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Low-dose adrenomedullin-2/intermedin$_{(8–47)}$ reduces pulmonary ischemia/reperfusion injury☆

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ABSTRACT

Adrenomedullin-2/intermedin stabilizes the pulmonary microvascular barrier challenged by application of thrombin ex vivo and by experimental ventilation in vivo. Here, we test the hypothesis that adrenomedullin-2/intermedin$_{(8–47)}$ protects mouse lungs from ischemia/reperfusion injury in vivo. C57BL/6 mice were anesthetized, intubated, ventilated, and heparinized. Blood vessels and the main bronchus of the left lung were clamped for 90 min. Thereafter, lungs were reperfused for 120 min. Five min before clamping and before reperfusion, mice obtained intravenous injections of adrenomedullin-2/intermedin$_{(8–47)}$. After reperfusion, mice were sacrificed and bronchoalveolar lavage of the left and the right lung was performed separately. The integrity of the blood–air barrier was investigated by electron microscopy using stereological methods. In response to ischemia/reperfusion injury, intraalveolar leukocytes accumulated in the ischemic lung. Two applications of 10 ng/kg body weight adrenomedullin-2/intermedin$_{(8–47)}$ dramatically reduced leukocyte infiltration to about 15% ($p \leq 0.001$). Also the proportion of the subpopulation of neutrophil granulocytes decreased (12% vs 5%, $p = 0.013$). Electron microscopy revealed a protection of the blood–air barrier by adrenomedullin-2/intermedin$_{(8–47)}$. Adrenomedullin-2/intermedin$_{(8–47)}$ ameliorates early ischemia/reperfusion injury in mouse lungs by protecting the integrity of the blood–air barrier and by potently reducing leukocyte influx into the alveolar space. Adrenomedullin-2/intermedin$_{(8–47)}$ might be of therapeutic interest in lung transplantation and cardiopulmonary bypass.

Introduction

Ischemia/reperfusion injury (IRI) of the lung is a severe and life threatening complication of lung transplantation and surgery involving cardiopulmonary bypass [3,5]. IRI results in contraction of pulmonary blood vessels, an impaired blood–air barrier, plasma extravasation, leukocyte infiltration, and in pulmonary dysfunction or failure [5,8]. During the first month after lung transplantation, graft failure is the most important reason for graft loss and recipient death. Furthermore, IRI is an important risk factor for the development of pulmonary infections, acute rejection episodes, and bronchiolitis obliterans syndrome, which limit the success of lung transplantation in the long run [15].

Increasing evidence suggests that adrenomedullin-2/intermedin (AM2/IMD) [24,26], a member of the calcitonin gene-related peptide (CGRP) family, may attenuate pulmonary IRI. CGRP, adrenomedullin (AM) and AM2/IMD bind to a receptor complex composed of the calcitonin-receptor-like receptor (CLR) and one of three receptor-activity modifying proteins (RAMP1, RAMP2 or RAMP3) [2,12]. AM2/IMD has been reported to bind to all three receptor complexes. There is, however, some controversy concerning receptor selectivity and affinity in comparison to CGRP and AM2 [2,12]. The existence of a fourth yet unknown AM2/IMD receptor was suggested based on pharmacological evidence [12].

☆ "Take home" message: Adrenomedullin-2/intermedin$_{(8–47)}$ ameliorates ischemia/reperfusion injury in mouse lungs and reduces leukocyte influx.

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CLR is a seven transmembrane G protein coupled receptor. Upon binding of an appropriate agonist to the CLR/RAMP complex, predominantly adenylate cyclase is activated, the cytosolic cAMP level rises followed by activation of protein kinase A [12]. There are three known isoforms cleaved from the prepro-peptide, which have different biological activities, IMD_{1-47}, Imd_{8-47} and Imd_{1-53} [2,12].

AM2/IMD is widely expressed including, but not limited to, the lung, and its expression is upregulated by hypoxia [23]. In the lung, it acts as a vasodilator lowering perfusion pressure [2,14,20], stabilizes endothelial permeability under conditions of raised venous pressure and ventilator-induced lung injury [20,23], and inhibits formation of lipid oxide products in oleic acid-induced acute lung injury [29]. Its effects in IRI yet have not been investigated in the lung, but it is markedly protective in cardiac IRI including reduction of infarct size, improvement of hemodynamic parameters and attenuation of apoptosis, oxidative and endoplasmic reticulum stress [22,25,27,28]. In analogy to CGRP and AM, anti-inflammatory effects of AM2/IMD can be expected, especially in mononuclear phagocytes [1,4,9,11], which play a central role in pulmonary IRI [5,17]. Mouse bone marrow macrophages carry AM2/IMD receptors, and osteoclast differentiation is inhibited by AM2/IMD [10] but specific effects on pulmonary macrophages have not been reported yet.

In this study, we investigate the therapeutic potential of exogenous $AM2/IMD_{8-47}$ in an in vivo model for acute IRI in the mouse, which involves 90 min of warm ischemia and 120 min of reperfusion of left lungs. We demonstrate that $AM2/IMD_{8-47}$ is highly effective in preventing leukocyte influx into the alveolar space and protects the integrity of the blood–air barrier.

