The contractile response of isolated small pulmonary arteries induced by activated macrophages.

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Running head: Pulmonary arterial vasoconstriction induced by macrophages

Summary

To test whether macrophages can play any role in hypoxic pulmonary vasoconstriction, we tested the in vitro response of rings from small pulmonary arteries to the activation of macrophages by FMLP, a substance stimulating predominantly membrane-bound NADPH oxidase. A small vessel myograph was used to measure the responses of rings from small pulmonary arteries (300-400 μm) isolated from rat lungs. Rings from 5 rats were placed into both chambers of the myograph. The vessels were stabilized for 40 min and then normalized by automatic stretching to a wall tension equivalent to the intravascular pressure 30 mm Hg. At the start of each experiment, vessels were exposed to 80 mM K^+ to obtain

maximal contractile response, which was used to normalize subsequent contractile responses. $2x10^6$ viable macrophages, obtained by peritoneal lavage, were added into one chamber, then 5 μM FMLP was administered to both chambers and the tension measurement was started. The hydrogen peroxide concentration produced by stimulated macrophages was measured luminometrically. The concentrations of H_2O_2 in specimens from chambers containing activated macrophages rose from 3.5±1.5 nM to 110±28 nM within 25 min of stimulation, while FMLP itself didn't increase the H_2O_2 concentration from the baseline value (4.5±3 nM) in samples from control chambers. After FMLP administration, the tension of the vessel rings in the presence of macrophages reached 0.23±0.07 of maximal contractile response, it did not change in controls. The addition of ROS scavenger 4-hydroxy-TEMPO blocked the contractile response to the activation of macrophages. We conclude that the activation of macrophages stimulates the contraction of small pulmonary arteries and that this contraction is probably mediated by reactive oxygen species.

Key words

Isolated pulmonary arteries, reactive oxygen species, macrophage.

Main body of the text

Acute hypoxia causes reversible vasoconstriction of peripheral pulmonary vessels – hypoxic pulmonary vasoconstriction (HPV) redirecting the blood flow to better-ventilated areas. The mechanism of this response still remains unclear. Experiments showing that hypoxia stimulates alveolar macrophages to produce, besides other substances, also reactive oxygen species (ROS) (Wilhelm *et al.* 1996) and that ROS in vitro experiments can contract pulmonary vessels (Sheehan *et al.* 1993; Tate *et al.* 1982), led us to speculate that macrophages can participate in a mechanism increasing vascular tone in hypoxia. We

therefore decided to test the response of vessel rings isolated from rat lungs to ROS released by macrophages.

Two sets of 5 adult male Wistar rats (234±75 g) were used. Experiments were performed in accordance with the European Community and NIH guidelines for using experimental animals. All procedures were approved by the Animal Studies Committee of the Second Medical School, Charles University, Prague.

Animals were euthanized by CO_2 inhalation. Heart and lungs were removed *en block* and placed into cold physiological salt solution (PSS). Two branches of the pulmonary arteries with internal diameters of 300-400 μm were isolated and dissected under microscopic control from each rat. The vessels rings (1.5-2.3 mm long) were then cleared of surrounding tissue and mounted by wires to the jaws of a small vessel myograph M 500A, Linton, Norfolk, GB. The tissue chamber contained 37 °C warm PSS (in mM): NaCl 118, $NaHCO_3$ 24, KCl 4, $CaCl_2$ 1.8, $MgSO_4$ 1, NaH_2PO_4 0.434, glucose 5.56 and was gassed continuously with 95% O_2 with 5 % CO_2. The pH of the mixture was maintained at ~7.4.

Rat peritoneal macrophages were isolated from each animal by peritoneal lavage as described in our previous study (Wilhelm *et al.* 1997). After the 10 min centrifugation at 2500 rpm, the supernatant was removed and cells dissolved in fresh cold PSS. The number of macrophages was counted under a microscope and their viability was assayed on the basis of Trypan Blue exclusion. The preparations contained 95±3% viable cells, 95% of which were positive for non-specific esterase, a marker of macrophage cell type (Hermanns 1987; Lavnikova *et al.* 1993).

The amount of hydrogen peroxide produced was assayed luminometrically during the whole experiment. This method is based on the reaction of H_2O_2 with luminol, catalysed by horse-radish peroxidase, as described by (Wilhelm *et al.* 1996). The maximum chemiluminescence (LDCL) was used to characterize H_2O_2 production. The background luminescence was tested

at the start of each measurement. The chemicals were obtained from Sigma–Aldrich Czech Republic.

