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Cyclooxygenase-2 promotes pulmonary intravascular macrophage accumulation by exacerbating BMP signaling in rat experimental hepatopulmonary syndrome

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ABSTRACT

Background and aims: One central factor in hepatopulmonary syndrome (HPS) pathogenesis is intravascular accumulation of activated macrophages in small pulmonary arteries. However, molecular mechanism underlying the macrophage accumulation in HPS is unknown. In this study, we aimed to explore whether elevated COX-2 induces the Bone morphogenic protein-2 (BMP-2)/Crossveinless-2 (CV-2) imbalance and then activation of BMP signaling pathway promotes the macrophage accumulation in Common Bile Duct Ligation (CBDL) rat lung.

Methods: The COX-2/PGE2 signaling activation, the BMP-2/CV-2 imbalance and the activation of Smad1 were evaluated in CBDL rat lung and in cultured pulmonary microvascular endothelial cells (PMVECs) under the HPS serum stimulation. The effects of Parecoxib (COX-2 inhibitor), BMP-2 and CV-2 recombinant proteins on 4-week CBDL rat lung were determined, respectively.

Results: The COX-2/PGE2 signaling pathway was activated in CBDL rat lung *in vivo* and PMVECs *in vitro*, which was due to the activation of NF-κB P65. The inhibition of COX-2 by Parecoxib reduced macrophage accumulation, decreased lung angiogenesis and improved HPS. Meanwhile, the CBDL rat lung secreted more BMP-2 but less CV-2, and the imbalance between BMP-2 and CV-2 exacerbated the BMP signaling activation thus promoting the macrophage accumulation and lung angiogenesis. The BMP-2/CV-2 imbalance is dependent on the COX-2/PGE2 signaling pathway, and thus the effects of this imbalance can be reversed by adminstration of Parecoxib.

Conclusion: Our findings indicate that inhibition of COX-2 by parecoxib can improve the HPS through the repression of BMP signaling and macrophage accumulation.

1. Introduction

Hepatopulmonary syndrome (HPS) is a liver dysfunction associated with abnormal gas exchange-induced hypoxia, which occurs in 15 to 30% of patients with liver cirrhosis [1–4]. The major pathology of HPS includes intrapulmonary vasodilation (IPVD), pulmonary vascular shunting and pulmonary angiogenesis [5–8]. At present, the pathophysiological mechanism underlying HPS is unclear, and liver transplantation is the only treatment option [9].

Previous studies have demonstrated that the accumulation of activated CD68(+) macrophages in CBDL rat pulmonary microvascular serves as a critical factor in HPS pathogenesis [10,11]. The accumulated macrophages exhibit the capacity to secrete

Abbreviations: HPS, hepatopulmonary syndrome; COX-2, cyclooxygenase-2; BMP-2, bone morphogenic protein-2; CV-2, crossveinless-2; CBDL, common bile duct ligation; PGE2, Prostaglandins E2; PMVEC, pulmonary microvascular endothelial cell; IPVD, intrapulmonary vasodilation; i-NOS, inducible nitric oxide synthase; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factors; PCNA, proliferating cell nuclear antigen; VWF, von Willebrand factor; EP, e-series of prostaglandin receptors.

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vasodilatory, angiogenic, and proliferative growth factors, including inducible nitric oxide synthase (i-NOS), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF), respectively. All these factors contribute to IPVD, lung angiogenesis and pulmonary vascular remodeling, thus leading to respiratory failure at the late stage of HPS. Consistently, the depletion of macrophages at the early stage strongly alleviates the HPS symptom, and therefore it might be considered as another potentially effective treatment for HPS [11]. But molecular mechanism underlying the macrophage accumulation in HPS is not defined yet.

Intravascular accumulated macrophages mainly differentiate from the circulating peripheral-blood monocytes, whose homing to vascular regions occurs in many pathological situations, including cutaneous wound healing, inflammation angiogenesis, tumors progression [12,13]. Several chemokine factors, such as CCL2, CXCL12, CX3CL, are able to regulate the homing of circulating monocyte by chemotaxis [14]. The expression of these chemokine factors is potentially regulated by Cyclooxygenase-2 (COX-2), a key enzyme in arachidonic acid metabolism. COX-2 and its downstream factors such as Prostaglandins E2 (PGE2) and Prostacyclin, are involved in the diverse physiological and pathophysiological processes, especially inflammation [15]. It has been implicated that COX-2 might indirectly promote the macrophage accumulation by regulating the CCL2 expression in acute hypoxia induced lung injury [16]. COX-2 is also known to be inducible in response to inflammatory stimuli, cytokines, or endotoxis [17]. Interestingly, the experimental HPS animals exhibit a high level of circulating endotoxis, which is due to the loss of liver function [18]. Thus, COX-2 might be a potential factor to regulate the macrophage accumulation involved in HPS.

