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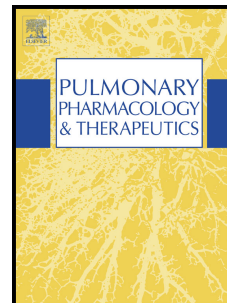
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**Title:**

Preserved pulmonary vasodilative properties of aerosolized Brain natriuretic peptide

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**Abstract****Introduction:**

Inhalation of vasoactive substances is an effective treatment of pulmonary hypertension. The B-type natriuretic peptide (BNP) leads to relaxation of smooth muscle cells, caused by an increased formation of cyclic guanosine monophosphate (cGMP). The biologic activity of BNP using an inhalative approach has not been addressed.

**Methods:**

In order to assess the vasorelaxing capacity of exogenous BNP in the isolated ventilated and buffer perfused rabbit lung model, a stable pulmonary vasoconstriction was established by either the application of endothelin-1 or the thromboxane A<sub>2</sub> mimetic U46619. This was followed by an intravascular or aerosol application of BNP. CGMP was measured in the recirculating buffer fluid using a radioimmunoassay.

**Results:**

During a stable plateau of U46619 induced pulmonary vasoconstriction (mean pulmonary artery pressure, PAP 25.5±0.23 mmHg), the intravascular administration of BNP induced a rapid vasodilation (mean PAP 18.13±0.95 mmHg, p<0.001). This vasodilation was dose dependent and was paralleled by a 6-fold increase of cGMP. When BNP was aerosolized, pulmonary vasoconstriction was also significantly alleviated in the U46619 model (mean PAP 22±2.1 mmHg) and during endothelin-1 induced vasoconstriction (mean PAP 17.1±2.47 mmHg). Correspondingly, inhalation caused a significant augmentation of cGMP levels was.

**Conclusion:**

The vasodilative capability of BNP as an indicator of the biologic activity of this peptide is preserved during its aerosolization. Presumably these vascular actions are caused at least in part by an increased availability of cGMP.

## Introduction

Different substances with acute vasodilative properties and antiproliferative action in long term have been used to achieve a reduction of pulmonary vascular resistance in experimental and clinical studies. The application of a vasoactive substance as an aerosol instead of its systemic use allows pulmonary and *intrapulmonary* selectivity; thereby a higher local deposition can be achieved while systemic side effects and aggravation of gas exchange disturbances can be avoided at the same time. This principle has been proven for prostacyclin and its stable analogs [1;2]. Antagonizing the deleterious effects of endothelin-1 is also a mainstay in the treatment of pulmonary hypertension which can be achieved by different endothelin receptor antagonists [3]. It has also been suggested that the therapeutic activity of such antagonists is preserved during aerosolization [4]. Due to the advances of an inhalative approach, additional substances that can be applied as an aerosol have been proposed in pulmonary hypertension [5;6]. However, not only since peptides are prone to proteolytic cleavage on the lung surface, it is not self-evident to assign observations from *in vivo* experiments or systemic applications to the inhalative use of the respective substance.

The B-type natriuretic peptide (BNP) is one of the main molecules of the natriuretic peptide system and relaxes smooth muscle cells by an increased formation of cyclic guanosine monophosphate (cGMP), generated by the particulate guanylate cyclase which itself is activated by the natriuretic peptide receptor A (NPR-A) [7]. The augmented availability of cGMP either by an increased formation or an inhibited degradation leads to relaxation of smooth muscle cells of airways and in the pulmonary vasculature [8-10]. Consequently, BNP is a vasodilator in various tissues [11]. The intravascular application of exogenous BNP has been shown to blunt acute hypoxic pulmonary vasoconstriction in rat lungs [12] and healthy volunteers [13]. In addition,

BNP caused pulmonary vasodilation in patients with chronic cor pulmonale [14]. Moreover, BNP exerts antiproliferative effects in vitro [15] and attenuates the remodelling process in chronically hypoxia exposed rats [16]. However, the biologic activity of BNP has never been assessed after aerosolization of the peptide to the lung. We used the isolated ventilated and buffer perfused lung model as a well established bioassay to investigate if the presumed pulmonary vasodilative effect of exogenous BNP is preserved during its aerosol application.

