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BASIC RESEARCH

Intestinal endotoxemia plays a central role in development of hepatopulmonary syndrome in a cirrhotic rat model induced by multiple pathogenic factors

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Abstract

AIM: To characterize the correlation between severity of hepatopulmonary syndrome (HPS) and degree of hepatic dysfunction, and to explore how intestinal endotoxemia (IETM) affects the development of HPS in cirrhotic rats.

METHODS: Male Wister rats were fed with a diet containing maize flour, lard, cholesterol, and alcohol and injected subcutaneously with CCl_4 oil solution every two days for 8 wk to induce typical cirrhosis and development of HPS. The animals were also given a nitric oxide (NO) production inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME) intraperitoneally, and an iNOS inhibitor, aminoguanidine hydrochloride (AG) via gavage daily from the end of the 4th wk to the end of the 6th or 8th wk, or a HO-1 inhibitor, zinc protoporphyrin (ZnPP) intraperitoneally 12 h prior to killing. Blood, liver and lung tissues were sampled.

RESULTS: Histological deterioration of the lung paralleled to that of the liver in the cirrhotic rats. The number of pulmonary capillaries was progressively increased from 6.1 ± 1.1 (count/filed) at the 4th wk to 14.5 ± 2.4 (count/filed) at the 8th wk in the cirrhotic rats. Increased pulmonary capillaries were associated with increased blood levels of lipopolysaccharide (LPS) (0.31 ± 0.08 EU/mL *vs* control 0.09 ± 0.03 EU/mL), alanine transferase (ALT, 219.1 ± 17.4 U/L *vs* control 5.9 ± 2.2 U/L) and portal vein pressure. Compared with normal control animals, the number of total cells in bronchoalveolar lavage fluid (BALF) of the cirrhotic rats at the 8th wk was not changed, but the number of macrophages and the ratio of macrophages to total cells were increased by nearly 2-fold, protein expression of inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) started to increase significantly at the 4th wk, and reached its peak at the 8th wk in the lung of cirrhotic rats. The increase of iNOS expression appeared to be quicker than that of eNOS. NO_2^-/NO_3^- was also increased, which was correlated to the increase of iNOS (r = 0.7699, P < 0.0001) and eNOS (r = 0.5829, P < 0.002). mRNA expression of eNOS and iNOS was highly consistent with their protein expression.

CONCLUSION: Progression and severity of HPS as indicated by both increased pulmonary capillaries and histological changes are closely associated with LPS levels and progression of hepatic dysfunction as indicated by increased levels of ALT and portal vein pressure. Intestinal endotoxemia plays a central role in the development of HPS in the cirrhotic rat model by inducing NO and/or CO.

Key words: Endotoxin; Alcohol; Nitric oxide synthase; Hemeoxygenase-1; Capillary; Macrophage; Cirrhosis; Hepatopulmonary syndrome

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INTRODUCTION

Hepatopulmonary syndrome (HPS) develops when arterial oxygenation deficiency occurs due to intrapulmonary vascular dilatations that are often associated with severe hepatic disease[1]. Recent studies support that the presence

of HPS significantly increases the mortality of cirrhotic patients, particularly those with decompensated liver disease[2,3].

The pathogenesis and precise mechanism of pulmonary vascular abnormalities in HPS are the fields of active investigation. So far, studies have been focused on characterizing the increased pulmonary production of vasodilator substances, most notably nitric oxide (NO). Pulmonary NO overproduction in human HPS, as assessed by exhaled NO levels, has also been reported[4,5]. It was reported that HPS patients showed short term improvements in oxygenation after acute administration of L-NAME (a specific nitric oxide synthase inhibitor) or methylene blue (an inhibitor of cyclic guanosine monophosphate generation)[6-8], indicating that pulmonary NO and cyclic guanosine monophosphate production can lead to changes in pulmonary vascular tone, thus affecting oxygenation. However, the role of pulmonary NO in lung oxygenation is still controversial. Inhalation of L-NAME could not acutely improve intrapulmonary vasodilatation[9], and sequential inhibition of the nitric oxide-cyclic guanosine monophosphate pathway by curcumin (diferuloylmethane), terlipressin and methylene blue could not improve the intrapulmonary shunt, and both hypoxaemia and orthodeoxia were substantially worsened after treatment with the above three durgs[10], strongly suggesting that factors other than nitric oxide synthase (NOS)-derived NO effects on vascular tone contribute to HPS. It has also been shown that the levels of COHb indicative of CO production are increased in cirrhotic patients who develop HPS compared to the cirrhotic patients who do not develop HPS and correlate with gas exchange abnormalities, suggesting that CO may also contribute to human HPS[11].

The sequelae of endotoxemia are often fatal. The lung appears to be an organ sensitive to endotoxemia and many distant-focus infections can be detected as a result of pulmonary dysfunction[12,13]. Patients with hepatic cirrhosis have an elevated plasma level of lipopolysacchride (LPS)[14], which is a leading causative factor for cirrhotic complications, such as hepatorenal syndrome[15-18] and hepatic encephalopathy[19].

We have previously developed a rat model characteristic of cirrhosis and HPS that can be non-invasively induced by multiple pathogenic factors including high fat diet, alcohol, cholesterol, corn flour and CCl_4[20]. A significantly increased level of LPS in plasma is closely related to the deceased blood oxygen content and intrapulmonary vascular dilatation in the rat model, suggesting that intestinal endotoxemia (IETM) and its accompanying cytokines, such as TNF-α, play a role in the pathogenesis of HPS.

This study was to further characterize the relation between severity of HPS and degree of hepatic dysfunction, and to explore how IETM affects the development of HPS in cirrhotic rats. The results show that the severity of HPS parallels to the levels of blood LPS and progression of hepatic dysfunction and IETM plays a central role in the development of HPS in cirrhotic rats.

