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Lack of synergistic effect of molsidomine and sildenafil on development of pulmonary hypertension in chronic hypoxic rats

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Abstract

The present study addressed whether combined treatment with a phosphodiesterase type 5 inhibitor, sildenafil, and a nitric oxide donor, molsidomine, prevents development of pulmonary hypertension in chronic hypoxic rats. Two weeks of hypoxia increased right ventricular systolic pressure, and right ventricular and lung weight. Treatment with either sildenafil (10 mg/kg/day) or molsidomine (15 mg/kg/day) in drinking water reduced right ventricular systolic pressure and weight, while lung weight was unchanged. Combining sildenafil and molsidomine did not have additional effects compared to molsidomine alone. The number of muscularized pulmonary arteries with diameters below 50 μm was increased in vehicle and sildenafil-treated, but not in molsidomine-treated hypoxic rats. Acetylcholine relaxation was blunted in arteries from vehicle and molsidomine-treated, but not in sildenafil-treated rats. In conclusion, both sildenafil and molsidomine blunts pulmonary hypertension and right ventricular hypertrophy in chronic hypoxic rats, but no synergistic effects were observed.

Keywords: Molsidomine; Sildenafil; Pulmonary hypertension; Acetylcholine; Atrial natriuretic peptide; (Chronic hypoxic rats)

1. Introduction

Pulmonary arterial hypertension is a severe disease characterised by vasoconstriction, intimal lesions, medial hypertrophy, and adventitial thickening of precapillary pulmonary arteries. The progressive pulmonary hypertension leads to increased afterload, right ventricular hypertrophy, right heart failure, and death. In primary pulmonary arterial hypertension the median survival is considered to be 2.8 years from the time of diagnosis. Therefore, a novel therapeutic strategy for pulmonary hypertension is desirable.

Short-term nitric oxide (NO) inhalation reduces pulmonary arterial pressure in various forms of pulmonary hypertension (Katayama et al., 1997; Roberts et al., 1997), whereas prolonged inhalation of NO attenuates pulmonary vascular remodelling induced by chronic hypoxia in rats (Roberts et al., 1995, 2000). However, the use of inhaled NO is hampered by the need for complicated delivery systems and possible toxic side effects (Weinberger et al., 2001), and NO donors could provide a means of improving NO treatment. Molsidomine, an orally active NO prodrug, reduced pulmonary pressure in patients with chronic obstructive pulmonary disease (Lampert et al., 1991), and improved hemodynamic parameters in 3 patients with pulmonary hypertension, secondary to Takayasu's arteritis (Lee et al., 2001). Molsidomine also lowered pulmonary arterial pressure in rats with pulmonary hypertension (Elmedal et al., 2004; Mathew et al., 1997), and reduced pulmonary vascular remodelling and endothelin-1 expression (Blumberg et al., 2001), but the effect was less pronounced after long-term treatment (Blumberg et al., 2001). In the liver, molsidomine is converted to the active metabolite, 3-morpholinosydnonimine (SIN-1), which releases NO (Megson, 2000), and an explanation for the lack of persistent effect of molsidomine could be due to alterations in the NO signal transduction pathways in pulmonary hypertension.

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NO decreases vascular resistance by stimulating the soluble guanylyl cyclase to produce cyclic guanosine monophosphate (cGMP) followed by relaxation of vascular smooth muscle cells, mainly through activation of cGMP-dependent protein kinase (Lucas et al., 2000). cGMP is primarily hydrolysed by phosphodiesterase type 5 (PDE5), which is highly expressed in lung tissue (Giordano et al., 2001; Hanson et al., 1998). Short-term studies in patients with pulmonary hypertension suggest that an inhibitor of PDE5, sildenafil, is an effective pulmonary vasodilator (Ghofrani et al., 2002b; Lepore et al., 2002; Michelakis et al., 2002), and it also improves exercise tolerance and cardiac index in these patients (Sastry et al., 2004). Recent studies show that sildenafil also reduces right ventricular hypertrophy in chronic hypoxic mice (Zhao et al., 2001, 2003), pulmonary arterial pressure and RhoA downregulation in chronic hypoxic rats (Sauzeau et al., 2003; Sebkhi et al., 2003), and improves survival rate in rats with pulmonary hypertension induced by monocrotaline injection (Itoh et al., 2004; Schermuly et al., 2004). Treatment with sildenafil only reduces pulmonary arterial pressure in patients with pulmonary hypertension by 15–20% (Ghofrani et al., 2002a), but vasodilator studies suggest that sildenafil enhances the lowering of pulmonary vascular resistance index evoked by inhaled prostacyclin analogues (Ghofrani et al., 2003), and the combination of oral sildenafil and the prostacyclin analogue, beraprost, was shown to improve pulmonary hypertension in rats (Itoh et al., 2004). Sildenafil also prolongs the acute lowering of pulmonary pressure by inhaled NO in patients with pulmonary hypertension (Lepore et al., 2002). Considering PDE5 is upregulated in pulmonary hypertension (MacLean et al., 1997; Murray et al., 2002; Sebkhi et al., 2003), combining sildenafil with an oral NO donor such as molsidomine would enhance the efficacy of the latter on pulmonary hypertension.

