Depletion of Alveolar Macrophages Attenuates Hypoxic Pulmonary Hypertension but not Hypoxia-Induced Increase in Serum Concentration of MCP-1

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Summary

Exposure to hypoxia, leading to hypoxic pulmonary hypertension (HPH), is associated with activation of alveolar macrophages (AM). However, it remains unclear how AM participate in this process. There are studies which imply that the AM product monocyte chemoattractant protein-1 (MCP-1) plays an important role. Thus we tested: 1. if the selective elimination of AM attenuates HPH in rats, 2. the correlation of MCP-1 plasmatic concentrations with the presence and absence of AM during exposure to hypoxia, 3. the direct influence of hypoxia on MCP-1 production in isolated AM. We found that experimental depletion of AM attenuated the chronic hypoxia-induced increase in mean pulmonary arterial pressure, but did not affect the serum MCP-1 concentrations. Furthermore, the MCP-1 production by AM *in vitro* was unaffected by hypoxia. Thus we conclude that AM play a significant role in the mechanism of HPH, but MCP-1 release from these cells is most likely not involved in this process. The increase of MCP-1 accompanying the development of HPH probably originates from other sources than AM.

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Introduction

Pulmonary hypertension is a condition that has been studied for decades because of its *exacerbating* effect on patients with chronic hypoxic diseases. Lung mast cells (MC) were implicated to participate in the development of hypoxic pulmonary hypertension (HPH) by several studies (Vajner *et al.* 2006, Mungall 1976), but not all (Zhu *et al.* 1983). In the systemic circulation, there is evidence that hypoxia can activate MC in vascular wall by circulating monocyte chemoattractant protein-1 (MCP-1) released from alveolar macrophages (Chao *et al.* 2009, Chao *et al.* 2011a). Because accumulation and activation of alveolar macrophages (AM) is characteristic of the development of hypoxic pulmonary hypertension (Frid *et al.* 2006, Hayashida *et al.* 2005, Madjdpour *et al.* 2003, Vergadi *et al.* 2011), we speculated that AM-derived MCP-1 could play a role in the activation of lung MC and thus in the mechanism of HPH. Therefore, we designed an experiment testing the role of AM and MCP-1 in HPH. We hypothesized that a selective elimination of AM by intrapulmonary application of clodronate-containing liposomes (Madjdpour *et al.* 2003) would result in decreased production of MCP-1 and attenuation of HPH.

Methods

Study design

Combinations of intratracheal clodronate

(AM-depleting agent) or vehicle administration with or without hypoxic exposure for 4, 10 or 15 days were used in adult male Wistar rats. For longer hypoxic exposures, the clodronate administrations were repeated every 5 days. The effectiveness of clodronate in reducing AM counts was assessed on day 4 of hypoxic exposure (4 days after clodronate administration). The effects of clodronate and/or hypoxia on serum MCP-1 levels were tested on day 4 and 10 of hypoxia. Finally, pulmonary hemodynamics and right heart ventricle weight were measured at the end of a 15-day hypoxic exposure. In a supplemental *in vitro* experiment, the ability of hypoxia (1 hour to 4 days) to stimulate MCP-1 production in the AM was tested.

All experimental procedures were performed in accordance with the European Community and NIH guidelines and approved by the Animal Studies Committee of the Second Medical School, Charles University, Prague.

Clodronate administration

Under brief ether anesthesia, a bolus (350 μl) of a suspension of liposome-encapsulated clodronate (Encapsula NanoSciences LLC, clodronate disodium, 5 mg/ml) in phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 140 mM NaCl at pH 7.4) was injected intratracheally under visual control (Gonzalez *et al.* 2007). Control groups were given the same volume of phosphate-buffered saline or a suspension containing empty liposomes (without clodronate) or received no treatment. All rats recovered within 20 min after the intratracheal instillation. In animals exposed to hypoxia, this procedure was performed just before the initiation of the exposure and was repeated on days 5 and 10 of hypoxia (the latter only in the group exposed to hypoxia for 15 days).