MATERIALS AND METHODS

Animals and reagents

Male Wister rats, weighing 220-250 g, were obtained from the Animal Center of Shanxi Medical University. All animals received human care during the study. The study was approved by the Ethical and Research Committee of the Shanxi Medical University. Experiments were performed following the institutional guideline for animal research. Cirrhosis and HPS were induced in the rats as previously described[20]. In brief, male Wister rats were fed with a complex diet containing maize flour, lard, cholesterol, and alcohol for 8 wk, and injected subcutaneously with CCl_4 oil solution every two days over the experimental period to induce typical cirrhosis and development of HPS. The animals were also given a NO production inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME) intraperitoneally, 5 mg/kg per day, or an inducible nitric oxide synthase (iNOS) inhibitor, aminoguanidine hydrochloride (AG) *via* gavage, 100 mg/kg per day from the end of the 4th wk to the end of the 6th or 8th wk, or 50 μmol/kg hemeoxygenase-1 (HO-1) inhibitor, zinc protoporphyrin (ZnPP) intraperitoneally 12 h prior to killing. The animals were randomized into four groups: (1) normal control group (fed with standard diet), (2) 4-wk group (fed with the complex diet for 4 wk), (3) 6-wk group (fed with the complex diet for 6 wk), and (4) 8-wk group (fed with the complex diet for 8 wk). Blood, liver and lung tissues were sampled from the rats.

Limulus amebocyte lysate (LAL) reagent, for determination of LPS in plasma, was provided by Shanghai Yi Hua Scientific, Inc (Shanghai, China). Tumor necrosis factor α (TNF-α) radioimmunoassay kit was provided by Radioimmunity Institute of PLA General Hospital (Beijing, China). Detecting kit for NO_2^-/NO_3^- was provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Polyclone anti- endothelial nitric oxide synthase (eNOS) and anti- inducible nitric oxide synthase (iNOS) were purchased from Beijing Zhongshan Biotic Reagent Company (Beijing, China). Immunohistochemistry kit for hemeoxygenase-1 (HO-1) was obtained from Wuhan Boshide Bioengeering Institue (Wuhan, China). TRIzol reagent was bought from the Life Technologies Company (California, USA). Reverse transcriptase polymerase chain reaction (RT-PCR) primers for β-actin, eNOS, and iNOS were synthesized by Shanghai Jiebeist Gene Company, LTD (Shanghai, China). NO inhibitor L-NAME, specific iNOS inhibitor AG, and HO-1 inhibitor ZnPP were obtained from Sigma Chemical Co (St. Louis, MO).

Measurement of LPS, TNF-α, alanine transferase, malondialdehyde *in plasma, NO_2^-/NO_3^- and COHb in pulmonary homogenate and portal vein pressure*

The contents of LPS, TNF-α, alanine transferase (ALT), malondialdehyde (MDA) in plasma, NO_2^-/NO_3^- (indirectly reflecting NO) and COHb (indirectly reflecting CO) in pulmonary homogenate were measured with the different kits following their manufacturers'instructions. The method described by MacPhail *et al*[21] was modified for

measurement of portal vein pressure (PVP) in which 18-gauge over-the-needle catheter was inserted into portal vein and connected to a water manometer.

Histology and calculation of capillaries

Samples from the liver and lung were collected and fixed immediately in 100 g/L phosphate-buffered formaldehyde overnight. After paraffin embedding, serial 4-μm thick sections were prepared and stained with hematoxylin and eosin (HE). Images (× 100) of liver and lung sections were captured with Olympus microscope equipped with a digital camera. The number of capillaries was counted in 6 fields per slide per group (n = 6) with the Image-Pro plus full automatism analysis system.

Number of cells in bronchoalveolar lavage fluid

Bronchoalveolar lavage was performed with 0.9% sterile NaCl. The number of total cells and macrophages in bronchoalveolar lavage fluid (BALF) was counted respectively with a hemocytometer and the ratio of macrophages to total cells was calculated.

Dynamic study of eNOS, iNOS and HO-1 protein expression in lung architecture

Samples from the right lobe of lung were collected into 100 g/L phosphate-buffered formaldehyde and fixed overnight. Serial 4-μm thick sections were prepared after the samples were dehydrated in graded ethanol solutions, cleared in chloroform, and embedded in paraplast. Expression of eNOS, iNOS and HO-1 was analyzed with immunohistochemical staining and quantified with the Image-Pro plus full automatism analysis system in a blind fashion.

Dynamic study of eNOS and iNOS mRNA expression in lung

RT-PCR was employed to detect the mRNA expression of eNOS and iNOS genes in lung. The primer sequences used are as follows: eNOS (435 bp)[22]: 5'-CTGCTGCCCG AGATATCTTC-3', 5'-AAGTAAGTGAGAGCCTGGCG CA-3'; iNOS (388 bp)[23]: 5'-AGCATCACCCCTGTGTTC CACCC-3', 5'-TGGGGCAGTCTCCATTGCCA-3'; β-actin (630 bp, as control)[24]: 5'-GATGGTGGGTAT GGGTCAGAAGGAC-3', 5'-GCTCATTGCCGATAG TGA TGA CT-3'.

Total RNA was extracted from 50-100 mg snap-frozen left lung tissue following TRIzol protocol. Following precipitation, the RNA was resuspended in RNase-free buffer, the concentration was determined by measuring the ultra-violet light absorbance at 260 nm and 280 nm and the purity of RNA was estimated from the ratio of A_{260}/A_{280}.

Single-stranded complementary DNA (cDNA) was synthesized from the total RNA. Two μg RNA was mixed with 0.5 μg oligo (dT), pre-incubated in 15 μL of diethylpyrocarbonate (DEPC) at 70℃ for 5 min, and rapidly chilled on ice. Five μL of M-MLV 5 × reaction buffer, 1.25 μL of dNTP (10 mmol/L, each), 25 units of rRNasin ribonuclease inhibitor, 200 units of M-MLV RT and DEPC-treated water were added into the annealed primer/template to a final volume of 25 μL. The reaction solution was incubated at 42℃ for 60 min and terminated by placing it on ice after denaturation at 85℃ for 5 min. The resulting cDNA was used as a template for subsequent PCR.