Thus, the purpose of this study was to investigate whether the combination of oral sildenafil and molsidomine ameliorates chronic hypoxia-induced pulmonary hypertension compared with each drug alone.

2. Methods and materials

2.1. Animal model

Ten weeks old male Wistar rats were kept for 2 weeks in a hypobaric chamber with a pressure of 550 mbar, thus halving the oxygen partial pressure (see Elmedal et al., 2004). Five groups were examined; (1) a normoxic control group, (2) a hypoxic control group, (3) a hypoxic group treated with molsidomine 15 mg/kg/day, (4) a hypoxic group treated with sildenafil 10 mg/kg/day, and (5) a hypoxic group treated with sildenafil and molsidomine.

The drugs were administered in the drinking water and initiated from the day the animals were placed in the chamber. The chamber was opened two or three times each week for renewal of water and cages, and the amount of consumed drinking water was measured by weighing the container. The rats had free access to chow.

All experiments conducted on animals in this study were performed with approval from the Danish Institutional Animal Care and Use Committee.

2.2. Hemodynamic measurements

Before and after the rats were placed in the chamber, they were weighed and blood pressures were measured by the tail-cuff method as previously described (Elmedal et al., 2004). Six measurements of blood pressure and corresponding pulse were obtained for each rat, and the average calculated.

On the day of experiment, the rat was anaesthetized with midazolam (4.1 mg/kg), fentanyl (0.26 mg/kg) and fluanisone (8.25 mg/kg), and if necessary the anaesthesia was supplemented with fentanyl (0.1 mg/kg) and fluanisone (3.3 mg/kg). Right ventricular systolic pressure was measured by catheterization of the right jugular vein as previously described (Elmedal et al., 2004).

2.3. Assessment of right ventricular hypertrophy

The rat was sacrificed by cervical dislocation, and the lung and heart were removed and kept in physiological salt solution (see composition below). The lungs, liver and kidneys were weighed and expressed as percent of body weight. The atria of the heart were removed and the right ventricle separated from the left ventricle and septum. Each part was weighed and the ratio of right ventricle to left heart ventricle and septum calculated.

2.4. Morphometric measurements

The lower segment of the left lung was isolated; a catheter inserted into the pulmonary artery and approximately 0.1 ml of heparin 100 IE/ml was injected. The lung segment was perfused with PSS containing albumin (2%) followed by PSS with papaverine (10^{-4} M) and finally with formalin 4%. A perfusion pressure close to mean pulmonary arterial pressure was aimed for each preparation and averaged 12–14±1–2 mm Hg in the different groups. Sections of the lung were stored in formalin buffer 4% or ethanol (70%) and embedded in paraffin or plastic, respectively. Paraffin embedded sections were stained with anti-α-smooth muscle actin (1:25, Dako, Denmark), and plastic embedded sections were stained with Giemsa. In paraffin embedded sections of rat lung stained for α-smooth muscle actin, arteries and veins were identified by media thickness compared to lumen by a blinded observer, and then number of arteries with lumen diameter below and above 50 μm, respectively, was counted and expressed as percentage of the number of alveoli. Finally, media area (delineated by the internal

and external elastic lamina) in arteries with diameter above 50 μm was measured in lung sections stained with Giemsa.

2.5. Dissection and mounting of arteries

Pulmonary arteries, which were third order branches of the main pulmonary artery, were dissected and kept in physiological salt solution. They were mounted on 40 μm thick wires in microvascular myographs for measurements of isometric tension, and stepwise stretched to a transmural pressure of 3.9 kPa as previously described (Elmedal et al., 2004). The bath was heated to 37 °C and bubbled with 5% CO_2 in air (20% O_2 and 75% N_2).

2.6. Functional measurements

The contractile function was tested using physiological salt solution with potassium (KPSS) (123.7 mM) and phenylephrine (10^{-5} M). If the arteries did not respond with a contraction corresponding to an increase in transmural pressure of 2.7 kPa or more, they were excluded (Elmedal et al., 2004).

In arteries contracted with U46619, concentration–response curves were performed for atrial natriuretic peptide (ANP, 10^{-10}–10^{-7} M) in the presence and absence of a blocker of the natriuretic peptide receptor subtypes A and B, HS-142 (10^{-7} M). Then curves were constructed for SIN-1 (10^{-8}–3×10^{-4} M) and acetylcholine (10^{-8}–10^{-5} M) in randomized order either in the absence or presence of 1*H*-[1.2.4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, 3×10^{-6} M), which was present for 30 min before construction of the concentration–response curve.

To study acute hypoxic contraction, the arteries were incubated with propranolol (10^{-6} M) and contracted with phenylephrine (10^{-5} M). Hypoxia was then induced for 1 h by changing the air to 5% CO_2 in nitrogen.