Hypoxic exposures

The rats were either bred air, or exposed to hypoxia (FiO_2 0.1) for 4, 10 or 15 days in a normobaric hypoxic chamber, as previously described (Hampl and Herget 1990).

The experimental groups are denoted with combinations of the letters c for clodronate, l for liposomes without clodronate, s for phosphate-buffered saline intratracheal instillation (without clodronate), N for normoxia and H4, H10 and H15 for 4, 10 and 15 days of hypoxic exposure, respectively (e.g. 15H-c for clodronate-treated rats exposed to hypoxia for 15 days).

Detection of AM depletion

The effectiveness of clodronate-containing liposomes in depleting AM was estimated four days after liposome administration by visually counting AM in suspension obtained using bronchoalveolar lavage (BAL) stained by hematoxylin-eosin and trypan blue techniques. The BAL was carried out on animals euthanized by sodium thiopental overdose (250 mg/kg i.p.). The trachea was cannulated and saline (10 ml) was instilled slowly into the lungs. The suspension was then removed and the washout was repeated 8 times. The BAL fluid was centrifuged for 10 min at 2500 rpm and the cell pellets were pooled and resuspended in 1 ml of saline. The total number of living cells in BAL was counted under a microscope using trypan blue staining dissolved in physiological salt solution. The number of macrophages in BAL was calculated from the percentage of AM in a smear fixed by ice-cold methanol and stained by hematoxylin eosin.

MCP-1 measurement

Serum concentrations of MCP-1 were detected by a standard sandwich ELISA kit from Invitrogen Corporation in serum obtained by centrifugation of blood acquired by syringe puncture of the descending aorta.

Hemodynamic and morphologic measurements

Mean pulmonary arterial blood pressure (MPAP, mm Hg) was recorded in rats anesthetized with sodium thiopental (50 mg/kg b.w. i.p.; ICN Czech Republic, Roztoky, Czech Republic) and spontaneously breathing room air. A polyethylene catheter was inserted in the pulmonary artery *via* the right jugular vein (Herget and Paleček 1972). A separate batch of identically treated rats was used to measure right ventricular weight. These rats were euthanized by an overdose of sodium thiopental (250 mg/kg i.p.) and the hearts were removed from the chest. The right heart ventricle (RV) and the left ventricle plus septum (LV+S) were separated, their wet weight was measured and the RV/LV+S ratio was calculated (Hampl and Herget 1990).

Supplemental in vitro experiment

The concentration of MCP-1 was measured in media of AM cultivated in normoxia or hypoxia (10 % O_2) after 1, 6, 24, and 96 hours, as well as in the lysate of AM after 4 days of normoxia or hypoxia. Additionally, MCP-1 concentration was assayed in the lysate of AM isolated immediately after a 4-day exposure

of rats to hypoxia (10 % O_2, n=8) or living in normoxia (n=6).

The AM were obtained by BAL as described above, except that 5 x 8 ml of Hanks buffered salt solution was used for washout. The suspension of cells was centrifuged for 10 min at 1500 g. The sediment was resuspended in 400 µl of Dulbecco's Modified Eagle's Medium. Half of the samples were then incubated in hypoxia, half in normoxia, and MCP-1 was periodically measured in the media of all samples as described above. After 4 days of hypoxia, the cell suspensions were lysed by freezing, resuspended in 150 µl of PBS and centrifuged at 20000 g. The pellets were removed and supernatants were used to measure MCP-1. The total protein concentration in the samples was assayed using a BCA protein assay kit (Pierce No. 23225) and did not differ between the groups.

All chemicals and kits, unless stated otherwise, were from Sigma-Aldrich, Prague, Czech Republic.

The statistical analysis was performed by ANOVA with Tukey's multiple comparisons post hoc test; all results are expressed as mean ± SEM. P<0.05 was considered significant.

Results

AM depletion

As expected, the administration of clodronate-containing liposomes reduced the AM count in BAL obtained from rats exposed to hypoxia for 4 days (Fig. 1).

