Pilot intervention: Aerosolized adrenomedullin reduces pulmonary hypertension

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d: List of nonstandard abbreviations:

ADM: adrenomedullin; L-NAME: N^G-nitro-L-arginine methylester;

eNOS: endothelial nitric oxide synthase; iNOS: inducible nitric oxide synthase;

ET-1: endothelin-1; ARDS: acute respiratory distress syndrome;

MAP: mean arterial pressure; MPAP mean pulmonary artery pressure.

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ABSTRACT

In pulmonary hypertension, systemic infusion of adrenomedullin (ADM), a potent vasodilator peptide, leads to pulmonary vasodilatation. However, systemic blood pressure declines alike. The present study investigated the effect of aerosolized ADM on pulmonary arterial pressure in surfactant depleted newborn piglets with pulmonary hypertension.

Animals randomly received aerosolized ADM (ADM, n=6), aerosolized ADM combined with intravenous application of N^G-nitro-L-arginine methylester to inhibit nitric oxide synthases (ADM+L-NAME, n=5), or aerosolized normal saline solution (control, n=6). Aerosol therapy was performed in 30 minute intervals for 5 hours. After a total experimental period of 8 hours, mRNA expression of NO synthases (eNOS and iNOS) and endothelin-1 (ET-1) in lung tissue was quantified using TaqMan real time PCR.

Aerosolized ADM reduced mean pulmonary artery pressure (MPAP) compared to control ($p<0.001$; at the end of the study: delta-MPAP –13.5 ± 1.4 vs. -6.2 ± 2.4 mmHg). PaO_2 significantly increased in the ADM (delta PaO_2: 243.3 mmHg) and the ADM+L-NAME group (delta PaO_2: 217.4 mmHg) compared to the control group (delta PaO_2: 82.9 mmHg; $p<0.001$). Aerosolized ADM did not influence mean systemic arterial pressure (baseline 63.2 ± 2.7 vs. end of the study 66.3 ± 6.5 mmHg, n.s.). NO synthases gene expressions were 20-30% lower with ADM compared to control. ET-1 gene expression was significantly reduced (>50%) after ADM aerosol therapy ($p<0.001$).

Aerosolized adrenomedullin significantly reduced MPAP without lowering the systemic arterial pressure and improved profoundly the arterial oxygen tension. This effect seems to be mediated at least in part by the reduction of ET-1.

Pulmonary hypertension due to acute respiratory distress syndrome (ARDS) or primary pulmonary hypertension of the newborn (PPHN) represent imbalanced homeostasis of vasoconstrictor and vasodilator peptides controlling pulmonary artery pressure (Keith, 2000). Pulmonary hypertension is associated with increased expression of endothelin-1 (ET-1), a potent constrictor, in pulmonary vascular endothelial cells. The local production of endothelin-1 is assumed to contribute to the vascular abnormalities associated with this disorder (Giaid et al., 1993). Adrenomedullin (ADM) is a potent vasodilator peptide. In the lung, ADM was shown to be present in several cells, such as endothelial and smooth muscle cells, alveolar macrophages and the columnar epithelium (Jougasaki and Burnett, Jr., 2000). ADM receptors are highly expressed in lung and heart tissue. Analysis of the ADM binding sites suggests a regulatory role of the peptide for the pulmonary circulation and cell differentiation (Kapas et al., 1995; Owji et al., 1995). ADM preferentially reduces pulmonary artery pressure through a decrease of pulmonary vascular resistance (Cheng et al., 1994; Lippton et al., 1994; Heaton et al., 1995). The marked elevation of ADM plasma levels in patients with pulmonary hypertension may represent a compensatory mechanism (Kakishita et al., 1999). Intravenous infusion of ADM in adult patients with precapillary pulmonary hypertension reduces the pulmonary vascular resistance significantly. However, systemic vascular resistance is simultaneously decreased (Nagaya et al., 2000). Similar systemic effects are seen with the application of ADM into the pulmonary artery of fetal sheep (Takahashi et al., 1999). Although pulmonary hypertension may respond to systemic vasodilator therapy, the treatment frequently fails because intravenous administration of vasodilators may increase blood flow in non-ventilated lung areas, increasing the ventilation/perfusion mismatch. In addition adverse effects on the systemic blood pressure are

common (Agusti and Rodriguez-Roisin, 1993). Administration of therapeutic drugs via inhalation is supposed to be more selective for the pulmonary vascular bed. This has been shown for inhaled nitric oxide (Nagamine et al., 2000; Demirakca et al., 1996) and for inhaled prostacyclin (Olschewski et al., 1999). To date, no studies on the efficacy and pulmonary selectivity of aerosolized ADM in pulmonary hypertension have been performed. We therefore examined the impact of aerosolized ADM on pulmonary artery pressure, systemic arterial pressure, and oxygenation in neonatal piglets with acute respiratory distress syndrome and pulmonary hypertension.

Intravenous infusion of ADM (50 ng/kg/min) showed significant effects after 15 minutes (Nagaya et al., 2000). Hence, in the present intervention 30 minutes inhalation intervals with incremental doses of ADM, followed by 30 minutes observation were used.

METHODS

Research animals:

Seventeen piglets of both sexes with a body weight of 3,5-4,3 kg were included in the study. The study was approved by the Animal Care Committee of the University of Erlangen and the government of Mittelfranken, Germany, and performed according to guidelines of the NIH. Animals randomly received aerosolized ADM (ADM, n=6), aerosolized ADM combined with intravenous application of N^G-nitro-L-arginine methylester to inhibit nitric oxide synthases (ADM+L-NAME, n=5), or aerosolized normal saline solution (control, n=6).

After a venous catheter had been placed into an ear vein, anesthesia was induced with midazolam (1 mg/kg), fentanyl (2,5 µg/kg) and ketamine (5 mg/kg) followed by continuous infusion of midazolam (1.5 mg/kg per hour), fentanyl (0.01 mg/kg per hour) and ketamine (15 mg/kg per hour) (Kandler et al., 2001; von der Hardt et al., 2002b). Piglets generally require high doses of anesthetics for narcosis. Therefore, the combination of three anesthetics was used. It was the intention to prevent adverse effects such as hyperthermia that occurs e.g. in inadequate sedation.

After tracheotomy, paralysis was induced with vecuronium 0.2 mg/kg i.v. and maintained with vecuronium 0.2 mg/kg per hour, to avoid any interference due to spontaneous breathing. that is thought to influence the efficacy of mechanical ventilation.

A sheath (4.5 F, Cook®, Mönchengladbach, Germany) was placed into the right jugular vein and a pulmonary artery catheter (4.0 F, Arrow®, Erding, Germany) was introduced into the pulmonary artery. After preparation of the left femoral artery, an arterial catheter (20 G, Arrow®, Erding, Germany) was placed and a sensor for online blood gas monitoring (Paratrend 7®, Philips®, Böblingen, Germany) was inserted for online registration of blood gases. The pulmonary artery and the femoral artery catheter were continuously rinsed, each by 2 mL normal saline containing 2.0 IU heparin per hour. The piglets received a

transcutaneous urinary catheter (Cystofix minipäd®, Braun, Melsungen, Germany). Heart rate, oxygen saturation, body temperature, central venous, pulmonary artery and systemic arterial pressure were continuously recorded (CMS 2001, Philips®, Böblingen, Germany). Arterial blood gas analysis was performed from arterial blood samples in 30 minutes intervals (ABL 330, Radiometer Copenhagen, Denmark). Intermittent mandatory ventilation (IMV) was performed with a neonatal respirator (Infant Star 950, Mallinckrodt, Hennef, Germany). Breath rate was 50 breaths/minute, peak inspiratory pressure (PIP) 32 cmH_2O, positive end expiratory pressure (PEEP) 8 cmH_2O and the inspiratory fractional oxygen concentration (FiO_2) 1.0 (100%).

Study protocol:

Lung injury with pulmonary hypertension was induced by surfactant depletion (repeated saline lung lavage (NaCl 0.9%, temperature 39 °C) using 30 ml/kg per side (Kandler et al., 2001; Lachmann et al., 1980). Piglets where included if lung injury was considered to be stable, defined as PaO_2 constantly remaining below 80 mmHg for a period of sixty minutes. If inclusion criteria failed, repeated lung lavages were performed until criteria had been met.