The PCR mixture contained 5 μL of 10 × Taq buffer, 1 μL of dNTP (10 mmol/L, each), 1 μL of gene specific primers, 2.0 units of Taq DNA polymerase and 1 μL of cDNA in a total volume of 50 μL. Thirty-five cycles of amplification were performed with an initial incubation at 94℃ for 3 min and a final extension at 72℃ for 7 min, each cycle consisted of denaturation at 94℃ for 1 min, annealing at 64℃ for 1 min and extension at 72℃ for 1 min. The quantities of cDNA producing equal amounts of β-actin-PCR-product were used in PCR with the primers for iNOS and eNOS. Following RT-PCR, 10 μL samples of amplified products was resolved by electrophoresis on 1.2% agarose gel and stained with ethidium bromide. The level of each PCR product was semi-quantitatively evaluated using a digital camera and an image analysis system (Alpha Innotech Co., USA), and normalized to β-actin.

Statistical analysis

Data were evaluated by analysis of variance, multiple comparisons between groups were performed with SPSS software. All values are reported as mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

Comparison of histological features of both liver and lung

Pulmonary pathological changes were consistent with hepatic pathological changes towards the same direction, namely, pulmonary pathological changes became progressively exacerbated as liver injury progressed. In normal liver architecture, sinusoids and cord located in order around central veins (Figure 1A). Derangement of hepatic cord and central lobular necrosis with infiltration of inflammatory cells, cytoplasm rarefaction in liver cells with balloon-like alteration and steatosis, and increased fiber amount in interlobular area were found in the 4th wk group (Figure 1B). Furthermore, more typical balloon-like alteration and steatosis, fiber network formation, further aggravated infiltration of inflammatory cells were observed in the 6th wk group (Figure 1C). False lobule formation, secondary degeneration and necrosis in liver cells with infiltration of inflammatory cells were demonstrated in the 8th wk group (Figure 1D). Thin alveoli wall with disseminated pulmonary phagocytes was observed in the normal control group (Figure 2A). Thick alveoli wall with infiltration of phagocytes and neutrophils was observed in the 4th wk group (Figure 2B). Dilated capillaries and increased capillary density in further wider septum, and partially decreased air space in their size with infiltration of phagocytes and neutrophils were found in the 6th wk group (Figure 2C). Obviously dilated capillaries and increased number of capillaries, massive enlarged phagocytes within wider septa and air spaces with focal narrower air spaces were found in the 8th wk group (Figure 2D).

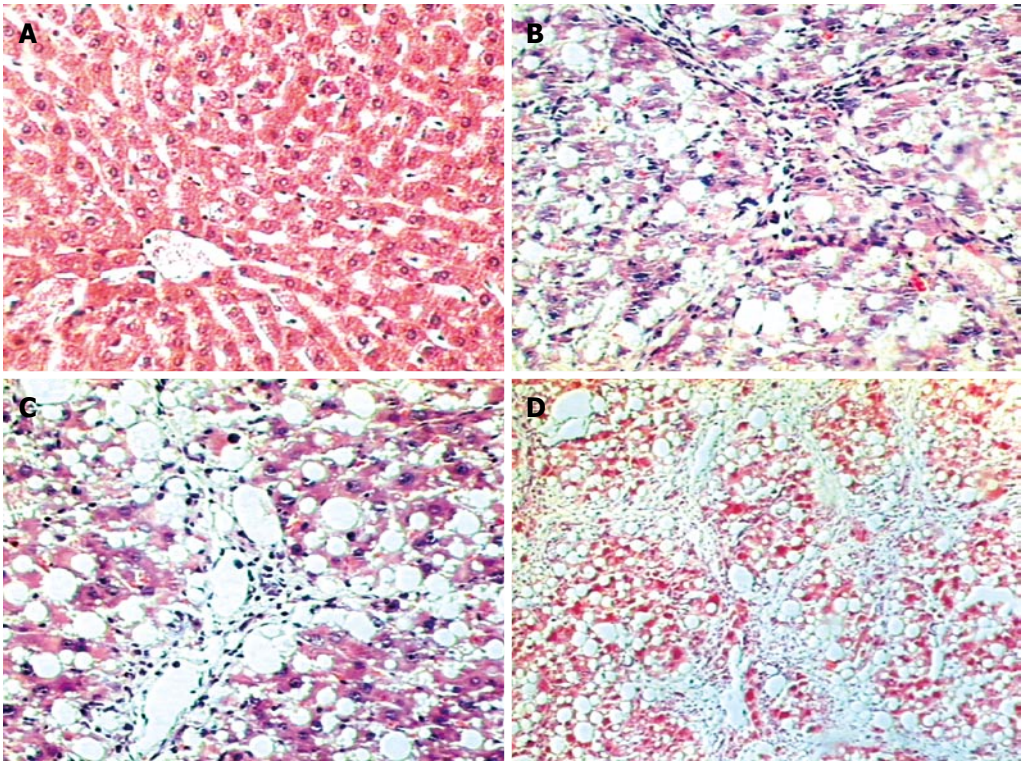

Figure 1 Liver histology of HPS (HE, × 100) in normal control group (**A**), 4th wk group (**B**), 6th wk group (**C**) and 8th wk group (**D**).

