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Dysregulation of the renin-angiotensin system during lung ischemia-reperfusion injury

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Abstract

Objectives: Activation of the renin-angiotensin system leading to increased angiotensin-(1-7) (Ang-(1-7)) and decreased angiotensin 2 (Ang 2) levels may be a new therapeutic approach to reduce acute lung injury. Prolyl carboxypeptidase (PRCP) and prolyl oligopeptidase (PREP) are capable of hydrolyzing Ang 2 into Ang-(1-7). However, their relation with circulating Ang 2 levels after lung ischemia-reperfusion injury (LIRI) has never been explored. This study determines whether the activity and expression of PRCP and PREP in plasma and lung tissue is related to circulating Ang 2 levels in a murine model of LIRI.

Methods: LIRI in Swiss mice (6 animals per group) was induced by temporary left lung hilar clamping (1 h) followed by 0, 1 or 24 h of reperfusion. Animals in the sham group received thoracotomy only. PRCP activity was measured via RP-HPLC, PREP activity using a fluorogenic substrate and plasma Ang 2 levels via ELISA. Western blotting was used to determine the PRCP and PREP protein expression profiles in left lung tissue.

Results: Plasma Ang 2 levels significantly rise after lung ischemia and remain increased after 1 h and 24 h of reperfusion compared to the sham group. While a significant decrease in plasma PREP activity was found after 24 h of reperfusion, a transient increase in plasma PRCP activity was observed after ischemia. However, no correlation with plasma Ang 2 levels could be demonstrated. The activity profiles of PRCP and PREP and the protein expression of PRCP in the lung tissues remained unchanged after LIRI.

Conclusions: LIRI causes a dysregulation of circulating Ang 2 levels and plasma PREP activity, although no direct link between both phenomena could be shown. The activity profile of pulmonary PRCP and PREP was not significantly changed after

LIRI, which implies a minor role for local PRCP and PREP in the ischemic lung itself.

Key words: lung ischemia-reperfusion injury, angiotensin, prolyl carboxypeptidase, prolyl oligopeptidase, angiotensin-converting enzyme

Introduction

Lung ischemia-reperfusion injury (LIRI) is an inevitable consequence of lung transplantation. There is always a period of time, however short, that a harvested donor lung does not receive adequate ventilation and perfusion. A similar pathophysiological mechanism is involved in inflammatory pulmonary reactions after cardiopulmonary bypass or experimental techniques such as isolated lung perfusion [1]. In extreme cases, this inflammatory process may lead to possibly life-threatening disease states, such as the adult respiratory distress syndrome (ARDS) [2]. Several reports indicate that the renin-angiotensin system (RAS) contributes to the development of ARDS and overall, to acute lung injury [3,4]. In the RAS, the role of angiotensin 2 (Ang 2) emerges to the forefront. This octapeptide, which is found in the circulation and in many vital organs including the lungs, carries out several physiological actions and is thereby involved in the pathogenesis of numerous diseases such as hypertension, atherosclerosis, thrombosis and pulmonary disorders [5]. Focusing on its pulmonary function, elevated levels of Ang 2 have been shown to increase oxidative stress, vascular permeability and infiltration of inflammatory cells [6,7]. The level of circulating and locally present Ang 2 in the lung is influenced by many enzymes among which the proline-specific peptidases named angiotensin-converting enzyme 2 (ACE2, EC:3.4.17.23), prolyl oligopeptidase (PREP, EC:3.4.21.26) and prolyl carboxypeptidase (PRCP, EC:3.4.16.2) [8–11]. As shown in Figure 1, these enzymes cleave Ang 2 at the C-terminus causing the release of a single amino acid phenylalanine, leading to the generation of a new peptide angiotensin-(1-7) (Ang-(1-7)). In comparison to Ang 2, this heptapeptide Ang-(1-7) tips the balance towards vasodilatation and an anti-inflammatory state [12]. Therefore the Ang 2 degradation and thus the formation of Ang-(1-7) by ACE2, PREP and

PRCP is supposed to be closely regulated to maintain RAS homeostasis in the lung. Wösten-van Asperen *et al.* already reported that reduced pulmonary ACE2 activity and Ang-(1-7) levels mediate an increased inflammatory response, leading to reduced lung function and eventually to the development of ARDS. Moreover, animals with ARDS seemed to benefit from the administration of a cyclic form of Ang-(1-7) [4]. This is in agreement with Chen *et al.* who found that the unbalanced expression of angiotensin-converting enzymes and angiotensins contributes to acute lung injury caused by hind-limb ischemia-reperfusion in mice [13]. However, these studies solely focused on the activity of ACE2, neglecting a possible role for PRCP and/or PREP in the cleavage of Ang 2. The relation between the Ang 2 levels and PRCP and PREP activity has never been explored in a LIRI model. This study determines for the first time whether the activity and expression of PRCP and PREP is associated with circulating Ang 2 levels in a murine model of LIRI.

