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The effect of exposure to hypoxia on superoxide formation by alveolar macrophages is indirect

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ABSTRACT

Aims: To elucidate the role of alveolar macrophages (AM) in the pathogenesis of hypoxic pulmonary hypertension (HPH), we tested the effects of sustained hypoxia on AM polarization and on the formation of superoxide by AM *in vivo* and *in vitro*.

Main methods: Rat AM were obtained by bronchoalveolar lavage. 4-day exposure to hypoxia (10% O2) was carried out *in vivo* (rats in isobaric hypoxic chamber, controls kept in air) or *in vitro* (control AM in 21% O2 and 5% CO2). Superoxide production was measured by luminol-orthovanadate chemiluminescence, AM polarization was detected immunocytochemically. To ascertain the effect of substances contained in the alveolar environment, we cultivated cells also in the presence of non-cellular components of the bronchoalveolar lavage fluid (BALF) either from controls or from rats exposed to 4 days of hypoxia.

Key findings: In vivo, but not *in vitro*, hypoxia increased AM superoxide production. Both types of hypoxia polarized AM into M2 (pro-proliferative) type. While the presence of control BALF attenuated superoxide production in AM cultivated in normoxia, BALF from the hypoxia-exposed rats had no effect. In AM cultivated in hypoxia, superoxide production was not altered by control BALF and elevated by BALF obtained from hypoxic rats.

Significance: Hypoxia does not influence superoxide production by AM directly but rather by modulating their milieu and their sensitivity to external influences.

1. Introduction

Oxidative stress during early phase of chronic hypoxia has been implicated as an important pathogenetic factor of hypoxic pulmonary hypertension (HPH) [1]. The increased formation of reactive oxygen species (ROS) in the lung tissue is well documented during the early phase of hypoxic exposure, as is the attenuation of HPH by antioxidant therapy [2]. Although the specific source of ROS activated by hypoxic exposure has not yet been determined, alveolar macrophages (AM) are prime candidates. AM activation has been shown as critical for later development of HPH [3,4], it is accompanied by increased ROS formation [5], and AM isolated from lungs of rats exposed to 3 days of hypoxia were shown to increase hydrogen peroxide formation [5]. However, the mechanism increasing the ROS formation by AM remains unclear.

Therefore, in the current study, we tested whether hypoxia affects the AM directly and whether the increase of ROS production (specifically, we looked at superoxide production) is due to polarization of AM into M1 type that is known to be able (in contrast to the M2 type) to increase superoxide production [6]. Because ROS formation in the lung tissue increases during first few days of hypoxia and this period seems to be critical for the initiation of the pulmonary vascular remodeling [7], we focused on a 4 days duration of the hypoxic exposure. To distinguish between direct effects of hypoxia on AM and effects mediated by alterations in AM milieu, we looked at differences between *in vivo* and in *in vitro* exposure to hypoxia. Because the final action of AM depends on their polarization status (M1 characterized as the pro-inflammatory type, associated with increased ROS production, M2 known as the “wound healing”, pro-proliferative type [6]), the changes in the proportion between M1 and M2 polarized AM in hypoxia were also studied.

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2. Methods

Twenty seven male Wistar rats were used. All experimental procedures were performed in accordance with the European Union and NIH guidelines and approved by the Animal Studies Committee of the Second Medical School, Charles University, Prague.

2.1. In vivo exposure to hypoxia

The experimental animals ($n = 6$) were exposed to hypoxia (FiO2 0.1) for 4 days in a normobaric hypoxic chamber [8], 6 control rats were kept in air. AM from both groups were harvested as described below [4]. Superoxide formation was measured immediately in the fresh AM obtained from bronchoalveolar lavage. One part of the samples was used for determination of the AM polarization.

2.2. In vitro exposure to hypoxia

AM from normoxic animals ($n = 15$) were exposed to hypoxia in Lab-Tek™ II Chamber Slide™ System (NUNC). 4 × 104 AM in 300 µl cultivation medium were seeded per chamber. The plates and slides were placed in two hypoxia incubator chambers (Stemcell Technologies); one chamber was filled with 21% O2 and 5% CO2 (normoxic conditions) and the other one with 10% O2 and 5% CO2 (hypoxic conditions [9]). Although the degree of hypoxia we used in our *in vitro* experiments may seem mild, it approximately corresponded to PO_2 in the environment of AM during *in vivo* hypoxia. Both chambers were cultivated for 4 days at 37 °C. A non-cellular part of the bronchoalveolar lavage fluid (BALF) from normoxic or hypoxia-exposed animals was administrated into the some plates. After completing the cultivation, the total amount of AM was counted in 5 plates per each group using haematoxylin eosin staining [10]. One part of the cultivated plates was used for the measurement of superoxide production, the other part was used for determination of the AM polarization.

