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Inhalation of high concentrations of hydrogen ameliorates liver ischemia/reperfusion injury through A_{2A} receptor mediated PI3K-Akt pathway

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ABSTRACT

Background and aims: This study explored the hepatoprotection of high concentrations of hydrogen (HCH) inhalation in a mouse hepatic ischemia/reperfusion (I/R) injury model and the potential mechanism.

Methods: To explore the role of the PI3K-Akt pathway in the hepatoprotection of HCH, C57BL/6 mice were randomly divided into five groups: Sham, I/R, I/R + HCH, LY294002 (PI3K inhibitor) + I/R + HCH, and LY + I/R groups. Mice received inhalation of 66.7% hydrogen and 33.3% oxygen for 1 h immediately after surgery. LY294002 was intravenously injected at 10 mol/kg. To explore whether PI3K-Akt pathway activation was mediated by the A_{2A} receptor, additional four groups were included: ZM241385 (A_{2A} receptor antagonist) + I/R + HCH, ZM241385 + I/R, bpv(HOpic) (PTEN inhibitor) + I/R, and ZM241385 + bpv + I/R + HCH. Six hours after I/R, serum biochemistry, histological examination, Western blotting, and immunohistochemistry were performed to evaluate the hepatoprotection of HCH and the role of the PI3K-Akt pathway and A_{2A} receptor in this protection.

Results: Liver dysfunction, hepatic pathological injury, infiltration of inflammatory cytokines, and hepatocyte apoptosis were observed after hepatic I/R, accompanied by inhibition of the PI3K-Akt pathway. HCH significantly improved liver function, reduced serum inflammatory cytokines, and inhibited hepatocyte apoptosis, and also induced the PI3K-Akt pathway activation. In the presence of LY294002 or ZM241385, the protective effects of HCH were markedly attenuated, but the effects of ZM241385 were reversed by bpv(HOpic).

Conclusion: Our findings indicate that HCH may protect the liver against I/R injury through the A_{2A} dependent PI3K-Akt pathway.

1. Introduction

Hepatic ischemia and reperfusion (I/R) injury is a phenomenon in which cellular damage is induced by hypoxia following the restoration of blood flow and oxygen delivery after transplantation surgery, tissue resections, and hemorrhagic shock [1]. Pathologically, liver I/R injury may cause hepatocyte swelling, hepatocyte vacuolization, endothelial cell disruption, neutrophil infiltration, and hepatocyte necrosis and apoptosis [2]. Hepatic I/R injury may significantly compromise graft survival and postoperative liver function, resulting in a high mortality. It affects liver function and significantly increases the risk to the circulatory system and respiratory system [3]. To date, numerous studies have explored the treatment and prevention of liver I/R injury [4–6], but no effec-

Abbreviations: ALT, alanine aminotransferase; Bad, Bcl-2-antagonist of cell death; Fas, tumor necrosis factor receptor superfamily member 6; Fas-L, tumor necrosis factor ligand superfamily member 6; GSK-3β, glycogen synthase kinase 3 beta; H_2, hydrogen; HCH, high concentrations of hydrogen; IL-6, interleukin-6; IL-1β, interleukin-1β; I/R, ischemia/reperfusion; JNK, c-Jun N-terminal kinase; MPO, myeloperoxidase; NF-κB p65, nuclear factor-κ-gene binding p65; O_2, oxygen; PI3K, phosphatidylinositol-3-kinase; WT, wild-type; ROS, reactive oxygen species; TNF-α, tumor necrosis factor α.

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tive strategies have been developed. Therefore, liver I/R injury is an important clinical problem that requires further study.

In the pathogenesis of hepatic I/R injury, oxidative stress and inflammation are the two major mechanisms, and some strategies targeting reactive oxygen species (ROS) and inflammation are used for the treatment of hepatic I/R injury [7,8].

