N-acetylcysteine inhibits hypoxic pulmonary hypertension most effectively in the initial phase of chronic hypoxia

Věra Lachmanová, Olga Hniličková, Viera Povýšilová, Václav Hampl, Jan Herget*

Department of Physiology, 2nd Medical School, Charles University and Center for Experimental Cardiovascular Research, Plzeňská 221, Prague 5, Czech Republic

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Abstract

Exposure to chronic hypoxia results in hypoxic pulmonary hypertension (HPH). In rats HPH develops during the first two weeks of exposure to hypoxia, then it stabilizes and does not increase in severity. We hypothesize that free radical injury to pulmonary vascular wall is an important mechanism in the early days of the hypoxic exposure. Thus antioxidant treatment just before and at the beginning of hypoxia should be more effective in reducing HPH than antioxidant therapy of developed pulmonary hypertension. We studied adult male rats exposed for 4 weeks to isobaric hypoxia (FiO2 = 0.1) and treated with the antioxidant, N-acetylcysteine (NAC, 20 g/l in drinking water). NAC was given “early” (7 days before and the first 7 days of hypoxia) or “late” (last two weeks of hypoxic exposure). These experimental groups were compared with normoxic controls and untreated hypoxic rats (3-4 weeks hypoxia). All animals kept in hypoxia had significantly higher mean pulmonary arterial blood pressure (PAP) than normoxic animals. PAP was significantly lower in hypoxic animals with early (27.1 ± 0.9 mmHg) than late NAC treatment (30.5 ± 1.0 mmHg, P < 0.05; hypoxic without NAC 32.6 ± 1.2 mmHg, normoxic controls 14.9 ± 0.7 mmHg). Early but not late NAC treatment inhibited hypoxia-induced increase in right ventricle weight and muscularization of distal pulmonary arteries assessed by quantitative histology. We conclude that release of free oxygen radicals in early phases of exposure to hypoxia induces injury to pulmonary vessels that contributes to their structural remodeling and development of HPH.

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Keywords: Hypoxic pulmonary hypertension; Oxidant stress; Vascular remodeling
Introduction

Exposure to chronic hypoxia causes hypoxic pulmonary hypertension (HPH). The rise in pulmonary peripheral vascular resistance in chronic hypoxia results from vasoconstriction and from structural remodeling of the walls of peripheral pulmonary arteries (Reid, 1979). Pulmonary arterial blood pressure (PAP) increases and consequently right ventricular hypertrophy develops. In rats exposed to an environment with 10% O2 HPH develops within 10 – 14 days. Then it essentially does not progress any further and remains steady during this adaptation phase (Herget et al., 1978; Reeves and Herget, 1984).

Exposure to hypoxia generates oxidative stress of lung tissue (Block et al., 1989; Chang et al., 1989; Nakanishi et al., 1995; Hoshikawa et al., 2001). We hypothesize that hypoxia-induced free radical injury to the walls of peripheral pulmonary arteries at the beginning of hypoxia is an important pathogenetic factor in the development of HPH. In the first few days of hypoxic exposure alveolar macrophages are activated and primed for an enhanced production of H$_2$O$_2$ (Wilhelm et al., 1996). Rats exposed to 3-days hypoxia have more H$_2$O$_2$ in their breath than normoxic animals. Then, after the first week of exposure, the concentration of H$_2$O$_2$ in expired air gradually decreases to normal values (Wilhelm et al., 1999). The concentrations of different biochemical indicators of lung tissue oxidative stress are increased in the first week of chronic hypoxia. After a more prolonged exposure, in the steady phase of HPH, the biochemical signs of lung tissue oxidative injury disappear (Wilhelm and Herget, 1999; Herget et al., 2000; Hoshikawa et al., 2001).

To test our hypothesis that oxidative injury is critical only in the initial development of HPH we compared the effects of antioxidant N-acetylcysteine (NAC) treatment, applied in rats just before and at the beginning of chronic hypoxia, with the results of its application in the fully developed HPH at steady state. It has been repeatedly reported that NAC given during the whole period of prolonged hypoxic exposure reduces the development of HPH (Hoshikawa et al., 1995; Herget et al., 1998; Hoshikawa et al., 2001).