During instrumentation and for the duration of the experiment, animals were in supine position. The animals were randomly assigned to three different therapy groups (adrenomedullin, ADM+L-NAME and control). In all animals, respiratory support was maintained constant at identical respiratory settings (PEEP 8 cm H_2O, PIP 32 cmH_2O, FiO_2 1.0, 50 breaths/minute). Before onset of treatment, baseline recordings of pulmonary and circulatory parameters were performed. Adrenomedullin (Bachem®, Germany) was applied in saline solution as aerosol (aerosol generator: Trudell Medical Inc.™, London, Canada (Kandler et al., 2001; MacIntyre et al., 1996; MacIntyre, 2001)). Five dose levels (6.25, 12.5, 25, 50 and 100 ng/kg/min) each for 30 min were applied, followed by 30 min inhalation free

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intervals over a total period of 5 hours (volume rate of 4 ml/h). The observation was continued for three hours after the inhalation procedure. To investigate the role of NO formation in the mechanism of ADM effects, NO synthases were inhibited in the ADM+L-NAME group. Piglets additionally received the inhibitor of the nitric oxide synthases L-NAME (Sigma, Steinheim, Germany) at a dose of 25 mg/kg/h intravenously 30 min before and continuously during the 8 h of the experiment. Piglets in the Control group received aerosolized saline solution (4 ml/h). After an additional observation period of 3 hours, animals were sacrificed by intravenous injection of 50 mg/kg methohexital and 20 ml potassium chloride 7.46%. Lungs and heart were removed en bloc.

Tissue processing:

The left lung was perfused with 5% buffered paraformaldehyde. For histological examinations from standardized sites, samples were taken from the peripheral upper and lower lobe. 5µm thick sections of paraffin embedded tissue sections were stained with hematoxylin-eosin for routine histo-pathologic evaluation. Chloracetate esterase histochemic reaction was performed to visualize neutrophil granulocytes. One blinded expert pathologist examined the sections for the items hyaline membranes, hyperemia, interstitial edema, intra-alveolar hemorrhage and neutrophil accumulation and attributed each item to a 4-point score: 0: None, 1: mild, 2: moderate and 3: severe. Lung injury score was calculated including all sites and items (Quintel et al., 1998). For each site, the score of all items was summarized. Data are presented as mean ± SEM of this summary score including all the sites.

RT-PCR

Standardized specimens (four from the inferior lobe (central and basal), two from the superior and two from the middle lobe) were taken from the native peripheral right lung and stored at –80°C until mRNA extraction was performed using guanidine-thiocyanate acid phenol (RNAzol, WAK Chemie®, Medical GmbH, Bad Homburg, Germany). One µg of RNA per

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tissue sample was reversely transcribed in a volume of 20 µl at 39°C for 60 minutes (all chemicals were obtained from Boehringer® Mannheim, Germany). The cDNA samples were stored at –20°C.Quantitative TaqMan real time PCR:

Efficiency and reliability of this method have been shown earlier (Dötsch et al., 1999; Heid et al., 1996; Schoof et al., 2002). The use of TaqMan real time PCR in this animal model was recently published (von der Hardt et al., 2002b; von der Hardt et al., 2003). Primers and TaqMan probes were elected for the porcine model (Table 1). This approach is based upon the 5' exonuclease activity of the Taq polymerase. Briefly, within the amplicon defined by a gene specific oligonucleotide primer pair an oligonucleotide probe labeled with two fluorescent dyes is designed. As long as the probe is intact, the emission of a reporter dye (i.e. 6-carboxy-fluorescein, FAM) at the 5'-end is quenched by the second fluorescence dye (6-carboxy-tetramethyl-rhodamine, TAMRA) at the 3'-end. During the extension phase of the PCR, the Taq polymerase cleaves the probe releasing the reporter dye. An automated photometric detector combined with a special software (ABI Prism® 7700 Sequence Detection System, Perkin-Elmer, Foster City, CA) monitors the increasing reporter dye emission. The algorithm normalizes the signal to an internal reference (ΔRn) and calculates the threshold cycle number (C_T), when the ΔRn reaches 10 times the standard deviation of the baseline. Commercial reagents (TaqMan PCR Reagent Kit, Perkin-Elmer) and conditions according to the manufacturer's protocol were employed. 2,5 µl of cDNA (reverse transcription mixture) and oligonucleotides with a final concentration of 300 nM of primers and 200 nM of TaqMan hybridization probe were added to 25 µl reaction mix. The thermocycler parameters were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. A serial dilution of known copy numbers of a PCR product served as reference providing a relative quantification of the unknown samples**.** Gene

expression was related to the housekeeping genes ß-actin (A) and hypoxanthine-guanine-phosphoribosyl-transferase (HPRT). Real-time PCR fragments of the measured porcine genes are shown in Fig. 7.

Data analysis and statistics: Values are expressed as mean ± SEM. After testing for Gaussian distribution, two-way ANOVA with Bonferroni post-hoc-test was utilized. To evaluate the data compared to the baseline one-way ANOVA with Dunett´s multiple comparison test was applied. A p-value of less than 0.05 was considered significant. For PCR data, depending on the presence of Gaussian distribution, either the ANOVA or Kruiskal-Wallis test were used for comparison of the groups. In case of significance, Bonferroni and Dunns post-hoc tests were applied. Comparing two groups, t-test or Mann-Whitney test were used, respectively.

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RESULTS

Mean pulmonary artery pressure

Bronchoalveolar lavage increased MPAP from 15.8 ± 1.1 mmHg to 37.5 ± 2.3 mmHg in the ADM, from 14.3 ± 0.9 mmHg to 38.8 ± 2.0 mmHg in the ADM+L-NAME group and from 16.0 ± 1.7 to 37.2 ± 2.2 mmHg in the saline control group (these post lavage values were defined as baseline). Aerosolized ADM reduced mean pulmonary artery pressure (MPAP) significantly compared to the control group (at the end of the study: (ADM) 21.5 ± 2.0 vs. (control) 28.7 ± 1.5 mmHg; $p<0.001$, Fig. 1). In addition, the decline of MPAP was significantly steeper in the ADM than in the control group. In animals continuously treated with L-NAME MPAP fell to 29.4 ± 2.1 mmHg (at the end of the study). Looking at the reduction of MPAP from the baseline of each group, ADM effect was unchanged when L-NAME was infused simultaneously (Fig. 1). The difference from baseline MPAP was significant after 2.5 hours in the ADM group and could not be distinguished from baseline before 4.5 hours in the control group.

Mean arterial pressure

During administration of aerosolized ADM and throughout the observation period, systemic mean arterial pressure (MAP) was not influenced compared to baseline (Fig. 2 and Fig. 3). There was no significant difference in MAP between the ADM and the control group (Fig. 3). In contrast, L-NAME increased MAP significantly and maintained higher levels throughout the course of the experiment ($p<0.001$; Fig. 3).

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Arterial oxygen tension:

Compared to the baselines (after establishment of pulmonary hypertension), PaO_2 significantly increased in the ADM group ($p<0.01$) and in the ADM+L-NAME group ($p<0.01$, Fig. 4). The arterial oxygen tension in both groups was significantly higher than in the control group ($p<0.001$). There was no significant difference in PaO_2 between the ADM and the ADM+L-NAME group during the treatment and the post treatment observation period (Fig. 4).

NO Synthases

iNOS mRNA expression was slightly but significantly lower in the ADM and the ADM+L-NAME group compared to the control group ($p<0.05$). These results were obtained irrespectively of whether gene expression was normalized to the housekeeping genes ß-actin or HPRT (Fig. 5). eNOS mRNA expression was significantly lower in the ADM group than in the control group only when normalized to ß-actin ($p< 0.01$; Fig. 5). eNOS mRNA expression was significantly lower in the ADM+L-NAME group than in the control group when normalized to both, ß-actin and HPRT ($p< 0.01$; Fig. 5).

Endothelin-1

ET-1 mRNA gene expression was significantly reduced after treatment with aerosolized ADM

compared to the control group (reduction: ET-1/A: 62.4% , ET-1/HPRT: 51.0%, $p<0.001$;

Fig. 6).

Histology

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The lung injury score (mean ± SEM) was not significantly different between the ADM and the control group 6.5 ± 0.6 vs. 5.3 ± 0.4.

