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Hepcidin protects pulmonary artery hypertension in rats by activating NF-κB/TNF-α pathway

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Abstract. – OBJECTIVE: The pulmonary artery hypertension (PAH) model was established in rats in this study. Therefore, we aimed to elucidate the protective role of Hepcidin in PAH rats and its underlying mechanism.

MATERIALS AND METHODS: 24 male Sprague Dawley (SD) rats were randomly divided into sham group, PAH group and Hepcidin group, with 8 rats in each group. After animal procedures, hemodynamic parameters and right ventricular hypertrophy indexes were determined in rats. Cytokines in serum samples of rats were detected by enzyme-linked immunosorbent assay (ELISA). Pathological lesions in lung tissues were observed by hematoxylin and eosin (H&E) staining. Finally, Western blot was conducted to detect the protein expressions of nuclear factor-kappa B (NF-κB), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), vascular cell adhesion molecule 1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1), and monocyte chemotactic protein-1 (MCP-1) in lung tissues of rats.

RESULTS: Compared with sham group, mean pulmonary artery pressure (mPAP) and right ventricular systolic pressure (RVSP) were significantly elevated in rats of PAH group ($p<0.05$). On the contrary, mPAP and RVSP in rats of Hepcidin group were both significantly lower than PAH group ($p<0.05$). Hepcidin treatment attenuated PAH-induced pathological lesions in lung tissues. ELISA results elucidated that Hepcidin treatment significantly decreased serum levels of TGF-β, TNF-α, IL-1β, and IL-6. In addition, Western blot results demonstrated that protein levels of NF-κB, TNF-α, IL-1β, VCAM-1, ICAM-1, and MCP-1 in Hepcidin group were remarkably lower than those of PAH group.

CONCLUSIONS: Hepcidin alleviates inflammatory response in PAH rats by inhibiting NF-kB/TNF-α pathway.

Key Words:
Hepcidin, PAH, NF-κB/TNF-α pathway.

Introduction

Pulmonary artery hypertension (PAH) is a pathophysiological syndrome characterized by pulmonary vascular resistance and elevated pulmonary artery pressure. PAH is the most common complication of congenital heart disease, which is also the leading cause of perioperative death[1-3]. As the systemic circulation of blood is diverted to the pulmonary circulation (left-to-right shunt), pulmonary vascular resistance increases and pulmonary vascular disease progressively aggravates. This may eventually develop into pulmonary hypertension right-to-left shunt syndrome, namely Eisenmenger's syndrome. These patients can only receive cardiopulmonary or lung transplantation without any chance of routine surgery. However, most of patients with Eisenmenger's syndrome die of respiratory and circulatory failure due to donor shortages and technical problems[4-6]. Therefore, how to effectively prevent the occurrence and development of PAH is an urgent problem to be solved.

Currently, it is believed that the occurrence of PAH involves multiple factors, including cells, circulatory mediators and genetic genes[7,8]. Dysfunctions of vascular endothelial cells, smooth muscle cells, fibroblasts and platelets are frequently observed in the pathological progression of PAH. Besides, the imbalance of vasoconstrictor and vasodilator factors, pro-coagulant and anti-coagulant substances promote the occurrence of PAH[6,9]. Under the action of the above stimulating factors, hyperplasia of pulmonary artery smooth muscle cells and endothelial cells can lead to increased blood flow resistance. Meanwhile, thickening or even occlusion of pulmonary arterioles and anterior capillaries walls occurs.

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As a result, pulmonary vascular remodeling further leads to PAH[10,11]. Previous researches[12,13] have demonstrated that the proliferation of endothelial cells and smooth muscle cells exert an important role in pulmonary vascular remodeling and PAH. Therefore, the regulation of proliferation is expected to be an effective way to control PAH progression. In this study, we suggested that regulatory proteins closely related to cell proliferation might contribute to alleviation or even reversion of PAH. Nuclear factor-κB (NF-κB) is one of the key factors regulating gene transcription, which is characterized by immediate transcription. NF-κB regulates the expression of multiple genes, especially those in early responses to the body's defense function and inflammatory response, including interleukin-1 (IL-1), IL-2, tumor necrosis factor-α (TNF-α), intercellular cell adhesion molecule-1 (ICAM-1), etc.[14-16]. Previous experimental investigations[17,18] has shown that the NF-κB pathway is involved in the production of reactive oxygen species (ROS) and infiltration of polymorphonuclear neutrophils during PAH. It is also reported that NF-κB exerts a regulatory role in the inflammatory response of PAH, which can eventually lead to the activation of downstream cytokines[19]. Hepcidin is a newly discovered small peptide hormone synthesized by liver. It is the main regulator of iron homeostasis *in vivo*. Current studies[20,21] have indicated that Hepcidin can be used as a "hormone-like substance" to relieve iron overload by inhibiting iron absorption in small intestinal epithelial cells. Great progress has been achieved on the roles of Hepcidin in hematological system diseases and nervous system diseases. Through literature review, Hepcidin has biological functions of anti-oxidation, anti-inflammation, blood lipid regulation and atherosclerosis prevention[22,23]. However, the potential effect of Hepcidin on PAH remains unclear. Therefore, the aim of this work was to elucidate the specific role of Hepcidin in PAH and the underlying mechanism. Our findings might provide new directions in clinical treatment of PAH.