To study the vasorelaxant effect of sildenafil in arteries from normoxic animals, vessels were contracted with the thromboxane-A_2 analogue, U46619 (3×10^{-8} M), and when the contraction was stable, increasing concentrations of sildenafil (10^{-10}–10^{-6} M) were added. In a number of arteries, the endothelial cell layer was removed by gently rubbing a human hair against the inside of the artery wall. Abolished acetylcholine relaxation was taken for successful removal of the endothelium. Some of the arteries with endothelium were incubated with an inhibitor of the soluble guanylyl cyclase, ODQ (3×10^{-6} M) before performing concentration–response curves for sildenafil, SIN-1, acetylcholine, and for an activator of adenylyl cyclase, forskolin (10^{-10}–10^{-5} M).

2.7. Drugs and solutions

The pulmonary arteries were dissected, mounted, and held relaxed in physiological salt solution of the following composition (mmol/l): NaCl 119, KCl 4.7, $MgSO_4\cdot7H_2O$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, glucose 5.5, $CaCl_2$ 1.6, and ethylenediaminetetraacetic acid (EDTA) 0.026. Potassium rich physiological salt solution was physiological salt solution in which NaCl was exchanged for KCl on equimolar basis.

The following drugs were used: acetylcholine hydrochloride, bovine albumin, phenylephrine hydrochloride, 9.11-dideoxy-11α9α-epoxymethanoprostaglandin F_{2a} (U46619), forskolin, papaverine, and propranolol from Sigma Aldrich (U.S.A.), molsidomine and 3-mopholino-sydnonimine hydrochloride from Alexis Biochemicals (San Diego, CA, U.S.A.), 1*H*-[1.2.4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) from Tocris Cookson (UK), rat atrial natriuretic peptide (ANP 1–28) and formaldehyde buffer 4% from Calbiochem Biochemicals (Bie and Berntsen, Denmark), fentanyl and fluanisone were from Jansen-Cilag (Denmark), HS-142 citrate was a gift from Kyowa Ltd. (Japan), sildenafil was a gift from Pfizer (UK), midazolam from Hoffmann-La Roche (Switzerland), and heparin from Leo Pharma (Denmark). Stock solutions of U46619 were prepared in 96% ethanol, ODQ, glibenclamide, and forskolin in DMSO, ANP in 5% acetic acid, and papaverine and albumin in PSS. Further dilutions were made in distilled water. Solvents in the concentrations applied did not affect the preparations. When sildenafil was administered in the drinking water pH was adjusted to 5.5 with 1 M NaOH.

2.8. Data analysis

The mechanical responses of arteries in myographs were measured as changes in force and expressed as wall tension, ΔT, which is the increase in measured force, ΔF, divided by twice the segment length (Mulvany and Halpern, 1976). Relaxations are expressed as percentage of U46619 contraction just before construction of the concentration–relaxation curves. By using a computer program (Graph Pad Prism, San Diego, CA, U.S.A.), the concentration–response curves were fitted to the classical Hill equation, as earlier described (Simonsen et al., 1997). Sensitivity to the agonists is expressed in terms of $pD_2=-\log(EC_{50})$, EC_{50} being the concentration (M) of agonist required to give half-maximal relaxation.

Area under the concentration–response curves was calculated by using a computer program (Graph Pad Prism) and compared by one-way analysis of variance (ANOVA) followed by Bonferroni complimentary analysis. To evaluate response to acute hypoxia, the area under the curve for the first 15 min and the following 45 min were calculated in Graph Pad Prism to represent the transient and sustained phase of the hypoxic response, respectively. Results are expressed as mean±S.E.M. Probability levels below 5% were considered statistically significant.

3. Results

3.1. Animal data

The initial body weight of rats was similar in all groups, but only the normoxic rats gained weight during the 2-week experimental period (Table 1). The lung weight was increased in all four hypoxic groups, and treatment with sildenafil and molsidomine did not prevent this increase (Table 1). Liver or kidney to body weight ratio was not changed by 2 weeks of hypoxia or treatment with sildenafil and molsidomine (Table 1).

3.2. Right ventricular systolic pressure and heart weight

In the chronic hypoxic rat right ventricular systolic pressure was significantly increased compared to the normoxic control group. Treatment with sildenafil, molsidomine, and the combination of molsidomine and sildenafil markedly prevented the increase in right ventricular systolic pressure (Fig. 1A). The heart rate was similar in animals exposed to normoxia and hypoxia (Table 1).

Heart weight expressed as percentage of body weight was increased in all the hypoxic groups (results not shown). Right ventricular hypertrophy was associated with chronic hypoxia as indicated by the significantly increased right ventricular to left ventricular plus septum ratio. This hypertrophy was significantly reduced by sildenafil and molsidomine treatment (Fig. 1B). The combined treatment with sildenafil and molsidomine had no additional effect on right ventricular hypertrophy (Fig. 1B). Neither hypoxia, nor any treatment had a significant effect on the systemic blood pressure (Fig. 1C).