DISCUSSION

The present study demonstrates a sustained decrease of MPAP by the repeated administration of aerosolized ADM (25 to 100 ng/kg/min) whereas mean systemic arterial pressure remained unchanged. This is markedly different from literature showing a significant reduction of systemic arterial pressure in addition to the intended reduction of pulmonary pressure when ADM was applied intravenously. The lower doses (6.25 and 12.5 ng/kg/min) did not show a significant effect (Fig. 1 and Fig. 2). In our study, a considerable improvement of oxygenation was observed by ADM inhalation. This suggests a sustained beneficial effect on ventilation perfusion mismatch, that is well known to be an important component of pulmonary vascular disease (Agusti and Rodriguez-Roisin, 1993). Similar effects were observed after inhalation of various vasodilators like nitric oxide, iloprost and sodium nitroprusside (Demirakca et al., 1996; Schutte et al., 2001; Olschewski et al., 1999). The positive effect of ADM on pulmonary vascular resistance might be even enhanced or perpetuated by the improvement of oxygenation. As histology of representative pulmonary specimens did not show significant differences between the groups, it seems unlikely that changes in lung injury led to consecutive reduction of vascular resistance. As far as safety issues are concerned, there were no expected or unexpected side effects of the therapy. In particular, none of the animals died during the use of inhaled ADM. Instead, an anti-inflammatory effect of inhaled ADM could be demonstrated by reduction of transforming growth factor-ß1 and interleukin-1ß gene expression (von der Hardt et al., 2002a). In prior studies, the vasodilating effect of ADM has been shown to be - at least in part - mediated by activation of nitric oxide synthases and enhancement of iNOS expression (Feng et al., 1994). Under physiological conditions, the ADM effect on pulmonary vascular resistance can be attenuated by NOS inhibitor administration to fetal sheep (Takahashi et al., 1999). The close interaction of ADM and nitric oxide in vascular cells also becomes obvious by an increase of

ADM gene expression and peptide synthesis after incubation of human umbilical venous cells (HUVEC) with different NO donors (Dötsch et al., 2002). In our present series of experiments, L-NAME did not attenuate the beneficial effect of inhaled ADM on pulmonary vasodilation and oxygenation. In addition, ADM did not increase iNOS gene expression. The reduced gene expression of iNOS and eNOS does not necessarily reflect enzyme activity. Nonetheless, it can be concluded from the L-NAME experiment that ADM does not work exclusively via NO synthase activation or expression. The fact that L-NAME does not influence ADM induced vasodilatation in this study implies that ADM might predominantly act via other pathways. For future experiments it might be useful to assess the effect of L-NAME on pulmonary arterial resistance in comparison to L-NAME+ADM. The effect of ADM might be mediated via the transmembrane ADM receptor by an increase in intracellular cyclic AMP concentration (Ishizaka et al., 1994; Shimekake et al., 1995), which is vasorelaxant itself (Maurice and Haslam, 1990). Furthermore, cAMP might impede the function of phosphodiesterase IIIa, which is responsible for the decay of cyclic GMP, the most important mediator of nitric oxide induced vasodilation (Shah and Kadowitz, 2002), this mechanism mimics NO mediated vasodilatation. To prove the specific intrapulmonary ADM effect manipulation of ADM receptor function e.g. by the infusion or inhalation of a specific antagonist such as ADM 22-52 might be included in future studies.

The reduction of ET-1 mRNA gene expression, following treatment with aerosolized ADM might be mediated by the suppressive effect of cAMP on ET-1 synthesis (Magnusson et al., 1994). Therefore, the vasorelaxant effect of ADM might be potentiated by the reduction of ET-1 synthesis. It appears unlikely that the mechanism of ADM action is via an increased activity of prostaglandins or is exerted by activation of calcitonin gene related peptide receptor (Takahashi et al., 1999).

Recently, a number of publications have addressed the systemic use of ADM in humans for the correction of various hemodynamic disorders. Apart from the improvement of pulmonary hypertension (Nagaya et al., 2000), cardiac afterload could be reduced (Del Bene et al., 2000). Interestingly the vascular effects of ADM are significantly attenuated in patients with chronic heart failure, in part because of impaired production of nitric oxide (Nakamura et al., 1997). In our model, animals have experienced serious pulmonary damage before the onset of ADM inhalation with probably severe impairment of NO synthases. Therefore, the effect of ADM on pulmonary artery pressure of newborn piglets might be even more pronounced if NO synthases function was maintained at a normal level. Nonetheless, the studies in humans are encouraging towards a potential use of inhaled ADM in man.

CONCLUSION

Aerosolized adrenomedullin leads to improved oxygenation, a reduction in ET-1 gene expression, and a selective reduction in pulmonary artery pressure, without lowering the systemic blood pressure.

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LEGENDS FOR FIGURES

Figure 1:

Mean and SEM of delta mean pulmonary artery pressure (Δ-MPAP) obtained after induction of lung injury (baseline), during therapy with ADM, ADM+L-NAME and with normal saline (control group) and during the post treatment observation time in surfactant depleted neonatal piglets. ADM vs. control: $_{*}$ $p<0.05$.

Figure 2:

Changes of MAP and MPAP (% Mean and SEM relation compared to baseline (0)) during therapy with ADM aerosol and during observation periods. ADM vs. control: $_{*}$ $p<0.05$; $_{**}$ $p<0.01$; $_{***}$ $p<0.001$.

Figure 3:

Mean and SEM of mean arterial pressure (MAP) obtained before (-4) and after induction of lung injury (0), during therapy with ADM, ADM+L-NAME and with normal saline (control group) and during the post treatment observation time in surfactant depleted neonatal piglets.

Figure 4:

Mean and SEM of arterial oxygen tension (PaO_2) obtained before (-4) and after induction of lung injury (0), during therapy with ADM, ADM+L-NAME and with normal saline (control group) and during the post treatment observation time in surfactant depleted neonatal piglets. ADM vs. control: $_{*}$ $p<0.05$; $_{***}$ $p<0.001$.

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Figure 5:

NO synthase gene expression (RU = relative units):

a) Inducible NO synthase/beta-actin mRNA expression (iNOS/A) and

b) iNOS/Hypoxanthine-guanine-phosphoribosyl-transferase (iNOS/HPRT) mRNA expression in the lung of surfactant depleted piglets after an interval therapy period of 5 hours with aerosolized adrenomedullin or with aerosolized normal saline (control group) during intermittent mandatory ventilation and an additional observation period of 3 hours. * $p<0.05$; *** $p<0.001$ vs. control.

c) Endothelial NO synthase/beta-actin mRNA expression (eNOS/A) and

d) eNOS/Hypoxanthine-guanine-phosphoribosyl-transferase (eNOS/HPRT) mRNA expression. ADM vs. control: * $p<0.05$; ** $p<0.01$; **** $p<0.001$.

Figure 6:

Endothelin-1 gene expression (RU = relative units):

a) ET-1/beta-actin mRNA expression (ET-1/A) and b) ET-1/Hypoxanthine-guanine-phosphoribosyl-transferase (ET-1/HPRT) mRNA expression in the lung of surfactant depleted piglets after an interval therapy period of 5 hours with aerosolized adrenomedullin or with aerosolized normal saline (control group) during intermittent mandatory ventilation and an additional observation period of 3 hours.

ADM vs. control: *** $p<0.001$.

Figure 7:

Real-time PCR fragments of the measured porcine genes inducible nitric oxide synthase (iNOS, 131 b), endothelial nitric oxide synthase (eNOS, 72 b), endothelin-1 (ET-1, 182 b), ß-actin (133 b) and hypoxanthine-guanine-phosphoribosyl-transferase (HPRT, 62 b).

REFERENCES

Agusti AG and Rodriguez-Roisin R (1993) Effect of pulmonary hypertension on gas exchange. *Eur.Respir.J.* 6:1371-1377.

Cheng DY, DeWitt BJ, Wegmann MJ, Coy DH, Bitar K, Murphy WA, and Kadowitz PJ (1994) Synthetic human adrenomedullin and ADM15-52 have potent short-lasting vasodilator activity in the pulmonary vascular bed of the cat. *Life Sci.* 55:L251-L256.

Del Bene R, Lazzeri C, Barletta G, Vecchiarino S, Guerra CT, Franchi F, and La Villa G (2000) Effects of low-dose adrenomedullin on cardiac function and systemic haemodynamics in man. *Clin.Physiol* 20:457-465.

Demirakca S, Dotsch J, Knothe C, Magsaam J, Reiter HL, Bauer J, and Kuehl PG (1996) Inhaled nitric oxide in neonatal and pediatric acute respiratory distress syndrome: dose response, prolonged inhalation, and weaning. *Crit Care Med.* 24:1913-1919.

Dötsch J, Nusken KD, Knerr I, Kirschbaum M, Repp R, and Rascher W (1999) Leptin and neuropeptide Y gene expression in human placenta: ontogeny and evidence for similarities to hypothalamic regulation. *J Clin Endocrinol Metab* 84:2755-2758.

Dötsch J, Schoof E, Hanze J, Dittrich K, Opherk P, Dumke K, and Rascher W (2002) Nitric oxide stimulates adrenomedullin secretion and gene expression in endothelial cells. *Pharmacology* 64:135-139.

