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Effect of DMPPO, a phosphodiesterase type 5 inhibitor, on hypoxic pulmonary hypertension in rats

[1]Saadia Eddahibi, [2]Bernadette Raffestin, [3]Anne-Charlotte Le Monnier de Gouville & [1,4]Serge Adnot

[1]Département de physiologie et INSERM U 492, CHU Henri Mondor, 94010 Créteil; [2]Département de physiologie, Université René Descartes, Hôpital Ambroise Paré, 92100 Boulogne; [3]Laboratoires Glaxo Wellcome, 91951, Les Ulis, France

1 Cyclic guanosine 3′–5′-monophosphate (cyclic GMP) is the second messenger of important physiologically active mediators controlling the pulmonary vascular tone. To potentiate the effects of cyclic GMP on the pulmonary vasculature, we used DMPPO, a new selective PDE-5 inhibitor, and examined its action in a rat model of hypoxic pulmonary hypertension.

2 Levels of cyclic GMP measured during baseline conditions at 5 and 60 min of perfusion were similar in the perfusate of isolated lungs from normoxic and chronically hypoxic rats and did not differ with time. Pretreatment with DMPPO (1 μM) induced a larger increase in cyclic GMP concentration in the perfusate from chronically hypoxic rat lungs (319 ± 36 at 5 min to 1821 ± 83 pmol ml^{-1} at 60 min) than in normoxic rat lungs (329 ± 20 to 1281 ± 127 pmol ml^{-1}, $P < 0.05$).

3 In isolated lungs preconstricted with U-46619, pretreatment with DMPPO (1 μM) potentiated the vasodilator effects of atrial natriuretic peptide (100 pM–10 nM) and sodium nitroprusside (1 pM–10 nM), but did not alter vasodilation to isoproterenol.

4 In conscious rats previously exposed to 15 days hypoxia and studied under 10% O_2, DMPPO (0.01, 0.05 and 0.1 mg kg^{-1}, i.v. bolus) caused a dose-dependent decrease in pulmonary arterial pressure (Pap) with no change in systemic artery pressure (Sap) and cardiac output.

5 Continuous infusion of DMPPO (0.1 mg kg^{-1} h^{-1} i.v. by osmotic pumps) in rats exposed to 10% O_2 during 2-weeks reduced the Pap ($P < 0.05$) and the degree of muscularization of pulmonary vessels at the alveolar wall ($P < 0.01$) and alveolar duct levels ($P < 0.05$) despite no significant change in right ventricular hypertrophy.

6 These results suggest that cyclic GMP phosphodiesterase inhibition may selectively dilate pulmonary circulation during chronic hypoxia.

Keywords: Phosphodiesterase-5 inhibitors; pulmonary hypertension; nitric oxide; cyclic GMP

Introduction

Endogenous mediators such as nitric oxide (NO) and atrial natriuretic peptide (ANP) are potent pulmonary vasodilators acting through accumulation of cyclic 3′–5′-guanosine monophosphate (cyclic GMP) within smooth muscle cells of pulmonary vessels. These endogenous mediators also share the ability to inhibit development of experimental pulmonary hypertension and attenuate remodelling of pulmonary arteries (Kouyoumdjian *et al*., 1994; Raffestin *et al*., 1992). Potentiation of the activity of endogenous ANP or NO on pulmonary vessels may therefore represent a therapeutic approach against pulmonary vasoconstriction and pulmonary vascular remodeling. This may be achieved at the level of cyclic GMP through inhibition of the nucleotide degradation by phosphodiesterases.

In smooth muscle cells, concentrations of cyclic GMP are established by the balance of production by guanylyl cyclase and degradation by phosphodiesterases (PDEs). Among the five isoforms of PDE now identified (1, 2, 3, 4 and 5) (Beavo, 1995; Polson & Strada, 1996), four have been shown to be present in pulmonary artery tissue from humans (1, 3, 4 and 5) (Dent *et al*., 1994). The cyclic GMP-specific isoform, PDE-5 is abundantly expressed in the lung and predominates in smooth muscle cells of pulmonary arteries (Rabe *et al*., 1994). Moreover, recent findings suggest an increase in PDE-5 activity in pulmonary arteries from rat with hypoxic pulmonary hypertension (MacLean *et al*., 1997). Isozyme-selective PDE inhibitors have been used to characterize the PDE activities in the pulmonary vasculature. In newborn lambs, zaprinast, a cyclic GMP PDE inhibitor, has been reported to reduce pulmonary artery pressure during hypoxic vasoconstriction or administration of the endoperoxide analogue U-46619 (Ichinose *et al*., 1995). Zaprinast also potentiates the vasodilating effects and prolongs the duration of action of NO inhalation in awake lambs with U-46619 induced pulmonary hypertension. In the ovine model of persistent pulmonary hypertension induced by ductal ligation, zaprinast enhances relaxation to sodium nitroprusside in isolated pulmonary arteries and potentiates pulmonary vasodilation induced by inhaled NO. Although this agent is a cyclic GMP-PDE inhibitor, it lacks sufficient potency and specificity to assess the role of cyclic GMP dependent PDE-5 activity in the pulmonary vasculature. More recently, E4021, a selective inhibitor of cyclic GMP-specific PDE-5 has been shown to be as effective as inhaled NO in inducing pulmonary vasodilation in rats exposed to acute hypoxia (Cohen *et al*., 1996). However, only acute effects were studied and no information was provided as to whether selective inhibition of cyclic GMP PDE-5 activity may protect against development and maintenance of pulmonary hypertension.