Materials and Methods

Chemicals and Reagents

Hepcidin was obtained from Sigma-Aldrich (St. Louis, MO, USA); relative determination kits of TGF-β, TNF-α, and IL-6 were obtained from Bio-Swamp (Beijing, China); ordinary light microscope and fluorescent inverted microscope were provided by Nikon (Tokyo, Japan); computer installed with Image J software was provided by Lenovo (Beijing, China); paraffin embedded microtome was provided by Hwotech (Shenzhen, China); electronic balance was provided by Mettler Toledo (Columbus, OH, USA); high-speed low-temperature desk centrifuge was provided by Heraeus (Hanau, Germany); digital gel imaging system was provided by Bio-Rad (Hercules, CA, USA).

Animals and Experimental Protocol

24 male Sprague-Dawley (SD) rats weighing 200±20 g were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). This investigation was approved by the Animal Ethics Committee of Qingdao University Animal Center. Rats were housed in an environment with 12 h/12 h cycle of light, with free access to water and food. Based on different interventions, rats were randomly divided into sham group, PAH group and Hepcidin group, with 8 rats in each group. Rats in Hepcidin group were intraperitoneally injected with 0.5 mL of Hepcidin pre-diluted with normal saline 10 minutes prior to hypoxia treatment. Hepcidin injection was performed every two days. Rats in both PAH group and Hepcidin group were induced by hypoxia. Nitrogen and oxygen were continuously injected into the atmospheric pressure animal chamber. Flow speed and rate of nitrogen and oxygen were adjusted using the oxygen meter (RSS-5100, Shanghai Leici Instrument Factory, Shanghai, China), so as to keep oxygen concentration stable at 10±0.5%. Water in the chamber was absorbed by silica gel and anhydrous calcium chloride, whereas carbon dioxide was absorbed by calx natrica. The animals were maintained in the chamber. Meanwhile, the cages were opened every 3 days for cleaning, food and water supplement within 30 min. Except for inhalation of air, rats in sham group were kept in the same condition as those in other groups. After sacrifice, blood samples were collected from the internal iliac vein of rats. Body weight and daily activities were observed throughout the administration period.

Determination of Hemodynamic Parameters and Right Ventricular Hypertrophy Indexes

At the end of animal procedures, rats were anesthetized by 1% pentobarbital sodium (60 mg/kg). A polyethylene catheter with a diameter of

1 mm containing 10 U/mL normal saline diluted heparin solution was inserted into the right external jugular vein of rats. A pressure sensor was used to monitor pressure changes by connecting a micro pressure sensor to the other end of the catheter. In addition, guided by the pressure waveform of the sensor, the catheter was inserted into the right atrium through the superior vena cava. Then, the catheter reached the right ventricle through the tricuspid valve. Right ventricular pressure was measured using a BL-420F biological and functional information collection system (Biolap 420F, Taimeng, Chengdu, China). Subsequently, the catheter was inserted into the trunk pulmonary, and the pulmonary arterial pressure was recorded. Mean pulmonary artery pressure (mPAP) and right ventricular systolic pressure (RVSP) were recorded by pressure sensor. After determining the RVP typical waveform based on oscilloscope waveform, the position and angle of the catheter were fixed. Relative indexes were recorded after 30 min of stabilization. Besides, cardiac functional indicators, such as right ventricular wall thickness were measured by echocardiography.

