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L-Arginine in combination with sildenafil potentiates the attenuation of hypoxic pulmonary hypertension in rats

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Short title:

L-Arginine and sildenafil attenuate of pulmonary hypertension

Summary

Chronic hypoxia induces an increased production of nitric oxide (NO) in pulmonary prealveolar arterioles. Bioavailability of the NO in the pulmonary vessels correlates with concentration of L-arginine as well as activity of phosphodiesterase-5 enzyme (PDE-5). We tested a hypothesis whether a combination of L-arginine and PDE-5 inhibitor sildenafil has an additive effect in reduction of the hypoxic pulmonary hypertension (HPH) in rats. Animals were exposed to chronic normobaric hypoxia for 3 weeks. In the AH group, rats were administered L-arginine during chronic hypoxic exposure. In the SH group, rats were administered sildenafil during chronic hypoxic exposure. In the SAH group, rats were treated by the combination of L-arginine as well as sildenafil during exposure to chronic hypoxia. Mean PAP, structural remodelling of peripheral pulmonary arterioles (%DL) and RV/LV+S ratio was significantly decreased in the SAH group compared to hypoxic controls even decreased compared to the AH and the SH groups in first two measured parameters. Plasmatic concentration of cGMP and NOx were significantly lower in the SAH group compared to hypoxic controls. We demonstrate that NO synthase substrate L-arginine and phosphodiesterase-5 inhibitor sildenafil administered in combination are more potent in attenuation of the HPH compared to a treatment by substances given alone.

Key words

pulmonary hypertension, chronic hypoxia, L-arginine, sildenafil.

Introduction

Morbidity and mortality of pulmonary arterial hypertension (PAH) still remains high. The production of nitric oxide (NO) in pulmonary arterioles increases during exposure to chronic hypoxia (Hampl et al. 1993; Xue et al. 1994). It suggests NO has an important role in the adaptation of the pulmonary circulation to elevated blood pressure. The production of NO in the pulmonary vessels is related to the concentration of L-arginine as a substrate for NO synthesis (Palmer et al. 1988; Stuehr et al. 1991). It is reported that administration of L-arginine increases NO production (Fike et al. 2000) as well as decreases pulmonary hypertension caused by chronic hypoxia (hypoxic pulmonary hypertension, HPH) (Mitani et al. 1997). Nitric oxide in smooth muscle cells binds to soluble guanylate cyclase and activates the production of cyclic guanosine monophosphate (cGMP). Cyclic GMP activates processes which result in the activation of voltage-gated potassium channels and inhibition of the depolarization of the smooth muscle cells and thus leads to vasodilation (Murad 1986; Archer et al. 1994). The soluble cGMP is degraded by the phosphodiesterase (PDE) enzyme (specific isoform number 5) in pulmonary arterioles. Inhibition of PDE activity by sildenafil has a significant vasodilatory effect in the developed HPH in rats (Cohen et al. 1996) and also in patients with pulmonary arterial hypertension (PAH) (Michelakis et al. 2002). The effective vasodilatory therapy has been considered an important factor in improving quality of life in patients with PAH (D'Alonzo et al. 1991). The PDE inhibitors are used most often in combination with other drugs in treatment of PAH: in combination with prostacycline analogues (Itoh et al. 2004; Clozel et al. 2006), in combination with endothelin receptor A inhibitors (Mouchaers et al. 2009), and in combination with HMG-CoA inhibitors (Satoh and Satoh 2008; Zhao et al. 2009). Every used combination has a moderate potentiation effect in decreasing the pulmonary artery pressure compared to the PDE inhibitor alone.

In the present study in rats, we tested the hypothesis whether the combination of L-arginine and the PDE-5 inhibitor sildenafil is more effective in reduction of the development of HPH than the drugs administered alone.

Methods

Experiments on five groups of adult male Wistar rats (BW 220-250g at the beginning of the experiment) were performed in accordance with the European Community and NIH guidelines for the

use of experimental animals. All experiments were approved by our institution's Animal Studies Committee.