## **Methods**

### The isolated ventilated and buffer perfused lung model:

This model has been used to characterize pulmonary vasodilators during intravascular and aerosol application because it facilitates a focussed investigation of substances that mediate, not only but also, pulmonary vasodilation in a quasi in vivo setting without interference of central or humoral influences (e. g. circulating blood cells). Recently we described our modifications of this isolated rabbit lung model [4;17]. Rabbits (New Zealand white bastards) of either sex, weighing 2.2-2.5 kg were anesthetized with ketamine (Ketamin Inresa®, Inresa, Freiburg, Germany) and xylazine (Rompun®, Bayer, Leverkusen, Germany). Subsequently the animals were anticoagulated with heparin (1000 U/kg body weight). After tracheotomy artificial ventilation was started with room air using a Havard respirator (tidal volume 8-10 ml/kg, frequency 30 breaths/min). Midsternal thoracotomy followed in a deep analgosedation and catheters were placed into the pulmonary artery and the left atrium. Lungs were perfused with sterile Krebs-Henseleit hydroxyethylamylopectine buffer (120 mM NaCl, 4.3 mM KCl, 1.1 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 2.4 mM CaCl<sub>2</sub>, 1.3 mM MgPO<sub>4</sub>, 2.4 g/L glucose) and 2.5% hydroxyethylamylopectine (mol weight 200,000) as an oncotic agent (Serag Wiesner,

Naila, Germany). All concentrations are given as the final concentration in the buffer fluid.

The isolated lungs were freely suspended from an electronic force transducer (Transducer U1, Hottinger Baldwin Messtechnik, Darmstadt, Germany) in a humidified and tempered glass thorax. Perfusion was performed at a constant rate of 120 mL/min in a recirculating manner (total perfusate volume 300mL), and the whole system was heated to 38°C. Room air, supplemented with ~ 4% carbon dioxide was used for artificial ventilation to reach a pH of 7.34 to 7.38 and a positive end expiratory pressure (PEEP) was set at 1-1.3 mmHg. Pressure in left atrium was set at 1.2 mmHg (reference point hilum). Pulmonary artery and ventilation pressures were continuously measured and are shown as mean values.

Lung preparations entered the experimental protocol if they had a homogenous white appearance, without signs of hemostasis or edema formation [no lung weight gain during baseline]. Since lungs were perfused using a constant flow rate of 120 ml/min, changes of the PAP directly reflected alterations in pulmonary vascular resistance.

All experiments were performed according to the Helsinki convention for the use and care of animals.

#### Study design:

In addition to controls, two different models of a non hypoxia mediated pulmonary vasoconstriction were established. The thromboxane mimetic U46619 [Paesel&Lorei, Frankfurt/ Main, Germany] was used to differentiate the vasodilating effects of intravascular and aerosolized BNP. In the endothelin model we aimed to reproduce the data during the aerosol application of BNP.

U46619 induced pulmonary vasoconstriction: the thromboxane A<sub>2</sub> mimetic U46619 (9,11-dideoxy-11,9-epoxy-methano-prostaglandin f 2) [Paesel&Lorei, Frankfurt/ Main, Germany] was individually titrated into the recirculating buffer fluid after a stable baseline period of 30 minutes. Once a PAP plateau of 25-28 mmHg was reached, the titration period was followed by a constant infusion rate (65 to 650 pmol\*kg<sup>-1</sup>\*min<sup>-1</sup>). This PAP plateau was stable when no further intervention was performed for 135 minutes. Otherwise, interventions were initiated after a stable plateau of 15 minutes. Dose response curves for bolus injected BNP into the buffer fluid were performed. In the definite experiments, 10 nM BNP was injected and all parameters were subsequently observed for 120 minutes. Besides continuous monitoring of the hemodynamic parameters and lung weight gain, repetitive samples for pH measurements and 2 ml samples for cGMP measurements were drawn every 15 – 30 minutes.

In the aerosol intervention, placebo (NaCl 0.9%) or BNP were nebulized over a time period of ~ 15 minutes with similar subsequent observation.

Control groups comprised four lung preparations. In each intervention group we performed six lung preparations, unless stated otherwise. All experiments (excluding dose response curves) were performed in a randomized order.

Endothelin-1 induced pulmonary vasoconstriction:

According to the U4619 model, 0.1 μM of endothelin-1 [Calbiochem, Germany] was bolus injected into the buffer fluid after a steady state period of 30 minutes. This dose provoked a constant increase of PAP over 60 minutes, followed by a plateau of 25 – 28 mmHg for another 135 minutes. Interventions were performed as described above.

Aerosolization



This aerosolization system has been described before [4]. Placebo (NaCl 0.9%) and BNP (total dose 200 nM) were aerosolized with an ultrasonic device (Optineb ®, Nebutech, Elsenfeld, Germany). In separate dose finding experiments 200 nM of BNP were found to cause a significant pulmonary vasodilation (data not shown). This nebulizer produces an aerosol with a mass median aerodynamic diameter of 4 µm and a geometric SD of 1.5. The nebulizer was placed into the inspiratory limb of the ventilatory system. A comparable nebulization system was evaluated by Schmehl and coworkers in an isolated lung model and an absolute deposition fraction of  $0.25 \pm 0.02$  by laser photometric technique was determined [18].

In separate experiments with a simple filter technique this nebulizer produced an aerosol deposition fraction of approximately 0.22 (data not shown).