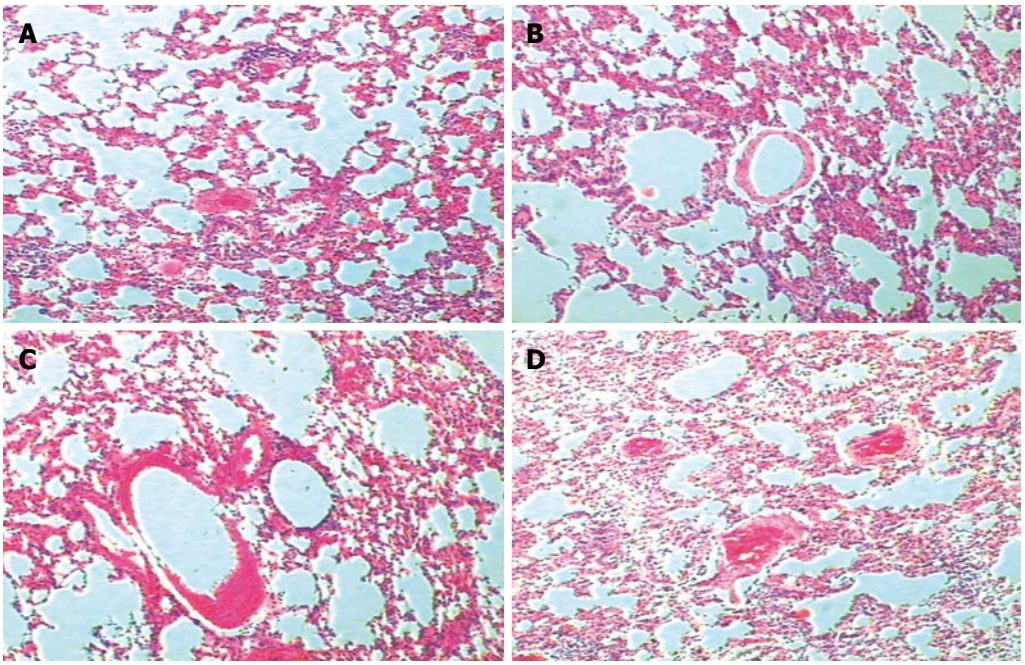

Figure 2 Lung histology of HPS (HE, × 200) in normal control group (**A**), 4th wk group (**B**), 6th wk group (**C**) and 8th wk group (**D**).

Changes in pathological parameters

The levels of LPS, TNF-α, ALT and MDA in plasma were progressively increased over the period of 8 wk. PVP was significantly elevated in the 4th wk and persisted up to the 8th wk (Table 1).

We have previously reported that the pulmonary capillaries are dilated in the lung of cirrhotic rats based on Tc99MAA labeling and scanning[20]. To know if proliferation of capillaries also occurs during the development of HPS, we counted the number of capillaries. The number of pulmonary capillaries was progressively increased in cirrhotic rats (Figure 3), which was positively related to the increased levels of LPS, TNF-α, ALT and MDA in plasma as well as to the NO and CO in pulmonary homogenate (Table 2).

The number of cells in bronchoalveolar lavage fluids could reflect the function of lung. Compared with normal control animals, the total number of cells was not changed. However, the number of macrophages and the ratio of macrophages to total number of cells were significantly increased in BALF of cirrhotic rats at the 8th wk, strongly suggesting that macrophages could play a role in the pathogenesis of HPS (Table 3).

Table 1 Levels of endotoxin, TNF-α, ALT and MDA in plasma and PVP (mean ± SD, *n* = 6)

Groups	Endotoxin (Eu/mL)	TNF-α (ng/mL)	ALT (u/L)	MDA (nmol/mL)	PVP (H_2O cm)
Normal	0.09 ± 0.03	0.53 ± 0.16	5.85 ± 2.24	0.06 ± 0.03	10.31 ± 1.16
4th wk	0.19 ± 0.03[a]	1.37 ± 0.35[a]	56.24 ± 14.73[a]	0.10 ± 0.05	17.54 ± 1.84[a]
6th wk	0.24 ± 0.04[a]	1.68 ± 0.23[a]	53.50 ± 22.10[a]	0.18 ± 0.03[a,c]	15.44 ± 3.11[a,c]
8th wk	0.31 ± 0.08[a,e]	2.42 ± 0.38[a,e]	219.10 ± 17.37[a,e]	0.26 ± 0.06[a,e]	18.38 ± 2.53[a,e]

[a]$P < 0.05$ *vs* normal group; [c]$P < 0.05$ *vs* 4th wk group; [e]$P < 0.05$ *vs* 6th wk group.

Table 2 Correlation analysis (*n* = 6)

Groups	*r*	*P*
LPS *vs* quantities of pulmonary capillaries	0.626	< 0.001
TNF-α *vs* quantities of pulmonary capillaries	0.644	< 0.001
ALT *vs* quantities of pulmonary capillaries	0.556	< 0.01
MDA *vs* quantities of pulmonary capillaries	0.691	< 0.0002
CO *vs* quantities of pulmonary capillaries	0.432	< 0.01
NO *vs* quantities of pulmonary capillaries	0.725	< 0.0001

Table 3 Changes of cell numbers in bronchoalveolar lavage fluid (mean ± SD, *n* = 6)

Groups	Total cell numbers (/μL)	Macrophages (/μL)	Ratio of macrophage/ total cell numbers
Normal control	582.7 ± 74.5	184.1 ± 5.4	0.32 ± 0.05
8th wk	619.2 ± 116.5	310.8 ± 27.5[a]	0.51 ± 0.06[a]

[a]$P < 0.05$ *vs* normal control group.

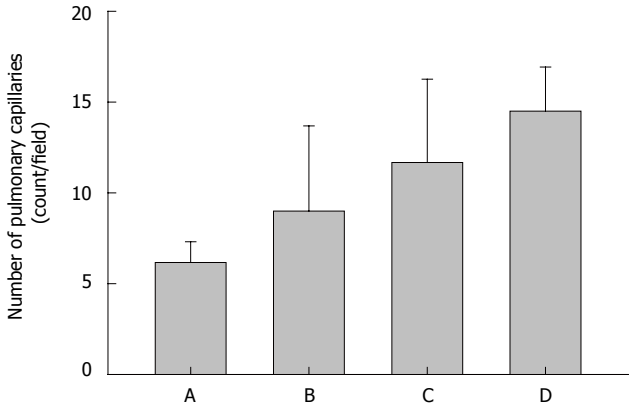

Figure 3 Change in the number of pulmonary capillaries in normal control group (A), 4th wk group (B), 6th wk group (C) and 8th wk group (D). [a]$P < 0.05$ B, C, D *vs* A; [a]$P < 0.05$ D *vs* B.

Levels of NO_2^-/NO_3^-, eNOS and iNOS in lung

Immunohistochemistry displayed that eNOS protein expression in pulmonary architecture started to increase significantly at the 4th wk and reached its peak at the 8th wk (Table 4). The change in iNOS protein expression was similar to that of eNOS, but significant differences were detected between the 4th and 6th wk as well as between the 6th and 8th wk, showing a progressive ascending. NO_2^-/NO_3^- was nearly increased by 3.5-fold at the 4th wk, by 5-fold at the 6th wk, and by more than 7-fold at the 8th wk.