Recently, Coste & Grondin (1995) described a new potent and selective PDE-5 inhibitor, 1.3 dimethyl-6-(2-propoxy-5-methane sulphonylamidophenyl)-pyrazolo[3,4-d]pyrimidin-4-(5H)-one (DMPPO). This compound is a competitive inhibitor with respect to cyclic GMP ($K_i = 3$ nM) and displays high

[4] Author for correspondence.

selectivity for PDE-5 when compared with other PDE isozymes ($K_i = 1$ mM, 3 mM, 10 mM, respectively for PDE-1, 2, 3 and 4). In cultured rat aortic smooth muscle cells, DMPPO is capable of increasing sodium nitroprussiate (SNP) and ANP stimulated cyclic GMP levels. In phenylephrine-precontracted rat aortic rings, DMPPO has also been shown to potentiate the relaxing effect of ANP or SNP and to cause dose-dependent relaxation in the presence of endothelium (Deply *et al*., 1996; Deply & Le Monnier de Gouville, 1996). Its high oral bioavailability and long duration of action makes it a potentially useful therapeutic agent in pulmonary hypertension.

In the present study, we examined the effects of DMPPO on cyclic GMP or cyclic AMP mediated pulmonary vasodilation using isolated lungs from normoxic or chronically hypoxic rats. We also examined acute hemodynamic effects of DMPPO in conscious chronically hypoxic rats. We then questioned whether continuous infusion of DMPPO could attenuate development of pulmonary hypertension and lessen severity of right ventricular hypertrophy and vascular remodelling in rats chronically exposed to hypoxia.

Methods

Chronic hypoxia

Male Wistar rats weighing 250–300 g at the start of the experiments, were exposed to chronic hypoxia (10% O_2) for 2 weeks in a ventilated chamber (500-litre volume, Flufrance, Cachan, France) as previously described (Adnot *et al*., 1991). To establish the hypoxic environment, the chamber was flushed with a mixture of room air and nitrogen, and the gas recirculated. Chamber environment was monitored with an oxygen analyzer (Servomex OA150, Crowborough, England). Carbon dioxide was removed by soda lime granules and excess humidity prevented by cooling of the recirculation circuit. The chamber temperature remained between 22–24°C. The chamber was opened every other day for 1 h to clean the cages and replenish food and water. Normoxic rats were kept in the same room, with the same light-dark cycle. Rat chow and tap water were provided ad libitum.

Studies in lungs isolated from normoxic and chronically hypoxic rats

Rats were anaesthetized with sodium pentobarbital (40 mg i.p.). After tracheal cannulation, they were ventilated with warmed normoxic gas (95% air, 5% CO_2) at 60 breaths min^{-1} with an inspiratory pressure of 9 cm H_2O and an expiratory pressure of 2.5 cm H_2O. A median sternotomy was performed and 100 IU heparin administered through the right ventricle. Heart and lungs were removed and suspended in an humidified chamber at 37°C. The lungs were perfused through a pulmonary arterial cannula with a peristaltic pump at a constant flow of 0.05 $\mu l\ g^{-1}$ body weight min^{-1}. The recirculated perfusate was a warmed (38°C) physiological salt solution (PSS) of the following composition (mM): NaCl, 116; KCl, 4.7; $NaHCO_3$, 19; $MgSO_4$, 0.83, $CaCl_2$, 1.8; H_2O, 2; NaH_2PO_4, 1.04; glucose, 5.5 and phenol red Na (0.11 g l^{-1}) as a pH indicator, ficoll (4 g 100 ml^{-1}, type 70, Sigma Chemical Co., St. Louis, U.S.A.). Meclofenamate (3.2 μM) was included in the perfusate at the start of experiments to inhibit cyclooxygenase. Effluent perfusate was drained from the left ventricular cannula into a reservoir. Mean perfusion pressure was measured from a side port of the pulmonary arterial line (P23 XL transducer; Gould, Ballainvilliers, France), the pulmonary venous pressure was assumed to be zero. Each lung preparation was used to study only one of the following procedures.

Measurement of cyclic GMP concentrations in the isolated lungs perfusate from normoxic and chronically hypoxic rats

The samples of perfusate (500 μl) were collected after 5 and 60 min of recirculation perfusion and quickly frozen. The frozen samples were assayed using a Biochemical Technologies Inc cyclic GMP RIA system. It uses a preconjugated double antibody separation system in an acetic buffer. Duplicate measurements were performed on all samples, including the standard curve, and all values are calculated and reported as picomoles per millilitre of cyclic GMP.

To assess the effect of DMPPO on cyclic GMP levels in the perfusate of isolated lungs, DMPPO (10^{-6} M) or its vehicle was added in the reservoir at the start of recirculating perfusion immediately after the first sampling of perfusate ($n = 5$ in each experiment except lungs from chronically hypoxic rats in the presence of DMPPO where $n = 4$).

To assess the role of endogenous NO in cyclic GMP accumulation, cyclic GMP concentrations in the perfusate were also measured in the presence of DMPPO after inhibition of NO production by L-nitroarginine methyl ester (L-NAME 100 μM, $n = 3$). To examine whether endogenous natriuretic peptides were involved in cyclic GMP production, we also measured cyclic GMP levels in the perfusate after inhibition of their degradation by the neutral endopeptidase inhibitor, thiorphan (10 μM, $n = 4$). Thiorphan or L-NAME was added concomitantly with DMPPO (1 μM) in the perfusate reservoir at the start of recirculating perfusion, immediately after the first perfusate sampling.

Effect of DMPPO on vasodilator responses to atrial natriuretic peptide (ANP), sodium nitroprusside (SNP) or isoproterenol

The effect of pretreatment with DMPPO or its vehicle on responses to ANP and SNP, two cyclic GMP dependent vasodilator agents was examined in lungs from normoxic and chronically hypoxic rats ($n = 5$ in each experiment). The effect of DMPPO or its vehicle was also tested on a cyclic AMP dependent vasodilator agent, isoproterenol, in lungs from normoxic rats ($n = 5$ in each experiment).

The endoperoxide analogue U-46619 was diluted in a 20-ml volume of physiological salt solution and infused into the pulmonary arterial line at a constant rate of 50 pmol min^{-1} with an infusion pump (Vial-Medical, Grenoble, France). Infusion of U-46619 was started after a 30 min equilibration period had elapsed. Pulmonary artery pressure increased gradually in response to U-46619 and did not reach a plateau. Atrial natriuretic peptide, SNP or isoproterenol was administered in the perfusate reservoir during U-46619 infusion when the increase in Pap had reached 6.5 to 9 mmHg. The vasodilator substances diluted in solutions of increasing concentrations, were injected as a 50-μl bolus at 3-min intervals to obtain increasing final concentrations in the recirculating perfusate.