Feng CJ, Kang B, Kaye AD, Kadowitz PJ, and Nossaman BD (1994) L-NAME modulates responses to adrenomedullin in the hindquarters vascular bed of the rat. *Life Sci.* 55:L433-L438.

Giaid A, Yanagisawa M, Langleben D, Michel RP, Levy R, Shennib H, Kimura S, Masaki T, Duguid WP, and Stewart DJ (1993) Expression of endothelin-1 in the lungs of patients with pulmonary hypertension. *N.Engl.J.Med.* 328:1732-1739.

Heaton J, Lin B, Chang JK, Steinberg S, Hyman A, and Lippton H (1995) Pulmonary vasodilation to adrenomedullin: a novel peptide in humans. *Am.J.Physiol* 268:H2211-H2215.

Heid CA, Stevens J, Livak KJ, and Williams PM (1996) Real time quantitative PCR. *Genome Res* 6:986-994.

Ishizaka Y, Ishizaka Y, Tanaka M, Kitamura K, Kangawa K, Minamino N, Matsuo H, and Eto T (1994) Adrenomedullin stimulates cyclic AMP formation in rat vascular smooth muscle cells. *Biochem.Biophys.Res Commun.* 200:642-646.

Jougasaki M and Burnett JC, Jr. (2000) Adrenomedullin: potential in physiology and pathophysiology. *Life Sci.* 66:855-872.

Kakishita M, Nishikimi T, Okano Y, Satoh T, Kyotani S, Nagaya N, Fukushima K, Nakanishi N, Takishita S, Miyata A, Kangawa K, Matsuo H, and Kunieda T (1999) Increased plasma levels of adrenomedullin in patients with pulmonary hypertension. *Clin.Sci.(Lond)* 96:33-39.

Kandler MA, von der Hardt K, Schoof E, Dotsch J, and Rascher W (2001) Persistent improvement of gas exchange and lung mechanics by aerosolized perfluorocarbon. *Am.J.Respir.Crit Care Med.* 164:31-35.

Kapas S, Catt KJ, and Clark AJ (1995) Cloning and expression of cDNA encoding a rat adrenomedullin receptor. *J.Biol.Chem.* 270:25344-25347.

Keith IM (2000) The role of endogenous lung neuropeptides in regulation of the pulmonary circulation. *Physiol Res.* 49:519-537.

Lachmann B, Robertson B, and Vogel J (1980) In vivo lung lavage as an experimental model of the respiratory distress syndrome. *Acta Anaesthesiol.Scand.* 24:231-236.

Lippton H, Chang JK, Hao Q, Summer W, and Hyman AL (1994) Adrenomedullin dilates the pulmonary vascular bed in vivo. *J.Appl.Physiol* 76:2154-2156.

MacIntyre N, Andjuval S, and Baran G (1996) Aerosol deposition from an intra-airway aerosol. *European Respiratory Journal* 9:P0963.

MacIntyre NR (2001) Intratracheal catheters as drug delivery systems. *Respir.Care* 46:193-197.

Magnusson A, Halldorsson H, Thorgeirsson G, and Kjeld M (1994) Endothelin secretion is regulated by cyclic AMP and phosphatase 2A in endothelial cells. *J Cell Physiol* 161:429-434.

Maurice DH and Haslam RJ (1990) Nitroprusside enhances isoprenaline-induced increases in cAMP in rat aortic smooth muscle. *Eur.J Pharmacol.* 191:471-475.

Nagamine J, Hill LL, and Pearl RG (2000) Combined therapy with zaprinast and inhaled nitric oxide abolishes hypoxic pulmonary hypertension. *Crit Care Med.* 28:2420-2424.

Nagaya N, Nishikimi T, Uematsu M, Satoh T, Oya H, Kyotani S, Sakamaki F, Ueno K, Nakanishi N, Miyatake K, and Kangawa K (2000) Haemodynamic and hormonal effects of adrenomedullin in patients with pulmonary hypertension. *Heart* 84:653-658.

Nakamura M, Yoshida H, Makita S, Arakawa N, Niinuma H, and Hiramori K (1997) Potent and long-lasting vasodilatory effects of adrenomedullin in humans. Comparisons between normal subjects and patients with chronic heart failure. *Circulation* 95:1214-1221.

Olschewski H, Ghofrani HA, Walmrath D, Schermuly R, Temmesfeld-Wollbruck B, Grimminger F, and Seeger W (1999) Inhaled prostacyclin and iloprost in severe pulmonary hypertension secondary to lung fibrosis. *Am.J.Respir.Crit Care Med.* 160:600-607.

Owji AA, Smith DM, Coppock HA, Morgan DG, Bhogal R, Ghatei MA, and Bloom SR (1995) An abundant and specific binding site for the novel vasodilator adrenomedullin in the rat. *Endocrinology* 136:2127-2134.

Quintel M, Heine M, Hirschl RB, Tillmanns R, and Wessendorf V (1998) Effects of partial liquid ventilation on lung injury in a model of acute respiratory failure: a histologic and morphometric analysis. *Crit Care Med.* 26:833-843.

Schoof E, von der HK, Kandler MA, Abendroth F, Papadopoulos T, Rascher W, and Dotsch J (2002) Aerosolized perfluorocarbon reduces adhesion molecule gene expression and neutrophil sequestration in acute respiratory distress. *Eur.J.Pharmacol.* %20;457:195-200.

Schutte H, Lockinger A, Seeger W, and Grimminger F (2001) Aerosolized PGE1, PGI2 and nitroprusside protect against vascular leakage in lung ischaemia-reperfusion. *Eur.Respir.J.* 18:15-22.

Shah MK and Kadowitz PJ (2002) Cyclic adenosine monophosphate-dependent vascular responses to purinergic agonists adenosine triphosphate and uridine triphosphate in the anesthetized mouse. *J.Cardiovasc.Pharmacol.* 39:142-149.

Shimekake Y, Nagata K, Ohta S, Kambayashi Y, Teraoka H, Kitamura K, Eto T, Kangawa K, and Matsuo H (1995) Adrenomedullin stimulates two signal transduction pathways, cAMP accumulation and Ca2+ mobilization, in bovine aortic endothelial cells. *J Biol.Chem.* 270:4412-4417.

Takahashi Y, de Vroomen M, Gournay V, Roman C, Rudolph AM, and Heymann MA (1999) Mechanisms of adrenomedullin-induced increase of pulmonary blood flow in fetal sheep. *Pediatr.Res.* 45:276-281.

von der Hardt K, Kandler MA, Fink L, Schoof E, Dotsch J, Bohle RM, and Rascher W (2003) Laser-assisted microdissection and real-time PCR detect anti-inflammatory effect of perfluorocarbon. *Am.J.Physiol Lung Cell Mol.Physiol* ..

von der Hardt K, Kandler MA, Popp K, Schoof E, Chada M, Rascher W, and Dötsch J (2002a) Aerosolized adrenomedullin suppresses pulmonary transforming growth factor-beta1 and interleukin-1beta gene expression in vivo. *Eur.J.Pharmacol.* 457:71-76.

von der Hardt K, Schoof E, Kandler MA, Dötsch J, and Rascher W (2002b) Aerosolized perfluorocarbon suppresses early pulmonary inflammatory response in a surfactant depleted piglet model. *Pediatr.Res.* 51:177-182.