Effect of AM depletion and hypoxia on the serum MCP-1 levels

Four days of exposure to hypoxia induced a rise in the serum level of MCP-1 (Fig. 2). However, in contrast to our hypothesis, this increase was unaffected by the AM depletion by clodronate. After ten days of hypoxia, the serum MCP-1 concentrations returned to the control level and, again, were not affected by the clodronate treatment (Fig. 2).

Effect of clodronate on the pulmonary artery blood pressure

MPAP was elevated in all rats exposed to 15 days of hypoxia (Fig. 3). This increase was significantly reduced (but not eliminated) in the hypoxic group repeatedly treated with clodronate-containing liposomes when compared with the hypoxic group treated with solvent only (Fig. 3).
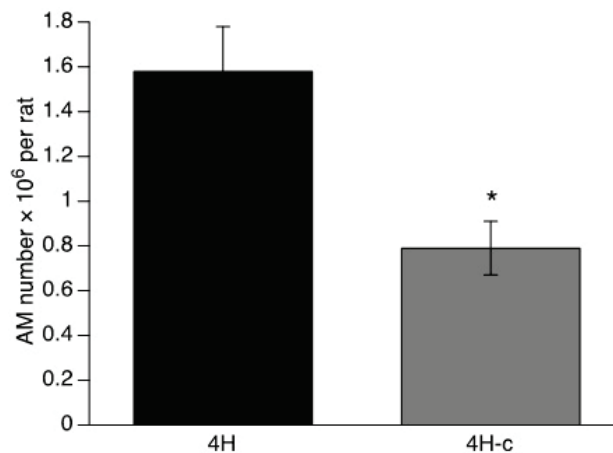

Fig. 1. Intratracheal instillation of clodronate-containing liposomes markedly reduces the number of alveolar macrophages in rats exposed to hypoxia for 4 days. 4H = rats exposed to 10 % O_2 for 4 days with no other treatment (n=6), 4H-c = rats exposed to 10 % O_2 for 4 days treated intratracheally with clodronate-containing liposomes (n=6). * P=0.0085.
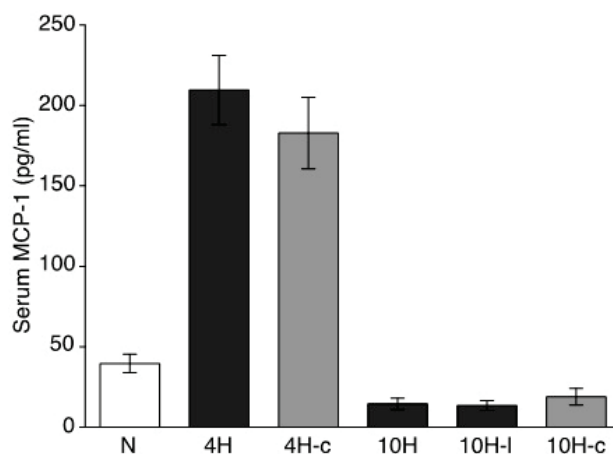

Fig. 2. Chronic hypoxia transiently elevates serum MCP-1 levels, and this effect is not affected by alveolar macrophages depletion by clodronate treatment. N = normoxic control rats (n=12), 4H = rats exposed to 10 % O_2 for 4 days with no other treatment (n=7), 4H-c = rats exposed to 10 % O_2 for 4 days treated intratracheally with clodronate-containing liposomes (n=7), 10H = rats exposed to 10 % O_2 for 10 days with no other treatment (n=8), 10H-l = rats exposed to 10 % O_2 for 10 days treated with clodronate-free liposomes (n=7), 10H-c = rats exposed to 10 % O_2 for 10 days treated with clodronate-containing liposomes (n=8). Only the 4H and 4H-c groups differ from all other groups (P<0.0001), but not from each other.

