monocrotaline induced pulmonary hypertension was sufficient to observe changes in the degree of pulmonary hypertension and vascular remodeling. We can not exclude the possibility that small changes in medial wall thickness between the groups were missed because of the sample size. However, since the infusion of CNP did not prevent the increase in the PA pressures and right ventricular hypertrophy it is unlikely that any meaningful changes in vessel muscularization occur with CNP treatment.

CONCLUSION

In conclusion, we found no evidence that continuous infusion of CNP has a mitigating effect on the development of pulmonary hypertension induced by exposure to chronic hypoxia or SU5416 plus hypoxia. This is an important observation despite being a negative study as the lack of consistency of the effects of CNP in different animal models of PH suggests that the attenuation of PH by CNP may be specific to models of pulmonary vascular inflammation and that CNP does not play an important role in modulating hypoxic pulmonary hypertension or pulmonary hypertension induced by VEGF receptor antagonists. Hence, the use of CNP as a therapeutic agent requires further evaluation prior to being considered for human PH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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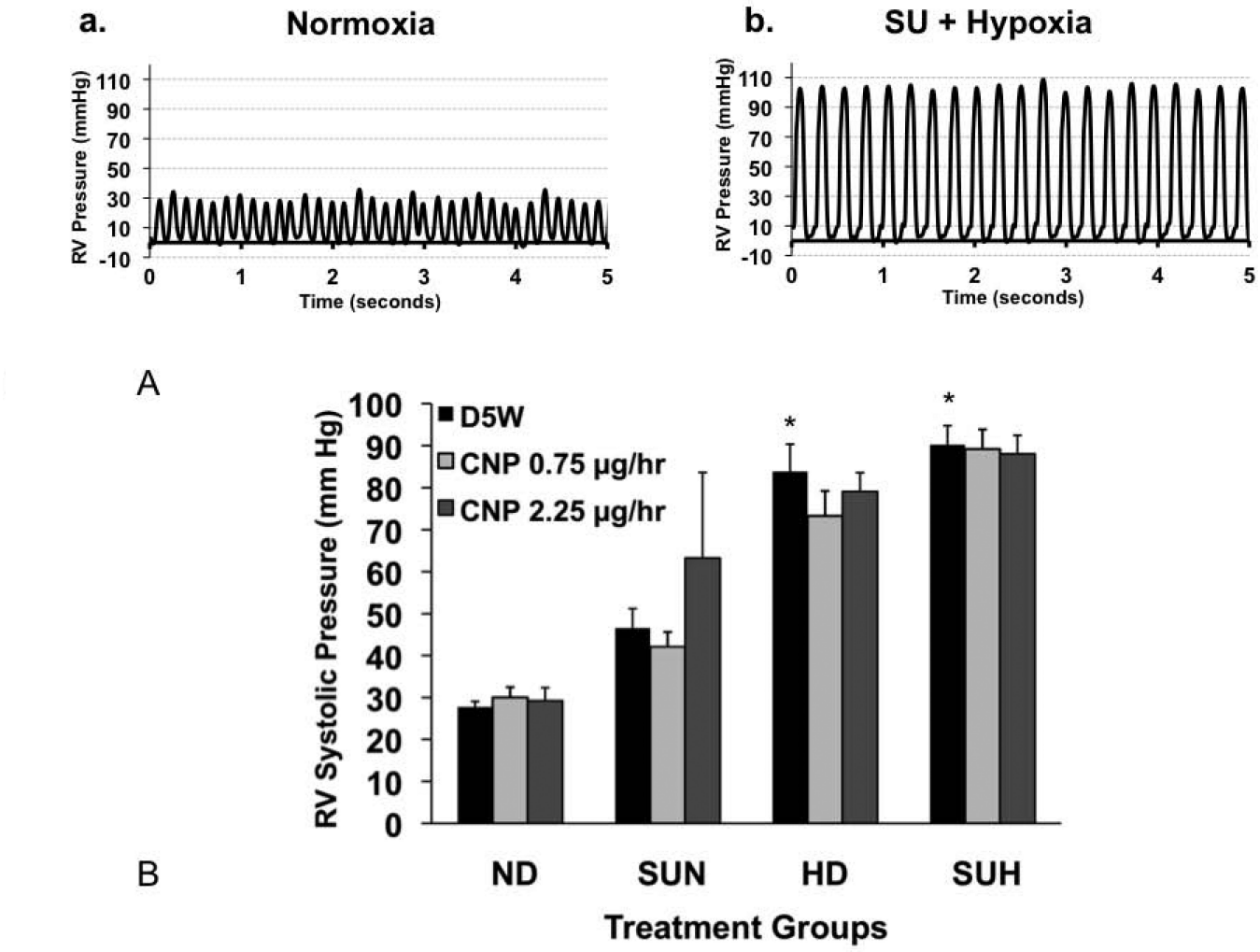