3.3. Morphometric measurements

Smooth muscle cells were identified by positive immunoreaction for smooth muscle actin (Fig. 2A). Based on smooth muscle actin staining, the number of arteries less than 50 μm in diameter expressed as percentage of the number of alveoles was significantly increased (Fig. 2B). This increase was prevented by molsidomine, but not affected by sildenafil (Fig. 2B). The combination of sildenafil and molsidomine did not have additional effect compared to treatment with only molsidomine (Fig. 2B). In arteries with a diameter greater than 50 μm, there was no

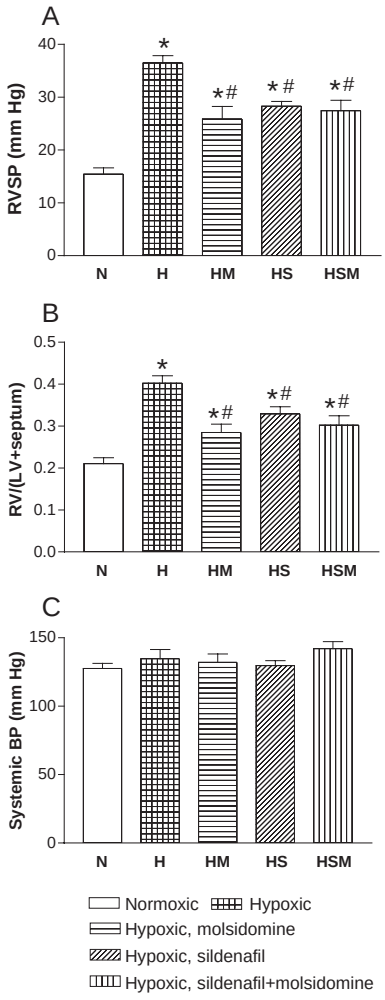

Fig. 1. Long-term in vivo treatment with sildenafil and molsidomine blunts right ventricular systolic blood pressure (RVSP) (A) and right ventricular hypertrophy (RV/(LV+septum)) (B) without any effect on systemic blood pressure (C) in chronic hypoxic rats. Measurements were performed in normoxic (N), vehicle-treated hypoxic (H), molsidomine (HM), sildenafil (HS) and molsidomine plus sildenafil (HMS) treated hypoxic rats. Results are means±S.E.M. of 8–12 measurements. Differences were evaluated by one-way ANOVA followed by a posteriori Bonferroni test in case of significance: *P<0.05 vs. N; #P<0.05 vs. H.

Table 1
Parameters describing the systemic and pulmonary impact of hypoxia (H), treatment of hypoxic rats with molsidomine (HM), sildenafil (HS), and the combination of molsidomine and sildenafil (HMS) compared to a normoxic control group (N)

	N	H	HM	HS	HMS
Body weight (g)	338±8	300±6[a]	295±5[a]	296±8[a]	280±7[a]
Heart rate (pr. min)	375±14	420±10	425±14	402±12	394±8
Lung weight (% of body weight)	0.52±0.03	0.75±0.02[a]	0.84±0.06[a]	0.78±0.03[a]	0.73±0.03[a]
Liver weight (% of body weight)	4.34±0.13	3.93±0.13	4.32±0.11	4.27±0.11	4.31±0.12
Kidney weight (% of body weight)	0.78±0.02	0.70±0.02	0.76±0.04	0.70±0.03	0.73±0.01

Values are mean±S.E.M. of 6–12 animals examined. Differences were evaluated by one-way analysis of variance (ANOVA) followed by a posteriori Bonferroni test in case of significance.

[a] P<0.05, parameter significantly different compared to N.

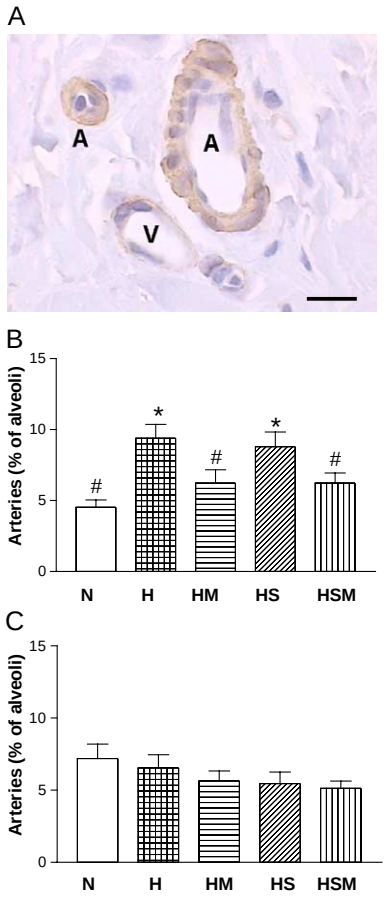

Fig. 2. (A) Section of rat lung stained for smooth muscle α-actin. Brown color indicate positive immunoreaction in artery (A) and vein (V). Bar corresponds to 10 μm. (B) Molsidomine abolished hypoxia-induced increases in number of smooth muscle α-actin immunoreactive small arteries, whereas sildenafil-treated animals had the same number of arteries with lumen diameter less than 50 μm. (C) Number of pulmonary arteries with diameters above 50 μm was unaltered in hypoxic animals. The number of arteries is expressed as percentage of alveoli in α-actin smooth muscle-stained lung sections from normoxic (N), vehicle-treated hypoxic (H), molsidomine (HM), sildenafil (HS) and molsidomine plus sildenafil (HMS) treated hypoxic rats. Results are means±S.E.M of sections from 4–8 animals. *P<0.05 vs. N; #P<0.05 vs. H.

difference in either number of arteries expressed as percentage of alveoli (Fig. 2C) or in media area (results not shown).