Methods

Four groups of adult male Wistar rats (Anlab, Czech Rep.) were studied. Experimental rats were exposed for 4 weeks to isobaric hypoxia (F$_{O2}$ = 0.1) (Hampl and Herget, 1990) and treated with NAC (20 g/l in drinking water). NAC was given before and at the beginning of exposure (7 days before and the first 7 days of hypoxia, n = 9, early NAC application) or in rats with developed HPH (last two weeks of hypoxic exposure, n = 8, late NAC application). The rationale for starting NAC treatment well before the beginning of the hypoxic exposure was to make sure that at the moment of onset of hypoxia, NAC had already reached an effective, stable level. Experimental groups were compared with normoxic controls (n = 9) and untreated rats exposed to hypoxia (3-4 weeks hypoxia, n = 9). Experiments were performed in accordance with the European Community and NIH guidelines for using experimental animals. All procedures were approved by our institution’s Animal Studies Committee.

In rats anesthetized with thiopental (30 mg/kg b.w. i.p.) left carotid artery was cannulated to measure systemic arterial blood pressure (SAP) and to obtain a blood sample for hematocrit determination. Right jugular vein was exposed, pulmonary artery was catheterized without opening the chest, and PAP was recorded (Herget and Paleček, 1972) in rats spontaneously breathing atmospheric air. Than tracheal cannula was introduced and the rats were mechanically ventilated with room air at 50 breaths/min by
positive pressure (peak inspiratory pressure 10 cm H2O, zero end-expiratory pressure). The chest was opened by sternotomy with extra care taken to minimize bleeding. Ultrasonic flow probe (2.5 mm SS-series with J reflector, Transonic Systems, Ithaca, NY, USA) was placed at the ascending aorta to measure aortic blood flow (T 106 flowmeter, Transonic Systems) as an estimate of cardiac output (CO) (Hampl et al., 1993). After the measurements were completed the heart and lungs were removed from the chest. Right and left ventricles plus septum were separated and weighed (Fulton et al., 1952). Lungs were filled with neutral formol solution through the trachea at a pressure of 12 cm H2O and then placed in the same solution for 3 – 4 weeks. Lung sections were then cut and stained by the hematoxylin resorcin fuchsin method. Remodeling of the walls of peripheral pulmonary arteries was assessed by counting distal vessels bound to alveolar ducts or to alveoli (≤ 300 μm) on one slide from each rat and determining how many of them were muscularized (Hunter et al., 1974; Herget et al., 2003). Counted as muscularized were those vessels that had internal and external elastic laminae separated at least in half of the vessel circumference. All peripheral pulmonary blood vessels found in sagittal sections through the hilus region of the right and left lungs were counted. All counting was performed by one person blinded to the group assignment of the slides. The number of vessels counted was 52 – 85 (range) in each rat. The result is reported as percentage of double-laminated (muscularized) peripheral vessels (%DL) (Herget et al., 1978).

**Statistical analysis**

The results were evaluated by ANOVA with Scheffe’s post-hoc test. Values of p < 0.05 were considered significant. The results are presented as means ± SEM.

**Results**

At the beginning of treatment, the groups did not differ in body weight. Rats exposed to chronic hypoxia gained body weight more slowly than controls, and consequently, their body weight at the end of the exposure was significantly less compared to normoxic controls (Table 1). Two rats from the group with the late NAC application died; there was no mortality in the other groups.

**Table 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body weight [g]</th>
<th>Cardiac output [ml/min]</th>
<th>SAP [mmHg]</th>
<th>Hematocrit [%]</th>
<th>RV/LV + S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>9</td>
<td>315±4</td>
<td>34.1±2.7</td>
<td>106±2</td>
<td>48.5±0.4</td>
<td>0.276±0.010</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>9</td>
<td>197±7 ***</td>
<td>25.1±1.2 *</td>
<td>109±3</td>
<td>78.3±1.2 **</td>
<td>0.510±0.042***</td>
</tr>
<tr>
<td>Hypoxia NAC early application</td>
<td>9</td>
<td>202±9 ***</td>
<td>23.3±2.4 *</td>
<td>94±4 ##</td>
<td>78.4±3.2 ***</td>
<td>0.430±0.026**</td>
</tr>
<tr>
<td>Hypoxia NAC late application</td>
<td>6</td>
<td>186±5 ***</td>
<td>25.4±1.8 *</td>
<td>103±6</td>
<td>80.5±2.1 ***</td>
<td>0.530±0.031***</td>
</tr>
</tbody>
</table>