Figure 1. CNP does not prevent the development severe pulmonary arterial hypertension

Animals were implanted with pumps that delivered i.v. infusion of 5% dextrose (Vehicle), low dose (0.75μg/hr) or high dose (2.25μg/hr) CNP, and placed in either normoxia or hypoxia (FiO2-10%) for 3 wks after receiving a s.c. injection of diluent or 20 mg/kg of SU5416, a VEGF receptor blocker. **Panel A:** Representative RV pressure tracings of animals exposed to 3 weeks of Normoxia/Vehicle and SU5416 + Hypoxia/ Vehicle. **Panel B:** RV systolic pressure in various treatment groups. (Mean ± SEM. n=4-11.* $p<0.05$ compared to Diluent + Normoxia treated with vehicle. ND: Diluent +Normoxia, SUN: SU5416 + Normoxia, HD: Diluent +Hypoxia, SUH: SU5416 + Hypoxia, D5W: 5% Dextrose)

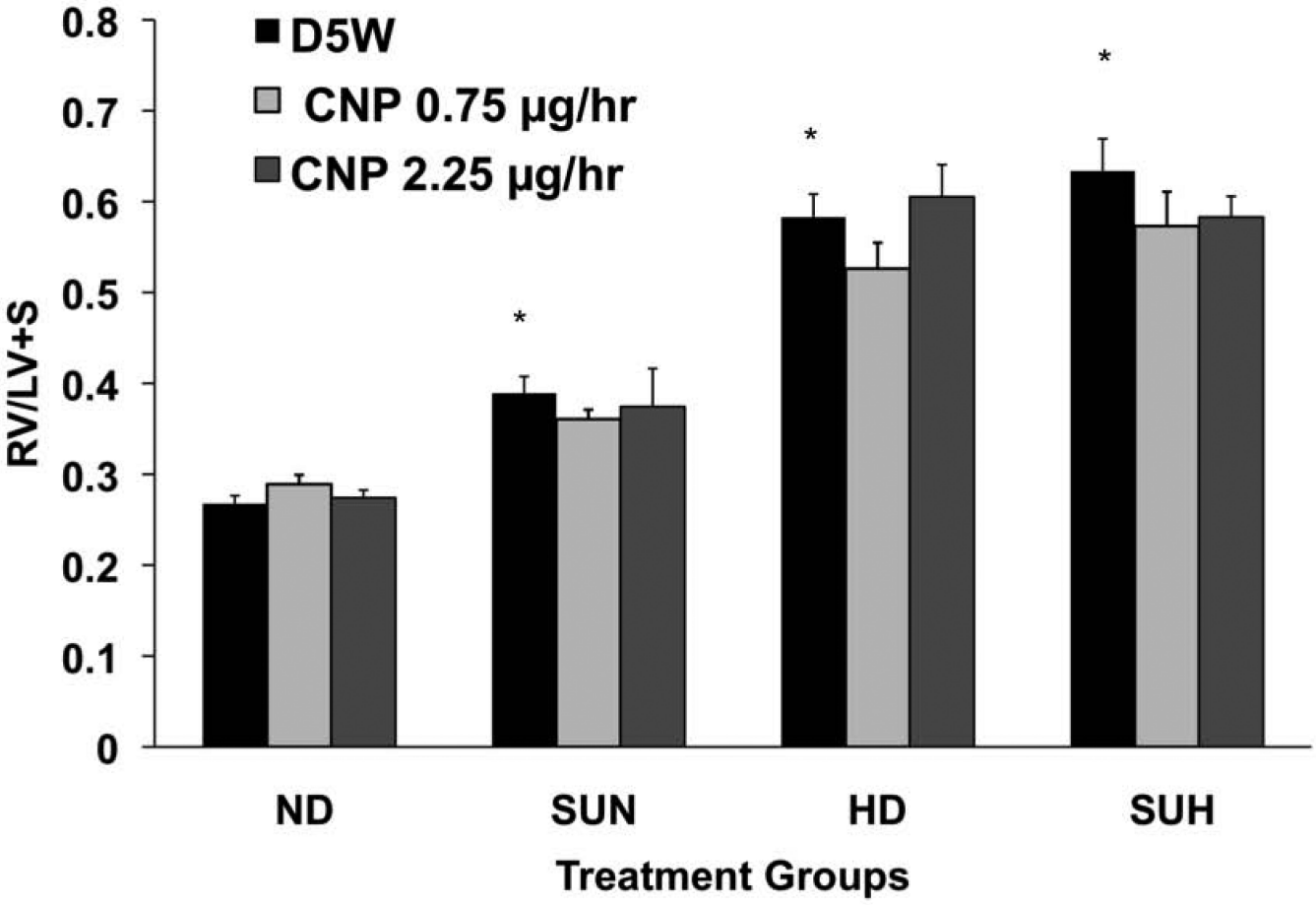

Figure 2. CNP does not prevent right ventricular hypertrophy in PH

Right ventricular weight normalized to LV + Septum (RV/LV+S) weight in rats implanted with subcutaneous pumps that delivered continuous i.v. infusion of 5% dextrose (vehicle), low dose (0.75 µg/hr) or high dose (2.25 µg/hr) CNP, and exposed to normoxia or hypoxia for 3 wks after receiving a s.c. injection of diluent or 20 mg/kg of SU5416, a VEGF receptor blocker. (n=4-11, Mean ± SEM. * $p < 0.05$ compared to Diluent + Normoxia treated with vehicle. ND: Diluent +Normoxia, SUN: SU5416 + Normoxia, HD: Diluent +Hypoxia, SUH: SU5416 + Hypoxia, D5W: 5% Dextrose)