The inhibitor of phosphodiesterase, DMPPO, or its vehicle was added into the perfusate reservoir at the end of the equilibration period to obtain a final concentration of 1 μM.

Acute hemodynamic effects of DMPPO treatment in rats previously exposed to chronic hypoxia

The day before the hemodynamic study, hypoxic rats were anaesthetized with an intramuscular injection of ketamine (20 mg) and xylazine (1 mg). After exposure of the right jugular vein, a polyvinyl catheter was inserted and manipulated through the right ventricle into the pulmonary artery, a second polyvinyl catheter was also inserted into the right jugular vein to allow injection of indocyanine green dye and DMPPO. A polyethylene catheter was inserted into the right carotid artery. A second polyethylene catheter was inserted into the left jugular vein for the return of blood to the rat during subsequent cardiac output measurements. Catheters were then sealed and tunnelled under the skin to the back of the neck, where they were exteriorized, secured and protected in a small plastic container.

Measurements were taken 1 day after surgery with the rat awake. Pulmonary and systemic artery pressures were measured using Gould P 23 ID transducers (Gould Electronics, Ballainvilliers, France), coupled to pressure modules and a Gould TA 550 multichannel recorder (Gould, Ballainvilliers, France). Cardiac output (CO) was measured with a dye dilution technique. A 50-μg bolus of indocyanine (1 mg ml^{-1}) was injected into the jugular vein. Blood (1 ml min^{-1}) was withdrawn from the carotid artery through a densitometer cuvette (Waters) and returned to the rat through the left jugular vein. Cardiac output was calculated from the dye dilution curve area after exponential extrapolation of the downslope. Calibration was performed at the end of each study with a known concentration of green dye (5 μg ml^{-1} rat blood).

On the experimental day, animals ($n=5$) were placed in a plexiglas box flushed with compressed air or an hypoxic gas mixture (10% O_2, 90% N_2). Hemodynamic variables were recorded first while the animal was breathing air. Then the plexiglas box was flushed with the hypoxic gas mixture. After 10 min of exposure to hypoxia, increasing doses of DMPPO (0.01, 0.05 and 0.1 mg kg^{-1}) were administered as 50-μl i.v. bolus at 30 min intervals. Pulmonary and systemic arterial pressures were continuously monitored and cardiac output measured before and 5, 15 and 30 min after each bolus administration.

Effect of continuous DMPPO infusion during chronic exposure to hypoxia

Two groups of rats were exposed to chronic hypoxia. One group was treated with DMPPO and the other group with its vehicle ($n=10$ in each group). One day before exposure to chronic hypoxia, miniosmotic infusion pumps (model 2002, Alzet, Charles River) containing DMPPO or its vehicle alone were implanted in the left jugular vein. The pumps delivered a volume of 0.5 μl h^{-1}, which was equivalent to 0.1 mg kg^{-1} h^{-1} of DMPPO.

After 15 days of continuous hypoxia and DMPPO infusion, rats were anaesthetized with ketamine and xylazine. Immediately after the insertion of catheters in the pulmonary and carotid arteries, pulmonary arterial (Pap) and systemic arterial pressure (Sap) were measured. Then, blood was sampled for hematocrit determination. Finally, after an intraperitoneal injection of sodium pentobarbital (20 mg kg^{-1}), the thorax was opened, and the heart was quickly removed, dissected and weighed. The ratio of right ventricular free wall weight to the sum of septum plus left ventricular free wall weight (fresh tissue) was used as an index of right ventricular hypertrophy.

The lungs were fixed in the distended state by infusion of 4% aqueous buffered formalin into the trachea at 25-cm H_2O pressure. The entire specimen was placed in a bath of the same fixative for a week. A midsaggital slice of the right lung including the apical, azygous and diaphragmatic lobes, was processed for paraffin embedding. Sections (5 μm thick) were cut for light microscopy and stained with hematoxylin phloxin saffron and orcein-picroindigo-carmine. In each rat, a total of 35–65 intraacinar vessels accompanying either alveolar duct or alveolus were analysed. Their type was identified as muscular, partially muscular and non muscular. Muscular arteries have a complete layer of smooth muscle cells bound by two orcein stained elastic lamina. Smooth muscle cells were identified as typical cells stained red by phloxin with elongated shape and square ends nuclei. They were seen in only part of the arterial circumference in partially muscular arteries and were lacking in non muscular arteries.

Drugs

Atrial natriuretic peptide (Sigma) dissolved in acetic acid (0.1 N) was stored as a stock solution at -30°C, and diluted in saline as required. Isoproterenol (Sigma) and sodium nitroprusside (Roche) were dissolved in saline. For *in vitro* experiments, DMPPO obtained from Glaxo Wellcome was dissolved in DMSO and diluted in saline. For *in vivo* experiments, DMPPO was dissolved in polyethylene glycol and N,N-dimethyl-acetamide (9v/v) and diluted in saline. Endoperoxide analogue U-46619 (Sigma) dissolved in ethanol was stored as a stock solution at -30°C, and diluted in saline as required.

In the isolated lung experiments, all concentrations are given as final concentrations in the recirculating perfusate.

Statistical analysis

All results are expressed as means $\pm$ s.e.mean.

Two-way repeated measures ANOVA were performed separately after vehicle or DMPPO pretreatment to compare cyclic GMP levels at 5 and 60 min of perfusion in normoxic and chronically hypoxic groups.

We also compared cyclic GMP levels at 60 min of perfusion in normoxic and chronically hypoxic groups after various pretreatments with two-way ANOVA.