3.4. Effect of a guanylyl cyclase inhibitor, ODQ on vasorelaxation

In U46619-contracted pulmonary arteries with endothelium from normoxic rats, sildenafil evoked concentration-dependent relaxations with pD_2-values and maximal effect of, respectively, 7.74±0.25 and 40±7% (n=10) (Fig. 3). In arteries without endothelium, sildenafil evoked significantly less relaxation (Fig. 3). In arteries with endothelium, incubation with an inhibitor of soluble guanylyl cyclase, ODQ (3×10^{-6} M), abolished sildenafil relaxation (Fig. 3), and rightward shifted concentration–response curves for acetylcholine and SIN-1 (Fig. 4A–D). In contrast, relaxations induced by an activator of adenylyl cyclase, forskolin, were not changed in the presence of ODQ (n=3, results not shown).

3.5. Acetylcholine, SIN-1, and ANP relaxation in arteries from normoxic and hypoxic animals

The diameters of the pulmonary arterial segments and U46619-evoked contraction was not different in arteries from hypoxic animals treated either with vehicle, molsidomine, sildenafil, or the combination of molsidomine and sildenafil (Table 2).

Acetylcholine relaxation was reduced in arteries from vehicle-treated and molsidomine-treated rats, while acetylcholine relaxation in arteries from sildenafil-treated rats was not different from those in arteries from normoxic animals (Fig. 4A). In arteries from rats treated with the combination of sildenafil and molsidomine, only the sensitivity for acetylcholine was changed (Table 3). In the presence of ODQ, relaxation was less in arteries from the vehicle, sildenafil, and molsidomine-treated animals compared to arteries from normoxic rats (Fig. 4B).

In U46619-contracted arteries, concentration–response curves for SIN-1 were leftward shifted in arteries from animals treated with sildenafil compared to vehicle-treated hypoxic rats (Fig. 4C, Table 3). In the presence of ODQ, SIN-1 induced comparable relaxations in arteries from normoxic and vehicle as well as drug-treated hypoxic rats (Fig. 4D).

ANP induced potent relaxations in pulmonary arteries both from normoxic and hypoxic rats. The concentration–response curves for ANP were not different in arteries isolated from the different groups even in the presence of the ANP receptor antagonist, HS-142 (Fig. 4E–F, Table 3).

3.6. Effect of treatments on acute hypoxic contraction

In phenylephrine-contracted arteries, acute hypoxia induced a transient contraction with duration of approx-

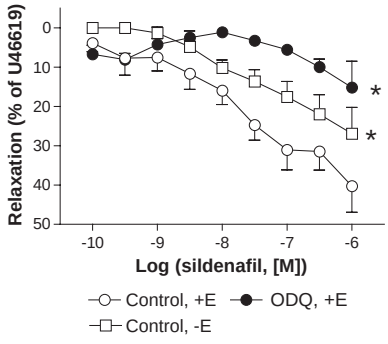

Fig. 3. Average concentration–response curves for sildenafil in rat pulmonary arteries. Sildenafil evoked relaxations were inhibited by endothelial cell removal and incubation with an inhibitor of guanylyl cyclase, ODQ (3×10^{-6} M). Results represent mean and vertical bars S.E.M. for 4–10 preparations. Significantly different responses compared to the parallel control curve: *P<0.05.