2.3. Isolation of AM

The bronchoalveolar lavage was carried out immediately after the rats were euthanized by sodium thiopental overdose (250 mg/kg i.p.). The trachea was cannulated and 10 ml of phosphate buffered saline (PBS, NaCl 137 mM, KCl 2.7 mM, Na2HPO4 10 mM. KH2PO4 1.8 mM) was instilled slowly into the lungs. The suspension was then removed and the washout was repeated 8 times. The BALF was centrifuged for 10 min at 1500 × *g* at 4 °C. The cell pellets were pooled and resuspended in 1 ml of PBS. The total number of living cells was counted under a microscope using Trypan blue staining dissolved in PBS. The number of macrophages in BALF was calculated according to the percentage of AM from the total cell number in a smear of BALF fixed by ice-cold methanol and stained by haematoxylin eosin [10].

2.4. Isolation of non-cellular part of BALF

The cell-free supernatant from the first bronchoalveolar lavage of normoxic or hypoxic rats was used after centrifugation at 15000 × *g* for 45 min at 4 °C (removing debris). BALF was concentrated by ultrafiltration on Amicon centrifugal filters (cut off 3 kDa). The resulting concentration of proteins was estimated from absorbance at 280 nm and pooled (normoxic or hypoxic) non-cellular parts of BALFs were diluted by PBS to concentration of 2.0 mg of protein/ml. These concentrated solutions were diluted 1:20 in cultivation media (thus, the concentration of surfactant proteins during cultivation was 100 µg/ml).

2.5. Measurement of superoxide production

Superoxide production experiments were performed in 96-well white sterile NUNCLON™ multidish plate (NUNC cat no 136101) and 2 × 104 AM in 200 µl cultivation medium were seeded per well. Superoxide production was measured by luminol-orthovanadate chemiluminescence [11] in a microtitre plate by luminometer Lumistar Optima and expressed in relative luminescence units (RLU). Briefly, a major part of the medium was gently aspirated from each well with the cultivated AM and only 50 µl of the medium was left in the well. Then warm 150 µl luminol reagent was added and the whole plate was immediately inserted in a luminometer and the measurement was started. Luminol reagent consists of 2 mM luminol and 1 mM sodium orthovanadate in Dulbecco's medium without phenol red, buffered by HEPES and adjusted to pH 7.4. Luminescence of the well was estimated each 300 s while the temperature was maintained at 37 °C during the measurement. The production of superoxide was calculated as the total sum of RLU registered within 5 h.

2.6. Determination of the AM polarization

The method of indirect immunocytochemistry was used for the detection of M1 and M2 markers in methanol-fixed smears or cell cultures. Mouse monoclonal antibody to rat M1 marker CD80 (BioLegend, San Diego, CA) and rabbit polyclonal antibody to rat M2 marker mannose receptor (Bioss, Woburn, MA) were diluted with saline 1:200 and 1:1600, respectively. The specimens were processed by the Expose rabbit-specific HRP/DAB detection IHC kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. Briefly, after blocking of endogenous peroxidase activity, the Protein Block was applied to prevent non-specific background staining. The 90 min room temperature incubation with mouse primary antibody was followed by the 10 min incubation with Mouse Specifying Reagent and 15 min incubation with HRP Conjugate. The 90 min room temperature incubation with rabbit primary antibody was followed by the 15 min incubation with HRP Conjugate only. After short visualization of the specimens with a mixture of DAB Chromogen and DAB Substrate, the nuclei were briefly counterstained with haematoxylin. The number of positively marked cells was counted from the random sample of 500 cells from each chamber box [10].

2.7. Statistical analysis

The statistical evaluation was performed using un-paired *t*-test or ANOVA followed by Fisher's post-hoc test as appropriate. The differences were considered significant when $p < 0.05$. The results are reported as means ± S.E.M.

3. Results

4 days of *in vivo* hypoxia resulted in higher superoxide formation by AM (Fig. 1a) and increased AM polarization to M2 type (Fig. 2a). The M1/M2 ratio was 2.7 in normoxia and 0.5 in hypoxia.