FOOTNOTES:

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Fig. 1

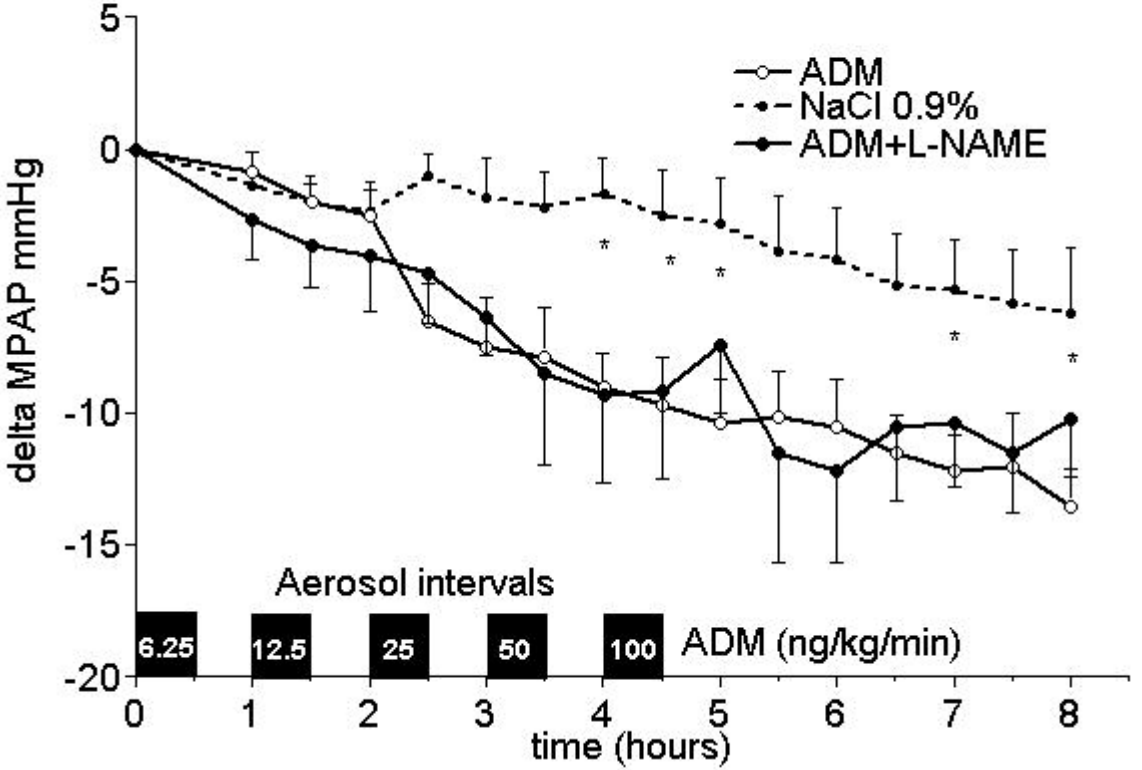
ADM
NaCl 0.9%
ADM+L-NAME
delta MPAP mmHg
5
0
-5
-10
-15
-20
Aerosol intervals
6.25
12.5
25
50
100
ADM (ng/kg/min)
0
1
2
3
4
5
6
7
8
time (hours)

Fig. 2

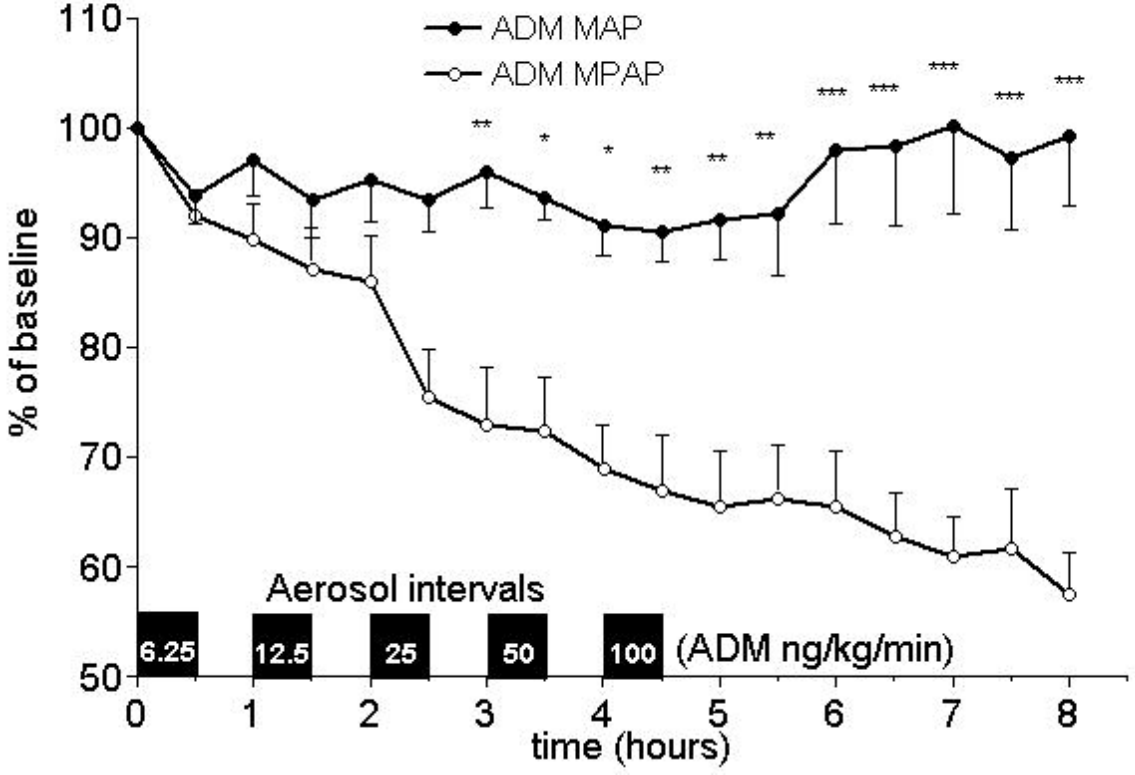
ADM MAP
ADM MPAP
% of baseline
110
100
90
80
70
60
50
0
1
2
3
4
5
6
7
8
time (hours)
Aerosol intervals
6.25
12.5
25
50
100
(ADM ng/kg/min)

Fig. 3

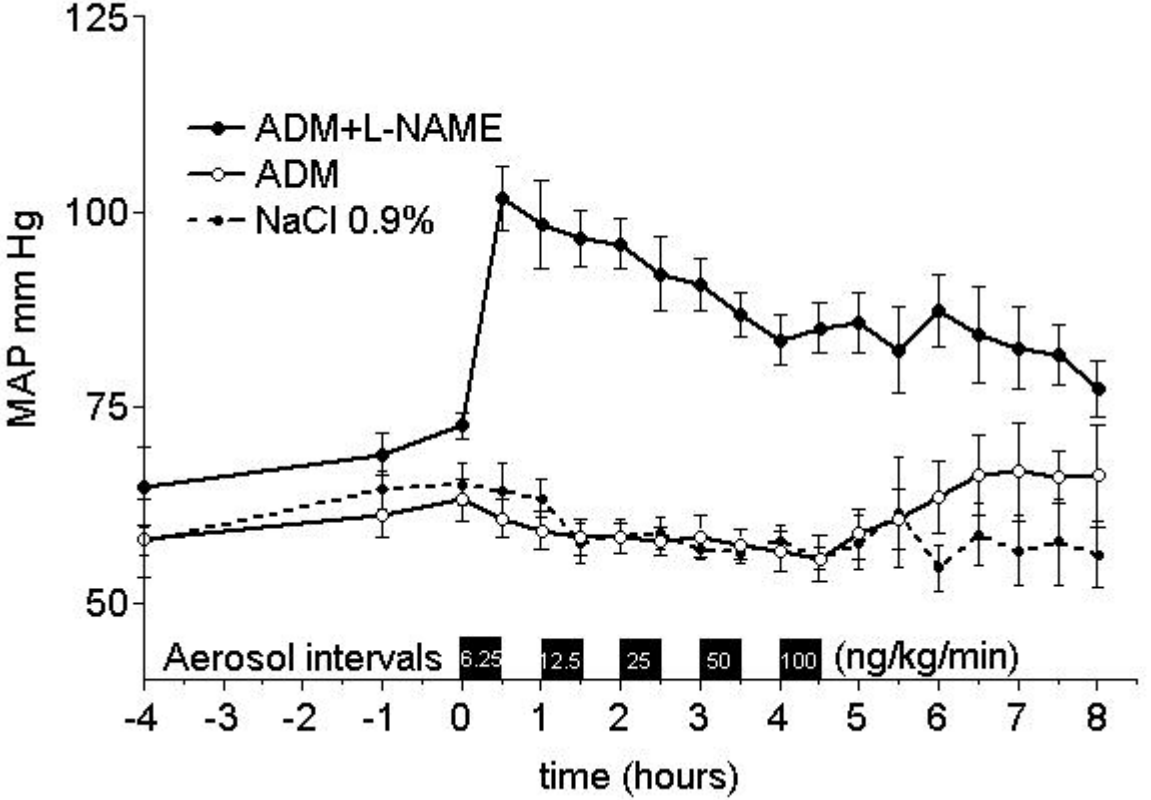
125
100
75
50
MAP mm Hg
ADM+L-NAME
ADM
NaCl 0.9%
Aerosol intervals
6.25
12.5
25
50
100
(ng/kg/min)
-4
-3
-2
-1
0
1
2
3
4
5
6
7
8
time (hours)