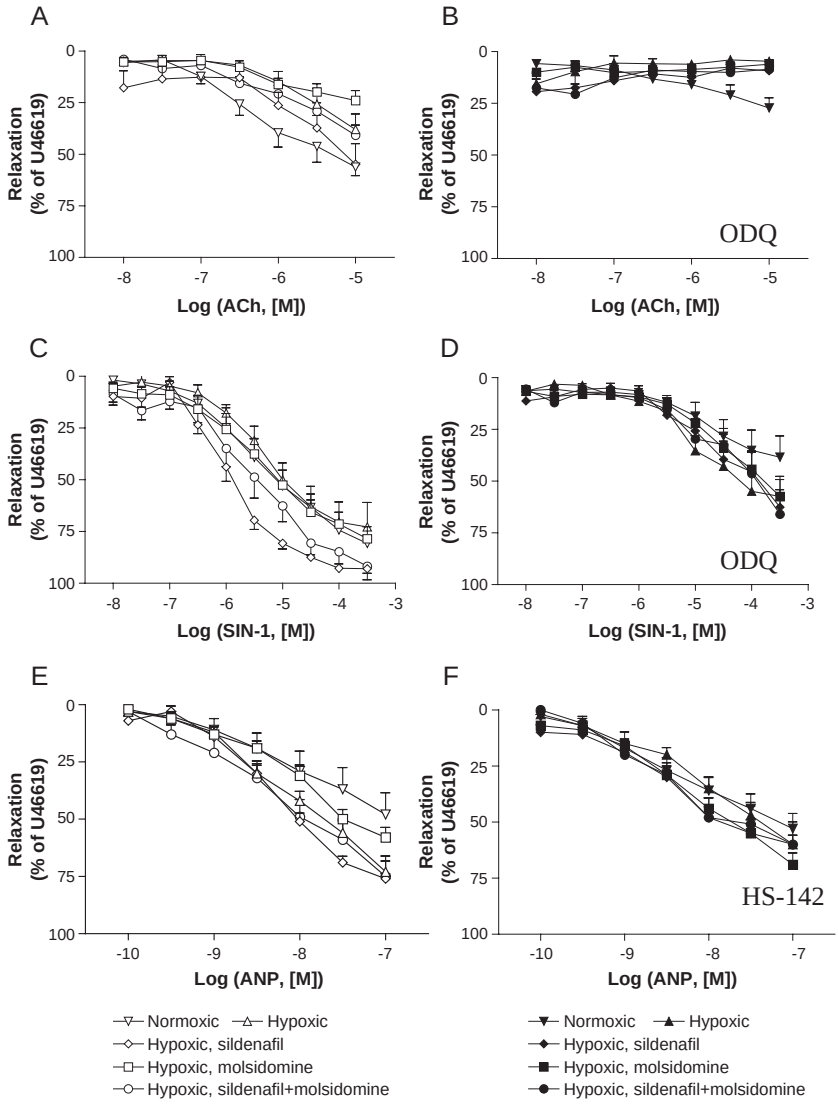

Fig. 4. Effect of hypoxia and treatment with molsidomine and sildenafil on responses to acetylcholine, SIN-1 and ANP in the absence and the presence of ODQ in pulmonary small arteries from normoxic (N), vehicle-treated hypoxic (H), molsidomine (HM), sildenafil (HS) and molsidomine plus sildenafil (HMS) treated hypoxic rats. (A) Concentration–response curves for acetylcholine (ACh) in absence of ODQ, and (B) in presence of ODQ. (C) Concentration–response curves for SIN-1 in absence of ODQ, and (D) in presence of ODQ. (E) Concentration–response curves for ANP in absence of HS-142, and (F) in presence of HS-142. The relaxations are expressed as a percentage of U46619-induced contraction. Results are means±S.E.M. of 5–10 preparations. Differences in area under concentration–response curves were evaluated by one-way ANOVA followed by Bonferroni test. Concentration–response curves for ACh obtained in the absence of the guanylyl cyclase inhibitor, ODQ, in pulmonary arteries from H and HM were significantly ($P<0.05$) different from the corresponding concentration–response curves in normoxic controls (A), and concentration–response curves for ACh obtained in the presence of ODQ in pulmonary arteries from H, HM, HS, HSM were significantly different from the corresponding curves in normoxic controls (B).

imately 15 min followed by a more sustained contraction (Fig. 5A). Phenylephrine contraction was enhanced in arteries from hypoxic rats treated with vehicle and molsidomine, and markedly increased in arteries from molsidomine plus sildenafil-treated rats (Table 2). The transient contraction evoked by acute hypoxia was only enhanced in arteries from chronic hypoxic rats treated with molsidomine (Fig. 5B). Treatment with sildenafil, molsidomine, or the combination did not inhibit the sustained contraction observed in the presence of acute hypoxia (Fig. 5B). These results suggest that none of the treatments impaired the physiological response to acute hypoxia.

4. Discussion

This is the first study which has addressed whether combination of sildenafil and molsidomine would enhance the efficacy of the latter on pulmonary hypertension. The main finding of the present study is that treatment with the PDE 5 inhibitor, sildenafil, as well as direct stimulation of soluble guanylyl cyclase by molsidomine substantially suppressed the effect of chronic hypoxia on pulmonary arterial pressure and right ventricular hypertrophy. However, the combination does not seem to have additional effects on right ventricular systolic pressure and hypertrophy,

Table 2
Contractile responses evoked by 125 mM K^+-rich solution with phenylephrine (PhE, 10^{-5} M), U46619 (10^{-7} M), and phenylephrine (10^{-5} M) in pulmonary arteries from normoxic (N), vehicle-treated hypoxic (H), and hypoxic rats treated with molsidomine (HM), sildenafil (HS), and sildenafil plus molsidomine (HMS)

	N (n=6)	H (n=8)	HM (n=10)	HS (n=7)	HMS (n=8)
Diameter (μm)	663±50	533±36	547±69	567±33	516±52
K^+ PhE (N/m)	2.8±0.7	2.0±0.3	2.7±0.3	1.9±0.2	1.3±0.2
U46619 (N/m)	2.4±0.9	1.4±0.2	2.0±0.3	1.2±0.2	1.2±0.2
PhE (N/m)	0.2±0.1	0.5±0.1[a]	0.7±0.1[a]	0.5±0.1[a]	1.2±0.1[a,b]

Values are mean±S.E.M., where (n) indicates number of animals. Differences were evaluated by one-way analysis of variance (ANOVA) followed by a posteriori Bonferroni test in case of significance.
[a] $P<0.05$ vs. arteries from normoxic animals.
[b] $P<0.05$ vs. arteries from hypoxic rats.

increased lung weight, and impaired acetylcholine relaxation in pulmonary arteries from hypoxic rats.