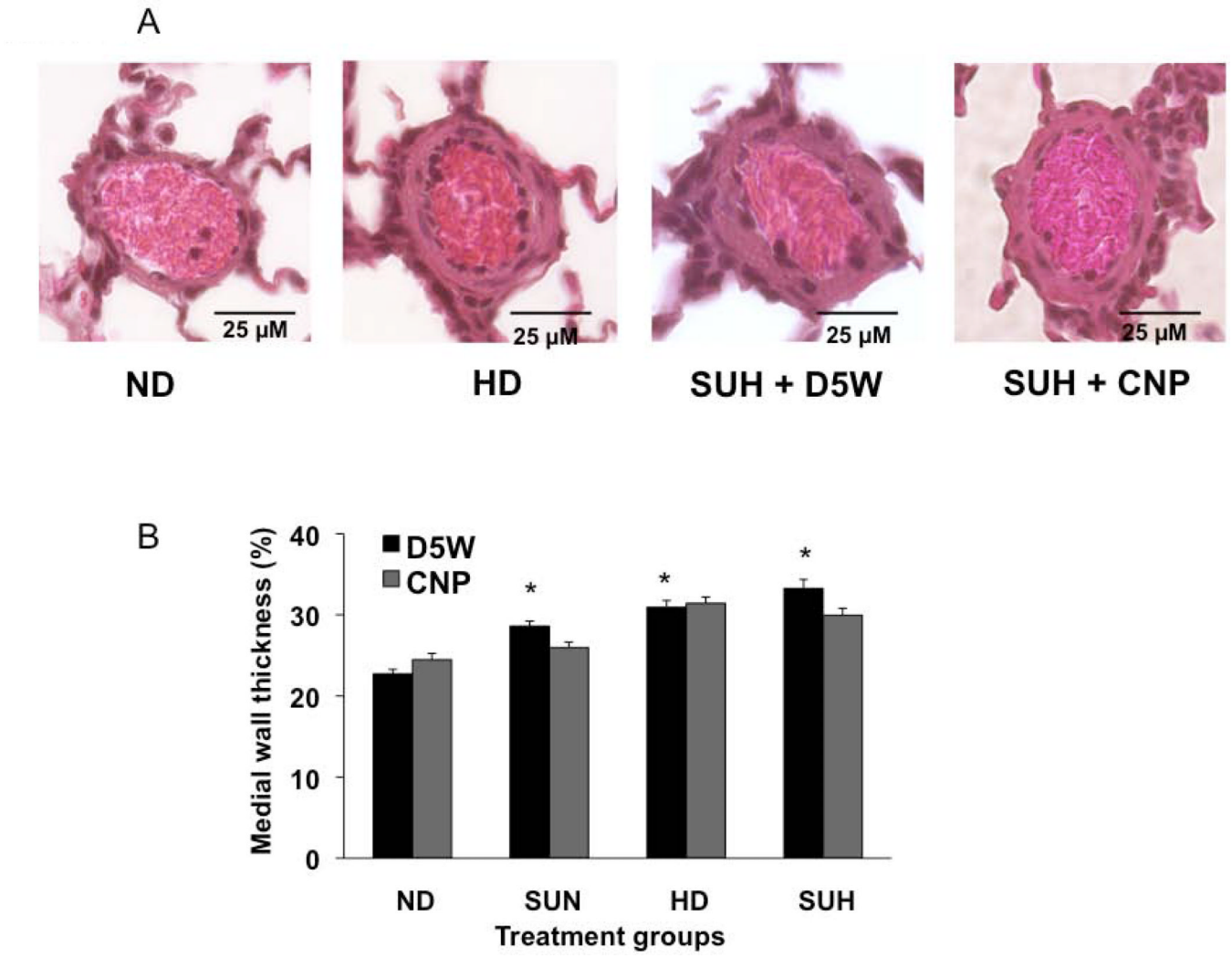

Figure 3. CNP does not inhibit microvascular remodeling in severe PH

Panel A Representative images of H&E stained lung sections of animals exposed to Normoxia, Hypoxia, SU5416 + Hypoxia and SU5416 + Hypoxia/ CNP showing vessels <150 μM in diameter at 40X magnification. **Panel B.** Medial thickness in <150 μM vessels expressed as percent of vessel diameter in animals delivered continuous i.v. infusion of 5% dextrose or 0.75 μg/hr CNP, and exposed to normoxia or hypoxia for 3 wks after receiving a s.c. injection of diluent or 20 mg/kg of SU5416. (n=3, Mean ± SEM. * p <0.05 compared to Diluent + Normoxia treated with vehicle. ND: Diluent +Normoxia, SUN: SU5416 + Normoxia, HD: Diluent +Hypoxia, SUH: SU5416 + Hypoxia, D5W: 5% Dextrose)

