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Protective Effects of Hydrogen Sulfide on Portal Hypertensive Vasculopathy in Rabbits by Activating AKT-NF-κB Pathway*

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Summary: The role of hydrogen sulfide (H_2S) in portal hypertension (PH)-induced esophagus-gastric junction vascular lesions in rabbits was observed. The rabbit PH models were established. The animals were randomly divided into the following groups: normal, PH, PH+sodium hydrosulfide (PH+S), PH+propargylglycine (PH+PPG). The plasma H_2S levels, apoptosis of esophageal-gastric junction vascular smooth muscle cells, and the expression of nuclear transcription factor-κB (NF-κB), p-AKT, IκBa and Bcl-2 were detected. The cystathionine γ lyase (cystathionine-gamma-splitting enzyme, CSE) in the junction vascular tissue was measured. The results showed that the plasma H_2S levels and the CSE expression levels had statistically significant difference among different groups (P<0.05). As compared with PH group, plasma H_2S levels were declined obviously (11.9±4.2 *vs.* 20.6±4.5, P<0.05), and CSE expression levels in the junction vascular tissue were notably reduced (1.7±0.6 *vs*. 2.8±0.8, P<0.05), apoptosis rate of vascular smooth muscle cells per unit area was significantly decreased (0.10±0.15 *vs*. 0.24±0.07, P<0.05), and the expression levels of p-AKT and NF-κB were significantly decreased (2.31±0.33 *vs*. 3.04±0.38, P<0.05; 0.33±0.17 *vs*. 0.51±0.23, P<0.05), however, IκBa and Bcl-2 expression increased obviously (5.57±0.17 *vs*. 3.67±0.13, P<0.05; 0.79±0.29 *vs*. 0.44±0.36, P<0.05) in PH+PPG group. As compared with PH group, H_2S levels were notably increased (32.7±7.3 *vs.* 20.6±4.5, P<0.05), the CSE levels in the junction vascular tissue were significantly increased (6.3±0.7 *vs*. 2.8±0.8, P<0.05), apoptosis rate of vascular smooth muscle cells per unit area was significantly increased (0.35±0.14 *vs*. 0.24±0.07, P<0.05), and the expression levels of p-AKT and NF-κB were significantly increased (4.29±0.49 *vs*. 3.04±0.38, P<0.05; 0.77±0.27 *vs*. 0.51±0.23, P<0.05), yet IκBa and Bcl-2 expression decreased significantly (3.23±0.24 *vs*. 3.67±0.13, P<0.05; 0.31±0.23 *vs*. 0.48±0.34, P<0.05) in PH+S group. It is concluded that esophagus-gastric junction vascular lesions happen under PH, and apoptosis of smooth muscle cells is declined. H_2S can activate NF-κB by the p-AKT pathway, leading to the down-regulation of Bcl-2, eventually stimulating apoptosis of vascular smooth muscle cells, easing PH. H_2S/CSE system may play an important role in remission of PH via the AKT-NF-κB pathway.
Key words: portal hypertension; hydrogen sulfide; vascular smooth muscle; apoptosis

Rupture of esophageal-gastric varices is one of the common and serious complications of portal hypertension (PH). Nitric oxide (NO) and carbon monoxide (CO) are accepted to be the important factors participating in PH and hyperkintis circulatory state[1, 2]. Our preliminary study found that hydrogen sulfide (H_2S) in patients with PH was significantly lower than in normal people, and is inversely proportional with the disease grading[3]. Studies have found that adding exogenous H_2S donor–NaHS can alleviate state of cirrhotic PH in rats[4–6]. In our experiment, we established the rabbit model of PH, observed the effects of H_2S on proliferation and apoptosis of vascular smooth muscle cells in stomach esophagus border area, and discussed the regulating mechanism of H_2S in vascular lesions of PH.

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1 MATERIALS AND METHODS

1.1 Materials

Animals care and the experimental scheme were in line with the animal management guidelines of China. Thirty healthy male big ear white rabbits were offered by Laboratory Animal Center of Tongji Medical College of Huazhong University of Science and Technology (weighing 2.5 to 3.2 kg). Using the method of abdominal patches, secondary infection was induced with schistosoma japonicum cercaria for 20 weeks. The PH model was established. According to the numbering method, the rabbits were randomly divided into following experimental groups: PH group, PH+NaHS (PH+S) group, PH+PPG group (n=10 each). Ten healthy white rabbits in experimental groups served as control group. NaHS and PPG were intraperitoneally injected at 21st week for 4 weeks[7].