The vessels were stabilized for 40 min and then normalized by the automatic stretching to a wall tension equivalent to the intravascular pressure 30 mm Hg. At the start of each experiment, vessels were exposed to 80 mM K^+ until a maximal contractile response was observed. K^+-rich solution was obtained by replacing an equimolar amount of NaCl for KCl in PSS. This maximal contraction served as a reference response and was used to normalize subsequent contractile responses. Resting tension remained unchanged throughout the experimental period. The 80 mM K^+ contraction was repeated at the end of each experiment to prove the viability of pulmonary arterial rings during the whole experiment.

In one test $2x10^6$ of viable macrophages was added to one of the chambers of the myograph, the other chamber was used as a control without macrophages. The chemotactic peptide N-formyl-methionyl-leucylphenylalanine (FMLP) was immediately administrated to reach its final concentration 5 μM. Contractile responses of both rings were registered for 25 min with the continuous measurement of hydrogen peroxide production in samples from both chambers of the myograph. In an additional experiment, FMLP administration was preceded by the administration of 0.58 mM 4-hydroxy-TEMPO.

All results are expressed as mean ± SEM. The statistical evaluation was performed using ANOVA and the Fishers *post-hoc* test.

FMLP administration to the experimental chambers (with macrophages) increased concentration of H_2O_2 and resulted in contraction of the vessel rings, while in control chamber H_2O_2 concentration as well as vessel rings tension remained unchanged (tab. 1). This response was abolished by preceding administration of 0.58 mM 4-hydroxy-TEMPO.

Our results show that rings from rat small pulmonary vessels contract in response to a mediator released by macrophages activated by FMLP. Because FMLP is known to activate

membrane-bound NADPH oxidase to increase the formation of superoxide (Wilhelm *et al.* 1996), which transformed immediately into H_2O_2 (El-Benna *et al.* 2010) and the contraction was blocked by the administration of ROS scavenger 4-hydroxy-TEMPO, we conclude that ROS are the relevant stimulus for the contraction. Because the main effect of 4-hydroxy-TEMPO is the stimulation of superoxide dismutase, we presume that the response is mediated by superoxide, which has been shown to increase Ca^{2+} sensitivity in smooth muscle cells via regulation of Rho-kinase activity (Knock *et al.* 2009). It has been repeatedly shown that ROS induce the contraction of small pulmonary vessels (Jones *et al.* 1997; Knock *et al.* 2009), although few authors reported vasodilatation (Burke and Wolin 1987). This controversy may result from differences in the concentrations of ROS used (which were also far from those seen "in vivo" (Perez-Vizcaino *et al.* 2010; Raj and Shimoda 2002)) and from the fact that some authors tested the response to H_2O_2, while others the response to superoxide. Because the H_2O_2 concentrations in our experiments varied in nanomolar values and were close to those found by (Raj and Shimoda 2002) in lungs in situ and the formation of ROS induced by FMLP starts with the production of superoxide, we believe that our way of stimulation mimics the in vivo situation better. We can exclude the direct effect of FMLP on the vessel rings reported by (Crowell *et al.* 1989) since in our experiment the vessel tone of control vessels did not change after FMLP application. Similarly, 4-hydroxy-TEMPO administration had no effect on the tonus of vessel rings. We cannot, of course, exclude the involvement of other substances possibly released by activated macrophages, because FMLP triggers the "respiratory burst", the process causing the production of many other mediators (Panaro and Mitolo 1999) . We used peritoneal macrophages, since, in contrast to alveolar macrophages, they are not activated by adherence and their baseline ROS production is therefore lower and steadier (Wilhelm *et al.* 1997; Wilhelm *et al.* 1996; Žaloudíková 2012).

For macrophages stimulation, we preferred FMLP to the other commonly used stimulant of ROS production – phorbol 12-myristate 13-acetate – because we found in experiments of (Barman and Ikeda 1995) that phorbol 12-myristate 13-acetate mediates pulmonary vascular reactivity.

Our results together with the fact that hypoxic macrophages release ROS (Wilhelm *et al.* 2003) suggest their possible role in acute and/or chronic lung vascular response to hypoxia.

Conflict of Interest

There is no conflict of interest.

Acknowledgments

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Group (n=5)	The contractile response (% of maximal contraction)	H_2O_2 (nM)
FMLP	0	4.5 ± 3
FMLP + macrophages	23 ± 7	110 ± 28
4-hydroxy-TEMPO + FMLP	0	2.8 ± 3
4-hydroxy-TEMPO + FMLP + macrophages	0	83 ± 21

Table 1: The concentrations of H_2O_2 in the bath of myograph and the responses of the pulmonary vessel rings. FMLP - N-formyl-methionyl-leucylphenylalanine. Values are means ± SEMs.

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