Effect of clodronate on right ventricular hypertrophy

Right ventricular hypertrophy, as assessed by significantly elevated RV/LV+S ratio, was induced by 15 days of hypoxia in rats treated with vehicle, but not in those treated with clodronate (Fig. 4). However, the difference in RV/LV+S between 4H-s and 4H-c did not reach statistical significance.
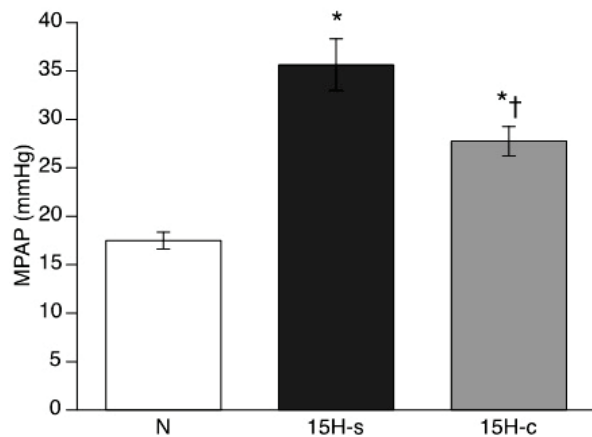
Fig. 3. Depletion of alveolar macrophages by clodronate reduces pulmonary hypertension induced by 15 days of hypoxia. MPAP = mean pulmonary arterial blood pressure, N = normoxic control rats (n=4), 15H-s = rats exposed to 10 % O_2 for 15 days treated intratracheally with phosphate-buffered saline (n=8), 15H-c = rats exposed to 10 % O_2 for 15 days treated with clodronate-containing liposomes (n=8). *$P<0.05$ vs. the N group.[†] $P≤0.05$ vs. the 15H-s group.

Fig. 5. MCP-1 accumulation in the media of cultured alveolar macrophages is unaffected by hypoxic conditions. The values at 1 hour after the onset of cultivation are below the detection limit (~15 pg/ml). * $P<0.05$ vs. relevant group at 1 hour. The differences between hypoxic and corresponding normoxic groups are not significant. n=4 per group, except for t=1 hr, where n=8 per group.

Discussion

This study was designed to explore the hypothesis that AM participate in the mechanism of HPH by activating MC through an increased production of MCP-1. In support of this hypothesis, we found that MCP-1 concentration in serum was elevated on the 4th day of hypoxic exposure and returned to control level by the 10th day of exposure. Furthermore, experimental depletion of AM by clodronate attenuated the chronic hypoxia-induced increases in MPAP and in the right heart ventricle relative weight. However, two findings contradict our hypothesis. First, AM depletion did not affect the serum MCP-1 concentrations in hypoxia. And second, MCP-1 production by AM *in vitro* was unaffected by hypoxia. Thus, the results taken together confirm that AM do have a significant role in the mechanism of HPH, but it is not mediated by MCP-1. Other sources than AM must participate in MCP-1 production in hypoxia, but regardless of its source, circulating MCP-1 does not participate in the mechanism of HPH.
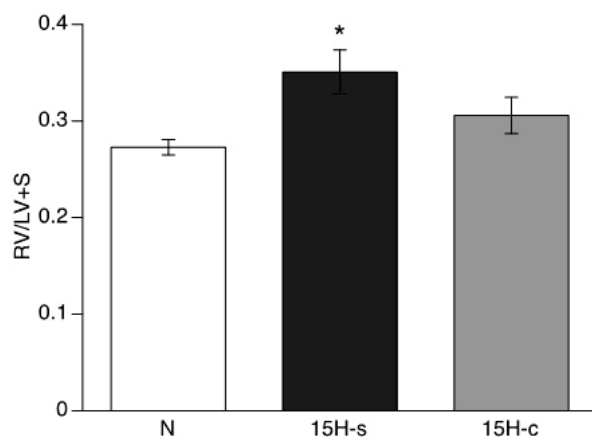

Fig. 4. Depletion of alveolar macrophages by clodronate prevents right ventricular hypertrophy during 15 days of hypoxia. N = normoxic control rats (n=6), 15H-s = rats exposed to 10 % O_2 for 15 days treated intratracheally with phosphate-buffered saline (n=6), 15H-c = rats exposed to 10 % O_2 for 15 days treated with clodronate-containing liposomes (n=6). * $P<0.05$ vs. the N group. All other differences are not significant.