Experimental groups and drug administration:

Experimental animals were exposed to chronic hypoxia in an isobaric hypoxic chamber ($F_{iO_2}=0.1$) for 3 weeks. Experimental group AH (n=10) were rats administered with L-arginine (Sigma-Aldrich[®], Czech Republic), 500mg.kg⁻¹ per day by gavage during hypoxia exposition. We used a similar effective dose of L-arginine as other researchers (Mitani et al. 1997). Group SH (n=10) were rats administered with the PDE-5 inhibitor sildenafil (Revatio 20mg tbl, Pfizer[®]), 25mg.kg⁻¹ per day by gavage during hypoxia. In the literature, the dosage of sildenafil varies from 1mg.kg⁻¹ to 100mg.kg⁻¹ per day or more (Preston et al. 2004; Clozel et al. 2006; Satoh and Satoh 2008; Zhao et al. 2009). We decided to use a lower dose of sildenafil (25mg.kg⁻¹ per day) to unmask the potentially amplifying effect of L-arginine. Group SAH (n=10) were rats given a combination of L-arginine and sildenafil during hypoxia. Group H (n=10) were rats exposed to chronic hypoxia with no drugs. Group N (n=10) were normoxic controls. All animals lived in 12 hours diurnal cycles and had free access to water and food. The gavage technique was used to avoid the influence of food and water intake decrease during hypoxic exposure (Singh and Selvamurthy 1993).

Experimental protocol and measurements:

After 3 weeks of hypoxia, rats were anesthetized with thiopental (30mg.kg⁻¹ b.w. i.p.). In closed-chest spontaneously breathing animals, we measured the mean pulmonary artery pressure (PAP) (Herget and Palecek 1972). After measurement of PAP, the animals were ventilated with air through a tracheal cannula (50 breaths.min⁻¹; peak inspiratory pressure 10 cmH₂O; positive end-expiratory pressure 2 cmH₂O). The chest was then opened by sternotomy and an ultrasound flow probe with J transducer (Transonic Systems Inc, Ithaca, NY, USA) was placed on the ascending aorta to measure cardiac output (Hampl et al. 1993). Subsequently, arterial blood samples were collected for measuring cGMP and hematocrit. The plasma concentration of NO and its oxidation products (nitrites and nitrates, NOx) were measured with a chemiluminescence NO analyzer using the methods described earlier (Sun et al. 2010). Finally, the heart and lungs were removed from the thoracic cavity. The heart was dissected and weighed in parts (Fulton et al. 1952). Lungs were filled with formol solution through the trachea for assessing the presence of hypoxia-induced structural remodelling of peripheral pulmonary arteries. The same person blinded to the study group from which the specimen originated calculated

all scores. The results are presented as the proportion of peripheral vessels with separated elastic laminae (double-laminated, %DL) (Herget et al. 1978).

Cyclic GMP was determined from blood samples which were frozen and stored at -80°C until measurement was performed by the radioimmunoassay kit (Immunotech Inc).

Statistical analysis:

Results were analyzed by 1-way ANOVA with Fischer *post-hoc* test. Values of $p < 0.05$ were considered significant. The results are presented as means \pm SEM.

Results

Body weight, cardiac output and hematocrit did not differ in any of the groups exposed to chronic hypoxia (tab. 1). Plasma concentration of cGMP was increased in all groups exposed to hypoxia compared to normoxic controls, however, rats administered by L-arginine and a combination of L-arginine with sildenafil had a significantly higher cGMP concentration than hypoxic controls (tab.1). The group with combined treatment had significantly higher concentrations of nitrites and nitrates (NO_x) compared to all other groups (tab.1).