4.1. Effect of molsidomine treatment on pulmonary hypertension

The effect of the orally active NO donor molsidomine on right ventricular systolic pressure and right ventricle hypertrophy in the present study is comparable to previous treatment studies of rats with pulmonary hypertension (Blumberg et al., 2001; Elmedal et al., 2004; Mathew et al., 1997). Molsidomine is converted in the liver to the active NO-releasing metabolite SIN-1, which evoked potent vasorelaxation in pulmonary arteries in the present study. Metabolization in the lungs of SIN-1 probably confers selectivity for pressure reduction in the pulmonary circulation and explains the lack of changes in systemic blood pressure and heart rate in molsidomine-treated hypoxic rats.

Treatment with molsidomine has either been reported not to affect the relation between media and lumen diameter in proximal pulmonary arteries (Elmedal et al., 2004) or partially prevent increased media area in pulmonary small arteries (Blumberg et al., 2001). In the present study, the increase in muscularized small arteries was less pronounced, but not normalized in lungs from molsidomine-treated animals. The effect of molsidomine was reported to disappear after 3 weeks treatment in patients with chronic obstructive pulmonary disease (Lampert et al., 1991) and chronic hypoxic rats treated for 4 weeks (Blumberg et al., 2001). Therefore, together with the observation that in the pulmonary arteries from the molsidomine treated rats phenylephrine contraction and the first phase of acute hypoxic vasoconstriction was increased, it cannot be excluded lack of complete reversal of arterial remodelling and increased propensity for vasoconstriction counteract the vasodilator effect of molsidomine, and hence contribute to the disappearance of the effect of molsidomine treatment on pulmonary hypertension.

4.2. Effect of sildenafil treatment on pulmonary hypertension

Sildenafil prevented the increase in right ventricular systolic pressure and right ventricle hypertrophy in chronic hypoxic rats in the present study, a finding which agrees with previous studies performed in chronic hypoxic mice (Zhao et al., 2001, 2003), chronic hypoxic rats (Sebkhi et al., 2003), and rats with pulmonary hypertension induced by treatment with monocrotaline (Itoh et al., 2004; Schermuly et al., 2004). The effect of sildenafil on right ventricular hypertrophy also supports the beneficial effect found in short-term studies in patients with pulmonary hypertension, where sildenafil is an effective pulmonary vasodilator (Ghofrani et al., 2002b; Lepore et al., 2002; Michelakis et al., 2002), and also improved exercise tolerance and cardiac index (Sastry et al., 2004).

The effect of sildenafil on vascular remodelling seems to be a question of dose. In chronic hypoxic mice treated with

Table 3
Relaxations induced by acetylcholine, SIN-1, and ANP in U46619 (30 μM)-contracted pulmonary arteries isolated from normoxic (N) and vehicle-treated hypoxic (H) rats, and hypoxic rats treated with molsidomine (HM), sildenafil (HS), and sildenafil plus molsidomine (HMS)

	Acetylcholine			SIN-1		ANP	
	n	pD_2	Max. relaxation (%)	pD_2	Max. relaxation (%)	pD_2	Max. relaxation (%)
N	6	7.00±0.38	56±5	5.40±0.39	81±4	8.01±0.12	52±10
H	6	5.49±0.16[a]	38±7	5.17±0.24	72±12	8.09±0.19	74±7
HM	9	5.63±0.25[a]	24±5[a]	5.40±0.21	78±6	8.01±0.12	58±4
HS	5	5.90±0.24	55±10	5.89±0.10[b]	93±2	8.19±0.08	77±2[a]
HSM	5	5.53±0.31[a]	41±5	5.48±0.19	92±7	8.35±0.20	75±7[a]

Values are means±S.E.M. of n number of preparations examined. pD_2=−logEC_{50}, where EC_{50} is the concentration of agonist producing half-maximal relaxation.
[a] $P<0.05$ parameter significantly different from normoxic animal.
[b] $P<0.05$ parameter significantly different from hypoxic animal.