Histological Examination

Lung tissues of rats were harvested and paraffin embedded. Subsequently, collected lung sections were dewaxed using Xylene I for 10 min and Xylene II for 5 min. Gradient dehydration was performed with 100%, 95%, 90%, 80%, 70%, and 50% alcohol, respectively, with 3 min for each. Then, the sections were stained with hematoxylin and eosin. Finally, the sections were sealed and observed under a microscope.

Determination of Inflammatory Factors

Serum levels of TGF-β, TNF-α, IL-1β, and IL-6 in rats were determined according to the instructions of enzyme-linked immunosorbent assay (ELISA) determination kit (R&D Systems, Minneapolis, MN, USA).

Western Blot

Tissues were added with lysis buffer and shaken on ice for 30 min. Total protein was separated after centrifugation at 14,000 g/min, 4°C for 15 min. The concentration of extracted protein was calculated by the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Extracted proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Western blot analysis was performed according to standard procedures.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for all statistical analysis. Quantitative data were represented as mean ± standard deviation ($\bar{x}\pm s$). Continuous variables were analyzed by the *t*-test, and categorical variables were analyzed by the χ^2-test or Fisher's exact test. ANOVA and SNK test was used to compare the difference among different groups, followed by post-hoc test. $p<0.05$ was considered statistically significant.

Results

Hepcidin Treatment Decreased Pulmonary Arterial and Right Ventricular Pressure

To determine whether Hepcidin alleviated PAH in rats, we measured hemodynamic parameters of each rat after animal procedures, including mPAP and RVSP. Rats in Hepcidin group presented significantly lower mPAP and RVSP than those of PAH group, which were similar as sham group (Figure 1A, 1B, $p<0.05$). It was concluded that Hepcidin treatment remarkably improved hemodynamic parameters in PAH rats.

Hepcidin Attenuated Right Ventricular Structural Remodeling in PAH Rats

Hepcidin treatment markedly attenuated the increase of RV/LVS and RV/TL (RV: right ventricular diameter, LVS: Left ventricular end systolic diameter, TL: True lumen) in rats of PAH group. Consistently, echocardiogram showed that the right ventricular wall in PAH group was significantly thicker when compared with sham group and PAH group (Table I).

In animal experiments, the P_{es} (end-systolic pressure) in rat lung tissues was remarkably increased after hypoxia treatment, indicating the successful construction of PAH model in rats. We found that the right ventricle afterload was significantly increased in Hepcidin group compared with that of PAH group. It was speculated that Hepcidin treatment had the tendency to improve the contractility of the right ventri-

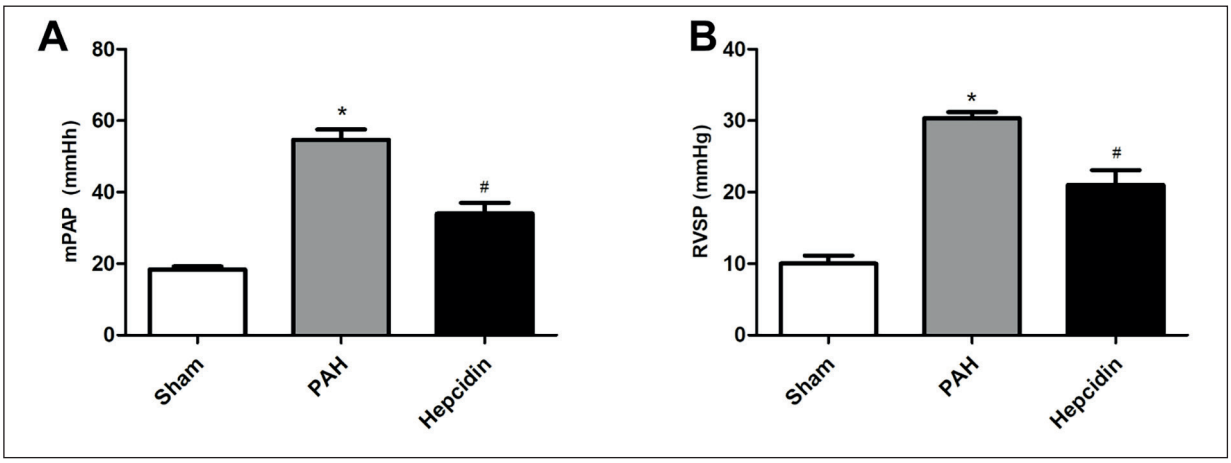

Figure 1. Effects of Hepcidin on mPAP and RVSP of PAH rats. Rats were randomly divided into sham group (n=8), PAH group (n=8) and Hepcidin group (n=8). ***A*,** MPAP in each group. ***B*,** RVSP in each group. Data were expressed as mean ± SD. *p<0.05 compared with sham group; $^{\#}p$<0.05 compared with PAH group.