Hydrogen is the simplest molecule in nature. It not only exists in nature but also can be generated in the human intestine. Traditionally, it is believed to function as an inert gas at body temperature in mammalian cells because it cannot react with biological compounds, including oxygen (O_2) gas, in the absence of catalysts at body temperature. Thus, hydrogen has been used during deep diving for the prevention of nitrogen narcosis [9]. In recent years, hydrogen has been found to protect against I/R injury to the brain [10], heart [11], kidney [12], liver [13,14], and retina [15], mainly by scavenging hydroxyl radicals, inhibiting inflammation, and suppressing cell apoptosis. However, in most studies, 2% or 4% hydrogen gas was used [10]. Recently, our group treated diseases with high concentrations of hydrogen (HCH) gas (67% H_2, 33% O_2) in animal models [15,16]. The mixed gas is produced using an AMS-H-01 hydrogen oxygen nebulizer (Asclepius, Shanghai, China), which can produce H_2 and O_2 by electrolyzing water [15,16]. Whether HCH is also protective towards hepatic I/R injury and, if so, the mechanism underlying the hepatoprotection of HCH remains unclear.

The phosphatidylinositol-3-kinase (PI3K)/Akt pathway is important for cell survival, and activation of the PI3K/Akt pathway has been found to protect cells against injury. Previous studies have shown that PI3K/Akt pathway activation is important for protection against I/R injury [17,18]. In addition, studies have shown that A_{2A} receptor is involved in hepatic protective effect [19], and the PI3K/Akt pathway can be regulated by A_{2A} receptor [20].

This study was performed to explore the protective effects of HCH on hepatic I/R injury and to examine the role of the A_{2A} receptor and PI3K/Akt pathway in the protective effects of HCH.

2. Materials and methods

2.1. Animals

A total of 96 male C57BL/6 wild-type (WT) mice aged 8–10 weeks and weighing 20–25 g were purchased from the Experimental Animal Center of the Second Military Medical University, Shanghai, China. Mice were housed in a specific pathogen free environment with a 12-h/12-h light/dark cycle and given *ad libitum* access to food and water. All procedures were performed according to the recommendations of the Committee of the Care and Use of Laboratory Animals at the Second Military Medical University. This study was approved by the Ethics Committee of the Second Military Medical University.

2.2. Establishment of liver I/R model in mice

Mice were intraperitoneally anesthetized with 10% chloral hydrate (SINOPHARM, Shanghai, China) at 5 mL/kg. A model of partial (70%) warm hepatic I/R was established as previously reported with minor modifications [21]. Briefly, after a midline laparotomy, the left porta hepatis was obstructed with a clamp for 60 min, after which the clamp was removed for reperfusion. The body temperature of mice was maintained at 37 °C during the operation with a heating pad.

2.3. Animal grouping

This study was divided into two experiments (Fig. 1), as follows:

Experiment 1: To investigate the hepatoprotective effects of HCH on hepatic I/R injury and the role of the PI3K-Akt pathway, animals were randomly divided into the following groups: a) Sham group: after intraperitoneal anesthesia, a midline laparotomy was performed without any other procedures; b) hepatic I/R group: I/R was induced as described above; c) I/R + HCH group: immediately after hepatic I/R, animals were exposed to a 66.7% hydrogen and 33.3% oxygen mixture for 1 h at normal pressure; d) LY (Akt inhibitor) + HCH + I/R group: LY294002 (Akt inhibitor, 10 mol/kg) (Sigma-Aldrich, America) was administered intravenously 10 min before ischemia; and e) LY + I/R group: LY294002 at 10 mol/kg was intravenously administered 10 min before ischemia.