By contrast, *in vitro* hypoxia did not affect superoxide production by AM (Fig. 1b). Nevertheless, the AM polarization to M2 type was similar as in *in vivo* hypoxia (Fig. 2b). The M1/M2 ratio was 1.2 in normoxia and 0.4 in hypoxia.

In our next experiment, the presence of non-cellular part of BALF obtained from normoxic animals (N-BALF) attenuated the superoxide production by AM cultivated *in vitro* in normoxia while the presence of the non-cellular part of BALF from rats exposed to hypoxia (H-BALF) did not significantly influence superoxide production in these cells (Fig. 3a). However the reducing effect of N-BALF on the superoxide production was not present in AM cultivated in hypoxia. Moreover, a combination of hypoxic cultivation and the presence of H-BALF caused a significant increase of AM superoxide production (Fig. 3b).

4. Discussion

Our results indicate that 1) hypoxia enhances AM superoxide

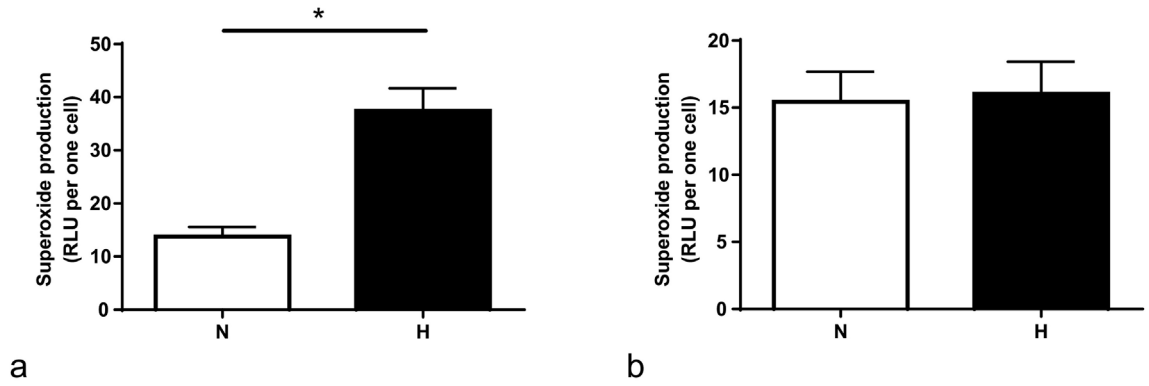

Fig. 1. a: Total amount of superoxide produced by non-stimulated alveolar macrophages exposed to *in vivo* hypoxia (AM harvested from rats exposed to 10% for 4 days, group H or from animals that lived in room air, group N). Expressed in relative luminescence units (RLU per one cell in 5 h) as the mean ± S.E.M. * $p < 0.01$.
b: Total amount of superoxide produced within 5 h from non-stimulated alveolar macrophages isolated from control animals. AM kept for 4 days in *in vitro* normoxia (N) or hypoxia (H). Superoxide production expressed as the mean ± S.E.M.

production indirectly by reducing the ihibiting effect of the alveolar environment and 2) the increased superoxide production does not result from elevated AM polarization to M1 type.

AM play important role in the development of HPH as indicated by HPH attenuation by AM depletion [4]. It is also well documented that early phase of hypoxic exposure is accompanied by increased ROS formation and subsequent oxidative injury [12]. This injury is believed to trigger HPH inasmuch antioxidant application attenuates HPH [13]. Although AM have already been shown to increase hydrogen peroxide formation in hypoxia [14] we decided to measure superoxide production as the first massive step of ROS formation. Although many methods of superoxide detection are put into practice [15,16], their use is very limited due to low specificity or sensitivity. Because our measurements required a sensitive method, we used luminol-orthovanadate chemiluminescence recommended by Sohn et al. [11].

Our finding of enhanced formation of superoxide after *in vivo* hypoxia contributes to the conclusion that ROS produced by AM participate in the pathogenesis of HPH. However, precaution is needed for two reasons: the proof that the detected change in ROS production is sufficient to cause oxidative injury is missing, and we measured superoxide production of isolated AM *in vitro,* not *in situ*. Altering factors possibly present in the pulmonary alveolar environment were thus eliminated in our measurements [17].