To compare in normoxic or hypoxic group, the effect of DMPPO versus its vehicle on vasodilator responses, two-way analysis of variance (ANOVA) with repeated measurements were performed. When interaction was significant, Mann–Whitney non parametric test was used to compare DMPPO and vehicle at each dose of the vasodilator.

To evaluate the acute hemodynamic effect of DMPPO administration, we used one-way repeated measures analysis of variance (ANOVA) followed by Dunnett's test.

Non parametric Mann–Whitney test was performed for comparisons of hemodynamic values between vehicle and DMPPO-chronically treated rats. Comparisons of ratio of right ventricle to left ventricle + septum weight and hematocrit between these two groups were performed with a similar statistical analysis after arcsine transformation of individual values (Zar, 1974).

To compare the effects of vehicle and DMPPO infusion on degree of muscularization of pulmonary vessels, vessels were ordinally classified as non muscular, partially muscular and muscular. Comparison of muscularization was performed separately at the alveolar duct and wall levels by a non parametric Mann–Whitney test.

Results

Experiments in isolated rat lungs

Effect of DMPPO on cyclic GMP levels in perfusate of isolated lungs Concentrations of cyclic GMP in the perfusate 5 min after starting recirculation of the perfusate were similar in lungs from normoxic and chronically hypoxic rats and remained stable with time after pretreatment with vehicle (Figure 1). After pretreatment with DMPPO (1 μM) alone, cyclic GMP levels were higher at 60 than at 5 min ($P<0.001$), levels at 60 min being higher in chronically hypoxic than in normoxic rats (1821 ± 83 *vs* 1281 ± 127 pg ml^{-1}, respectively, $P<0.05$).

In lungs from normoxic and chronically hypoxic rats, L-NAME, did not abrogate the increase in perfusate cyclic GMP levels which was observed at 60 min after pretreatment with DMPPO (1 μM).

The increase in cyclic GMP levels observed at 60 min in the perfusate from normoxic rat lungs after pretreatment with DMPPO was not altered by thiorphan (10 μM). However, there was a trend for thiorphan to potentiate the increase in cyclic GMP levels caused by pretreatment with DMPPO in lungs from chronically hypoxic rats but due to a small number of experiments, there was no statistically significant difference between values obtained after pretreatment with DMPPO alone and DMPPO associated with thiorphan.

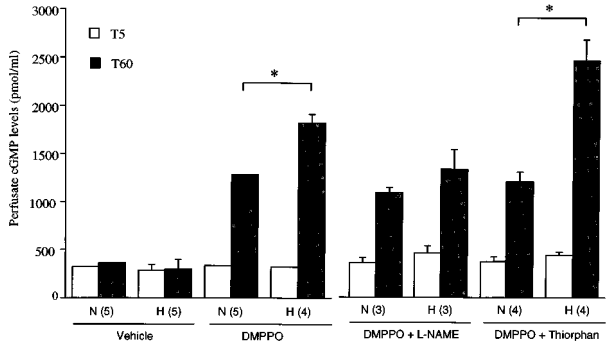

Figure 1 Levels of cyclic GMP in the perfusate from normoxic (N) and chronically hypoxic (H) rat lungs. Perfusate was sampled 5 min and 60 min after pretreatment with DMPPO (1 μM) alone, DMPPO associated with L-NAME (100 μM), DMPPO associated with thiorphan (10 μM) or vehicle alone. In the presence of DMPPO, cyclic GMP levels differed with time ($P<0.001$) and between groups (normoxic *vs* hypoxic, $P<0.05$) with a significant interaction between time and groups ($P<0.05$). Values are means$\pm$s.e.mean. Number between parentheses indicate number of experiments. *$P<0.05$.

Effect of DMPPO on vasodilator responses to ANP, SNP and isoproterenol in isolated rat lungs In lungs from normoxic rats, mean baseline Pap was 7.5 ± 0.8 mmHg. Administration of ANP, SNP or isoproterenol 15 min after starting U-46619 infusion caused a dose-dependent decrease in Pap (Figure 2). Pretreatment of the lungs with DMPPO did not alter baseline Pap and pressor response to U-46619 but potentiated the vasodilator responses to ANP and SNP. In contrast, isoproterenol-induced pulmonary vasodilation was unaffected by pretreatment with DMPPO.

In lungs from chronically hypoxic rats, baseline Pap was 12.2 ± 0.5 mmHg (significantly higher than in normoxic rats, $P<0.01$). Administration of ANP or SNP in the perfusate 15 min after starting U-46619 infusion induced a dose-dependent decrease in Pap which was similar to the vasodilator response obtained in normoxic rat lungs. In these lungs, pretreatment with DMPPO (1 μM), did not alter baseline Pap and vasoconstrictor response to U-46619 but potentiated the dilator responses to both ANP and SNP (Figure 3).

Acute hemodynamic response to i.v. administration of DMPPO in rats previously exposed to chronic hypoxia and studied in an hypoxic environment

In conscious rats previously exposed to chronic hypoxia, basal Pap while breathing room air was 29.7 ± 0.6 mmHg. This value