Table 4 Levels of NO2-/NO3-, expression of eNOS and iNOS in lung (mean ± SD, *n* = 6)

Groups	NO_2^-/NO_3^- (μmol/g protein)	eNOS (IOD)	iNOS (IOD)
Normal control	3.6 ± 1.7	2385.6 ± 752.4	4214.8 ± 1783.7
4th wk	12.6 ± 3.7[a]	6448.9 ± 922.7[a]	8498.7 ± 2703.7[a]
6th wk	18.9 ± 5.5[a,c]	8008.9 ± 2812.7[a]	12831.6 ± 4901.2[a,c]
8th wk	26.9 ± 6.4[a,e]	14704.7 ± 5779.9[a,e]	19173.4 ± 2401.2[a,e]

[a]$P < 0.05$ *vs* normal group; [c]$P < 0.05$ *vs* 4th wk group; [e]$P < 0.05$ *vs* 6th wk group.

Table 5 Levels of eNOS and iNOS mRNA expression in lung (mean ± SD, *n* = 6)

Groups	eNOS	iNOS
Normal control	0.62 ± 0.07	0.22 ± 0.01
4th wk	0.65 ± 0.08[a]	0.98 ± 0.03[a]
6th wk	0.68 ± 0.07[a]	1.23 ± 0.03[a,c]
8th wk	0.75 ± 0.08[a,e]	1.46 ± 0.05[a,e]

[a]$P < 0.05$ *vs* normal group; [c]$P < 0.05$ *vs* 4th wk group; [e]$P < 0.05$ *vs* 6th wk group.

The increased NO_2^-/NO_3^- was more relevant to the expression of iNOS ($r = 0.7699$; $P < 0.01$) than to that of eNOS ($r = 0.5829$, $P < 0.002$).

The expression of eNOS and iNOS was distributed differently in the lung tissue stained with immunohistochemistry. In normal lung, eNOS was expressed in pulmonary capillary endothelium. In contrast, eNOS was expressed not only in the endothelium but also in type Ⅰ and Ⅱ epithelial cells and macrophages, and the expression was progressively increased in the lung of cirrhotic rats over the experimental period (Figure 4). iNOS was located mainly in macrophages and type Ⅱ epithelial cells during the development of HPS.

At mRNA levels, the expression of eNOS and iNOS in the lung was highly consistent with their protein expression (Table 5, Figure 5). Plasma endotoxin was significantly correlated with the expression of eNOS and iNOS over the 8 wk period (Table 6), suggesting that IETM might induce the expression of eNOS and/or iNOS in the development of HPS.

Effects of NOS or HO-1 inhibitors on expression of HO-1 or NOS in lung

The level of COHb and expression of HO-1 in the lung of cirrhotic rats began to increase at the 4th wk and reached their peak at the 8th wk (Table 7). The location of

Table 6 Analysis of correlation (n = 6)

Pairs	r	P value
Plasma endotoxin *vs* eNOS protein	0.6348	$P < 0.01$
Plasma endotoxin *vs* eNOS mRNA	0.7490	$P < 0.01$
Plasma endotoxin *vs* iNOS protein	0.5710	$P < 0.01$
Plasma endotoxin *vs* iNOS mRNA	0.8980	$P < 0.01$
Plasma TNF-α *vs* eNOS protein	0.8354	$P < 0.01$
Plasma TNF-α *vs* iNOS protein	0.8538	$P < 0.01$

Table 7 Changes of COHb and HO-1 protein in lung (mean ± SD, n = 6)

Groups	COHb (mg/g protein)	HO-1 protein (IOD)
Normal Control	0.13 ± 0.06	1515.18 ± 981.50
4th wk	0.27 ± 0.06[a]	7391.47 ± 674.97[a]
6th wk	0.29 ± 0.06[a]	11 157.03 ± 6093.39[a]
8th wk	0.43 ± 0.98[a,e]	24 867.59 ± 11 054.95[a,e]

[a]$P < 0.05$ *vs* normal group; [e]$P < 0.05$ *vs* 6th wk group.