Animals exposed to hypoxia with administration of drugs had significantly lower mean PAP compared to non-treated hypoxic rats. Mean PAP in the SAH group was significantly lower compared to the groups administered with L-arginine or sildenafil alone (fig.1). We found the right heart ventricle weight was significantly reduced in the SAH group compared to the H group, but not in the AH and SH groups (fig. 2). Presence of the double laminated pulmonary arterioles was significantly lower in the groups exposed to hypoxia with administration of drugs compared to hypoxic controls, but the SAH group had significantly lower %DL compared to the AH and SH groups (fig. 3).

Discussion

We demonstrate that the NO synthase substrate L-arginine and the phosphodiesterase-5 inhibitor sildenafil added in combination are more potent in the attenuation of HPH compared to treatment by substances given alone. Combined administration was more potent in decreasing mean PAP; it reduced the hypoxia-induced remodelling of the prealveolar pulmonary arterioles and

significantly reduced right heart hypertrophy. Adding L-arginine together with sildenafil resulted in increased plasma concentrations of NO_x and cGMP compared to hypoxic non-treated rats.

In rats treated with L-arginine alone, we found slightly, but significantly attenuated HPH which coincides with increased plasma concentration of cGMP. These data are consistent with the other researchers (Mitani et al. 1997; Fagan et al. 1999). In the present experiment we did not find, however, any effect of L-arginine on the right heart hypertrophy induced by chronic hypoxia. Mitani et al. (Mitani et al. 1997) reported that right heart ventricle hypertrophy reduction during chronic hypoxia with L-arginine administered intraperitoneally was dose dependent: 500 mg.kg⁻¹ of L-arginine was effective but 200mg.kg⁻¹ had no effect. In our experiment we used 500 mg.kg⁻¹ of L-arginine, but it was given by a gastric gavage. The amount of L-arginine absorbed in the animal gastrointestinal tract could be lower than after intraperitoneal application. The release of insulin and growth hormones by administration of parenteral L-arginine may be considered also (Giugliano et al. 1997). **The effect of the L-arginine administered could be explained by its bioavailability and/or by competition with the endogenous asymmetric methylarginines (ADMA). The increased concentration of ADMA was found in patients with pulmonary hypertension (Gorenflo et al. 2001). ADMA acts as an endogenous inhibitor of the NO synthase (Vallance et al. 1992). In conditions of oxidant stress L-arginine supplementation may exacerbate NOS uncoupling, production of superoxide prevails NO and the radical tissue injury worsens (Druhan et al. 2008)**

Results of the studies where authors administered the PDE-5 inhibitor sildenafil alone in HPH are partly controversial. We found lower mean PAP and reduced remodelling of the peripheral pulmonary arterioles compared to hypoxic controls. These results are in accordance with the other researchers (Hanasato et al. 1999; Sebkhi et al. 2003). On the other hand we did not find a reduction of the right heart hypertrophy and we did not find a difference in plasma concentration of the cGMP compared to hypoxic rats. Similar results were found in mice in experiments by Zhao *et al.* (Zhao et al. 2001). They found significantly increased cGMP concentration in lung homogenate but not in plasma in chronic hypoxia exposed mice administered with 25mg.kg⁻¹ per day of sildenafil. In the case of right heart hypertrophy, we found even more controversies in the literature. No comparable experiment was reported which would directly compare the effect of sildenafil administration on hypoxia induced right

heart hypertrophy in rat species. Zhao *et al.* (Zhao *et al.* 2001) found significantly decreased right heart hypertrophy in mice after 25mg per kg of sildenafil during chronic normobaric hypoxia. Hanasato *et al.* (Hanasato *et al.* 1999), used a different PDE-5 inhibitor: E-4010, hypobaric hypoxia and more importantly, they used pretreatment with a PDE-5 inhibitor for 1 day before hypoxia exposition. The other study suggested that sildenafil efficacy on the right heart hypertrophy is not dose-dependent in doses of 3mg.kg⁻¹ per day and more (Hanasato *et al.* 1999).