Materials and methods

Animals and experimental model

Animal care and animal experiments were performed following the current version of the German Law on the Protection of Animals as well as the NIH "Principles of laboratory animal care". Male C57BL/6 mice weighing 23–27 g were raised under pathogen free conditions and provided by Charles River (Sulzfeld, Germany). Anesthesia was induced by intraperitoneal injection of 50 mg ketamine (Inresa, Freiburg, Germany) and 20 mg xylazine hydrochloride (Bayer Vital, Leverkusen, Germany) per kg body weight. Mice were tracheotomized, intubated, and ventilated (MiniVent mouse ventilator, Hugo Sachs Elektronik, March, Germany) with air containing 1% isoflurane (Baxter, Unterschleissheim, Germany) at a respiratory rate of 150 strokes per min and a positive end expiratory pressure of 1 cm H_2O. The tidal volume was 220 μl when both lungs were ventilated and 150 μl after clamping the left hilum. Body temperature was kept at 37 °C during the procedure and 100 IU heparin (Ratiopharm, Ulm, Germany) was injected into the tail vein. The hilum of the left lung was exposed via the fifth intercostal space, carefully dissected and clamped for 90 min. During ischemia, the left lung was inflated. Thereafter, the clamp was removed and the lung was reperfused for 120 min. Five min before clamping the hilum and, in addition, five min before reperfusion, mice obtained injections of mouse $AM2/IMD_{8-47}$ (0.1–1000 ng per kg body weight, Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) into the tail vein. $AM2/IMD_{8-47}$ is the active fragment of the full-length AM2/IMD. Control treatment consisted of saline alone. During the period of ischemia as well as during reperfusion, the chest was temporarily closed. After reperfusion, the abdomen was opened and mice were exsanguinated. Bronchoalveolar lavage (BAL) of the left and the right lung was performed separately using 400 μl 0.9% saline at room temperature. Thereafter, lungs were excised and instilled with 4% freshly dissolved paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at a pressure of 15 cm H_2O for 15 min, followed by 24 h immersion in the same fixative at 4 °C and embedded in paraffin. Control lungs were taken from healthy untreated C57BL/6 mice.

Analyses of BAL fluid (BALF)

BAL fluid (BALF) of individual lungs was spun down at 1200 × *g* for 10 min and the protein content of the supernatant was determined using the Micro BCA™ protein assay (Thermo Fisher Scientific Inc., Rockford, IL, USA). Total cell number was determined and differential cell counting was done on cytospins stained according to Pappenheim.

Immunohistochemical detection of active caspase-3

De-waxed and rehydrated paraffin sections underwent antigen retrieval by digestion with 0.5 mg/ml protease type XIV (Sigma–Aldrich, Taufkirchen, Germany) in 50 mM Tris–HCl buffer pH 7.6, 0.9% NaCl, for 15 min at room temperature followed by 1% H_2O_2 in PBS for 30 min. Unspecific binding of proteins was blocked by application of PBS, pH 7.2, 1% bovine serum albumin (BSA) (Serva, Heidelberg, Germany), 0.1% NaN_3 (p.a. Merck, Darmstadt, Germany) (PBS/BSA/NaN_3) for another 30 min at room temperature. Rabbit anti-active caspase-3 (ab2302, abcam, Cambridge, UK) was diluted in PBS/BSA/NaN_3 1:200 and incubated over night at 4 °C. Bound primary antibodies were detected using the mouse EnVision™ peroxidase system (Dako Cytomation, Glostrup, Denmark) in the presence of 5% heat inactivated normal rat serum (Harlan Winkelmann, Borchem, Germany). Peroxidase was detected with 3,3′-diaminobenzidine (DAB, Sigma–Aldrich). Sections were evaluated with an Olympus BX51 (Hamburg, Germany) microscope. Active caspase-3-positive cells were counted in at least seven optical fields per specimen at a 200-fold original magnification.

Stereology

For light (LM) and electron microscopic (EM) qualitative and stereological analysis, lungs were either fixed by vascular perfusion (edema quantification) or by instillation (analysis of blood–air barrier integrity) at hydrostatic pressure of 20 cm. The fixative contained 2% paraformaldehyde and 1.5% glutaraldehyde in phosphate buffer. After systematic uniform random sampling, the samples were osmicated, stained with uranyl acetate, dehydrated in an ascending ethanol series and finally embedded in glycol methacrylate (for LM) or in epoxy resin (for EM). For LM, 1.5 μm thick sections were prepared and stained with toluidine blue. For EM, ultrathin sections were cut and post-stained with uranyl acetate and lead citrate. At LM level, the point counting method was used to estimate the volume fraction of intra-alveolar edema and of the peribronchovascular space as described previously [19]. At EM level, the surface fraction of normal, swollen and fragmented blood–air barrier in relation to its total surface area was estimated by the intersection counting method as described earlier [18].

Statistics

Data are presented in box plots and when appropriate analyzed by the Kruskal–Wallis test followed by the Mann–Whitney rank sum test using SPSS software (SPSS software, Munich, Germany).