Online supplement

Chymase: a multifunctional player in pulmonary hypertension associated with lung fibrosis

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Methods

Study design

Pulmonary hypertension (PH) was induced in adult Syrian hamsters by intratracheal instillation of bleomycin. Healthy control group received only NaCl. Bleomycin-instilled hamsters were randomized into two groups and treated orally by gavage in the therapeutic approach manner (started a week after the bleomycin instillation) once per day either with chymase inhibitor BCEAB (4-[1-{[bis-(4-methyl-phenyl)-methyl]-carbamoyl}-3-(2-ethoxy-benzyl)-4-oxo-azetidine-2-yloxy]-benzoic acid) (Bleo-BCEAB) at the dose of 300 mg/kg body weight/day or placebo (vehicle) (Bleo-placebo). The dose used in the study was selected based on previous publication [1]. The detailed pharmacological characteristics of BCEAB were also previously described [1].

Hemodynamics and right ventricular hypertrophy measurements

Hemodynamic and right heart hypertrophy (RHH) measurements were performed two and a half weeks after bleomycin instillation. *In vivo* hemodynamics and RHH measurements were carried out similarly as previously described [2]. Briefly, right ventricular systolic pressure (RVSP) was measured by a catheter inserted through the jugular vein to the right ventricle (RV). For the measurement of systemic arterial pressure (SAP) the left carotid artery cannulation was used. Hemodynamic measurements were followed by determination of RV hypertrophy. RV wall was separated from left ventricular (LV) wall and ventricular septum (S),

and RV hypertrophy was expressed as the weight ratio of the RV and LV+S (Fulton´s index).

Lung tissue processing and plasma collection

After the *in vivo* measurements, the left lungs from different experimental groups were formalin-fixed and paraffin-embedded. The right lungs were snap-frozen in liquid nitrogen and stored at -80^{0}C until subsequent use. Additionally, the plasma isolated from blood was stored at -20^{0}C until subsequent use.

Morphometric assessment of pulmonary vascular remodeling

Pulmonary vascular remodeling was assessed by performing the morphometric analysis of the medial wall thickness and degree of muscularization, as previously described [3]. Briefly, the 3µm thick formalin-fixed and paraffin-embedded lung tissue sections were stained with Elastica van Gieson for measurement of medial wall thickness, or double-immunostained with an anti-α-smooth muscle actin antibody (dilution 1:900, Sigma) and anti-von Willebrand factor antibody (dilution 1:900, Dako) for measurement of degree of muscularization.

Lung fibrosis assessment

For histological assessment of lung fibrosis, Hematoxylin-Eosin (HE) stained slides were scanned by using of computer software for image analysis under light microscope (QWin, Leica, Wetzlar, Germany). The images were reviewed and

degree of fibrosis was assessed according to Ashcroft's fibrosis score system with modifications, in accordance to the literature [4].

Mast cells and chymase analysis in the lungs

Histological analysis of mast cell number and activity (index of granulation) was performed as described in the literature [5]. Furthermore, the lungs from different experimental groups were immunostained to detect chymase (dilution 1:100, Abcam), followed by counting of chymase-positive cells under light microscope. The results were expressed as chymase-positive immunoreactivity/mm^2 (in %).

Morphometric and stereological analysis of the right heart

Formalin-fixed and paraffin-embedded right ventricular (RV) tissues were stained with 0.1% Sirius red F3B (Niepoetter, Bürstadt, Germany) in picric acid (Fluka, Buchs, Germany) for detection of collagen, as described previously [3]. Photomicrographs were further quantified to determine the interstitial collagen fraction by using of computer-assisted image analysis software (QWin, Leica, Wetzlar, Germany). Additionally, 3µm thick formalin-fixed and paraffin-embedded RV sections (3 sections of 20µm apart from each right ventricle) were stained with fluorescein isothiocyanate (FITC)-conjugated wheat germ agglutinin (Sigma-Aldrich) to outline the cell membrane, tetramethylrhodamine-5-(and 6)-isothiocyanate (TRITC)-conjugated isolectin B4 (Sigma-Aldrich) to stain the capillary endothelial cells, and diamidino phenylindole (DAPI) (Invitrogen) for nuclear staining. Cardiomyocyte cross sectional area was determined by using

Leica QWin software. For capillary length, volume based stereological tool was applied. Total volume (reference volume, V) of the tissue was determined by dividing with its density (widely used density for cardiac tissue is 1.06). To count the capillaries the STEPanizer stereological tool developed by Stefan A Tschanz, University of Bern, was used [6]. Total profile count (Q) per defined area i.e. within counting frame was performed using STEPanizer software and total length of capillary was estimated by multiplying volume density (Lv) with reference volume (V).

Big endothelin (ET)-1-induced vasoconstriction in isolated pulmonary artery rings

3 ring segments of the hamster pulmonary arteries were isolated per animal, cleaned of fat and connective tissues and mounted between two stainless-steel wires in 10-ml organ baths (EMKA technologies, France) containing a Krebs solution at 37°C. The arterial segments were allowed to recover for 30 minutes. Following this recovery period, stepwise increases in tension up to 1 g were applied to each segment. Two responses to KCl 60 mmol/l were tested in order to establish maximal contraction. After a 30 minutes resting period, concentration–response curves for big-ET-1 (Sigma, 1 nmol/l to 1 µmol/l) were obtained in absence or presence of neutral endopeptidase (NEP)/endothelin converting enzyme (ECE) inhibitor phosphoramidon (Sigma) or combination of

phosphoramidon and chymase inhibitor chymostatin (Sigma). Data are presented as % of maximal contraction to KCl 60 mM.