Table I. Morphological and hemodynamic parameters measured using echocardiography in live rats.

	Sham	PAH	Hepcidin
h, mm	0.62 ± 0.06	0.86 ± 0.04*	0.76 ± 0.03
SV, lL	214.7 ± 9.8	208.0 ± 10.1*	176.2 ± 12.2$^{\#}$
CO, mL/min	71.5 ± 3.9	69.3 ± 4.6	70.7 ± 5.5
CI, mL/min/kg	213.6 ± 10.4	212.3 ± 12.7	218.0 ± 20.3
IVRT, ms	29.4 ± 1.2	34.7 ± 1.7*	29.7 ± 1.7$^{\#}$
ET, ms	77.2 ± 2.9	67.6 ± 1.5*	72.8 ± 4.9$^{\#}$

Data are expressed as mean ± SEM. n=8 in each group. h, ventricular wall thickness; SV, stroke volume; CO, cardiac output; CI, cardiac index; IVRT, isovolumic relaxation time; ET, ejection time. *p < 0.05 *vs*. Sham, $^{\#}p$ < 0.05 *vs*. PAH.

cle. Ventricular-vascular coupling efficiency was maintained in rats of Hepcidin group; however, no statistical significance was detected. Neither SV (stroke volume) nor CO (cardiac output) was affected by PAH or Hepcidin treatment. Furthermore, CI (cardiac index) in rats of Hepcidin group was remarkably higher than that of PAH group (Table II).

Table II. Hemodynamic parameters measured using right heart catheterization in live rats.

	Sham	PAH	Hepcidin
sys Ps, mmHg	112.8 ± 6.9	132.6 ± 5.1*	119 ± 6$^{\#}$
Pes, mmHg	24.2 ± 1.1	45.6 ± 5.6*	40.6 ± 3.7$^{\#}$
dP/dt max, mmHg/s	2719 ± 168	4066 ± 262*	3273 ± 310$^{\#}$
dP/dt min, mmHg/s	-2064 ± 137	-3426 ± 290*	-2664 ± 460$^{\#}$
EF, %	56 ± 3	54 ± 3	52 ± 5
HR, bpm	350 ± 10	341 ± 13	338 ± 17
SV, uL	283.5 ± 15.5	246.8 ± 14.6	226.5 ± 16.2
CO, mL/min	98.7 ± 4.9	83.2 ± 3.8	76.2 ± 6.1
CI, mL/min/kg	293.3 ± 14.0	252.4 ± 8.6*	360.9 ± 22.8$^{\#}$
SV/RV PP, uL/mmHg	13.3 ± 1.0	6.3 ± 0.5*	6.4 ± 0.7$^{\#}$
Hct, %	49 ± 1	48 ± 1	41 ± 2

Data are expressed as mean ± SEM. n=8 in each group. sys Ps, peak systemic pressure; Pes, end-systolic pulmonary pressure; dP/dt max and dP/dt min, maximal and minimal pressure gradient; HR, heart rate; SV, stroke volume; CO, cardiac output; CI, cardiac index; SV/RV PP, ventricular compliance; Hct, hematocrit. *p < 0.05 *vs*. Sham, $^{\#}p$ < 0.05 *vs*. PAH.

Hepcidin Treatment Attenuated Medial Wall Thickness of Muscular Pulmonary Arteries

The pulmonary arteriolar wall of rats in sham group was relatively thin. By comparison, rats in PAH group showed evident characteristics of pulmonary arteriole remodeling. Briefly, the pulmonary arteriolar wall (especially the arterial smooth muscle layer between the inner and outer elastic fibers) was significantly thickened, and the lumen of blood vessels was significantly narrowed. In addition, the pulmonary arteries of PAH rats showed mild hyperplasia of endothelial cells, enlarged nuclei, as well as hyperplasia of smooth muscle cells. The lumen was asymmetrical and significantly narrowed. Non-muscular arterioles were severely musculocutaneous and close to lumen occlusion. Rats in Hepcidin group showed markedly milder pathological lesions when compared with PAH group. They presented normal microstructure, with exudation of pink protein mucus. Myocardial cell edema and inflammatory cell infiltration showed no statistically significance (Figure 2A-2C). Besides, PAWT level in Hepcidin group was markedly decreased compared with PAH group (Figure 2D).