The increase in NO production during the exposure to chronic hypoxia is consistent with several of our former studies (Hampl *et al.* 2006; Hodyc *et al.* 2012) and studies of other research groups (see review (Hampl and Herget 2000)). NO plays an important role in chronic hypoxia induced structural and functional changes in prealveolar vessels. In the early phase of hypoxic exposure, increased NO production participates with oxygen radicals in the pathogenesis of HPH (Herget *et al.* 2000; Hampl *et al.* 2006). In the later phase of hypoxic exposure, increased NO production restricts already developed hypertension (see review (Hampl and Herget 2000)). Nitric oxide is not only a vasodilator and smooth muscle cells remodelling inhibitor, it also inhibits platelet aggregation (Adams *et al.* 1995), as well as decreases endothelial activation of adhesion molecules and proinflammatory cytokines (De Caterina *et al.* 1995). The other, additional, mechanisms which may be involved in our study should also be considered. The antiaggregatory effect of L-arginine on blood platelets and PDE-5 in pulmonary circulation is thought to limit the vasodilator and antiproliferative effects of the NO (Sebkhi *et al.* 2003).

In the present study, we focused on the administration of minimal but still effective doses of L-arginine and the PDE-5 inhibitor sildenafil to reveal a potentially synergistic effect of the combination of drugs. Our data clearly presents a pure synergistic effect of the combination of the L-arginine and the PDE-5 inhibitor sildenafil in rat model on attenuation of the development of HPH, characterized by significantly decreased mean PAP, significantly decreased remodelling of the peripheral pulmonary arterioles and significantly reduced right heart hypertrophy. **Present experimental study suggests novel direction how to enhance the therapeutic effects of PDE-5 inhibitors in hypoxic pulmonary hypertension.**

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	BW [g]	CO [mL.min ⁻¹]	HTC [%]	cGMP in plasma [pM.L ⁻¹]	NOx in plasma [μM.L ⁻¹]
N	405.6±33.3	34.3±7.2	43.4±3.7	0.1±0.04	21.4±2.8
H	274.4±22.4 *	20.0±3.7 *	68.4±4.6 *	73.5±27.8 *	27.3±4.7
AH	263.4±24.3 *	21.7±1.5 *	64.8±7.5 *	101.6±31.6 **	27.8±2.2
SH	253.2±11.6 **	25.8±5.9 #	72.5±3.8 *	95.6±26.1 *	22.5±1.5
SAH	258.0±18.0 *	22.6±6.0 *	70.0±5.8 *	113.3±29.5 **	38.0±4.4 ‡

Tab.1: BW – body weight in time of measurement; CO – cardiac output; HTC – hematocrit; cGMP – cyclic guanosine monophosphate. * - P<0.0001 vs. group N; # - P<0.01 vs. group N; + - P<0.05 vs. group H. ‡ - P<0.05 vs. all other groups.