#### Cyclic GMP Measurement

Buffer samples were drawn from the left atrial catheter at predefined time points, every 30 minutes in the control group and every 15 minutes in the intervention groups. Samples were immediately frozen and stored at -80° Celsius. Analysis of cGMP was performed using a commercially available radio immuno (RIA) assay kit (Amersham, Bioscience, Germany). This assay system utilizes a high specific activity cGMP [<sup>125</sup>I] 2'-O-succinyl cGMP tyrosine methyl ester tracer, together with a high specific and sensitive antiserum. Separation of the antibody bound from free fraction is achieved with a second antibody Amerlex-M preparation, thus allowing a simple magnetic separation. Cross reactivity with cyclic AMP is < 0.001. The sensitivity, defined as the amount of cGMP needed to reduce zero dose binding by two standard deviations was 0.5 fmol. All samples were analyzed in the same kit.

#### Experimental protocol:

During the control experiments hemodynamics, ventilation pressure and lung weight were observed for a total of 180 minutes. Samples for cGMP measurements were drawn in 30 minutes intervals.

### Data analysis

All data are shown as mean and standard error of mean (SEM). For primary data analysis, comparison was performed between the U46619 groups and intravascular administered BNP. Second, the BNP aerosol group was compared to the respective placebo inhalation group in U46619 and endothelin-1 induced pulmonary vasoconstriction.

Comparison between groups was performed for all parameters at the end of inhalation (t=45min in the U46619 group) and the end of the experiment. For comparison of statistical difference between groups we performed either an unpaired students't-test or a one factorial analysis of variance with the Bonferroni correction. Significance was assumed when  $p < 0.05$ .

## **Results**

### Baseline condition

After completion of 30 minutes steady state period, all lungs had a mean PAP of 5 - 6 mmHg without any lung weight gain and a mean ventilation pressure of 2 - 4 mmHg. In this group no significant changes were observed with regard to PAP, ventilation pressure and lung weight during the whole observation period up to four hours (data not shown in detail).

Cyclic GMP levels increased in an almost linear fashion over time from  $0.83 \pm 0.2$  pmol/ml at the end of the baseline period to  $5.07 \pm 1.38$  pmol/ml at the end of the experiment.

Dose response curves for BNP (Figure 1):

During U46619 infusion, increasing doses of 5, 10 and 100nM BNP were administered under conditions of stable pulmonary vasoconstriction. All doses led to a rapid and dose dependent decline of PAP without affecting ventilation pressure or lung weight.

Pharmacodynamics of a single dose of intravascular administered BNP in U46619 induced pulmonary vasoconstriction (Figure 2):

After application of 10nM BNP PAP decreased immediately from  $25.5 \pm 0.32$  to  $18.13 \pm 0.95$  mmHg within 15 minutes ( $p < 0.01$ ). During the subsequent observation period PAP further declined to  $16.5 \pm 1.59$  mmHg and increased again to  $18.48 \pm 3.72$  mmHg at the end of the experiment, when the PAP lowering effect was still significant ( $p < 0.05$ ).

This PAP decline was paralleled by a rapid increase of cGMP that reached a more than 6 fold augmentation of cGMP at the end of the experiment as compared to the U 46619 control group ( $p < 0.001$ ).

These results were observed irrespective of the exposure of the BNP solution to the ultrasound nebulizer for 15 minutes before intravascular administration or not, suggesting stability of the peptide.

Aerosolization of placebo and BNP in U46619 induced pulmonary vasoconstriction (Figures 3, 4a and 4b):

In the placebo inhalation group, continuous infusion of U46619 provoked an increase of PAP to  $25.5 \pm 0.26$  mmHg within 15 minutes. This increase was followed by a stable plateau phase for another 135 minutes ( $25.53 \pm 0.9$  mmHg at the end of the experiment). Aerosolization of placebo (NaCl 0.9%) had no influence on any of the registered hemodynamic parameters (plateau  $26.5 \pm 0.56$  mmHg, end of experiment  $27.12 \pm 1.06$  mmHg). Neither the ventilation pressure nor the lung weight changed significantly during and after inhalation of NaCl 0.9% which served as the placebo.

Cyclic GMP increased during U46619 induced pulmonary vasoconstriction. This was comparably observed with and without the placebo aerosol. However, this effect was not significantly different as compared to the control group.

Administration of the BNP aerosol [200 nM] under conditions of a sustained pulmonary vasoconstriction led to a rapid and significant decrease of PAP ( $25.96 \pm 0.55$  mmHg to  $23.52 \pm 0.65$  mmHg) within 15 minutes ( $p < 0.05$ ) and further decreased during the observation period to a minimum of  $22 \pm 2.1$  mmHg at the end of the experiment. This PAP lowering effect was accompanied by a rapid increase of cGMP already immediately after the inhalation procedure. A 3 fold higher cGMP level was noted at the end of the experiment ( $p < 0.05$ ) as compared to the placebo inhalation group. However, the PAP reduction and cGMP augmentation was not as strong as observed during intravascular administration of BNP.

Lung weight and ventilation pressure were not significantly affected.