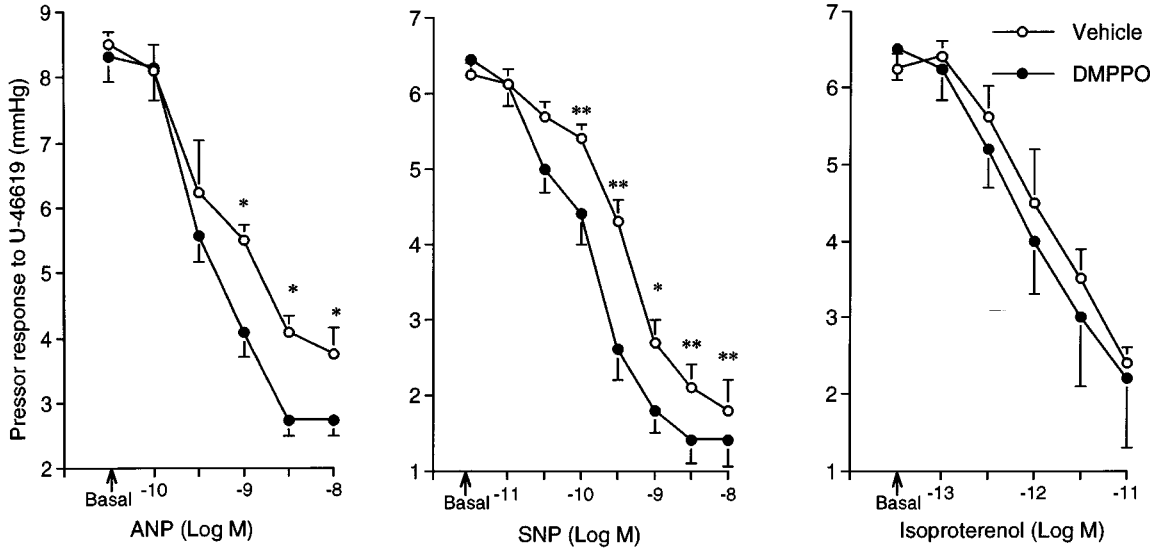

Figure 2 Vasodilator responses to atrial natriuretic peptide (ANP), sodium nitroprusside (SNP) and isoproterenol in lungs from normoxic rats in the presence of DMPPO (1 μM). The inhibitor or its vehicle was added in the perfusate reservoir 10 min before starting infusion of U-46619. Basal indicates perfusion pressure during U-46619 infusion immediately before administration of the vasodilators. Values are means$\pm$s.e.mean of $n=5$. *$P<0.05$ and **$P<0.01$ as compared with corresponding dose of vasodilator after pretreatment with DMPPO.

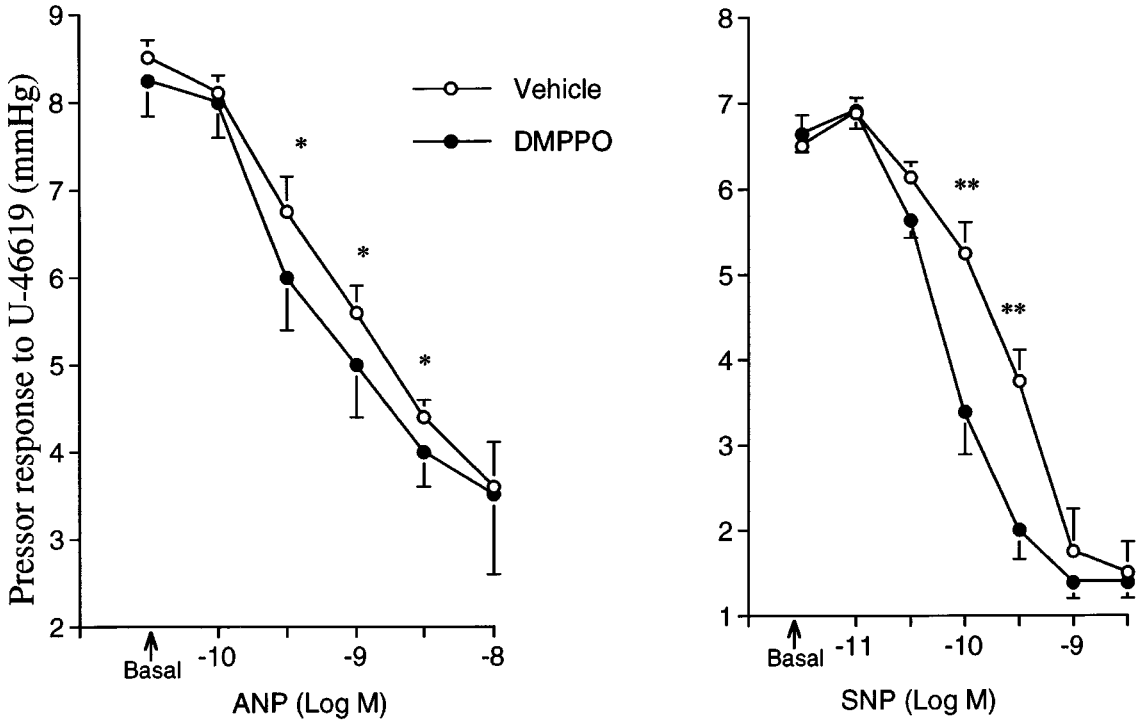

Figure 3 Vasodilator responses to ANP and SNP in lungs from chronically hypoxic rats in the presence of DMPPO (1 μM). The inhibitor or its vehicle was added in the perfusate reservoir 10 min before starting infusion of U-46619. Basal indicates perfusion pressure during U-46619 infusion immediately before administration of the vasodilators. Values are means$\pm$s.e.mean of $n=5$. *$P<0.05$ and **$P<0.01$ as compared with corresponding dose of vasodilator after pretreatment with DMPPO.

is significantly higher than Pap during similar conditions in control normoxic rats (18.3$\pm$0.5 mmHg). Reexposure to hypoxia was associated with an increase in Pap to 42.3$\pm$1 mmHg. In these conditions, administration of DMPPO at increasing doses (0.01, 0.05 and 0.1 mg kg^{-1} bolus i.v.) induced a dose-dependent decrease in Pap, with no change in Sap, CO (Figure 4). There was also no significant change of heart rate during DMPPO administration, heart rate being 310$\pm$30 b.p.m in baseline condition and 330$\pm$20 b.p.m. after the largest dose.