Fig. 4

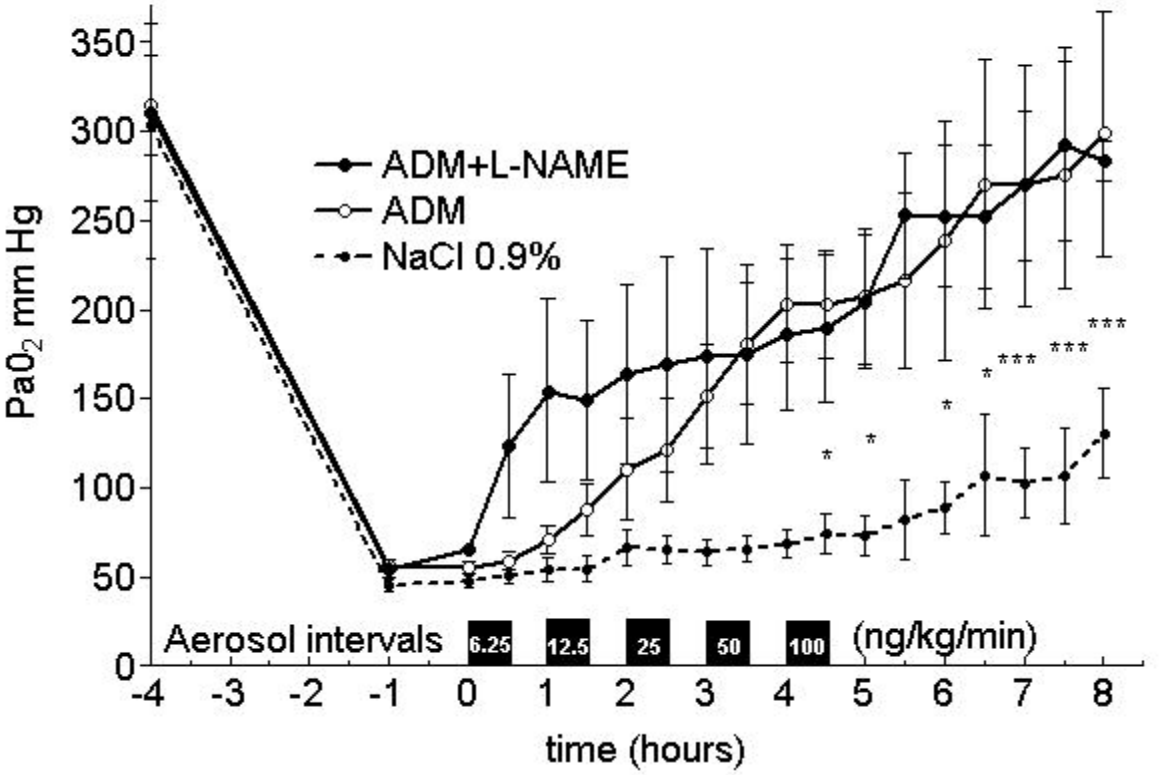
ADM+L-NAME
ADM
NaCl 0.9%
PaO2 mm Hg
350
300
250
200
150
100
50
0
Aerosol intervals
6.25
12.5
25
50
100
(ng/kg/min)
-4
-3
-2
-1
0
1
2
3
4
5
6
7
8
time (hours)
*
*
*
*
***
***
***