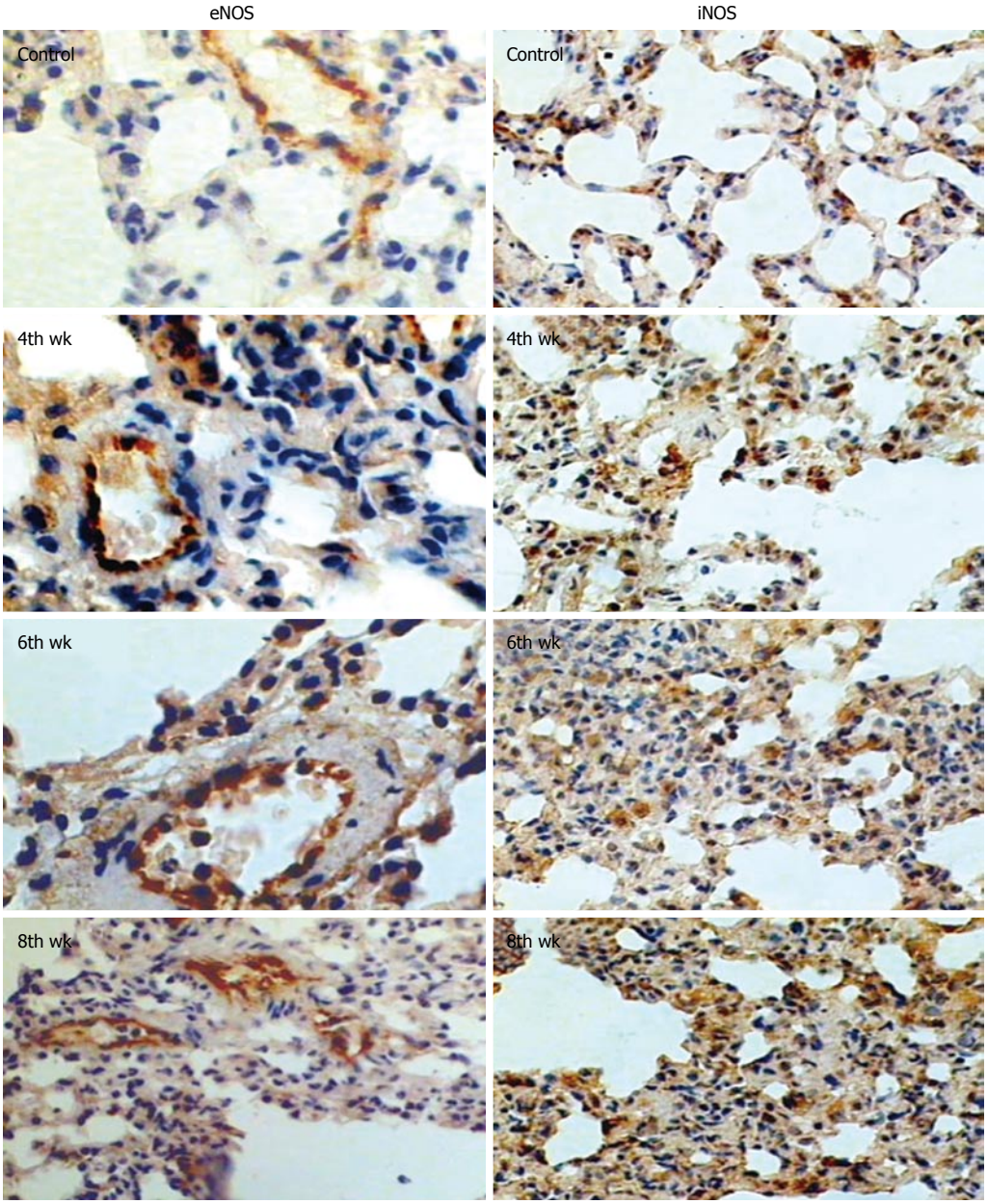

Figure 4 Immunohistochemistry of NOS expressions in the lung of rats with their eNOS and iNOS stained brown (× 200).

HO-1 was mainly found in pulmonary type Ⅱ epithelial cells, macrophages, and capillary endothelium (Figure 6). The levels of plasma LPS and TNF-α were closely correlated with COHb and expression of HO-1 (Table 8).

The expression of HO-1 in cirrhotic rats was decreased by 3-fold at the 6th wk when the non-specific inhibitor (L-NAME) of NOS was administrated and by 5-fold when the specific inhibitor (AG) of iNOS was administrated (Table 9). Similar effects were also detected at the 8th wk. Inhibition of HO-1 expression specifically by ZnPP in cirrhotic rats decreased significantly the expression of eNOS and iNOS (Table 10), indicating that both CO and NO were involved in the development of HPS.

DISCUSSION

In this study, dynamic alterations in plasma endotoxin were found to be closely associated with the expression

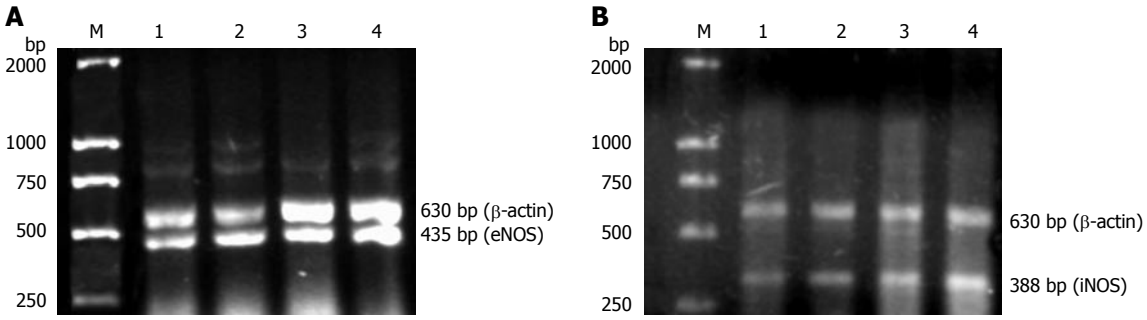

Figure 5 Expression of eNOS mRNA (**A**) and iNOS mRNA (**B**) in rat lung. M: DNA size marker; 1: control; 2: 4th wk group; 3: 6th wk group; 4: 8th wk group.