Materials and Methods

Animals

24 adult female Swiss-Webster mice (Janvier Laboratories, Saint-Berthevin, France) aged 6-8 weeks were kept in standard cages with an inverted 12 hour day/night cycle and *ad libitum* access to food and water. The study was approved by the ethical committee of the University of Antwerp (file number 2012-36). A sample-size calculation was performed based on a pilot experiment to determine the amount of animals in each group. Animals were treated according to the guidelines provided by our institution and the European Directive for Laboratory Animal Care (Directive 2010/63/EU of the European Parliament).

Surgical procedure

The microsurgical method has been extensively described previously [14]. In brief, the animal was anesthetized, intubated and ventilated with a positive end-expiratory pressure (PEEP) of 2 cm H₂O. A muscle-sparing left thoracotomy exposed the left pulmonary hilum, which was temporarily clamped with an atraumatic microvessel clamp (Fine Science Tools, Heidelberg, Germany). Animals in the sham group received thoracotomy only, without placement of the clamp. Induction of ischemia was confirmed by visible cessation of respiratory movement (cessation of ventilation) and whitening of the left lung (cessation of perfusion). Also, a steadily increasing resorption atelectasis was observed during ischemia. After the designated ischemia time of one hour, the animal was either immediately euthanized by cervical dislocation or its wound was closed in layers after removal of the clamp. It was then allowed to recover for the designated reperfusion time (1 h or 24 h), after which it was euthanized. The designated reperfusion times were chosen based on the results of earlier experiments [14], which showed an increase in inflammatory parameters after 1 hour of reperfusion which were gradually attenuated after 24 hours of reperfusion. Animals in the sham group were euthanized after one hour of ventilation. The left lung was subsequently excised and blood samples were collected, snap-frozen in liquid nitrogen and stored at -80 °C until analysis. The experimental design is shown in table 1.

Histological analysis

After resection, left lungs were cut in pieces of equal size and randomly allotted to different analysis techniques. Specimens marked for histological analysis were briefly rinsed in cold phosphate-buffered saline, fixated for 2 h in a 4% formaldehyde solution, embedded in paraffin, and cut in 5- μ m sections. Subsequently, standard haematoxylin-eosin staining was performed for histologic analysis of the

morphological changes occurring during LIRI and to evaluate neutrophil sequestration in the tissue. Other sections were used for lymphocyte detection with immunohistochemistry. This was performed using a rabbit polyclonal antibody to CD3 (Abcam, Cambridge, UK) in accordance with the manufacturer's instructions. Secondary antibody binding reaction (alkaline phosphatase) was visualized with a BCIP/NBT Substrate System (DAKO, Heverlee, Belgium). The number of infiltrated lymphocytes and recruited neutrophils was determined as the average count of five high power fields. A blinded experienced technical assistant who had no knowledge of the experimental design performed histologic analysis.

Blood gas analysis

Oxygen tension (pO_2), carbon dioxide tension (pCO_2), acidity (pH) and bicarbonate (HCO_3^-) concentration were analyzed in arterial blood in order to evaluate the clinical relevance of the LIRI animal model. At the end of the surgical procedure 200 μ L of arterial blood was drawn from the aorta. The needle and syringe were heparinized to prevent clotting. The blood sample was immediately analyzed using a calibrated ABL™ 77 series analyzer from Radiometer (Copenhagen, Denmark).

Sample preparation

The left lung was first cut into small pieces and homogenized (Polytron 1200E, Kinematica, Luzern, Switzerland) on ice in a lysis buffer for western blot (1% Triton X-100, 150 mM NaCl, cOmplete protease inhibitor cocktail tablet (Roche Diagnostics, Vilvoorde, Belgium) in a 50 mM Tris buffer, pH 7.6) and a lysis buffer to measure enzyme activity (1% octylglucoside, 10 mM EDTA, 70 μ g/mL aprotinin in a 50 mM Tris buffer, pH 8.3). After homogenization, the samples were left on ice for 1 hour and centrifuged at 4 °C at 12 000 g for 15 min. The supernatant was stored

at -80 °C until all samples were collected and western blot experiment and/or enzyme activity measurement could be performed.