Aerosolization of placebo and BNP in endothelin -1 induced pulmonary vasoconstriction (Figures 5a and 5b):

Endothelin-1 administration into the buffer fluid caused a constant rise of PAP over 60 minutes, levelling off in a stable plateau phase. When the placebo aerosol was started, PAP measured  $27 \pm 2.16$  mmHg. This plateau was constant for another 120 minutes when the aerosol was stopped, and only a mild decrease occurred over time to  $25.8 \pm 1.71$  mmHg at the end of the experiment.

While ventilation pressure and lung weight did not differ significantly over time, the cGMP levels increased comparable to that seen during U46619 infusion alone. Again, there was no significant difference as compared to the control groups.

Administration of the BNP aerosol during the stable plateau phase of pulmonary vasoconstriction led to a rapid and significant reduction of PAP from  $24.88 \pm 2.13$  mmHg to  $21.8 \pm 2.13$  mmHg within 15 minutes, further decreased  $19.9 \pm 2.51$  mmHg 105 minutes after endothelin-1 administration and reached a minimum PAP at the end of the observational period of  $17.1 \pm 2.47$  mmHg ( $p < 0.05$ ).

Again, lung weight and ventilation pressure were not significantly affected.

## Discussion

In this study we demonstrate that the pulmonary vasodilative potential of exogenous BNP, as an indicator of the biologic activity of BNP, is maintained during the aerosolization of the peptide. In two models of non hypoxia mediated pulmonary vasoconstriction BNP induced cGMP and led to vasodilation either during intravascular or aerosol application.

In order to achieve a stable pulmonary vasoconstriction, either a continuous infusion of the thromboxane mimetic U46619 or a single bolus injection of endothelin-1 were used.

Both mediators are potent pulmonary vasoconstrictors and have been shown to induce increased pulmonary vascular resistance with a predominant precapillary component [19;20]. Indeed, vasoconstriction resulted in a stable pulmonary hypertension with a ~ 4 fold increase of the pulmonary artery pressure without any significant lung edema formation.

Incremental doses of BNP were applied into the recirculating buffer fluid in the pre-treated lungs, and a dose dependent decrease of pulmonary artery pressure occurred within a few minutes after its administration. Although a direct BNP induced vasodilation has never been described in a quasi in vivo setting in non hypoxia mediated pulmonary vasoconstriction, this observation is in accordance to the previously reported data from acute and chronic hypoxia. For example, intravascular application of BNP partially antagonised acute hypoxia induced pulmonary vasoconstriction in an experimental study [21] and in healthy volunteers [13]. In addition, mice lacking the natriuretic peptide A receptor encoding gene have an increased susceptibility to hypoxia mediated pulmonary vascular remodelling [22]. Finally, chronic BNP infusion ameliorated pulmonary vascular remodelling in chronic hypoxia exposed rats [16]. Nevertheless, hypoxia induce pulmonary vasoconstriction is a physiologic reflex that leads to a redistribution of the pulmonary blood flow from alveoli with low oxygen content to well ventilated areas, and antagonizing this reflex alone may have questionable benefit. However, in a small study in patients with cor pulmonale an infusion of exogenous BNP produced dose- related pulmonary vasodilatation, without worsening oxygen saturation or systemic blood pressure [14].

Interestingly, experimental data suggest a direct inverse relation between the oxygen dependent nitric oxide pathway and the natriuretic peptides system in the systemic circulation [23]. In light of these investigations, we therefore sought to characterize the

vasodilatory capacity of BNP in the pulmonary vasculature in two non hypoxia mediated models of pulmonary vasoconstriction, comprising two pivotal mediators of acute and chronic pulmonary hypertension *in vivo*.

More importantly, we demonstrate a maintained vasodilative capability of BNP during its application as an aerosol. Inhalation of vasodilators has been suggested before in pulmonary hypertension in order to reach preferential distribution into ventilated areas of the lung, to gain pulmonary and *intrapulmonary* selectivity and consequently to reduce systemic side effects and to avoid further gas exchange disturbance [19;24]. However, it is not self-evident that a peptide which is exposed to a nebulizer-technique and proteolytic enzymes on the lung surface preserves its biologic activity. This is of special interest, since all previous studies employing BNP have used a direct *in vitro* approach in cell cultures, isolated arteries or during intravenous application of the substance *in vivo*. But again, these application modes resemble para- and/or endocrine action of the peptide and translation of these results into an aerosol approach is not self-explanatory. To our knowledge there is only one other peptide that has been administered as an aerosol in pulmonary hypertension so far, namely the vasoactive intestinal peptide [5;6]. Thus, BNP is only the second peptide with preserved biologic activity for this application.