Hepcidin Decreased Levels of Inflammatory Factors Relative to NF-κB in PAH Rats

To explore the protective role of Hepcidin in PAH, we collected serum samples of rats in PAH group and Hepcidin group, respectively. Serum levels of TNF-α, TGF-β, IL-1β, and IL-6 were detected by ELISA. Our data revealed that Hepcidin treatment remarkably decreased levels of TNF-α, TGF-β, IL-1β, and IL-6 when compared with PAH group (Figure 3A-3D, $p<0.05$).

Hepcidin Down-Regulated Expressions of NF-κB and its Downstream Genes in PAH Rats

Lung tissues in rats of sham group, PAH group and Hepcidin group were harvested. Western blot

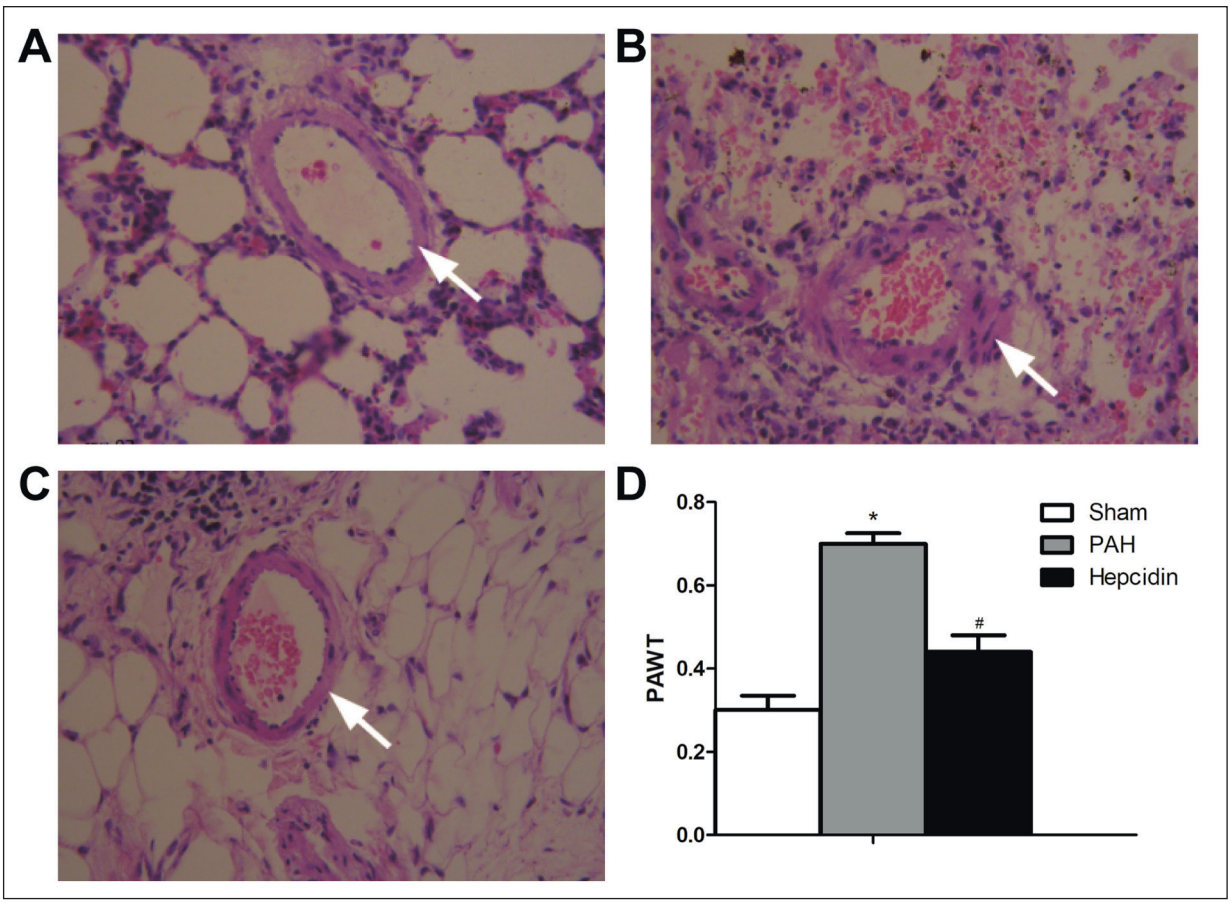

Figure 2. Hepcidin attenuated right ventricular structural remodeling in PAH rats. ***A-C,*** Pathological changes of lung tissues in sham group (n=8), PAH group (n=8) and Hepcidin group (n=8). ***D,*** Quantification of PAWT in each group. Magnification was set at 200×. Data were expressed as mean ± SD. *$p<0.05$ compared with sham group; $^{\#}p<0.05$ compared with PAH group