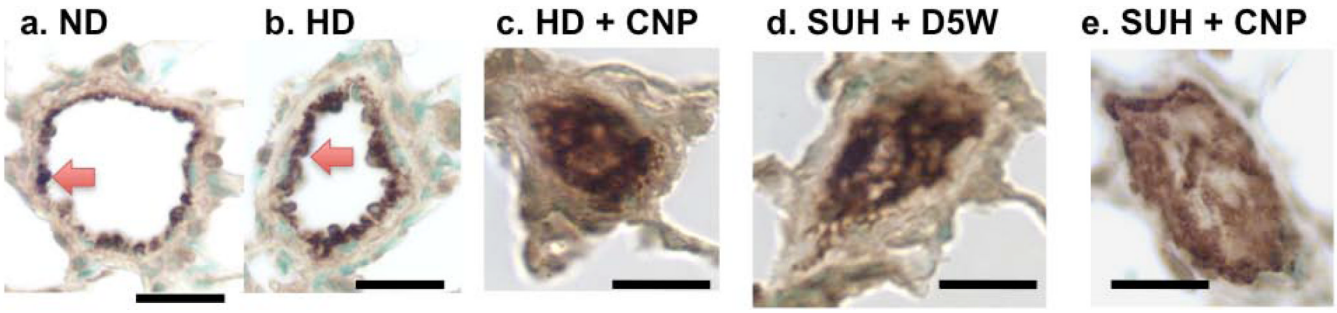

Figure 4. Effect of CNP on endothelial proliferative lesions in severe PH

Representative images of immunoperoxisae stating using anti-vWF antibody showing endothelial cells in microvasculature of lung sections from animals exposed to (a) diluent + normoxia treated with vehicle, (b) Diluent + Hypoxia treated with vehicle. Representative images of endothelial proliferative lesions resulting in vessel occlusion noted in animals exposed to (c) Diluent + Hypoxia treated with CNP, (d) SU5416 and Hypoxia treated with vehicle or (e) SU5416 and Hypoxia treated with CNP. (Scale bar= 50 μm) Magnification 40X. Endothelial cells are stained brown. ND: Diluent +Normoxia, SUN: SU5416 + Normoxia, HD: Diluent +Hypoxia, SUH: SU5416 + Hypoxia, D5W: 5% Dextrose