Effect of continuous treatment with DMPPO during exposure to chronic hypoxia

Body weight after 15 days of exposure to hypoxia was similar in DMPPO and vehicle-treated rats (358$\pm$9 *vs* 353$\pm$18 g, respectively). Hematocrit was also similar after DMPPO chronic infusion and vehicle treatment (46.1$\pm$4.6 *vs* 42.5$\pm$5.2%, respectively). Systemic artery pressure measured at the end of the hypoxic period in anaesthetized rats spontaneously breathing room air did not differ between DMPPO and vehicle-treated rats (90$\pm$13 *vs* 97$\pm$6 mmHg). Although the ratio of right ventricle to left ventricle plus septum weight tends to be lower in DMPPO than in vehicle-treated rats (42.0$\pm$5 *vs* 47.7$\pm$7%), the difference did not reach statistical significance (Figure 5). However, the Pap was lower (22.2$\pm$2 *vs* 28.6$\pm$1.6 mmHg, $P<0.05$) and the degree of muscularization of distal pulmonary arteries less severe at both the alveolar duct ($P<0.05$) and alveolar wall ($P<0.01$) levels in the DMPPO than in the vehicle-treated group (Figure 6).

Discussion

The present results show that inhibition of cyclic 3′–5′-guanosine monophosphate-specific phosphodiesterase with DMPPO induces selective vasodilation of the pulmonary vascular bed in chronically hypoxic rats. Chronic administration of DMPPO also protects against development of pulmonary vascular remodelling in chronically hypoxic rats. This suggests that cyclic GMP plays an important role in modulating vasoconstrictor and vascular remodelling processes during exposure to hypoxia.

DMPPO is a novel potent and highly selective PDE-5 inhibitor. This compound acts in a reversible and competitive manner towards cyclic GMP with an apparent K_i of 3 nM (Coste & Grondin, 1995). In previous studies, DMPPO has been shown to selectively inhibit PDE-5 activity and to increase SNP and ANP-stimulated cyclic GMP levels in cultured vascular smooth muscle cells. In addition, DMPPO potentiates the relaxant effects of exogenously added activators of guanylyl cyclase, ANP and SNP, in rat aortic rings (Deply *et al*., 1996; Deply & Le Monnier de Gouville, 1996). Moreover, DMPPO has been shown to relax phenylephrine-precontracted rat aortic rings when a functional endothelium is present but fails to relax preparations devoid of endothelium. This suggests that DMPPO potentiates the relaxant effects of cyclic GMP generated in smooth muscle cells by the constitutively released endothelium-derived NO.

In our studies performed in isolated rat lungs during conditions of increased tone by U-46619 infusion, DMPPO potentiated the vasodilation induced by ANP and SNP, two agents acting through cyclic GMP formation. In contrast, it did not affect isoproterenol-induced vasodilation, an effect

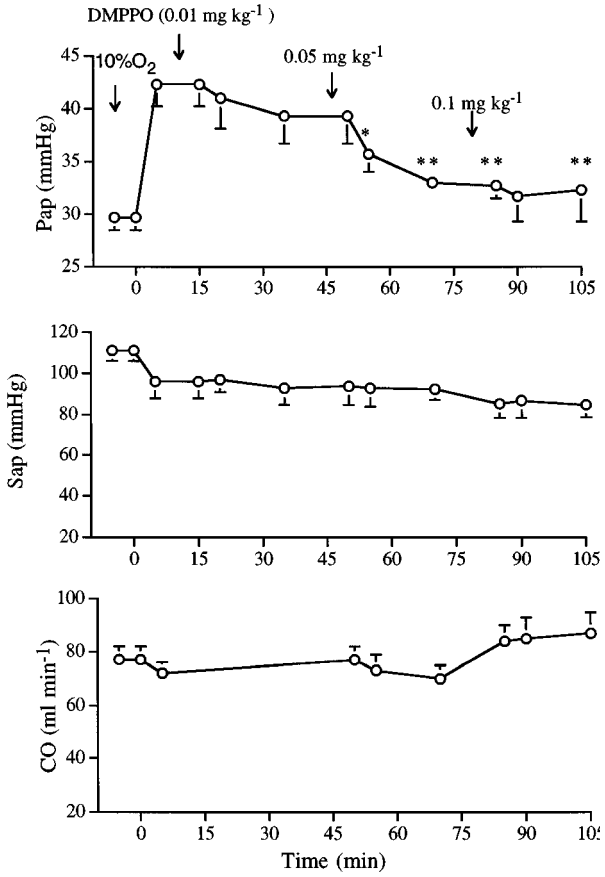

Figure 4 Pulmonary artery (Pap), systemic artery (Sap) pressure and cardiac output (CO) in response to DMPPO (0.01, 0.05 and 0.1 mg kg^{-1}, i.v. bolus as 30 min intervals) in conscious chronically hypoxic rats. Baseline pressure was first measured in normoxia, then DMPPO was administered during exposure to continuous hypoxic environment (10% O_2). Measurements were performed at 5, 15 and 30 min after each bolus injection ($n=5$). *$P<0.05$ and **$P<0.01$ as compared with baseline value during hypoxia.

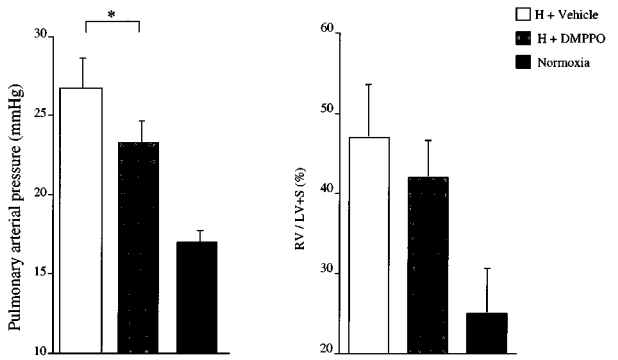

Figure 5 Pulmonary artery pressure (left panel) and ratio of right ventricle/left ventricle + septum weight (RV/LV + S) (right panel) in rats exposed to hypoxia (10% O_2) for 15 days and treated with DMPPO (H + DMPPO, $n=8$) or vehicle (H + vehicle, $n=8$). Values in untreated rats exposed to normoxia are also indicated ($n=10$)). Results are expressed as means ± s.e.mean, *$P<0.05$.

which has been shown to be mediated by stimulation of adenylyl cyclase. Contrasting with previous lung studies using another inhibitor of PDE-5, E4021, and showing pulmonary vasodilation as well as inhibition of hypoxic pulmonary vasoconstriction (Cohen *et al.*, 1996), we found that DMPPO did not alter the baseline tone of our lung preparations from either normoxic or hypoxic rats (results not shown). This difference may be related to differences in lung preparations since, in our hands, neither SNP nor ANP did affect baseline tone, a finding consistent with previous observations that there is no basal tone in isolated lungs studied during normoxic conditions (Adnot *et al.*, 1991).