Data are means ± SEM, *: p < 0.05, **: p < 0.02, ***: p < 0.001 hypoxic groups differ from a normoxic group, #: p < 0.02 early NAC treated group differs from all other groups, +: p < 0.05 NAC early NAC treated hypoxic group differs from late NAC treated hypoxic group. SAP = systemic arterial blood pressure.
Chronic hypoxia induced pulmonary hypertension characterized by a significant increase in PAP (Fig. 1) and increased right to left heart ventricle + septum weight ratio (RV/LV + S, Table 1). All animals kept in hypoxia had significantly higher PAP than normoxic animals. However, PAP was significantly lower in the group of hypoxic rats with early NAC treatment than in rats with late application (Fig. 1). The same applied for the RV/LV + S ratio (Table 1). In all groups of rats exposed to hypoxia, the relative

Fig. 1. Pulmonary mean arterial blood pressure in rats exposed to hypoxia and treated with N-acetylcysteine. Hypoxia + NAC early = rats exposed to hypoxia and treated with N-acetylcysteine one week before and during the 1st week of exposure to hypoxia. Hypoxia + NAC late = rats exposed to hypoxia and treated with N-acetylcysteine during the last two weeks of exposure to hypoxia. All groups exposed to hypoxia are significantly different from controls (Normoxia). $P < 0.05$ = statistical difference of groups treated early and late with N-acetylcysteine.

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Fig. 2. Percentage of muscularized (double laminated, DL) peripheral pulmonary arteries in rats exposed to hypoxia and treated with N-acetylcysteine. For the groups denomination see Fig. 1. All groups exposed to hypoxia are significantly different from controls (Normoxia). $P < 0.05$ = statistical difference of groups treated early and late with N-acetylcysteine.
weight of right ventricle was larger than in normoxic controls. In the group exposed to hypoxia with the early NAC treatment the relative right ventricle weight was significantly lower (95.9 ± 4.4 mg/100 g of b. w.) than in hypoxic rats with the late NAC application (118.2 ± 5.7 mg/100 g of b. w., P < 0.01). The value in hypoxic rats with the late NAC application did not differ from that in unterated hypoxic rats. The relative weight of the left ventricle was slightly, but significantly, higher in all rats exposed to hypoxia. We did not observe any effect of NAC treatment on the left heart ventricle weight (data not shown). Chronic hypoxia induced the typical remodeling of peripheral pulmonary arteries with its characteristic feature – muscularization of walls of prealveolar vessels. The percentage of muscularized peripheral lung vessels was significantly higher in all groups of hypoxic rats than in normoxic controls. Vascular muscularization was slightly but significantly lower in rats with early NAC treatment than in hypoxic rats with late NAC treatment and in not treated hypoxic rats (Fig. 2). The animals in the group with early NAC treatment had significantly lower SAP than other groups. CO was significantly lower and hematocrit significantly higher in all hypoxic groups than in the groups kept in normoxia, but there were no differences in CO and hematocrit attributable to NAC treatment.