In various tissues BNP has been shown to exert its biologic effects via the NPR-A, which activates the particulate guanylyl cyclase, leading to an enhanced cGMP generation [7]. This cyclic nucleotide has many biologic effects within the lung. For example, NO, which is the prototype of a vasodilator, activates the soluble guanylyl cyclase, leading to increased levels of cGMP and consequently relaxation of smooth muscle cells [9]. However, this effect is not restricted to the pulmonary vascular site and an

augmentation of cGMP within airway smooth muscle cells could be a therapeutic target in obstructive airway diseases [8;25].

We observed a dramatic augmentation of the circulating cGMP levels both after a single intravascular bolus and when BNP was aerosolized to the lung. This increase was accompanied by a significant drop in pulmonary artery pressure in both series. Although we can not conclude on the exact source of cGMP within the lung, these observations underline the potential of the lung to produce significant amounts of cGMP in response to an activation of the NPR-A, and that this activation can be achieved by aerosolized BNP. The intravascular application mode of the type A natriuretic peptide urodilatin has been investigated in another model of acute pulmonary hypertension in intact rabbits before. During the infusion of urodilatin Schermuly et al observed increasing *systemic* cGMP levels which were accompanied by a drop of the blood pressures preferentially in systemic and to a minor extend in pulmonary circulation. However, the co-application of an inhaled phosphodiesterase inhibitor led to an amplification of the pulmonary vasodilation, and the authors concluded that the lung vasculature was a source of cGMP generation [26]. Nevertheless, in our isolated lung model extra pulmonary cGMP generation in response to NPR-A activation by an BNP aerosol can be excluded.

The inhalative administration of BNP could potentially lead to beneficial effects within the lung as the target organ by high regional production of cGMP, making it also preferred combination partner for phosphodiesterase inhibitors [10;27]. However, as stated above obstructive lung diseases may also be a target for the use of cGMP enhancing therapies, such as BNP aerosol. For example, in a recent study, the infusion of recombinant BNP (neseritide) led to a significant alleviation of pulmonary obstruction in asthmatic patients [25].



Our study has clear limitations. One is that we can only describe acute effects of BNP administration and we are not able to conclude on a chronic treatment effect. However, we demonstrate a mechanism that involves cGMP generation. It has been shown before that the beneficial effects of cyclic nucleotides are not limited to acute vasoactive properties, but also has antiremodeling effects in experimental pulmonary hypertension [28]. Based on our current pathophysiologic understanding on the biology of BNP, together with our actual findings, we think that it is reasonable to assume that the increased availability of cGMP caused the vascular actions of exogenous BNP application. Although we did not inhibit the formation of cGMP in our model, we measured cGMP in the perfusate as an indirect indicator of an augmented intracellular production. However, since we used an isolated lung model we can not exclude that increased cGMP levels would have caused systemic side effects, especially hypotension. Also, we did not measure ventilation/perfusion distribution and so we can only speculate from earlier studies with inhaled vasodilators that BNP aerosol has a beneficial effect on pulmonary gas exchange. Finally, we did not combine exogenous BNP with inhibitors of phosphodiesterase V. However, in extensive experimental and clinical studies the beneficial role of co-application of inductors of cyclic nucleotides and the inhibition of its degrading enzymes has been shown [29-31].

Despite these limitations, the present study is the first to demonstrate that the pulmonary vasodilative property as an indicator of cGMP mediated activity of BNP is preserved during its aerosol application.

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**Figure legends:**

Figure 1: Dose response curve for intravascular administration of BNP in stable pulmonary hypertension.

During stable pulmonary vasoconstriction increasing doses of BNP of 5, 10 and 100nM were administered into the recirculating buffer fluid.

Figure 2: Effect of a single dose of intravascular administered BNP in U46619 elicited pulmonary hypertension.

A single bolus application of 10 nM BNP led to an immediate vasodilation, which was accompanied by a significant rise of cGMP in the buffer fluid.

Figure 3: Effect of placebo aerosol, intravascular administration of BNP and BNP aerosol on cGMP levels in U46619 elicited pulmonary hypertension.

While the induction of a U 46619 induced pulmonary vasoconstriction did not cause a significant change in the cGMP concentration, a dramatic increase of cGMP was observed when BNP was bolus applicated into the buffer fluid or during the aerosol application of BNP.

Figure 4 A and B: Effect of placebo and BNP aerosol on pulmonary artery pressure and lung weight gain in U46619 elicited pulmonary hypertension

During a stable pulmonary vasoconstriction BNP was applicated as an aerosol and led to a rapid and significant vasodilation. Neither the aerosol application of placebo, nor the BNP aerosol led to a significant lung edema in the U 46619 model.

Figure 5 A and B: Effect of placebo and BNP aerosol on pulmonary artery pressure and lung weight gain in endothelin-1 elicited pulmonary hypertension

During a stable pulmonary vasoconstriction BNP was applicated as an aerosol and led to a rapid and significant vasodilation. Neither the aerosol application of placebo, nor the BNP aerosol led to a significant lung edema in the endothelin-1 model.