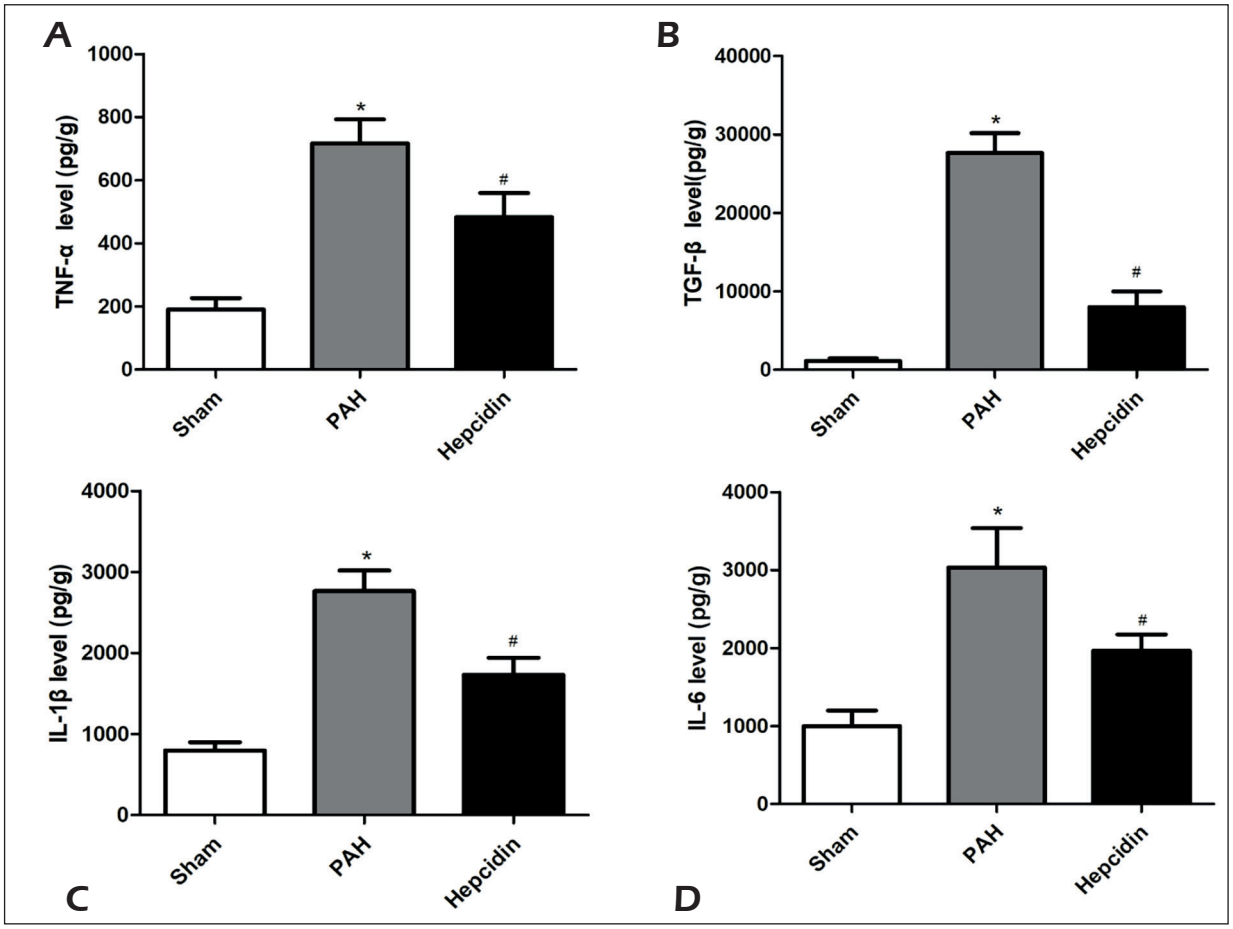

Figure 3. Hepcidin decreased levels of inflammatory factors relative to NF-κB in PAH rats. ***A,*** Serum level of TNF-α in each group. ***B,*** Serum level of TGF-β in each group. ***C,*** Serum level of IL-1β in each group. ***D,*** Serum level of IL-6 in each group. Data were expressed as mean ± SD. *$p<0.05$ compared with sham group; $^{\#}p<0.05$ compared with PAH group.