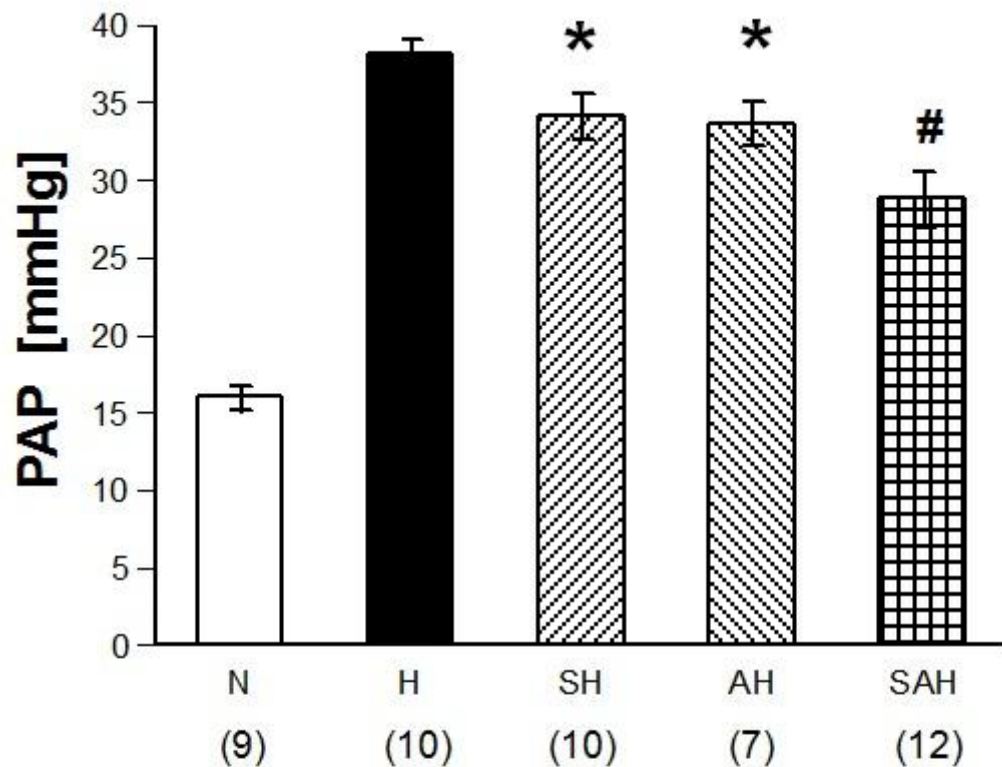


Fig. 1: Pulmonary arterial mean pressure

N – normoxic controls, ambient air H – hypoxic controls, 3 wks of hypoxia, SH – hypoxia exposure for 3wks and sildenafil administration; AH – hypoxia exposure for 3 wks and L-arginine administration; SAH – hypoxia for 3 wks and sildenafil plus L-arginine administration.

* - $p < 0.05$ vs. group H; # - $p < 0.0001$ vs. group H and $p < 0.05$ vs. groups SH and AH.

Data are means \pm SEM, numbers of rats are in parentheses.

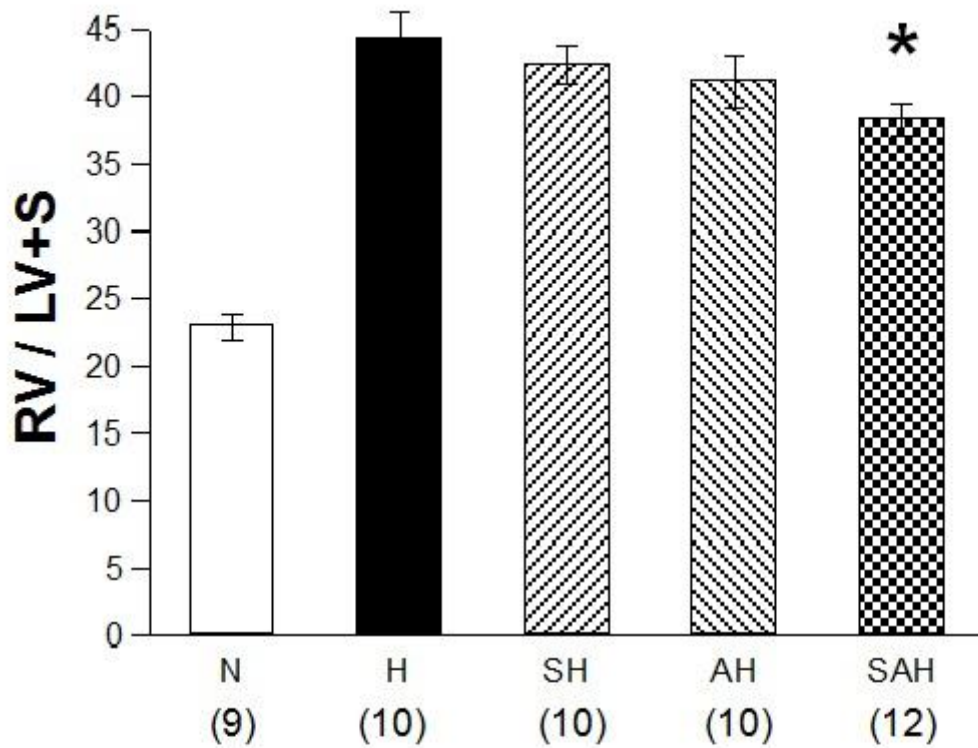


Fig. 2: Right ventricular heart weight to the sum of weights of left heart ventricle and septum ratio.