Central venous blood was drawn into prechilled citrated tubes (20% v/v) and mixed by inversion. After centrifugation at 4 °C at 2000 g for 15 min the plasma was stored immediately at -80 °C.

Angiotensin 2 plasma levels

Ang 2 levels were determined in citrate plasma according to the manufacturer's instructions via a competitive ELISA kit (ADI-900-204, Enzo Life Sciences, Antwerp, Belgium), based on colorimetric detection. Absorbances ($\lambda = 450$ nm) were measured with a Versamax Microplate Reader (Molecular Devices, Berkshire, United Kingdom). The limit of detection for Ang 2 is defined as 4.9 pg/mL and very low cross-reactivity for Ang-(1-7) (0.053%) is described. The plasma samples were diluted to ensure that the measured Ang 2 levels fell within the standard range.

Enzyme Activity measurements

An in-house reversed-phase high-performance liquid chromatography technique was used to measure the PRCP activity in left lung homogenates and citrate plasma samples, as previously described [15]. PRCP activity was determined by measuring the hydrolysis of N-benzyloxycarbonyl-Pro-Phe (Z-Pro-Phe, Bachem, Bubendorf, Switzerland). Lung homogenates and plasma samples were incubated for 2 h with Z-Pro-Phe at 37 °C. The enzymatically formed Z-Pro was tracked by its UV absorbance at 210 nm, and quantification was performed by peak height measurements.

Specific PREP activity was measured in plasma after activation with DTT using a fluorogenic substrate as described before [16]. In brief, plasma samples were diluted 6 times in PREP assay buffer (100 mM potassium phosphate buffer pH 8.0 (ambient temperature), 1 mM sodium azide, 1 mM EDTA) supplemented with and without

(blank) 10 mM DTT. Each sample was supplemented with 220 μ M benzyloxycarbonyl-Gly-Pro-7-amido-4-methylcoumarine final concentration (in 4.7% dimethyl sulfoxide) and incubated for 2 h at 37 °C. Reaction was stopped with 1.5 M acetic acid and fluorescence was measured ($\lambda_{\text{ex}} = 370$ nm, $\lambda_{\text{em}} = 440$ nm). In these assay conditions the measured activity after correction by the sample blank was attributable to PREP. Inter- and intra-assay CV's were 1.2 % and 5.5 % respectively. The long incubation time was acceptable because of the linearity in function of the incubation time. Lung homogenates were diluted 21 times and analysed in the same way as the plasma samples. Enzyme activity was expressed as units per gram (U/g) protein, where 1 unit defines the amount of enzyme that hydrolyzes 1 μ mol of substrate per minute. Protein concentration of the lung homogenates was determined via the Bradford method using bovine serum albumin (Sigma-Aldrich, Diegem, Belgium) as a standard [17]. Enzyme activity measured in plasma was compensated for citrate dilution.

Western blot

Left lung homogenates lysed in western blot buffer were further diluted in Laemmli sample buffer (4x) before loading onto a 10% (PRCP) or 7.5% (PREP) SDS-PAGE gel to allow protein separation. Thereafter, the proteins were transferred to a nitrocellulose (0.45 μ m) or PVDF membrane (0.2 μ m) by electroblotting. Non-specific binding sites on the membranes were blocked by incubation in 5% skimmed milk or 4% BSA in washing buffer (0.05 M Tris, 0.15 M NaCl, 0.01% Tween 20) for 1 h at ambient temperature, followed by overnight incubation at 4 °C with primary antibodies against PRCP (bs-1873R, 1:500 dilution, Gentaur, Kampenhout, Belgium), PREP (P48147, 1:10000 dilution, R&D Systems, Abingdon, United Kingdom), and vinculin (ab129002, 1:20000 dilution, Abcam, Cambridge, United Kingdom), which

was used as loading control. Next, the membranes were incubated with HRP-goat anti-rabbit (656120, 1:5000 dilution, Invitrogen, Ghent, Belgium) and HRP-chicken anti-goat secondary antibodies (HAF019, 1:1000 dilution, R&D Systems, Abingdon, United Kingdom) for 2 h at ambient temperature. SuperSignal West Femto substrate kit (Thermo Scientific, Erembodegem, Belgium) allowed protein band detection, while visualization was done using the OptiGo (Isogen Life Sciences, De Meern, The Netherlands) and Proxima AQ-4 software (Isogen Life Sciences, De Meern, The Netherlands). TotalLab TL100 (TotalLab Ltd) software was used for image quantification.