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Figure 1: Dose response curve for intravascular administration of BNP during the U46619 induced vasoconstriction (n=4)

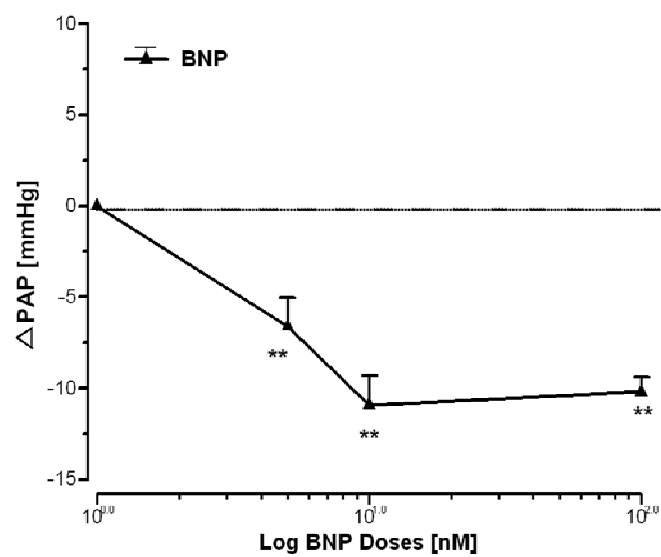


Figure 2: Effect of a single dose of intravascular administered BNP in U46619 elicited pulmonary vasoconstriction (n=6)

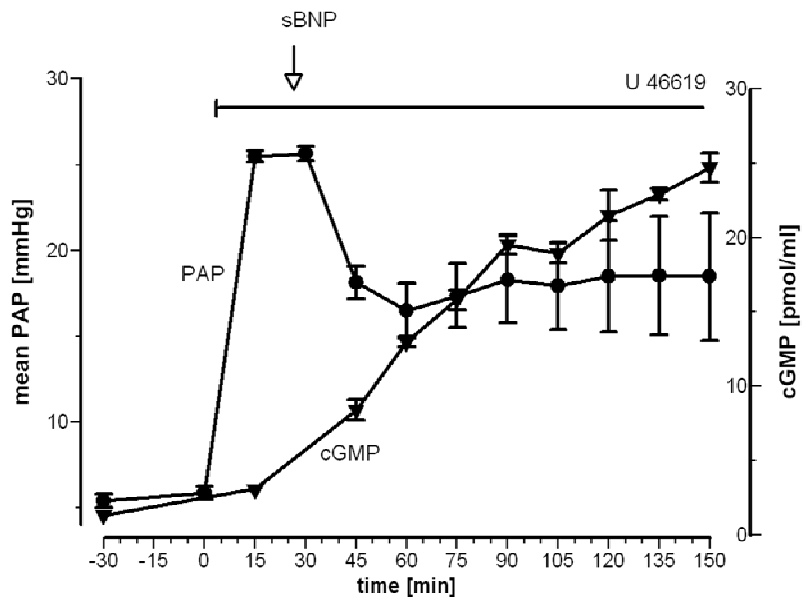


Figure 3: Effect of placebo aerosol, intravascular administration of BNP and BNP aerosol on cGMP levels in U46619 elicited pulmonary vasconstriction (each n=6)