N – normoxic controls, ambient air H – hypoxic controls, 3 wks of hypoxia, SH – hypoxia exposure for 3wks and sildenafil administration; AH – hypoxia exposure for 3 wks and L-arginine administration; SAH – hypoxia for 3 wks and sildenafil plus L-arginine administration.

*- $p < 0.05$ vs. group H.

Data are means \pm SEM, numbers of rats are in parentheses.

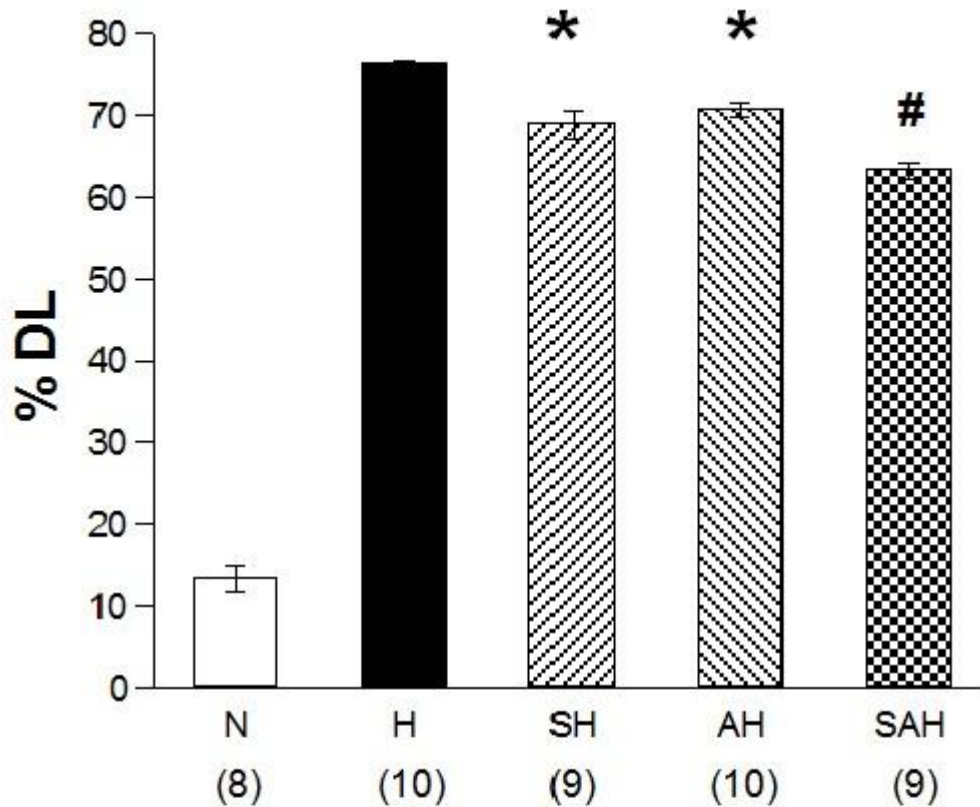


Fig. 3: Ratio of hypertrophied (double laminated) peripheral pulmonary arteries and total number of peripheral pulmonary arteries on section of the right lung.

N – normoxic controls, ambient air H – hypoxic controls, 3 wks of hypoxia, SH – hypoxia exposure for 3wks and sildenafil administration; AH – hypoxia exposure for 3 wks and L-arginine administration; SAH – hypoxia for 3 wks and sildenafil plus L-arginine administration. * - $p < 0.001$ vs. group H; # - $p < 0.001$ vs. group H, group SH and group AH.

Data are means \pm SEM, numbers of rats are in parentheses.