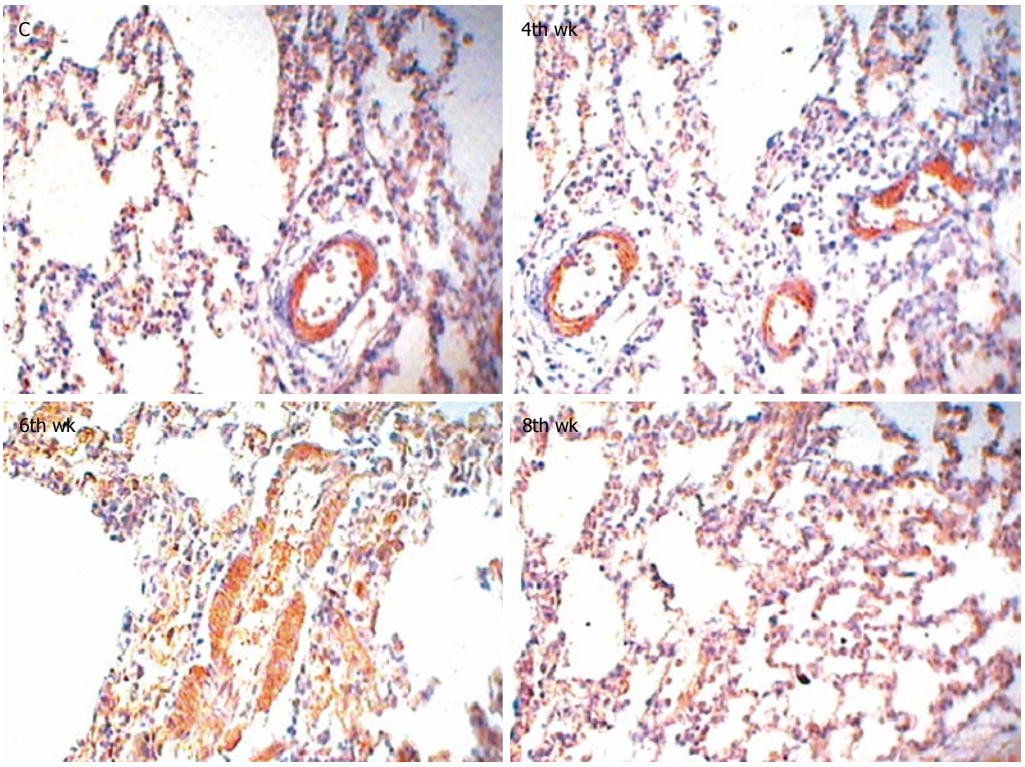

Figure 6 Immunohistochemistry of HO-1 expressions in lung of rats with HO-1 stained brown (× 200).

Table 8 Correlation analysis (n = 6)

Groups	r	P
LPS *vs* COHb	0.876	< 0.01
LPS *vs* HO-1	0.802	< 0.01
TNF-α *vs* COHb	0.755	< 0.01
TNF-α *vs* HO-1	0.796	< 0.05

Table 9 Effect of L-NAME and AG on HO-1 expression (mean ± SD, n = 6; IOD)

Groups	6th wk	8th wk
Cirrhotic control	11157.03 ± 6093.39	24867.59 ± 11054.95
L-NAME + cirrhosis	3455.13 ± 1370.03[a]	11063.48 ± 426.48[a]
AG + cirrhosis	2286.59 ± 6181.20[a]	9828.32 ± 799.51[a]

[a]P < 0.05 *vs* cirrhotic control group.

Table 10 Specific effect of ZnPP on expression of eNOS and iNOS (mean ± SD, n = 6)

Groups	eNOS (IOD)	iNOS (IOD)
6th wk cirrhotic control	13206.7 ± 8490.9	9401.1 ± 691.9
6th wk ZnPP	2025.7 ± 6097.1[a]	3500.3 ± 237.4[a]
8th wk cirrhotic control	21198.1 ± 11865.3	26419.8 ± 3560.5
8th wk ZnPP	2406.3 ± 9117.9[a]	3522.8 ± 356.5[a]

[a]P < 0.05 *vs* cirrhotic control group.

of eNOS, iNOS, HO-1, the number of capillaries, the histological changes characteristic of HPS in cirrhotic rats, demonstrating that the function of lung and liver is simultaneously deteriorated, both CO and NO are involved in pulmonary vascular abnormalities, pulmonary macrophages play a role in HPS of hepatic cirrhosis rats.

Whether the presence and severity of HPS are associated with the severity of underlying liver disease remains unclear. It was reported that HPS occurs more common in advanced cirrhosis[25-29]. HPS can also occur in acute[30] and chronic noncirrhotic hepatitis[31,32]. In patients with hypoxic hepatitis, intrapulmonary vasodilatation indicative of HPS frequently occurs and can be reversed

after normalization of hepatic dysfunction[33], indicating that HPS is interrelated with underlying liver disease. In contrast, other studies reported that HPS occurs both in extrahepatic portal venous obstruction[34] and in hepatic venous outflow obstruction without hepatic cirrhosis[35], suggesting that severe liver dysfunction and cirrhosis are not absolutely associated with the development of HPS. In the present study, the characteristic histological changes in both liver and lung during the development of HPS were observed in cirrhotic rats, indicating that there is a paralleling deterioration of lung and liver function, which supports our hypothesis that hepatic pathological alteration is a substantial basis for the development of HPS in cirrhotic rats.

Oxygenation of red blood cells occurs in the alveolar-capillary network where the pulmonary capillary bed and alveoli come together in the alveolar wall achieving optimal gas exchange. Because the diameter of pulmonary capillaries (about 6 μm) is slightly smaller than that of red blood cells (8 μm), red blood cells must flow through in a single file and change their shape as they pass through the pulmonary capillaries. This shape change ensures a minimal distance between oxygen in alveoli and hemoglobin in red cells. Passive changes in the diameters of pulmonary capillaries are caused by alterations in pre- and post-capillary resistance. Injection of micro-opaque gelatin into pulmonary arteries of autopsy specimens from patients with cirrhosis has documented the presence of pre-capillary pulmonary vascular dilatations and direct arteriovenous communications[36]. The resulting consequence is the distending of pulmonary capillaries with increased blood leading to incomplete penetration of oxygen through dilated vessels that abut upon alveoli and true anatomic shunts in which deoxygenated blood is directly shunted into arterial blood bypassing gas exchange units. Therefore, it is believed that hypoxemia occurs as a result of one (or the combination of several) of the following mechanisms: (1) ventilation-perfusion mismatching (reflecting excess perfusion for a given ventilation), (2) diffusion-perfusion impairment (due to an increased oxygen diffusion distance from alveoli to hemoglobin across the dilated vessels), and (3) true intrapulmonary anatomical shunts[37-42]. It was reported that vascular abnormalities in HPS are associated with the increased number of dilated pre-capillaries, capillaries and pre-capillary arteriovenous communications[43]. In experimental HPS, the increased number of pulmonary vascular capillaries can be observed under electron microscope[44], but no detailed analysis is available. Lung perfusion imaging with Tc99MAA can show broadened pulmonary capillaries in rats with HPS[20]. Furthermore, in the present study, a number of progressively increased pulmonary capillaries were found as cirrhosis progresses in rats, confirming the probability of remodeling, angiogenesis or vasculogenesis proposed by Gomez *et al*[9]. The increased number of pulmonary capillaries is positively correlated with both dynamically increased levels of LPS, TNF-α, and MDA in plasma, further demonstrating that LPS plays a central role in the development of HPS in cirrhotic rats[20]. The increased number of pulmonary capillaries is also positively correlated with dynamically increased ALT. Moreover, a clinical syndrome similar to HPS has been observed in congenital disorders without liver injury in which either hepatic venous blood flow does not reach the lung [45] or portal venous blood reaches the inferior vena cava without passing through the liver[46], indicating that factors either produced or metabolized in the liver can modulate the pulmonary vasculature and play a key role in maintaining the normal pulmonary vascular integrity. In addition, liver transplantation can reverse the ventilation-perfusion mismatch seen in HPS and restore normoxaemia[47]. These findings further support our notion that hepatic pathological alteration is a substantial basis for the development of HPS.