Endothelin (ET)-1 and big-ET-1 measurements in the lungs and plasma

Lung tissues were snap-frozen and protein was extracted from homogenized tissue in 1M acetic acid at 100^0C for 15 minutes. The homogenate was then centrifuged at 14000g for 15 minutes at 4^0C. The supernatant (tissue extract) or plasma was applied to SepPak C18 cartridges (Waters, the Netherlands) and the ultimate eluate was dried in a centrifugal concentrator. The dried samples were reconstituted in assay buffer for ET-1 radioimmunoassay (RIA) and big-ET-1 enzyme-linked immunoassay (EIA). ET-1 and big-ET-1 levels were measured by commercially available RIA-kit (S2024, Bachem, Basel, Switzerland) and EIA-kit (ENZO LifeSciences, Belgium), respectively. Tissue levels of ET-1 and big-ET-1 were normalized to total protein concentration (fmol/mg protein) as determined using the BCA-method. The results were expressed as ET-1/Big-ET-1 ratio. Plasma concentration of ET-1 was expressed in fmol/ml.

Analysis of transforming growth factor-β1 (TGF-β1) and matrix metalloproteinase (MMP)-2 in the lungs

Immunohistochemistry with anti-TGF-β1 antibody (dilution 1:50, Abcam) was performed on lung tissue sections from different experimental groups. Also, the lungs were immunostained with anti-MMP-2 antibody (dilution 1:50, Abcam). The quantification of the stainings, taking into consideration both intensity and area of

the immunoreactivity, was done by using of a special computerized software based morphometric analysis (QWin, Leica, Wetzlar, Germany). The results were expressed as score value (%). The representative photomicrographs were taken by usage of the special prisms for the microscope (Interferenz-Kontrast, Kondensorprisma, Objectivprisma, Leica, Wetzlar, Germany) that exhibited 3D effect [7].

Immunohistochemistry of chymase and transforming growth factor (TGF)-β1 in human lung tissues

Human lung tissues were obtained from patients with idiopathic pulmonary arterial hypertension (IPAH) and idiopathic pulmonary fibrosis (IPF) undergoing lung transplantation. After explantation, lung tissues were formalin-fixed and paraffin-embedded, as previously reported [5]. Finally, double-immunostaining with anti-chymase and anti-TGF-β1 antibodies (dilutions 1:200 and 1:50, both from Abcam) was performed and photomicrographs were taken by usage of the special prisms for the microscope (Interferenz-Kontrast, Kondensorprisma, Objectivprisma, Leica, Wetzlar, Germany) that resulted in 3D effect [7].

All histological measurements were done in a fashion blinded to the physiological results and treatment assignment.

Discussion

In our attempt to summarize the potential signaling pathways involved in pathology of PH associated with LF (supplemental fig.1), we would like to include two additional possibilities connected with chymase and suggested to play a role in PH and PF conditions, such as Ang II and ILs [8-14]. In this context, chymase is indeed involved in activation of IL-18 and IL-1β pro-forms, and in conversion of Ang I to Ang II [10, 15-22]. Taking into count a clear distinction between PH secondary to or disproportionate to the existing PF, our scheme (supplemental fig.1) includes different molecular culprits that may lead to: 1) development of PF and consequently to PH, or, 2) as many of the mediators are shared between these two diseases, pathogenesis of disproportionate PH [23].

In the context of PH associated with LF, our study provided another novel aspect, such as in-depth analysis of the RV remodeling process with focus on RV fibrosis and cardiomyocyte size that may represent underlying histopathological attributes of RVH. We would like to highlight one important issue that may arise regarding these beneficial effects of chymase inhibition on right heart pathology during PH development. It was shown previously that altered chymase activities are implicated in other heart medical conditions, such as myocardial infarction and cardiac fibrosis in cardiomyopathy [1, 24, 25]. Even more, the chymase inhibitor that we used in our study BCEAB showed a prominent inhibitory effect on heart chymase activities and exerted beneficial properties on reduction of cardiac fibrosis in the hamster model of cardiomyopathy and in the context of myocardial infarction [1, 24, 26]. Therefore, one can expect that observed

beneficial effects of BCEAB on RVH, fibrosis and cardiomyocyte size, may also appear as a direct influence of this chymase inhibitor on the right heart. However, whether the attenuation of the RV remodeling and hypertrophy in the present study was exclusively the consequence of improved pulmonary hemodynamics or there was a contribution from direct effect of chymase inhibition on the right heart, was not further analyzed and future studies may be useful to fully resolve this issue.

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Figure legend

Supplemental figure S1. Potential role of chymase in the pathology of pulmonary hypertension associated with lung fibrosis. The summary of the findings (blue letters) together with current knowledge from the literature (black letters) is schematically presented. Legend: Ang – angiotensin, Coll – collagen, MMP – matrix metalloproteinase, TGF – transforming growth factor; IL – interleukin, ET – endothelin, PF – pulmonary fibrosis, PH – pulmonary hypertension.