One question addressed in our experiment concerned the possibility that the hypoxia-induced increase in superoxide production was due to polarization of AM. When activated, AM typically polarize into M1 or M2 type [6]. While M1 macrophages are characterized as pro-inflammatory with increased expression of systems producing ROS, M2 type is defined as pro-proliferative type without the augmented ability to form ROS [6]. The immunohistochemical staining of AM markers CD80 for M1 [18] and mannose receptor (also known as CD206) for M2 [19] is a generally accepted method for evaluating macrophage polarization [20,21]. Because we observed a reduction in M1/M2 ratio in hypoxia (both *in vivo* and *in vitro*), the hypoxic increase in superoxide production cannot be assigned to polarization of AM to M1 type. In fact, the increased proportion of pro-proliferative M2 AM suggests their involvement in the remodeling of pulmonary vessels associated with HPH [22].

Surprisingly, exposure of isolated AM to hypoxia *in vitro* did not exhibit any effect on their production of superoxide. This implies that the effect of hypoxia is not direct, but mediated by some change in their environment. It is interesting that although *in vitro* hypoxia did not affect the superoxide production by AM, the M1/M2 ratio of AM was the same after both types of hypoxia, indicating that the polarization of AM into M2 type is a direct effect of hypoxia [3]. Although Vergadi et al. [3] used a different model of hypoxia, the exposure to hypoxia resulted in the same type of polarization as in our study.

To elucidate the influence of AM environment on their superoxide formation, we tested the effect of a fluid obtained by bronchoalveolar lavage from lungs of either controls or hypoxia exposed rats on AM

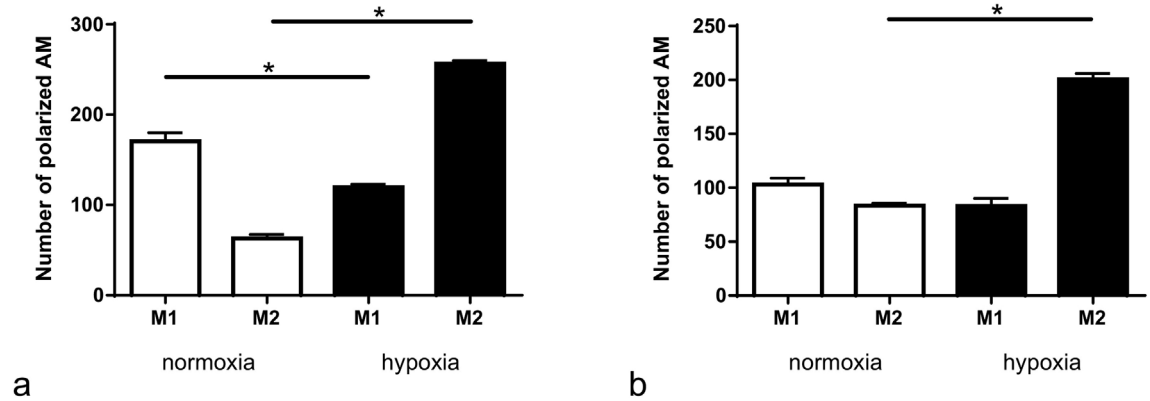

Fig. 2. a: Counts of M1 and M2 types of AM from rats exposed to 4 days of hypoxia and normoxic controls. Counted from the random sample of 500 cells from each animal. Expressed as the mean ± S.E.M. * $p < 0.01$.
b: Counts of M1 and M2 types of AM when AM were kept *in vitro* in normoxia or exposed to 4 days to hypoxia. Counted from a random sample of 500 cells from each chamber. Expressed as the mean ± S.E.M. * $p < 0.01$.