Statistical Analysis

Data were analysed using the statistical SPSS software version 22 (IBM, New York, United States). The PRCP and PREP activity measured in lung homogenates and plasma samples and the PRCP protein expression data followed a normal distribution. The Ang 2 levels were Log-transformed to obtain normality and to allow parametric testing. Significant differences between the groups were examined with ANOVA, followed by the Tukey's HSD post-hoc test. Bivariate (Pearson) correlations were used to analyze the relation between the enzymatic activity (PREP and PRCP) and plasma Ang 2 levels. Since parametric tests were used, all data are presented as mean \pm SEM. A P-value < 0.05 was considered significant and indicated in the graphs by an asterisk (* $P < 0.05$, ** $P < 0.01$).

Results

Validity of the LIRI surgical model

The surgical LIRI model was evaluated and validated morphologically as shown in Figure 2 and is described in more detail in [14]. Histological analysis showed a very early increase of lymphocyte recruitment after 1 h of reperfusion, followed by

neutrophil sequestration after 4 h of reperfusion (Figure 3A and 3B). After 24 h of reperfusion, inflammatory cell count returned to baseline. Oxidative stress as a marker of reperfusion injury was measured using electron paramagnetic resonance. This showed an increase in superoxide radical formation in peripheral blood after 1 h of ischemia and a steep increase in nitric oxide and superoxide radical after 1 h of reperfusion. After 24 h of reperfusion, oxidative stress levels returned back to normal [18]. Blood gases (pO_2 , pCO_2 , HCO_3^-) were measured as a functional marker of lung function and to evaluate the degree of LIRI (Figure 4).

Angiotensin 2 plasma levels

The Ang 2 levels were measured in the plasma of the mice from each group (sham, 1I0R, 1I1R, 1I24R). Compared with the sham group, the plasma Ang 2 levels significantly rise after 1 h of lung ischemia induced through temporary left lung hilar clamping ($P = 0.031$). This increase remains significant after 1 h ($P = 0.011$) and 24 h ($P = 0.044$) of reperfusion. The results are shown in Figure 5.

PRCP and PREP activity in plasma

The PRCP activity showed a trend towards an increase after ischemia with subsequent decrease after 24 h of reperfusion. However, none of these transient changes in PRCP activity between the different groups reached statistical significance ($P > 0.05$). As for the plasma PREP activity, a significant decrease was observed after 24 h of reperfusion compared to the sham group ($P = 0.031$). The results are summarized in Figure 6A and 6B. Since decreasing activity of PRCP and PREP in plasma is consistent with decreased Ang 2 degradation and thus with rising plasma Ang 2 levels, correlation tests were performed. Nonetheless, statistical correlation could not be proven between the decreasing plasma PRCP activity ($r = 0.367$, $P = 0.085$) or plasma PREP activity ($r = -0.101$, $P = 0.645$) and the increasing plasma Ang 2 levels.

PRCP and PREP activity and protein expression in lung tissue

The PRCP and PREP activity were determined in the left lung of the mice after homogenization in a lysis buffer for enzyme activity measurements. The activity profile of PRCP and PREP in the left lung tissue was not significantly changed after ischemia neither after 1 h nor after 24 h of reperfusion compared to the sham group ($P > 0.05$). Large inter-individual variations for the specific lung PRCP and PREP activity were observed in the different animal groups. The results are summarized in Figure 7A and 7B.

In each group, the protein expression profile of PRCP and PREP in the left lungs of the mice was assessed through western blotting. Semi-quantitative analysis was done using vinculin, a cytoskeletal protein, as a loading control. The results are shown in Figure 8. In accordance with the activity measurement, the protein expression of PRCP remains rather stable after LIRI ($P > 0.05$). Despite all our efforts, western blotting of PREP was unsuccessful since the results were not reproducible and faint protein bands with high background signal were not reliable. Murine kidney homogenate and recombinant PREP were used as a positive control and the specificity of the secondary antibodies was examined before use.

Discussion

Interest has recently been focused on the possible role of the RAS in the development of pulmonary diseases. Previous studies suggest that dysregulation of the RAS, resulting in increased ACE activity and decreased ACE2 activity in bronchoalveolar lavage fluid (BALF), might lead to ARDS. These reports also indicate that the changes in enzyme activity cause an increase in Ang 2 levels and a decrease in Ang-(1-7) levels in BALF, thereby generating a peptide balance favoring an inflammatory response. Unsurprisingly, the administration of a synthetic form of Ang-(1-7) has

emerged as a novel therapeutic strategy to treat ARDS, as up to now no actual drug has been shown beneficial or solely in a particular subset of patients and supportive care has been the only feasible approach [4,19].