1.2 Determination of Plasma H_2S Levels

Sensitive sulphur electrode method was used for

determining the plasma H_2S levels (μmol/L)[3].

1.3 Western Blotting of P-AKT, IκBa and CSE

The blood vessels from the stomach-esophagus border area in each rabbit were homogenized in 0.5 mL RIPA buffer overnight at 4°C. The samples were centrifuged at 10 000 r/min for 30 min. The protein samples were loaded at 30 μg/well, separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane. The membranes were incubated with primary antibody, p-AKT (1:500 dilution, Santa, USA) and IκBa (1:500 dilution, Santa, USA), subsequently with secondary antibody (horseradish peroxidaselabeled IgG, 1:3000 dilution, Cell Signal Co., USA). After rinses with PBS, the solution was then treated for color using the ECL kit. The bands were visualized and subsequently analyzed using Quantity One software.

1.4 Detection of Vascular Smooth Muscle Cell Apoptosis in Stomach-esophagus Border Area by TUNEL Method

TUNEL kit was purchased from Santa Cruz (USA), and TUNEL was done according to the manual instructions. Dark blue particles were seen in the nucleus of apoptotic cells. Four perpendicular fields of vision were observed, and the apoptosis index (AI) was calculated.

1.5 Detection of Nuclear Factor-kappa B (NF-κB) and Bcl-2 by Immunohistochemical Staining

The immunohistochemical staining was performed by the ABC kit. The rat-anti-rabbit polyclonal antibody Bcl-2 was purchased from Beijing Zhongshan Co. (China) in a dilution of 1:100, and NF-κB mouse-anti-rabbit polyclonal antibody from Cell Signaling Co. (USA) in a dilution of 1:100. The orange-brown staining of cytoplasma (nuclei) presented the positive signals of the protein expression. Ten small blood vessels and 10 medium-sized vessels at a diameter of 20–50 μm and 51–150 μm respectively were selected in each rabbit. Semi-quantitative integral analysis for NF-κB and Bcl-2 was done under the light microscope. The positive expression rate of NF-κB and Bcl-2 was assessed respectively, and the protein expression intensity was: －, +, ++, representing the positive expression cells of 0%, 1%–50% and 51%–100% respectively. The integral absorbance values of vessels were measured by Leica Q550CW image acquisition and analysis system.

1.6 Statistical Processing

Data was expressed as $\bar{x}\pm s$, and analyzed by SPSS17.0 statistical software package. The single factor analysis of variance (one-way ANOVA) was applied.

2 RESULTS

2.1 Levels of Plasma H_2S and CSE

The levels of plasma H_2S and CSE were shown in table 1.

As compared with control group, levels of H_2S and CSE in PH, PH+S and PH+PPG groups were significantly decreased (P<0.05). After treatment with NaHS, levels of H_2S and CSE in PH+S group were significantly increased as compared with those in the rest 3 groups (P<0.05). After given PPG, levels of H_2S and CSE in PH+S group showed a significant decrease as compared with PH group (P<0.05). The details are shown in fig. 1.

Table 1 Comparison of plasma H_2S and CSE among different groups (n=10 each)

Groups	H_2S (μmol/L)	CSE
Control	38.5±5.2	8.6±0.9
PH	20.6±4.5[a]	2.8±0.8[a]
PH+S	32.7±7.3[ab]	6.3±0.7[ab]
PH+PPG	11.9±4.2[ab]	1.7±0.6[ab]

[a]P<0.05 *vs*. control group, [b]P<0.05 *vs*. PH group

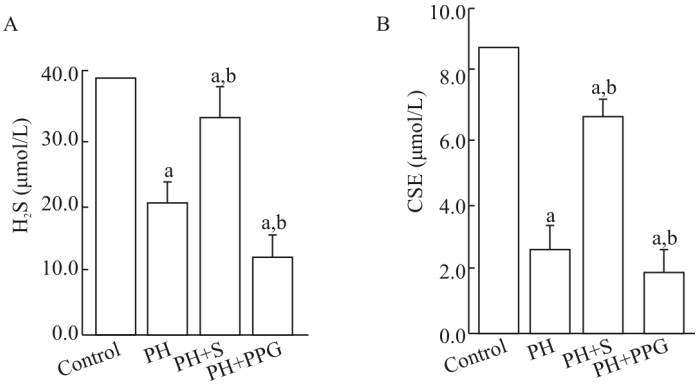

Fig. 1 Levels of H_2S (A) and CSE (B) in different groups
[a]P<0.05 *vs*. control group, [b]P<0.05 *vs*. PH group

2.2 Apoptosis of Vascular Smooth Muscle Cells in Stomach-esophagus Border Area and p-AKT, IκBa, Bcl-2, NF-κB Expression

Apoptosis of vascular smooth muscle cells in stomach-esophagus border area and p-AKT, IκBa, Bcl-2, NF-κB expression levels are shown in table 2.