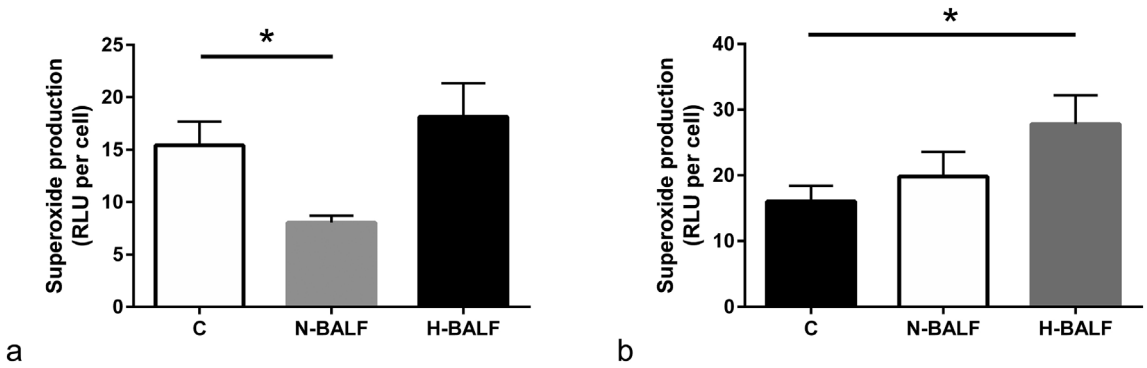

Fig. 3. a: Total amount of superoxide produced per one cell within 5 h from non-stimulated alveolar macrophages cultivated in normoxia in presence of: 1) the cultivation medium only (C), 2) non-cellular part of BALF isolated from normoxic animals (N-BALF), 3) non-cellular part of BALF isolated from animals exposed to hypoxia (H-BALF). Expressed as the mean ± S.E.M. * $p < 0.02$.
b: Total amount of superoxide produced per one cell within 5 h from non-stimulated alveolar macrophages cultivated in hypoxia in presence of: 1) the cultivation medium only (C), 2) non-cellular part of BALF isolated from normoxic animals (N-BALF), 3) non-cellular part of BALF isolated from animals exposed to hypoxia (H-BALF). Expressed as the mean ± S.E.M. * - $p < 0.05$.

superoxide production *in vitro*. First, we verified that the presence of substances contained in N-BALF or H-BALF did not affect the chemiluminescence detection (data not shown). Second, we showed that the presence of non-cellular part of BALF from normoxic animals attenuated the superoxide production by AM in normoxia. This shows that non-cellular components of BALF attenuate superoxide production by AM in physiologic situations. It is in agreement with the previous observations of Hayakawa [23]. However, the same non-cellular part of BALF from normoxic animals did not affect superoxide production in AM cultivated in hypoxia. This suggests that hypoxia decreases the sensitivity of AM to the superoxide production-lowering effect of non-cellular part of BALF from normoxic animals. Alternatively, it is possible that the properties of N-BALF could be altered during the 4 days of hypoxic incubation with the AM. Third, we found that the presence of non-cellular part of BALF from hypoxic animals was without effect in AM cultivated in normoxia and increased superoxide production in AM cultivated in hypoxia. This implies that hypoxia alters the properties of BALF in such a way that it gains a stimulatory effect on the superoxide production in the hypoxia-exposed AM.

An important substance interacting with AM in the alveolar space is the pulmonary surfactant [24,25]. It is thus possible that the modulation of AM superoxide production is mediated by binding of free fatty acids contained in the surfactant to the PPAR-γ receptors on the AM surface [26,27], resulting in attenuation of the respiratory burst in these cells [23,28,29]. The composition of surfactant is modified by hypoxic exposure [30]. Thus, hypoxic alterations of the surfactant might be underlying the altered properties of H-BALF that we observed.

Although we demonstrate in this study that exposure of rats to hypoxia results in increased superoxide formation by AM, and we and others have shown previously that ROS contribute significantly to the mechanism of HPH [12], it remains unclear whether the ROS production by AM in hypoxia is quantitatively sufficient to instigate the tissue injury resulting in HPH. However, the effect of ROS produced by AM does not have to lay only in the direct cytotoxicity of ROS. Instead AM may exert their role through a local signaling function of ROS (for which very small quantities are sufficient) [31]. More experiments are needed to clarify this issue.

5. Conclusions

Our study shows that the effect of hypoxia on AM is dual. While the polarization of AM into the M2 type is induced by hypoxia itself, the increased superoxide formation is mediated by modification of the AM environment and altered AM sensitivity to it. The alveolar milieu is altered by hypoxia in such a way that its normal inhibitory effect on the AM superoxide production is reduced or even reversed. In addition, hypoxia also diminishes the sensitivity of AM to the inhibiting effect of their environment on the superoxide formation. These changes together bring about a net increase in superoxide generation by AM in hypoxia.

Declaration of competing interest

There is no conflict of interest.

Acknowledgments

This paper is dedicated to the memory of the late Professor Jan Herget, our colleague who had initiated the idea of hypoxic alteration of surfactant as a possible pathogenetic mechanism in HPH. He has passed away on March 14, 2019.

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