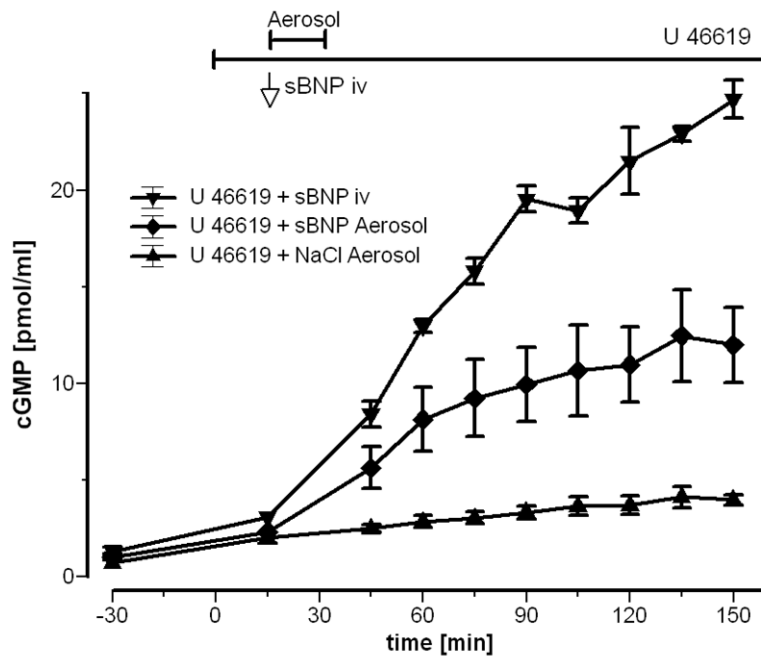
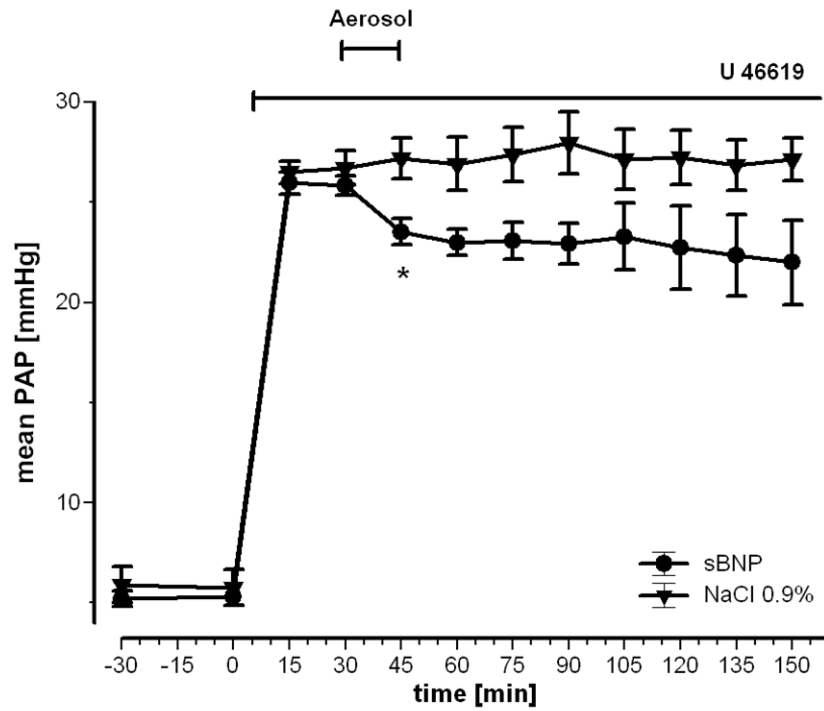
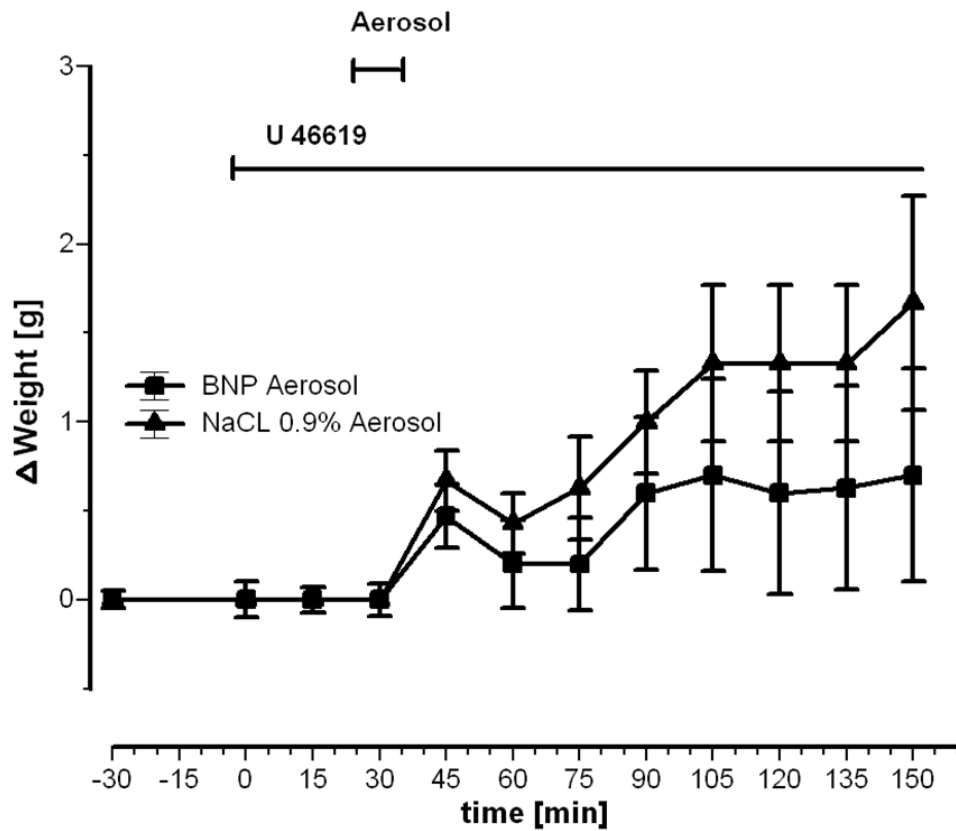


Figure 4a: Effect of placebo and BNP aerosol on pulmonary artery pressure in U46619 elicited pulmonary vasoconstriction



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Figure 4b: Effect of placebo and BNP aerosol on lungweight in U46619 elicited pulmonary vasoconstriction