In the presence of DMPPO and without any other pharmacological stimulation, cyclic GMP concentration in the lung perfusate increased during the 60 min time period of the study whereas in the absence of DMPPO, it remained steady with time and similar in the perfusate from normoxic and chronically hypoxic rat lungs. Presence of cyclic GMP in the lung perfusate is a consequence of its export across cellular membranes. We are not aware of any effect of DMPPO on cyclic GMP egression. Although levels in the perfusate is only an indirect reflect of cyclic GMP levels in smooth muscle cells, the fact that cyclic GMP levels increased in the lung perfusate only when the degradation was abolished is consistent with an equilibrium between production and degradation of cyclic GMP levels in isolated lungs from both normoxic and chronically hypoxic rats during baseline conditions. These results appear in discrepancy with those of Cohen *et al.*, 1996). They found a marked time-dependent accumulation of cyclic GMP in the perfusate from chronically hypoxic rats which was further increased in the presence of the PDE-5 inhibitor, E4021 (Cohen *et al.*, 1996). However their protocol differs from ours. It is possible that in their experiments, the successive hypoxic challenges which preceded sampling of perfusate caused enhancement of cyclic GMP formation. Nevertheless, in the present study, pretreatment with DMPPO caused a greater cyclic GMP accumulation in the perfusate from chronically hypoxic than in normoxic rat lungs. This suggests that an increased production of cyclic GMP is balanced by its increased degradation through PDE in the hypertensive lungs. Supporting this idea, development of pulmonary hypertension in the chronic hypoxic rat has been shown to be associated with a decrease in cyclic nucleotide levels (MacLean *et al.*, 1996) and an increase in the activity of PDE 1, 3 and 5 in the pulmonary arteries (MacLean *et al.*, 1997).

At this point, we can only speculate about the mechanisms leading to formation of cyclic GMP by the lungs in these *ex-vivo* experiments. Among endogenous substances, NO is a potent stimulus of soluble guanylyl cyclase (Gary & Hassid, 1989; Murad, 1986). Although controversial, we have previously found that endothelium-dependent NO release is abolished in the pulmonary vasculature of chronically hypoxic (Adnot *et al.*, 1991; Carville *et al.*, 1993). However, several laboratories, including ours, have also found some evidence for induction of NO synthase in lung tissue from rats exposed to chronic hypoxia (Carville *et al.*, 1997; Xue *et al.*, 1994). In the present study, cyclic GMP accumulation in the perfusate of lungs pretreated with DMPPO was not inhibited by L-NAME, an inhibitor of NO synthesis. These results are in accordance with the previous report of Muramatsu *et al.*, (1997). These authors also found that pretreatment with the L-arginine analogue, nitro-L-arginine, did not alter cyclic GMP accumulation in the perfusate of isolated lungs from chronically hypoxic rats. It is therefore likely that cyclic GMP production does not result from NO-mediated stimulation of soluble guanylyl cyclase. Other candidates for cyclic GMP production are natriuretic peptides. In these *in vitro* experiments,

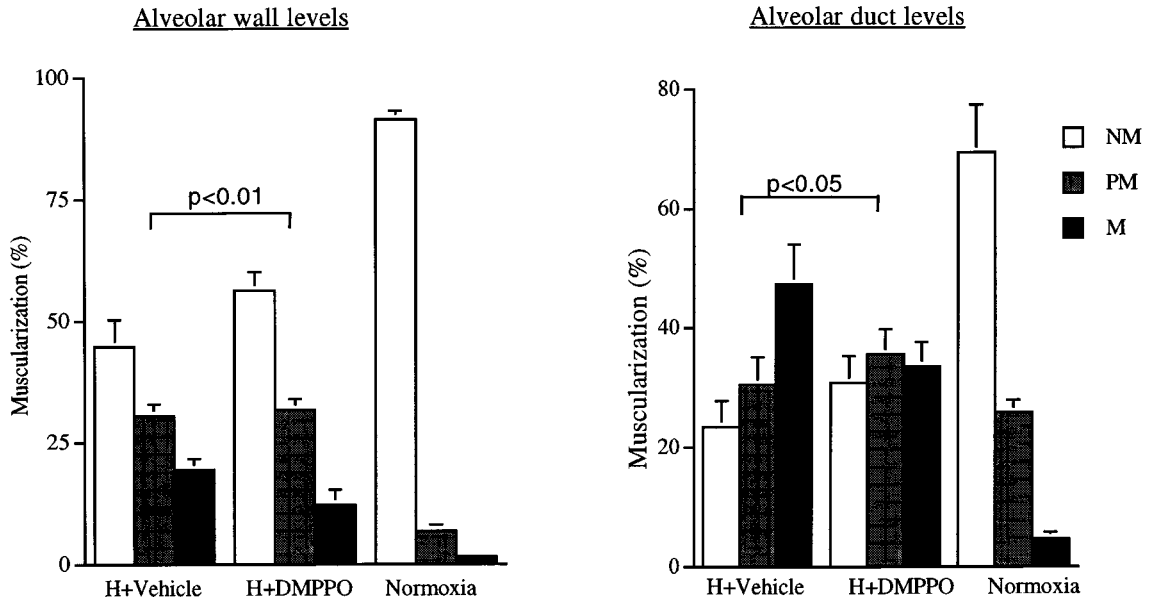

Figure 6 Bar graphs showing the percentage of muscularized (M), partially muscularized (PM), and non muscularized (NM) arteries at the alveolar duct and alveolar wall levels in rats exposed to hypoxia (10% O_2) for 15 days and treated with DMPPO (H + DMPPO, $n=9$) or vehicle (H + vehicle, $n=9$). Values in untreated rats exposed to normoxia are also indicated ($n=10$). Results are expressed as means ± s.e.mean. Degree of muscularization of distal pulmonary arteries was less severe at both the alveolar duct ($P<0.05$) and alveolar wall ($P<0.01$) levels in the DMPPO than in the vehicle-treated group.