Effect of hypoxia on AM production of MCP-1 in vitro

In vitro cultivation of AM resulted in an increase in MCP-1 concentration in media that reached a plateau after 24 hours. This pattern, however, was unaffected by cultivation under hypoxic conditions (Fig. 5). The content of MCP-1 in AM lysate after 4 days of hypoxic cultivation was under the detection limit of the ELISA method used (~15 pg/ml), as was that of cells cultivated under normoxic conditions. The same was true for lysates of AM freshly obtained from rats at the end of their 4-day exposure to hypoxia and from normoxic control rats.
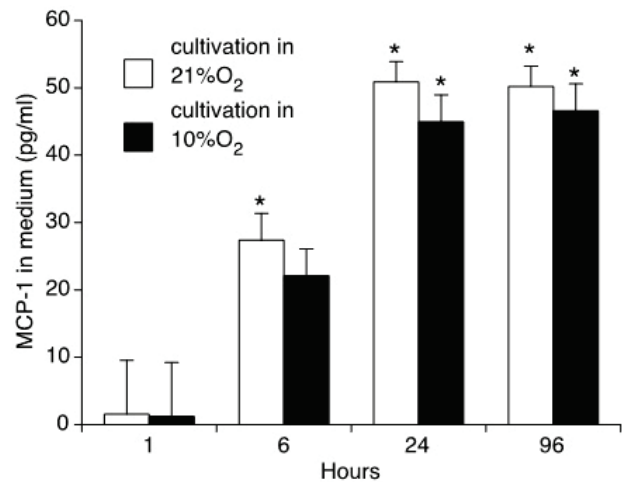
The mechanism/s underlying the development of hypoxic pulmonary hypertension is still far from being clear, even though an increasing number of studies imply the involvement of inflammation and monocytes/macrophages. Interestingly, Chao *et al.* (2011) reported that hypoxia-induced systemic inflammation is mediated by mast cells activated by AM-derived MCP-1. The

increase in plasmatic concentration of MCP after 4 days of hypoxia expanded on the results of Chao, who found a similar increase 60 min after hypoxic exposure. Surprisingly, AM depletion did not affect this increase. This fact can be explained by the duration of hypoxic exposure. Chao *et al.* (2011) found different concentrations of MCP between control and AM-depleted rats after 90 min, it is therefore possible that prolonged hypoxia stimulated MCP production also in other cells. The production of MCP-1 by other cells than AM, including endothelial, fibroblasts, epithelial or smooth muscle, is well documented (Deshmane *et al.* 2009), however, information about the effects of hypoxia on this production are scarce and controversial (Bosco *et al.* 2004, Chao *et al.* 2011).

The reducing effect of AM depletion on MPAP corresponds well with reports showing that the development of HPH is preceded and/or accompanied by an inflammatory response involving AM (Minamino *et al.* 2001, Frid *et al.* 2006, Hayashida *et al.* 2005). More recently, Vergadi *et al.* (2011) demonstrated a link between M2 polarized AM and the promotion of HPH.

The finding that *in vitro* hypoxia did not affect MCP-1 production by isolated AM contrasted with the results of Chao *et al.* (2009), who reported an elevation of MCP-1 production by hypoxia in comparable settings. On the other hand, Bosco *et al.* (2004) reported that hypoxia inhibits MCP-1 production in cell lines of mouse macrophages and T-lymphocytes.

Our results strongly refute the role of circulating MCP-1 in the HPH mechanism. The possibility that MCP-1 participates in inducing HPH has also been questioned recently by a report that mice with a CCR2 (MCP-1 receptor) deficiency displayed more severe HPH than those with CCR2 intact (Yu *et al.* 2013).

In conclusion, we have shown that AM participate significantly in the mechanism of HPH and in accordance with Hashimoto-Kataoka *et al.* 2015 they are supposed to be a potential target for treating of pulmonary hypertension. However their involvement is not mediated by MCP-1, despite the fact that the levels of circulating MCP-1 transiently increase in hypoxia (from other sources than AM).

Conflict of Interest
There is no conflict of interest.

Acknowledgements

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