If COX-2 is a critical factor in regulating macrophage accumulation, what is the downstream signaling pathway of COX-2 in this regulation? In our previous study, we found that the BMP-2 signaling pathway is activated in PMVECs under the condition of HPS serum [19], indicating the importance of BMP-2 in HPS. Local BMP activity is tightly regulated by its extracellular BMP modulators, such as Crossveinless-2 (CV-2) [20]. CV-2 binds directly to BMPs, and this interaction inhibits the BMP activity by its blocking the binding site of BMP receptors [21]. It has been known that inflammatory stimuli, such as TNF-α and LPS, is able to cause the abnormal balance between BMP-2 and CV-2 in vascular regions, and thus the induced BMP signaling pathway contributes to the monocyte homing by increasing the VCAM-1 and ICAM-1 expression in vascular endothelial cells [22]. In addition, the BMP activation also upregulates the CCL2 expression in human melanoma cells [23]. Thus, it is possible that the BMP-2/CV-2 balance might be disrupted to result in the macrophage accumulation in HPS lung. Furthermore, a recent report demonstrated that cyclic tensile force upregulates the expression and activity of BMP-2 through the COX-2/PGE2 signaling pathway in human periodontal ligament cells [24], which implies a molecular link between the BMP signaling pathway and COX-2. However, the effect of COX-2 on the BMP-2/CV-2 imbalance is unknown. Therefore, the aim of our present study is to define if COX-2 influences the macrophage accumulation by regulating the BMP-2/CV-2 imbalance in the experimental HPS.

2. Materialand methods

2.1. Animal experiments

All animal experiments were approved by the Ethics Committee of Animal Experiments of Third Military Medical University. Sprague-Dawley rats (220–250 g) were used for experiments. The CBDL and Sham Sprague-Dawley rats (10 rats in each group) were performed as described previously [25]. Sham rat underwent mobilization of common bile duct without ligation. 10 mg/kg Parecoxib (Hisun-Pfizer Pharmaceuticals Co) was intraperitoneally injected after the CBDL surgery on the 1st day and then injection was repeated for 28 consecutive days. The Pareoxib-Sham rats also received the same IP injection as a control group. In other animal experiments, 20 μg/kg recombinant BMP-2 or CV-2 protein (R&D systemsInc) was injected from 2 to 4 weeks for 14 consecutive days, based on the first appearance of BMP-2/CV-2 imbalance *in vivo*. Finally, the rats received terminal anesthesia for all analyses, and 1 mL blood was immediately collected through abdominal aorta for blood gas analysis.

2.2. Cell culture

PMVECs were isolated from rat lung as previously reported [26]. PMVECs were cultured in DMEM medium (Life Technologies), and were maintained at 37 °C in a humidified atmosphere in 5% CO2. Subsequently, cells were divided into the following two groups: group C is consisted of PMVECs cultured in DMEM supplemented with normal rat serum (5%), whereas Group HPS is consisted of PMVECs incubated in DMEM containing CBDL-rat serum (5%). The pharmacological reagents used to evaluate the potential roles of various signaling pathways following exposure to the HPS serum included the P65 inhibitor SN-50 (50 mM) (Sigma Chemical Co), the COX-2 inhibitor Parecoxib (100 mM), and PGE2 (50 ng/ml) (Sigma Chemical Co). They were added to and remained in the media of cultured PMVECs throughout the experimental period of 36 h.

2.3. Western blotting

Lung tissues and PMVECs were lysed in the RIPA buffer containing 1% protease inhibitor. The lysates were centrifuged and then supernatants were collected. Equal proteins from tissues and PMVECs were added on an 8–12% Bis-Tris gel and then transferred to a polyvinylidene difluoride membrane. After transfer, membrane was treated with blocking solution (5% dry milk in Tris buffered saline with 0.1% Tween-20) for 2 h and probed with primary antibody against COX-2 (Abcam, Cambridge, USA), CCL2 (Abcam, Cambridge, USA), i-NOS (Abcam, Cambridge, USA), PCNA (Abcam, Cambridge, USA), VWF (Abcam, Cambridge, USA), BMP-2 (Abcam, Cambridge, USA), CV-2 (Sigma Chemical Co), P-Smad1 (Abcam, Cambridge, USA) overnight at 4 °C, followed by horseradish peroxidase-conjugated secondary antibody for 1 h. The loading control was the constitutively expressed protein β-actin (Sigma Chemical Co). The blots were visualized with enhanced chemiluminescence system and the density of autoradiographic signals was assessed with GeneSnap (Syngene, Cambridge, UK).

2.4. Immunofluorescence and immunohistochemistry

The fixed PMVECs and 5-mm-thick paraffin sections of fixed lung tissue were blocked by 10% goat serum for 1 hour, and then incubated overnight at 4 °C with primary antibody. For immunofluorescence staining of COX-2, BMP-2, CV-2, P-Smad1, CD68, PCNA, VWF, fluorescence-tagged secondary antibody and DAPI were used, and then examined by using an Olympus BX40 microscope. For immunohistochemistry staining of i-NOS and VEGF, DAB Peroxidase Substrate Kit and hematoxylin were employed, and microphotographs of the sections were taken with a light microscope. The images were counted in a blinded manner by two pathologists. All positive signals of these images were quantitated with ImageJ software.