Not only in ARDS but also in acute lung injury Chen *et al.* reported the involvement of the RAS. A hind-limb ischemia-reperfusion model in mice was used to induce acute lung injury. Again, decreased ACE2 activity and expression in lung tissue was found with high levels of Ang 2 and low levels of Ang-(1-7) in lung tissue and serum. The findings of this study support the assumption that RAS homeostasis is important to maintain stable lung function and may help to reduce lung injury after trauma [13]. These studies, however, suggest that changes in ACE2 activity lie at the basis of unbalanced angiotensin levels, disregarding the fact that other angiotensin metabolizing enzymes may also be involved. To our knowledge, PRCP and PREP are possible candidate enzymes of which the expression and/or activity might also be dysregulated when acute lung injury occurs. Already in 1980, Kumamoto *et al.* discovered that PRCP activity could be measured in human lungs and hypothesized a possible role for the enzyme in angiotensin cleavage [20]. Moreover, this is supported by Tamaoki *et al.* who published that endothelial PRCP regulates contractile responses of pulmonary blood vessels induced by Ang 2 [21]. Local elevation of PREP activity has been shown in small cell lung cancer, presuming that the enzyme may participate in the proliferation and regeneration of tissues after injury and/or malignancy. Also, a correlation between PREP and ACE activity in the sera of hypertensive patients has been demonstrated, assuming a function for PREP within the RAS [22]. Upregulation of PREP, which is highly expressed in the inflammatory cells in the lung tissue, has been described in a murine model of cigarette-smoke induced lung emphysema [23].

Not only ACE2, but also PRCP and PREP are capable of metabolizing Ang 2 into Ang-(1-7) in the circulation and the lung. Regarding this fact, we are the first to investigate the relation between circulating Ang 2 levels and PRCP and PREP activity in a murine model of pulmonary ischemia and reperfusion. This model focuses on the acute effect of pulmonary ischemia itself, rather than the effect of peripheral limb ischemia on lung tissue in a hind-limb ischemia-reperfusion model. Therefore, this model may be better suited to evaluate the immediate pathophysiological effect of lung transplantation or cardiopulmonary bypass. The one-hour duration of ischemia time was elected in order to compare results with previous research in the literature. Reperfusion times were evaluated in the acute phase up until 24 h.

Our results indicate that also in the LIRI model used here, the Ang 2 levels significantly rise after ischemia and reperfusion, a finding consistent with prior publications [13]. High levels of Ang 2 lead to increased production of superoxide radicals by NADPH oxidase and free radicals may be important inflammatory mediators in the pathogenesis of LIRI. Therefore, the impaired angiotensin system and simultaneous burst of free radicals after ischemia may be related [18,24]. Despite the fact that plasma PREP activity is significantly decreased in the I124R group compared to the sham group, no correlation with the plasma Ang 2 levels was found. Moreover, the activity profile of PRCP and PREP in the lung tissue is not significantly altered after LIRI, implying a minor role for local PRCP and PREP in the ischemic lung itself. In addition, the protein expression of PRCP measured in the lungs harvested from the different groups remained rather unchanged. A limitation of the present study is the large inter-individual variation that was observed at the activity and protein expression level in the harvested lungs. A possible explanation could be the use of outbred mice. Normalization through right lung measurements

was not appropriate since the right lung is also susceptible to the consequences of ischemia-reperfusion injury. We attempted to measure specific ACE2 activity in plasma and lung homogenates via a fluorometric assay [25]. Unfortunately, these values fell below the limit of detection of the assay and no suitable antibodies against ACE2 were found to give satisfactory results via western blotting. Therefore, all the details concerning the ACE2 assays are described in the supplementary data.

This study describes the activity profile of PRCP and PREP in plasma and lung tissue in a murine model of LIRI in addition to plasma Ang 2 levels. We conclude that LIRI causes a dysregulation of circulating Ang 2 levels and plasma PREP activity, whilst PRCP activity and expression in plasma and lung tissue stays unaffected. Future studies are necessary to examine the link between Ang 2 and the generation of free radicals and to define whether PREP and PRCP in addition to ACE2 are important players in LIRI.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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