Table 2 Comparison of AI and apoptosis related proteins in different groups (n=10)

Groups	AI	p-AKT	IκBa	Bcl-2	NF-κB
Control	0.45±0.16	0.21±0.13	0.19±0.13	0.25±0.26	0.28±0.30
PH	0.24±0.07[a]	3.04±0.38[a]	3.67±0.13[a]	0.44±0.36[a]	0.51±0.23[a]
PH+S	0.35±0.14[ab]	4.29±0.49[ab]	3.23±0.24[ab]	0.31±0.23[ab]	0.77±0.27[ab]
PH+PPG	0.10±0.15[ab]	2.31±0.33[ab]	5.57±0.17[ab]	0.79±0.29[ab]	0.33±0.17[ab]

[a]P<0.05 *vs*. control group; [b]P<0.05 *vs*. PH group

As compared with control group, the AI, expression levels of p-AKT and NF-κB in PH, PH+S, and PH+PPG groups were decreased obviously (P<0.05). But IκBa and Bcl-2 showed a more significant increase (P<0.05). After treatment with NaHS, the AI, expression levels of p-AKT and NF-κB in PH+S group were increased significantly as compared with those in PH group (P<0.05), yet IκBa and Bcl-2 decreased significantly (P<0.05). After given PPG, the AI, expression levels of p-AKT and NF-κB decreased significantly as compared with PH group (P<0.05), but AI, expression of IκBa and Bcl-2 increased significantly (P<0.05). The details are shown in fig. 2.

2.3 Expression of p-AKT, IκBa and CSE Proteins

The expression levels of p-AKT, IκBa and CSE proteins are shown in fig. 3.

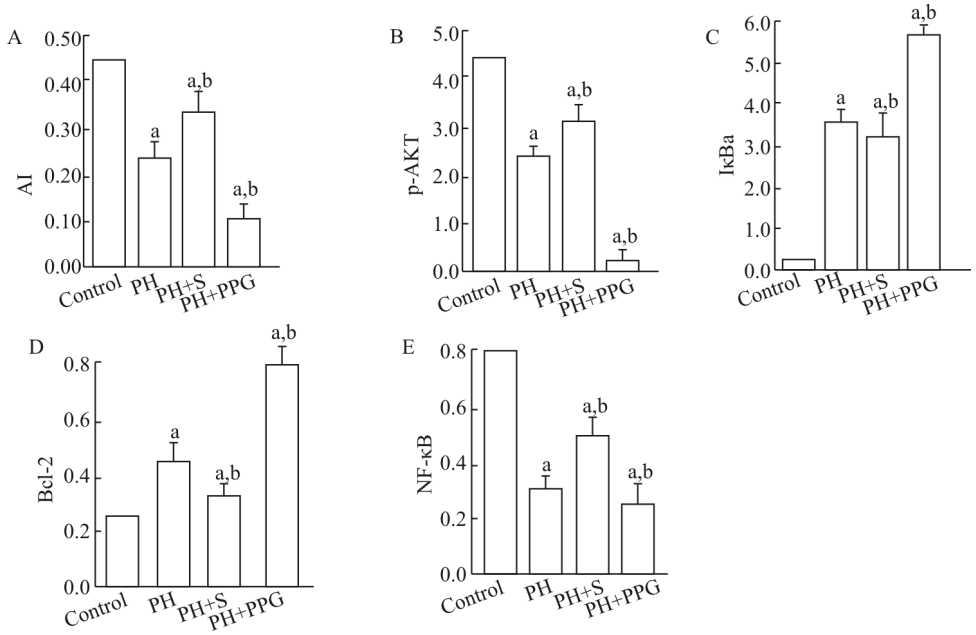

Fig. 2 AI (A), p-AKT (B), IκBa (C), Bcl-2 (D) and NF-κB (E) in different groups
[a]P<0.05 *vs*. control group, [b]P<0.05 *vs*. PH group