Discussion

The main finding of our study is that the antioxidant NAC given to rats just before and during the first few days of hypoxic exposure reduces the development of HPH. It contrasts with the lack of effect of NAC applied in the late phase of exposure (3rd and 4th week of hypoxia). This observation strongly supports our hypothesis that the development of HPH proceeds in two phases. The initial phase is characterized by injury to the walls of peripheral pulmonary arteries that stimulates vascular smooth muscle and fibroblast proliferation. Structural remodeling of peripheral pulmonary arteries and increase in smooth muscle tonus result in the increase in peripheral pulmonary vascular resistance and prealveolar vessels become less compliant. While the role of free radicals in this process has been emerging during the recent years (for review, see Hampl and Herget, 2000) the inhibition of HPH only by the early NAC treatment in our present study suggests that release of free radicals participates in the pathogenesis of HPH specifically at the very onset of HPH. In the rat species the pulmonary hypertension is fully developed by the third week of hypoxia. Then it becomes stable and does not progress any further (Hunter et al., 1974; Herget and Paleček, 1978; Herget et al., 1978; Reeves and Herget, 1984). The release of oxygen radicals declines to normal values (Wilhelm et al., 1996; Wilhelm et al., 1999; Wilhelm and Herget, 1999) and, therefore, the antioxidant treatment did not influence the HPH in this stable stage.

An important feature of the early phase of HPH is the presence of pulmonary vasoconstriction which contributes to the increase in pulmonary vascular resistance. This increase in vascular tension does not appear to be a simple extension of acute hypoxic pulmonary vasoconstriction inasmuch the latter is blunted in chronic hypoxia (McMurtry et al., 1978; Hampl and Herget, 1990). In another study in isolated rat lungs we found that NAC treatment prevented the blunting of hypoxic pulmonary vasoconstriction brought about by 5-d hypoxia (Lachmanová and Herget, 2002). Therefore, it is unlikely that the reduction of HPH by NAC was due to inhibition of hypoxic pulmonary vasoconstriction.

The dose of NAC, which was dissolved in drinking water, depended on water intake by individual animals. We estimated water consumption roughly by weighing water bottles in each animal cage every other day. Watter consumption was lower in the group with the early NAC administration (17 ml/rat/day,
range: 16–19 ml, 1st week of hypoxic exposure) than in rats with the late NAC treatment (27 ml/rat/day, range 20 – 31 ml, last week of exposure). Therefore the actual NAC intake was probably higher in the group where we did not observe any effect of NAC on HPH. We did not measure biochemically the effect of NAC administration on markers of oxidative stress. Hoshikawa and co-workers (Hoshikawa et al., 2001) used 1% of NAC in drinking water (50% of our dose) in a very similar experimental arrangement. They found that NAC administration significantly attenuated the increase in phosphatidylcholine hydroperoxide concentration during the first week of hypoxia.

A surprising finding is the slightly but significantly reduced SAP in the group exposed to hypoxia with the early NAC treatment, especially as SAP was normal in both the untreated rats exposed to hypoxia and in the group with the late NAC treatment. In our previous study we did not observe any effect of chronic NAC treatment on SAP in normoxic rats (Herget et al., 1998) While we do not have an explanation for this finding, it seems to indicate that radicals somehow counterbalance some hypotensive effect of early hypoxia on the systemic circulation. Thus, free radicals may have an important role during the first days of hypoxia in both the pulmonary and systemic vessels.

The mechanism of how the radical injury to pulmonary vascular wall initiates the remodeling of prealveolar vessels may be linked to the changes of matrix proteins. In the first week of exposure to hypoxia the peripheral pulmonary arteries are surrounded by numerous mast cells positive to immunostaining for interstitial collagenase (Vajner et al., 2003). The collagenolytic activity in the walls of peripheral pulmonary arteries is increased (Novotná and Herget, 1998) and it is particularly high at the beginning of exposure to hypoxia (Novotná and Herget, 2001). ROS and peroxynitrite (product of superoxide and nitric oxide interaction) are potent activators of collagenases (Rajagopalan et al., 1996) and NAC inhibits this effect (Tyagi, 1998). Collagen breakdown products that accumulate in the vascular wall (Novotná and Herget, 1998) may stimulate fibroproduction and smooth muscle proliferation (Gardi et al., 1990, 1994; Bačáková et al., 2003) in the walls of peripheral pulmonary arteries. Various cytokines and other regulatory molecules are involved in this cascade. Their interaction with oxidative stress induced by hypoxia is complex.

Conclusion

We conclude that antioxidant, applied in the early phase of exposure to hypoxia reduces the development of HPH. We propose protection of matrix proteins of prealveolar vessels from free radical-induced increase in collagenolysis as a possible mechanism.

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References