Experiment 2: To explore the role of A_{2A} receptor in HCH-induced PI3K-Akt activation, additional four groups were included: f) ZM (ZM241385, A_{2A} receptor antagonist) (Sigma-Aldrich, America) + HCH + I/R + group: A_{2A} receptor antagonist ZM241385 (0.2 mg/kg) was intravenously administered 10 min before ischemia; g) ZM + bpv(HOpic) + HCH + I/R group: ZM241385 (0.2 mg/kg) and bpv(HOpic) (0.05 mg/kg) were intravenously administered 10 min before ischemia; h) ZM + I/R group: ZM241385 (0.2 mg/kg) was intravenously administered 10 min before ischemia; and i) bpv(HOpic) + I/R group: bpv(HOpic) (0.05 mg/kg) was intravenously administered 10 min before ischemia.

After 6-h reperfusion [20], blood was collected for the detection of alanine aminotransferase (ALT), tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), and IL-1β, and the liver was harvested for detection of the injured area ratio after HE staining. Protein expression of Akt, p-Akt, JNK, p-JNK, GSK-3β, p-GSK-3β, Bad, p-Bad, Fas, Fas-L, NF-κB p65, and cleaved caspase-3 was determined by Western blotting of the liver, and p-Akt, MPO, cleaved caspase-3, and NF-κB p65 were analyzed using immunohistochemistry.

2.4. HCH treatment

For HCH treatment, animals were placed in a chamber that was flushed with 66.7% hydrogen and 33.3% oxygen produced with the AMS-H-01 hydrogen producer (Asclepius, Shanghai, China), which was designed to electrolyze water to produce mixed gas. In the control group, 33.3% oxygen with 66.7% nitrogen was used to treat animals. HCH treatment last for 60 min.

2.5. Sample collection

After 6-h reperfusion, blood was collected from the left ventricle and liver tissues were harvested for further detection. The serum was separated and stored at 4 °C, followed by detection of alanine aminotransferase (ALT), tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), and IL-1β. The liver tissues were divided into two parts: one was stored at −80 °C and processed for Western blotting and the other was fixed for histological examination and immunohistochemistry.

2.6. Evaluation of liver injury

The degree of liver injury was evaluated by measuring serum ALT level with an automatic biochemistry analyzer according to the manufacturer's instructions in the Department of Inspection, Eastern Hepatobiliary Surgery Hospital. (ALT Activity Assay, Sigma-Aldrich).

2.7. Histological examination

Liver tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 4-μm sections. Liver sections were subjected to hematoxylin and eosin (H&E) staining. The necrotic area was