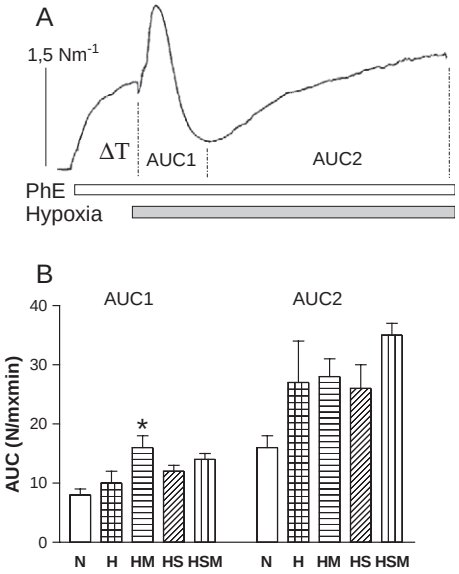

Fig. 5. Effect of chronic hypoxia on acute hypoxic contraction in rat pulmonary arteries. (A) Original recording showing the increase in tension (ΔT) evoked by phenylephrine (PhE, 10 μM) in pulmonary artery from chronic hypoxic rat, and the effect of changing bubbling with 5% CO_2 in air to 5% CO_2 in nitrogen (hypoxia). Hypoxia resulted in a transient contraction with duration of 0–15 min (area under curve, AUC1) followed by a sustained contraction (AUC2). (B) AUC for transient contraction (AUC1) and sustained contraction (AUC2) in response to acute hypoxia in phenylephrine-contracted pulmonary arteries from normoxic (N), vehicle-treated hypoxic (H), molsidomine (HM), sildenafil (HS) and molsidomine plus sildenafil (HMS) treated hypoxic rats. Results are means±S.E.M. of 5–10 preparations. Differences in responses were evaluated by one-way ANOVA followed by Bonferroni test in case of significance: *P<0.05 vs. N; #P<0.05 vs. H.

a low dose (25 mg/kg/day), sildenafil had only minor effect on muscularization of lung arteries smaller than 50 μm (Zhao et al., 2003). However, in rats with pulmonary hypertension injection of high doses (75 to 100 mg/kg/day) of sildenafil partially prevented vascular remodelling (Itoh et al., 2004; Schermuly et al., 2004; Sebkhi et al., 2003). To avoid lowering of systemic blood pressure by combination of molsidomine and sildenafil (Rehse et al., 1999), we chose a dosage of sildenafil, which would result in approximately the same plasma concentration as 50 mg in man (Walker et al., 1999). In the present study, treatment with this lower dose of sildenafil (10 mg/kg/day) in chronic hypoxic rats prevented development of right ventricular hypertrophy, but failed to prevent muscularization, measured as smooth muscle α-actin immunoreaction, of the pulmonary small arteries.

In rat intrapulmonary arteries, sildenafil evoked potent relaxations which were inhibited by endothelial cell removal and in the presence of an inhibitor of guanylyl cyclase. The dose of sildenafil applied in the treatment of the rats in the present study corresponds approximately to plasma concentrations of 10^{-7} M, and would hence suggest sildenafil reverses right ventricular hypertrophy and lowers right ventricular systolic pressure by inhibition of PDE5 and by increasing cyclic GMP. This is supported by the observation that sildenafil treatment normalized guanylyl cyclase-dependent vasorelaxation evoked by acetylcholine in pulmonary arteries from hypoxic rats. However, in the present study concentrations above 10^{-7} M sildenafil were still able to induce relaxation in pulmonary arteries even in the presence of an inhibitor of guanylyl cyclase. In addition to inhibition of PDE5, sildenafil at higher plasma concentrations either through the increases in cyclic GMP and inhibition of PDE3 or direct inhibition of phosphodiesterases also increases cyclic AMP (Itoh et al., 2004; Schalcher et al., 2002; Stief et al., 2000). Therefore, it cannot be excluded higher concentrations of sildenafil associated with both increased cyclic GMP and cyclic AMP are necessary for prevention of pulmonary arterial remodelling in pulmonary arterial hypertension.

4.3. Effect of the combination of sildenafil and molsidomine on pulmonary hypertension

Considering PDE5 is degrading cyclic GMP and is upregulated in pulmonary hypertension in rats (MacLean et al., 1997; Murray et al., 2002; Sebkhi et al., 2003), suggest that inhibitors of PDE5 would enhance the effect of NO donors. Indeed, sildenafil prolongs the effect of cyclic GMP elevating agent such as inhaled NO in patients with pulmonary hypertension (Lepore et al., 2002). However, in the present study there was a lack of additional effect of the combination of sildenafil and an oral NO donor, molsidomine, on right ventricular systolic pressure and hypertrophy compared to each of the drugs administered alone.

Other mechanisms of action of the molsidomine metabolite, SIN-1, may explain the lack of synergistic effect of SIN-1 and sildenafil on pulmonary arterial hypertension in chronic hypoxic rats. Thus, SIN-1 relaxation persisted in the presence of inhibition of guanylyl cyclase in the rat pulmonary arteries in the present study, and in rat mesenteric arteries SIN-1 was found to cause relaxation by opening of calcium-activated K^+ channels (Plane et al., 2001). However, SIN-1 releases NO (Simonsen et al., 1997), and in the present study an inhibitor of guanylyl cyclase caused significant rightward shifts in the concentration–response curves for SIN-1. Moreover, sildenafil leftward shifted concentration–response curves for SIN-1 suggesting a synergistic mechanism of action of the two drugs. Therefore, a more likely explanation for the lack of synergistic effect of the two drugs on pulmonary hypertension is the interaction of SIN-1 with endogenous sources of cyclic GMP. Thus, vasorelaxation evoked by acetylcholine is impaired in pulmonary arteries from molsidomine treated animals, and it is likely impaired endothelial cell function would counteract an eventual beneficial effect of combining molsidomine and sildenafil.

In summary, this study shows that molsidomine and sildenafil attenuate development of pulmonary hypertension

in chronic hypoxic rats, with no side effects such as systemic hypotension. The effect on pulmonary pressure is probably caused by arterial relaxation via guanylyl cyclase-dependent pathways. However, there were no synergistic effects of the doses of molsidomine and sildenafil applied in the present study.

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