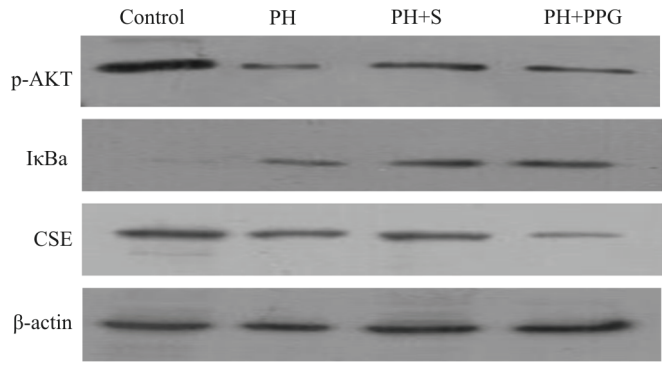

Fig. 3 Expression of p-AKT, IκBa and CSE proteins in different groups
As compared with control group, the expression levels of p-AKT and CSE were decreased obviously, and those of IκBa were significantly increased in PH, PH+S and PH+PPG groups (P<0.05). After treatment with NaHS, the expression levels of p-AKT and CSE in PH+S group were increased significantly, but those of IκBa decreased significantly as compared with PH group (P<0.05). After given PPG, the expression levels of p-AKT and CSE were decreased significantly, and those of IκBa significantly increased as compared with PH group (P<0.05).

3 DISCUSSION

The rabbit schistosomiasis cirrhosis-induced PH model is stable, and in accordant with the natural development of human schistosomiasis liver cirrhosis PH[8]. Currently, it is believed that the mechanical obstruction of portal vein system blood flow and the metabolic disorders of vasoactive substances in liver cirrhosis are involved in the pathogenesis of PH, leading to the hyperkintis circulatory state of the internal organs, and subsequently causing PH. During this process, the remodeling of vascular structure in the portal vein system is closely associated with PH. Studies have found that vascular remodeling is a result of co-regulation of cell proliferation and apoptosis, therefore, cell apoptosis plays an important role in vascular remodeling of portal vein system[9].

Endogenous H_2S comes from catalytic metabolism of sulfur-containing amino acids in the body by 5' phosphopyridoxal dependent enzymes including cystathion-

ine-beta-synzyme (CBS) and CSE, which can inhibit the proliferation and induce the apoptosis of smooth muscle cells, dilate the vascular vessels and can adjust the tension of vascular smooth muscle[10, 11]. The main expression in the portal vein and liver is CSE. Studies have found that with the development of liver cirrhosis, the expression of H_2S in portal venous blood is reduced gradually[3, 12, 13]. After administration of exogenous H_2S donor—NaHS, H_2S/CSE system is up-regulated, and PH has a certain remission, indicating that the endogenous H_2S/CSE plays a certain regulation role in cirrhotic PH disease. PI3K/AKT is a signaling pathway, involved in the regulation of cell proliferation and apoptosis, and widely exists in cells[14–16]. H_2S can induce the activation of PI3K, thus prompting the activation of its downstream key factor—AKT through a series of cascade reaction. The activated AKT can influence a series of downstream substrates, such as NF-κB, which can regulate the cell proliferation, differentiation, apoptosis and migration, etc[17–19].

Esophageal and gastric varices bleeding caused by PH is a common but serious complication, which can even cause death. In rabbit schistosomiasis cirrhosis model, PVP is elevated, AKT, IκBa, NF-κB and Bcl-2 expression levels in blood vessels of stomach-esophagus border area were significantly up-regulated, and H_2S/CSE expression level was reduced. Simultaneously, the apoptosis of vascular smooth muscle cells was decreased significantly, indicating that the elevated PVP is closely correlated to the apoptosis reduction of vascular smooth muscle cells in the stomach-esophagus border area. When given H_2S donor–NaHS, the CSE protein expression and H_2S *in vivo* were increased, the expression levels of p-AKT and NF-κB of the blood vessels in stomach-esophagus border area were increased significantly, and the AI increased, but the expression of IκBa and Bcl-2 deduced obviously, suggesting that H_2S can promote the remodeling of vascular vessels in the stomach-esophagus border area in PH, thus, alleviating PH. The involved mechanism may be that the endogenous H_2S/CSE system plays its role by influencing the p-AKT-NF-κB pathway or dilating the blood vessels of portal system directly. Our study provides certain theoretical basis for the prevention and treatment of the stomach-esophagus border area varicosity, ruptured hemorrhage and seeking for a new target for drug treatment.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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