Fig. 5a

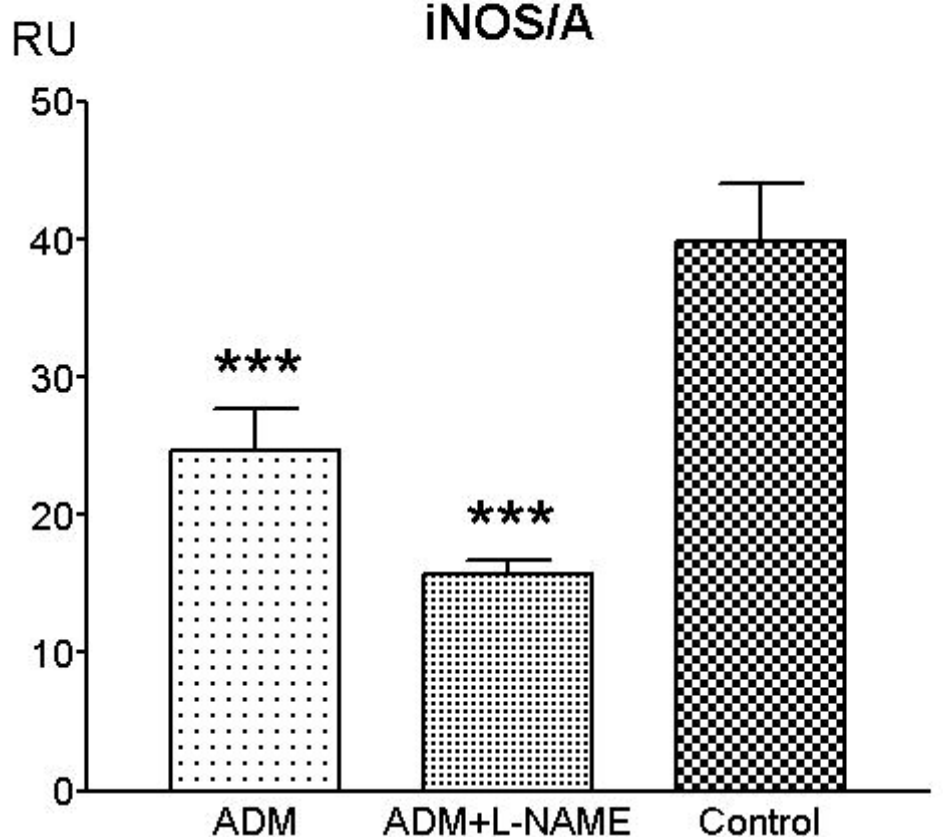

Fig. 5b

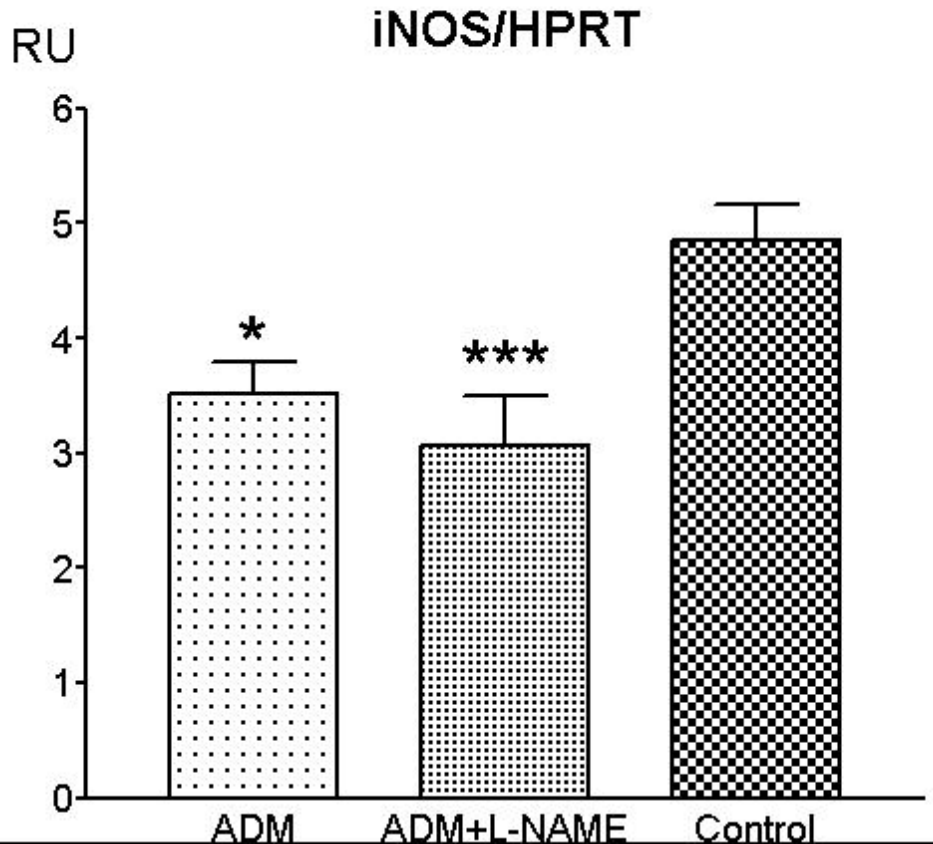

Fig. 5c

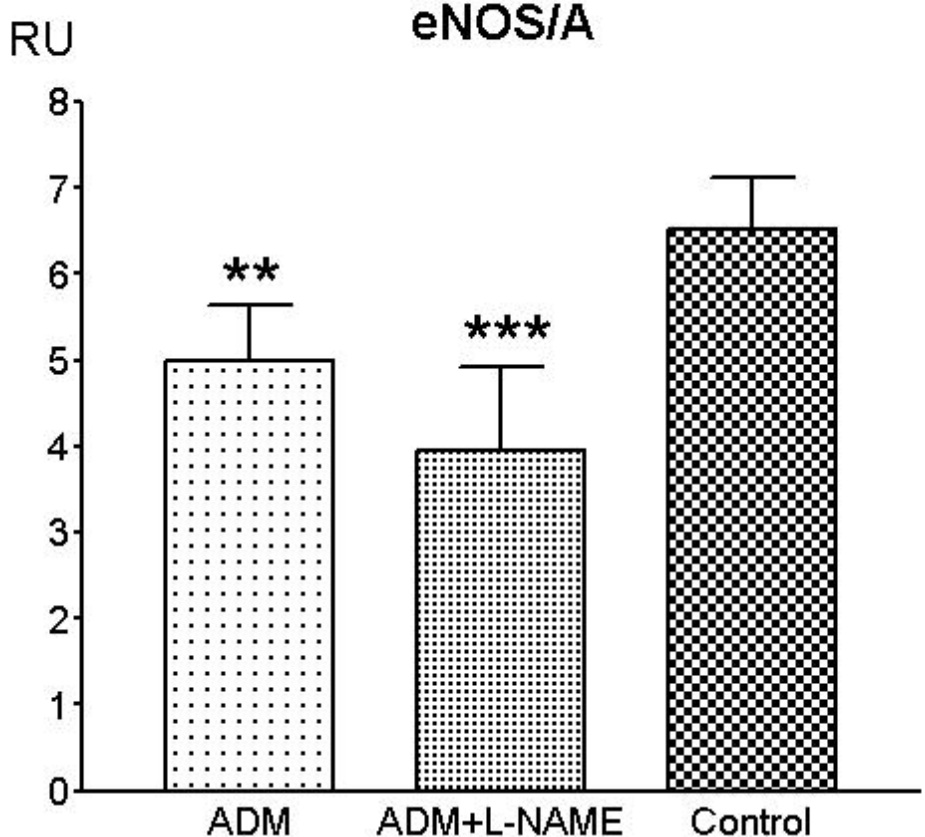

Fig. 5d

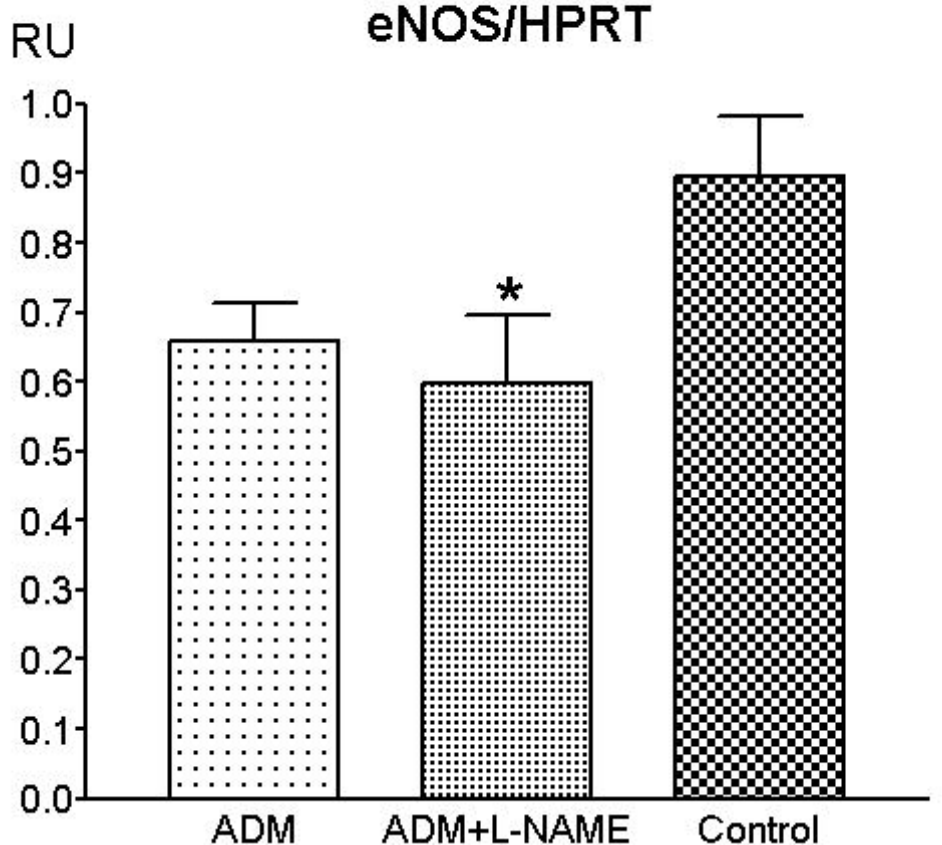
eNOS/HPRT
RU
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
ADM
ADM+L-NAME
Control