endogenous ANP is unlikely to play a significant role since ANP secreted from the right ventricle was excluded from the circuit of our lung preparation and because only low levels of ANP are synthetized by the pulmonary vascular cells. Another member of the natriuretic peptides family, C-type natriuretic peptide, is a vasodilator that is synthetized by vascular endothelial cells and may also play a role in modulating pulmonary vascular tone through cyclic GMP formation (Furuya *et al*., 1990; Suga *et al*., 1992). Thiorphan has been shown to inhibit neutral endopeptidase, an endogenous substance involved in the degradation of natriuretic peptides (Lafferty *et al*., 1989). The fact that cyclic GMP accumulation in the perfusate from chronically hypoxic rats tends to be potentiated in the presence of thiorphan suggests that during chronic hypoxia, endogenous natriuretic peptides such as C-type natriuretic peptide may exert a compensatory role by stimulating guanylyl cyclase activity. However, since thiorphan may inhibit degradation of other non related peptides, we cannot rule out stimulation of guanylyl cyclase by another non-identified mediator.

Bolus i.v. administration of the cyclic GMP-PDE inhibitor, induced dose-dependent and selective pulmonary vasodilation in conscious rats previously exposed to 2 weeks of hypoxia and studied in an hypoxic environment. This potent vasodilator effect occurred in response to doses varying from 0.01 to 0.1 mg kg^{-1}. Pulmonary vasodilation developed within 5 min and lasted 30 to 60 min. In contrast, we observed no significant change in Sap, CO and heart rate. These results are in accordance with previous studies examining the acute effects of cyclic GMP-PDE inhibitors on pulmonary and systemic hemodynamics in rats. In chronically hypoxic rats, the selective cyclic GMP-PDE inhibitor, E4021, was shown to decrease Pap with no significant effect on CO, systemic pressure and resistance. Comparison of E4021 to inhaled nitric oxide demonstrated that cyclic GMP-PDE inhibition with this compound was as selective and effective as inhaled NO. Similar results can be drawn with DMPPO since we previously found that acute inhalation of NO (40 ppM) reduces Pap by 10–15 mmHg in chronically hypoxic rats, a decrease similar to that induced by DMPPO in the present study. The mechanism by which DMPPO selectively induced pulmonary vasodilation in conscious instrumented rats can only be suspected from the present studies. As discussed above, the role of endogenous NO in dilating pulmonary vessels during chronic hypoxia is still debated. *In vivo*, DMPPO may potentiate the vasodilatory action of endogenous ANP. Indeed, increased circulating levels of ANP have been documented in animals exposed to chronic hypoxia (Winter *et al*., 1989) and in a previous study, we found that blockade of endogenous ANP with monoclonal antibody causes a selective increase in pulmonary vascular resistance in conscious chronically hypoxic rats (Raffestin *et al*., 1992). In our study, no effects on Sap and CO were seen with the doses of DMPPO we used (0.01–0.1 mg kg^{-1}). Decrease of Sap have been observed in anaesthetized rats but with doses 20 fold more important (2 mg kg^{-1}). These results provide evidence for a selective effect of DMPPO on the pulmonary circulation.

The development of hypoxic pulmonary hypertension is associated with hypertrophy and hyperplasia of smooth muscle cells in normally muscularized arteries and the appearance of new smooth muscle cells in non muscular and partially muscularized segments of the intraacinar circulation. Concomitant with the decrease in Pap, muscularization of distal pulmonary arteries at alveolar duct and wall levels although still significant in comparison with the normoxic group, was less severe in chronically hypoxic rats subjected to treatment with DMPPO. Chronic treatment with DMPPO, therefore partially prevented pulmonary vascular remodelling caused by hypoxia. It is tempting to speculate that these effects were

related to the antiproliferative and antihypertrophic properties of cyclic GMP. Indeed, 8-bromo-cyclic GMP has been shown to inhibit DNA synthesis and proliferation of rat aortic muscle cells in culture (Gary & Hassid, 1989). The ability of NO-generating vasodilators and ANP to exert antimitogenic effects, also favours the idea that this effect, similar to the relaxant action of NO and ANP, is mediated by cyclic GMP as the second messenger. Therefore, it is possible that, in chronically hypoxic rats, DMPPO attenuated the muscularization of pulmonary arteries by a direct inhibitory influence on smooth muscle growth. Another mechanism may be related to the relaxant properties of DMPPO. Indeed, in both the pulmonary and systemic circulations, proliferation of smooth muscle may be viewed as an adaptative process in response to increased arterial wall stress. Since acute administration of DMPPO reduced the vasoconstrictor component of hypoxic pulmonary hypertension and since pressure has a direct role in the remodeling of the pulmonary arterial wall during chronic hypoxia, DMPPO could have attenuated the extension of muscularization to the distal arteries by reducing the pressure.

Inhaled NO is a potent and selective pulmonary vasodilator (Kouyoumdjian *et al*., 1994) but its short duration of action is a disadvantage in patients with chronic pulmonary hypertension who may require continuous vasodilator therapy. The ability of the inhibitor of PDE-5, DMPPO, to induce selective pulmonary vasodilation and to attenuate pulmonary vascular remodelling confers to this molecule potential for treating pulmonary hypertension. Moreover, the ability of DMPPO to potentiate the vasorelaxant effect of agents stimulating guanylyl cyclase may be clinically relevant in patients with chronic pulmonary hypertension and high circulating levels of ANP. During inhalation of NO, PDE-5 inhibitors such as DMPPO may also allow to reduce concentrations of NO and therefore minimize the risk of NO toxicity. Further experiments are needed to examine if DMPPO is as efficient orally as intravenously.

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