The role of NO in cirrhotic hyperdynamic circulation and induction of iNOS in endothelial cells by cytokines and endotoxin has been suggested as the mechanism[48], which soon appears as a very likely candidate for HPS[49]. However, the involvement of NOS isoform in increased NO production in the lung is controversial[50-53]. In the present study, the expression of eNOS and iNOS started to increase at the 4th wk and was closely related to the increased plasma LPS. Interestingly, the expression of iNOS not eNOS was significantly increased at the 6th wk compared to that at the 4th wk and the increased levels of nitric and nitrate in pulmonary homogenate exhibited a more significant linear correlation with iNOS than with eNOS, suggesting that iNOS may be more important in the development of HPS. In addition, a significantly increased quantity of macrophages in bronchoalveolar lavage fluid was observed during HPS in cirrhotic rats. These results demonstrate that the elevated NO results preferably from iNOS of pulmonary macrophages and, to a lesser extent, from eNOS in the lung of cirrhotic rats. Pulmonary macrophages may be a leading contributor to the development of HPS. Meanwhile, COHb in homogenate and HO-1 expression in the lung were increased over the 8 wk period of experiment. The increased COHb and HO-1 were positively correlated with the increased plasma LPS and TNF-α level. Since increased HO-1 expression in intravascular macrophages and carbon monoxide overproduction are also reported in a model of common bile duct ligation in rats[52], it would be interesting to investigate the interplay of NOS/NO pathway and HO-1/CO pathway in pulmonary vascular abnormalities in cirrhotic rats. For this purpose, L-NAME (a non-selective inhibitor of NOS), AG (a selective inhibitor of iNOS), or ZnPP (a selective inhibitor of HO-1) were administrated in cirrhotic rats. Interestingly, we observed a mutual inhibition, i.e., L-NAME or AG inhibited HO-1 expression, whereas ZnPP inhibited eNOS and iNOS expression. Histologically, AG inhibitor likely inhibited the enlargement of diameters of the dilated pulmonary capillaries at the 6th wk but there was no obvious change in the number of capillaries (data not show). Therefore, CO may be another important mediator in the development of HPS, HO-1/CO and NOS/NO pathways may be interrelated in pathogenesis of intrapulmonary vascular abnormalities in cirrhotic rats.

The underlying mechanisms are required to be further investigated.

Hypoxia is very important in inducing expression of the vascular endothelial growth factor (VEGF)[54], which specifically regulates endothelial cell growth and differentiation and also acts as a survival factor for endothelial cells[55]. It is generally believed that hypoxia is a consequence of intrapulmonary vascular dilatation in the presence of IETM. Since hypoxia induces NO generation[56-58] and NO induces expression of VEGF[59-62], it is conceivable that blood vessel remodeling and angiogenesis or vasculogenesis occurs during the development of HPS in cirrhotic rats. However, it is worth mentioning that CO may also be a contributing factor in regulating the expression of VEGF since CO levels are correlated with the number of capillaries.

In summary, IETM plays a central role in the development of HPS under conditions of hepatic cirrhosis[63,64]. Therefore, strategies against LPS should be made for preventing the development of HPS.

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COMMENTS

Background

Hepatopulmonary syndrome (HPS) develops when arterial oxygenation deficiency occurs due to intrapulmonary vascular dilatation often associated with severe hepatic disease. Recent studies support that the presence of HPS significantly increases mortality in cirrhosis patients, particularly in those with decompensated liver disease. The pathogenesis of HPS remains to be elucidated. Some complications of cirrhosis, including hepatorenal syndrome, hepatic encephalopathy and HPS are closely associated with intestinal endotoxemia (IETM) in both experimental and clinical investigations. We further explored the pathogenesis of HPS on the basis of the preceding research in a cirrhotic rat model induced by multiple pathogenic factors.

Research frontiers

One of the characteristics of hepatopulmonary syndrome is intrapulmonary vascular dilation which severely affects pulmonary gas exchange leading to hypoxia and increased mortality of cirrhotic patients. The present study focused on clarifying the pathogenesis of HPS, particularly the mechanism of intrapulmonary vasodilation. Clinically, many studies have been performed on evaluating the prevalence, etiology, clinical features, early diagnosis, treatment and prognosis of this syndrome worldwide.

Innovations and breakthroughs

In this study, we further characterized the relation between severity of HPS and degree of hepatic dysfunction, and explored how IETM affects the development of HPS in cirrhotic rats. Our results indicate that the severity of HPS is directly associated with the level of blood LPS and progression of hepatic dysfunction, and IETM plays a central role in the development of HPS.

Applications

All of our findings in the present study further confirm that IETM plays a central role in the development of HPS under conditions of hepatic cirrhosis. Therefore, the strategy against LPS is certainly effective both in preventing the development of HPS and in its treatment.

Terminology

Angiogenesis: a natural process in the body that involves the growth of new blood vessels. It can occur in many diseases, such as coronary artery disease, peripheral artery disease and stroke when blood supply and oxygen are insufficient in tissues. It denotes the formation of new blood vessels from pre-existing ones. Vasculogenesis: the process of blood vessel formation occurring by a *de novo* production of endothelial cells. Microvasculature remodeling: alterations in a blood vessel network resulting from arteriogenesis and angiogenesis. Briefly, arteriogenesis is an increased arterial diameter while angiogenesis is an increased number of capillaries either by sprouting from or splitting existing capillaries. External events stimulate these two types of vessel growth through a combination of mechanical and chemical pathways.

Peer review

This manuscript is very interesting. The topic is original for a basic science study and the structure of material and methods is outstanding. Results are clearly explored and discussion widely covers the topic even for humans.

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