Fig. 6a

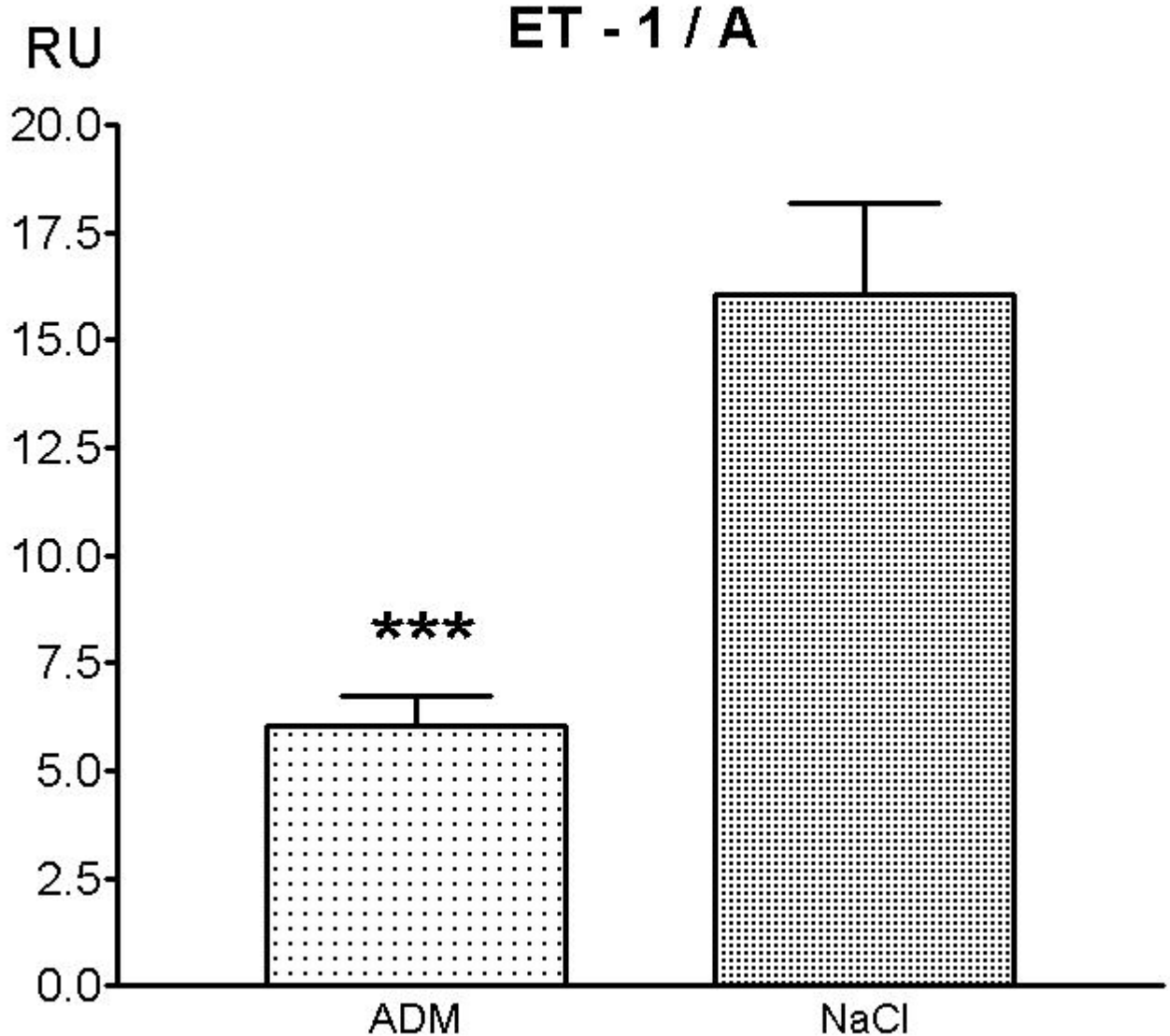

Fig. 6b

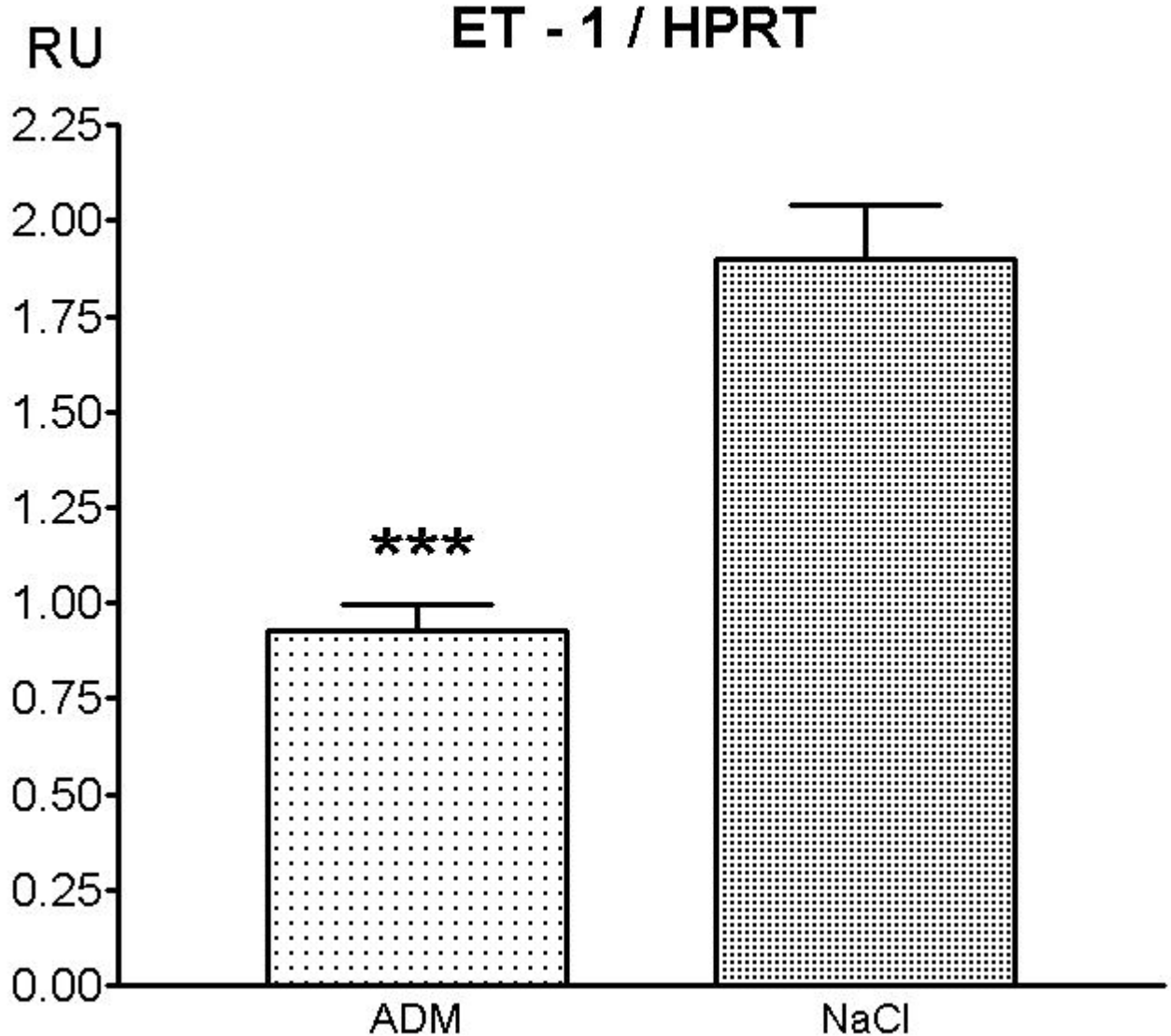

Fig. 7

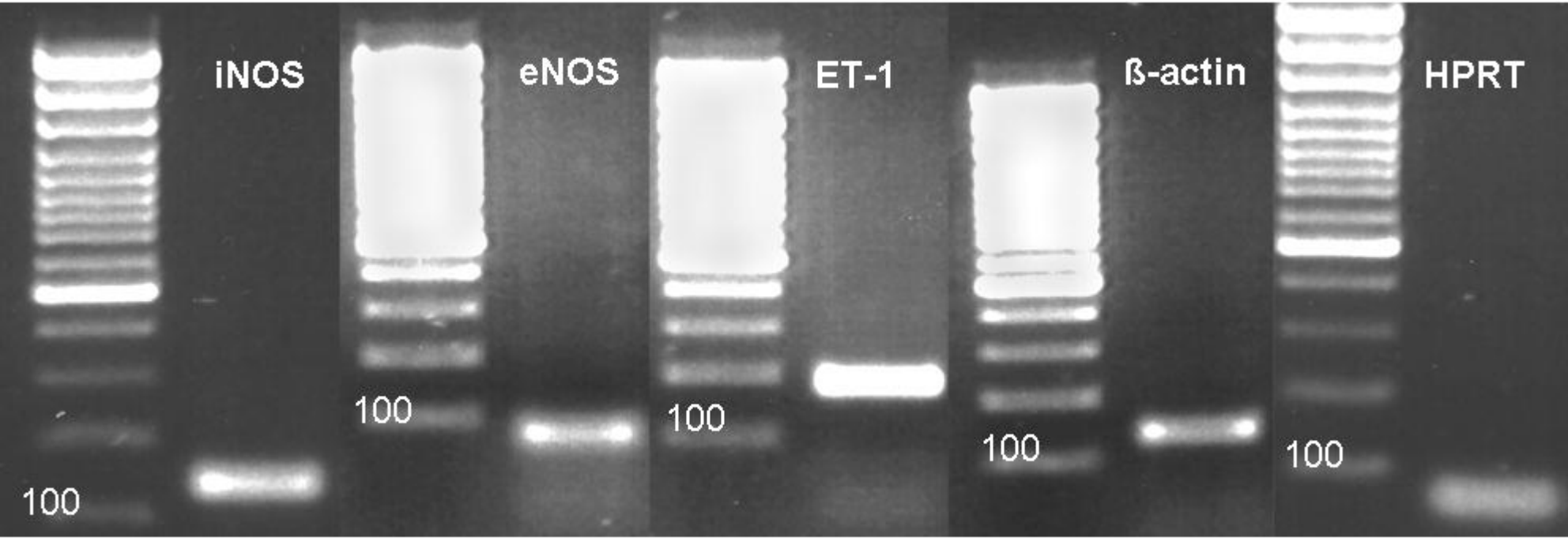

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TABLE 1:

The following primers and TaqMan probes were used:

Hypoxanthine-guanine-phosphoribosyl-transferase :

Forward: 5‘- TGGAAAGAATGTCTTGATTGTTGAAG -3‘

Reverse: 5‘- ATCTTTGGATTATGCTGCTTGACC -3‘

TaqMan probe: 5‘ (FAM)-ACACTGGCAAAACAATGCAAACCTTGCT -(TAMRA)3‘

ß-Actin:

Forward: 5`- TCATCACCATCGGCAACG –3´

Reverse: 5`- TTCCTGATGTCCACGTCGC –3

TaqMan probe: 5` (FAM)- CCTTCCTGGGCATGGAGTCCTGC -(TAMRA)3´

Endothelial nitric oxide synthase (eNOS):

Forward: 5`- GCATCGCCAGAAAGAAGACG -3`

Reverse: 5`- CGGTGGCCATGAGTGAGG -3`

TaqMan probe: 5` (FAM)- TTAAGGAAGTGGCCAACGCGGTGAA -(TAMRA)3`

Inducible nitric oxide synthase (iNOS):

Forward: 5`- GAGCACATCTGCAGGCACC -3`

Reverse: 5`- GCATACCGGATGAGCTGAGC -3`

TaqMan probe: 5` (FAM)- TGGCAAGCACGACTTCCGGGTG -(TAMRA)3`

Endothelin-1

Forward: 5´- CTCCTGCTCTTCCCTGATGG -3´

Reverse: 5´- TGGCACACTGGCATCTATCC -3´

TaqMan probe: 5´(FAM)- TTCTGCCACCTGGACATCATTTGGG -(TAMRA)3´