results elucidated that NF-κB was significantly down-regulated in lung tissues of Hepcidin group than that of PAH group (Figure 4A and 4B). Subsequently, cytoplasmic samples of rat lung tissues were extracted. Similarly, cytoplasmic levels of NF-κB, TNF-α, IL-1β, VCAM-1, ICAM-1, and MCP-1 in Hepcidin group were significantly lower than those of PAH group (Figure 4A, 4C-G).

Discussion

PAH is characterized by elevated pulmonary vascular resistance and pulmonary pressure[1-3]. So far, the specific pathogenesis of PAH has not been fully demonstrated. Studies have shown that main characteristics of PAH are the proliferation of pulmonary vascular endothelial cells and smooth muscle cells, as well as pulmonary vascular remodeling[11-13]. Excessive proliferation further narrows the lumen of blood vessels and aggravates blood flow resistance, eventually exacerbating PAH[12,13]. Our previous results found that expressions of genes relative to NF-kB/TNF-α pathway were upregulated after hypoxia induction in important organs of rats, such as heart, liver, spleen, lung, kidney, brain and muscle. With the prolongation of hypoxia induction in lung tissues, expression levels of these relative genes were gradually increased[24-26].

As a central regulator of intracellular inflammatory mediator signaling, NF-κB regulates expressions of multiple genes involved in immune responses, inflammatory responses, cell proliferation, and tumorigenesis[14-17]. The NF-κB family includes five members, namely p65 (RelA), RelB, cRel, NF-κB1 (p50), and NF-κB2 (p52). They present a common feature that their amino ter-