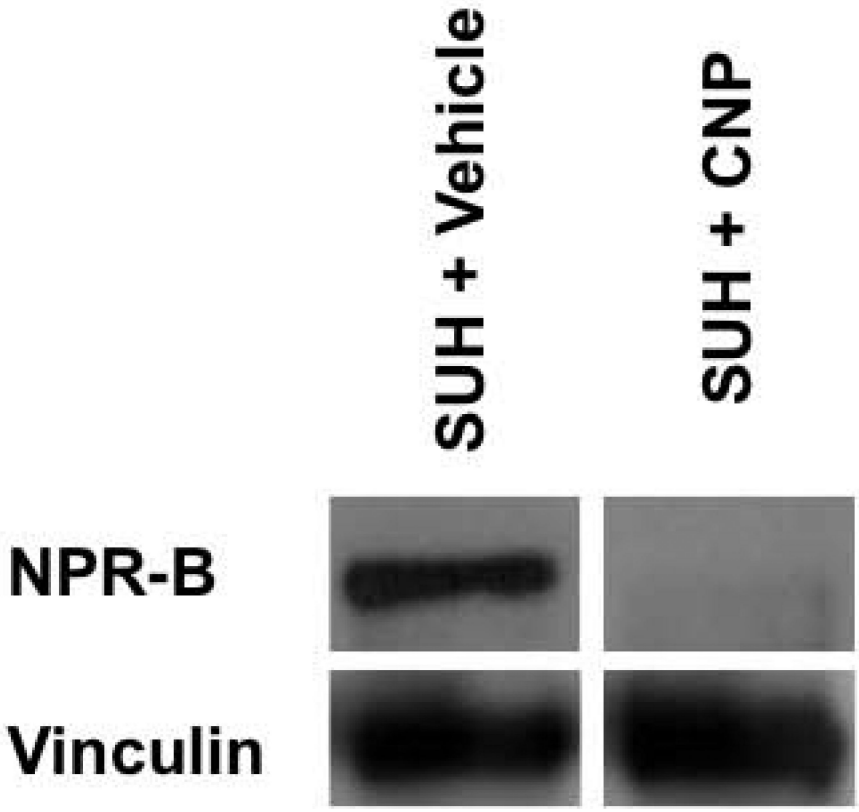

Figure 5. Diminished NPR-B expression in SU5416 + hypoxia animals infused with CNP

Representative immunblot of the NPR-B receptor expression in the whole lung lysates of SU5416 + hypoxia (SUH) animals treated with 3 weeks of CNP (2.25μg/hr) or vehicle. Equal amount of protein lysates from homogenized lungs were resolved on SDS PAGE and probed for NPR-B. The membranes were subsequently stripped and reprobed for vinculin. (n=3)

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Table 1

Echocardiographic measurements in CNP and Vehicle-Treated Rats Exposed to Normoxia, Chronic Hypoxia and Chronic Hypoxia Plus SU5416.

	Normoxia		Hypoxia		SU5416 + Hypoxia	
	Vehicle	**CNP**	**Vehicle**	**CNP**	**Vehicle**	**CNP**
Heart Rate (bpm)	282 ± 18	294 ± 13	267 ± 17	288 ± 17	288 ± 14	295 ± 30
RVOT Diameter (cm)	0.32 ± 0.02	0.30 ± 0.00	0.28 ± 0.01	0.31 ± 0.01	0.32 ± 0.01	0.30 ± 0.01
RVOT Velocity Time Integral (cm)	4.74 ± 0.30	5.15 ± 0.71	4.38 ± 0.45	5.36 ± 0.42	3.57 ± 0.24	3.86 ± 0.37
Pulmonary Acceleration Time (ms)	39 ±3	41 ± 2	25 ± 3 *	22 ± 2	22 ± 3 *	28 ± 4
Stroke Volume (μL/gm)	1.60 ± 0.26	1.69 ± 0.25	1.18 ± 0.13	1.91 ± 0.19	1.29 ± 0.12	1.28 ± 0.17
Cardiac Index (cc/gm)	0.49 ± 0.12	0.50 ± 0.07	0.32 ± 0.04	0.56 ± 0.08	0.38 ± 0.05	0.38 ± 0.07

RVOT: Right Ventricular Outflow Tract; CNP infusion – 2.25 ug/hr

* p<0/05 compared to normoxia/diluent n=4

Table 2

Percentage of total vessels with partial or complete occlusion with endothelial proliferative lesions.

	Vehicle	CNP
Diluent + Hypoxia	0%	7.9% ± 4.6%
SU5416 + Hypoxia	3.4% ± 0.9% *	7.8% ± 2.4%

* $p < 0.05$ compared to diluent + hypoxia/vehicle, n=4-9