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Figure 5a: Effect of placebo and BNP aerosol on pulmonary artery pressure in Endothelin 1 elicited pulmonary vasoconstriction

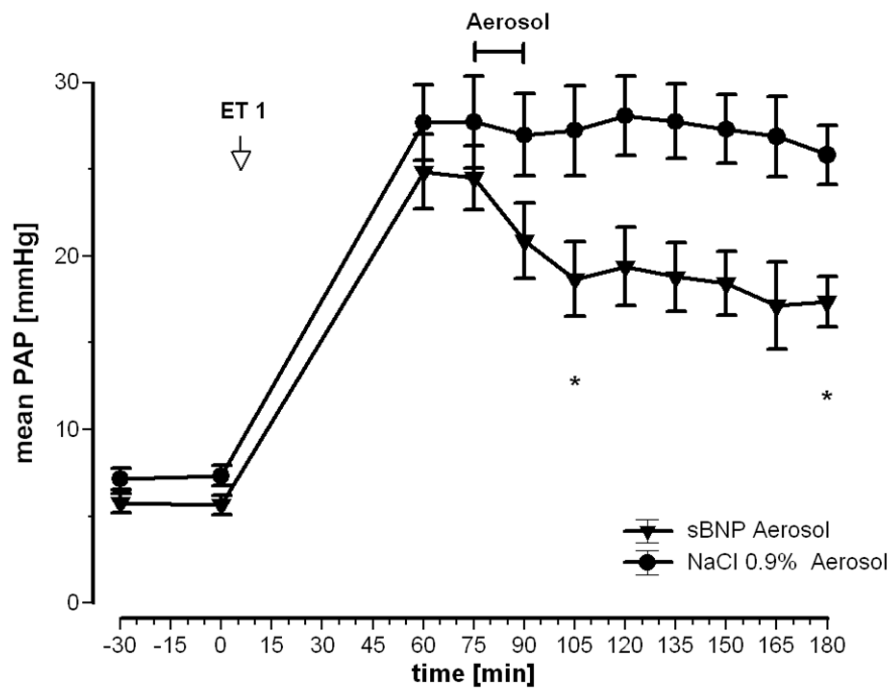
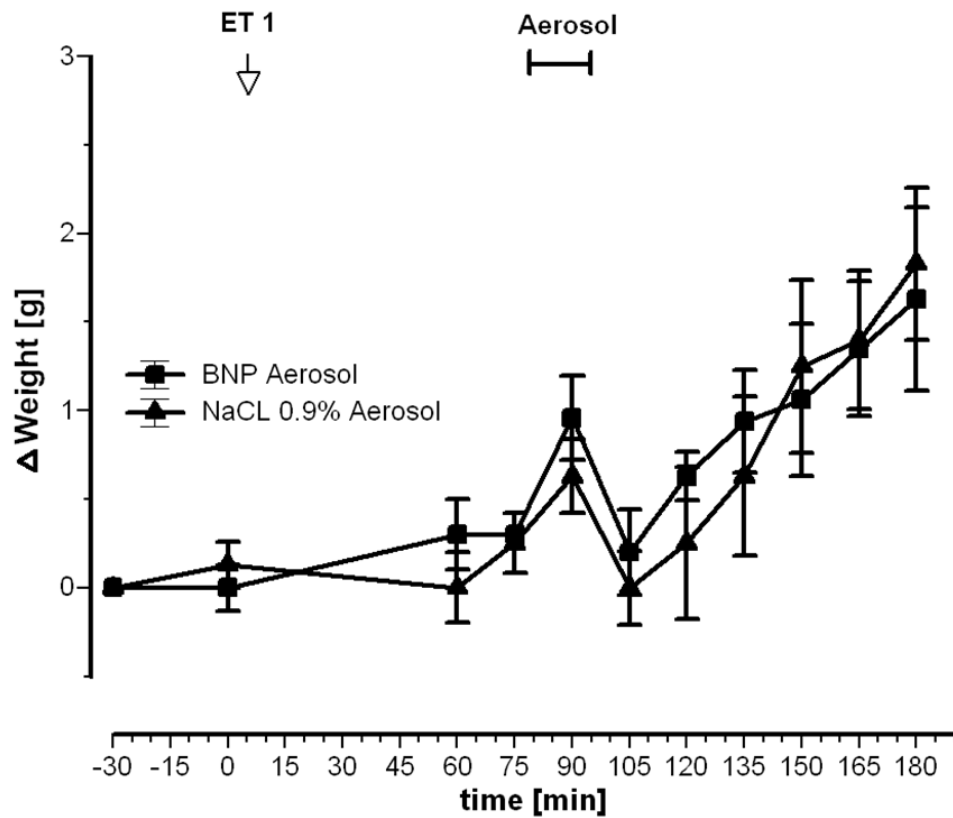


Figure 5b: Effect of placebo and BNP aerosol on lung weight in Endothelin 1 elicited pulmonary vasoconstriction



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