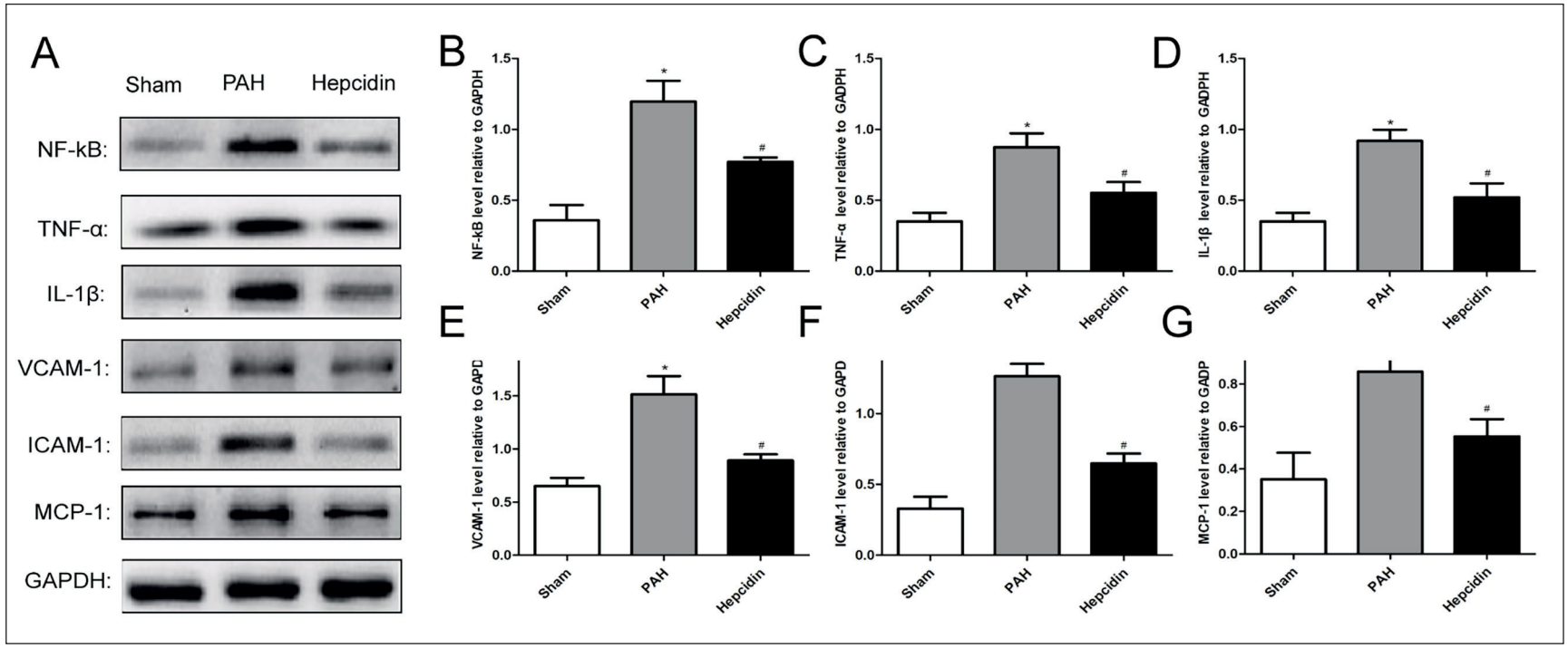

Figure 4. Hepcidin down-regulated expressions of NF-κB and its downstream genes in PAH rats. ***A,*** Protein levels of NF-κB, TNF-α, IL-1β, VCAM-1, ICAM-1, and MCP-1 in each group. ***B-G,*** Quantification of protein levels of NF-κB, TNF-α, IL-1β, VCAM-1, ICAM-1, and MCP-1. GAPDH was used as an internal reference. Data were expressed as mean ± SD. *$p<0.05$ compared with sham group; $^{\#}p<0.05$ compared with PAH group.

minus consists of a conserved region containing approximately 300 amino acids (Rel homology domain, RHD). The functional region within RHD can bind to DNA and dimerize, thereby regulating the expression of target genes[14,16]. These members can bind in the form of homodimers or heterodimers. Moreover, the common NF-κB dimers are p50/p65, p50/cRel, and p52/RelB. The NF-κB inhibitory protein (inhibitor of κB, IκB) family includes eight members in mammals, namely IκBII, IκBβ, IκBY, IκB £, IκB-R, Bcl-3, p105, and p100. Ank repeated sequence of IκB interacts with RHD of NF-κB, thereby inhibiting NF-κB activity and retaining it in cytoplasm[15,19]. NF-κB has two distinct activation pathways, including classical and non-canonical NF-κB pathways. The classical NF-κB pathway can be rapidly and transiently activated by various mitogens, cytokines or certain microbial components, which are dependent on IκB degradation[13,17]. IKK contains two catalytic subunits (IKKα and IKKβ) and one regulatory subunit NEMO (IKKY). Activated IKK can phosphorylate, ubiquitinate and degrade the specific sites of IκB. This may result in the release and nuclear translocation of NF-κB dimers, eventually activating NF-κB pathway[18,19].

Relative studies have showed that Hepcidin is a potent anti-inflammatory drug that has been successfully synthesized. Other *in vitro* experiments have pointed out its anti-inflammatory, anti-oxidative and anti-apoptotic biological functions. Meanwhile, it is presumed to exert a protective effect on PAH[22,23]. Clinically, the mortality of PAH is relatively high, which also consumes huge medical resources. Hence, it is of great significance to elucidate the molecular mechanism of PAH and search for novel therapeutic targets[11-13]. So far, many basic researches on PAH have revealed a deeper understanding of its causes, pathogenesis and clinical manifestations. In addition, some beneficial prevention strategies have gradually applied in clinical practice[9,13].

Hepcidin has been considered to influence bone metabolism and mineral metabolism[20,21]. However, in recent years, diverse functions of Hepcidin have been explored, including anti-tumor, renin-angiotensin system inhibition, cardio-protection, anti-inflammatory and atherosclerosis prevention. In particular, the relationship between Hepcidin and fibroblasts has been well concerned[20-22]. Animal experiments have detected the protective effect of Hepcidin on PAH[22,23]. In this study, rats in PAH group showed significantly histopathological changes, higher oxidative stress level and weaker anti-inflammatory ability when compared with those of sham group. These results suggested that oxidative stress induced by PAH largely produced inflammatory response that could impair tissues and cells. Besides, the expression of NF-κB in PAH group was significantly higher than that of Hepcidin group, showing an inhibitory effect of Hepcidin on PAH-induced NF-κB activation.

Conclusions

Hepcidin can significantly reduce PAH-induced inflammatory response by inhibiting NF-kB/TNF-α pathway. Moreover, it can be used as a potential treatment therapy for PAH in the future.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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