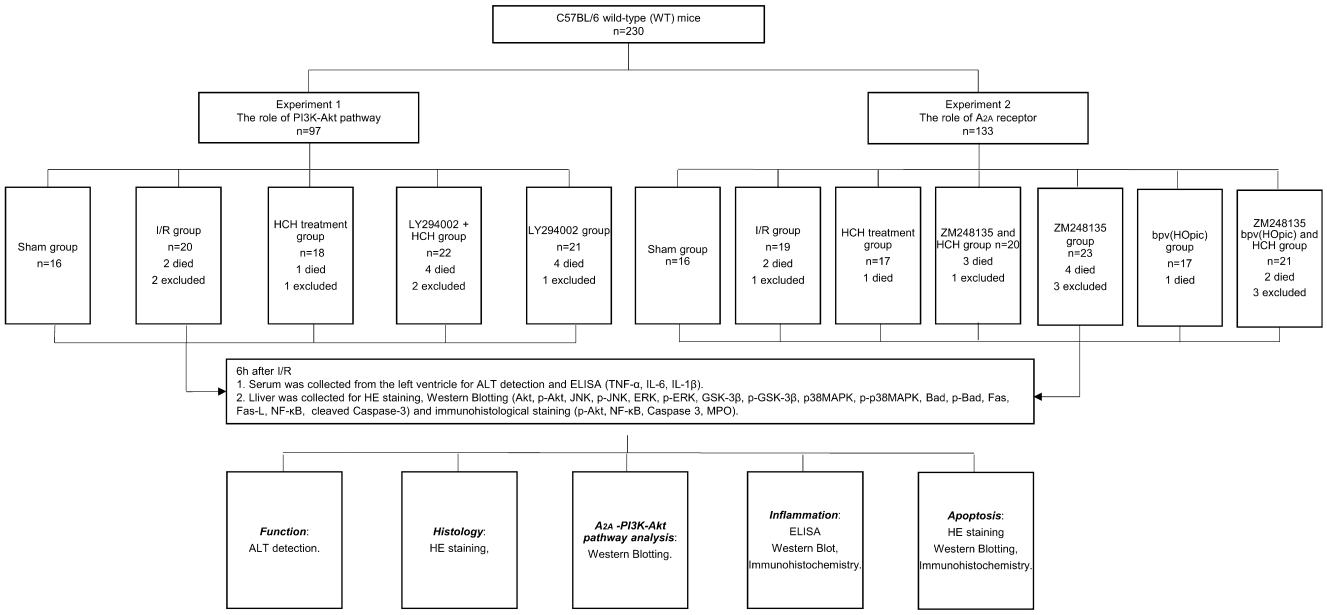

Fig. 1. Study flow chart and graphic abstract of the study.

measured under a light microscope at a magnification of 100× in 10 randomly selected fields per liver.

2.8. Immunohistochemistry

Liver tissues were processed for immunohistochemistry with the following antibodies: p-Akt (Abcam, 1:1000), NF-κB p65 (Abcam, 1:800), cleaved caspase 3 (Cell Signaling Technology, 1:1000), and myeloperoxidase (MPO; Abcam, 1:1000). Briefly, the liver sections were de-paraffinized, re-hydrated with de-ionized water, and washed three times with phosphate-buffered saline (PBS). Sections were then incubated overnight at 4 °C with primary antibodies. Sections were washed with PBS and incubated with secondary antibodies at 37 °C for 30 min. After washing three times with PBS for 5 min, sections were incubated with 0.05% (w/v) 3,3-diaminobenzidine tetrahydrochloride dehydrate, counterstained with hematoxylin, dehydrated, and mounted for microscopic examination (Carl Zeiss Jena, Oberkochen, Germany). Photographs were captured at a magnification of 400×, and positive cells were counted by two investigators in a blind manner. Positive cells were expressed as the percentage of positive cells per field.

2.9. Western blotting

Western blotting was performed as described previously with the following antibodies: Akt, p-Akt, JNK, p-JNK, GSK-3β, p- GSK-3β, Bad, p-Bad, Fas, Fas-L, NF-κB p65, and cleaved caspase-3 (Abcam, UK). Briefly, liver tissues were homogenized followed by protein extraction. Nuclear protein was extracted with a nuclear protein extraction kit (CelLytic™ NuCLEAR™ Extraction Kit, Sigma-Aldrich). After measurement of protein concentration using the BCA method, proteins in each group (50 μg) were loaded and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were independently incubated with primary antibodies at 4 °C. After washing, membranes were incubated with horseradish peroxidase conjugated secondary antibodies (1:2000) in 5% non-fat milk in TBS-T at room temperature for 2 h. Bands were visualized with chemiluminescence detection reagents (Bestbio, Shanghai, China) according to the manufacturer's instructions and then quantified with the Quantity One Analysis Software (Bio-Rad). The optical density (OD) of each band was normalized to that of GADPH as an internal reference [22].

2.10. Enzyme-linked immunosorbent assay

The serum was separated and used for the measurement of TNF-α, IL-1β, and IL-6 using an enzyme-linked immunosorbent assay (ELISA) with the corresponding commercial kits (R&D Systems, Minneapolis, MN, USA), following the manufacturer's instructions. The absorbance was measured at 450 nm with a microplate reader (ELx800, BioTek).

2.11. Statistical analysis

Statistical analyses were performed using SPSS version 16.0 software (SPSS, Chicago, IL, USA). Comparisons were performed with one-way analysis of variance (ANOVA) among groups, followed by a SNP test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Inhibition of the PI3K/Akt pathway activation attenuates the hepatoprotective effects of HCH on hepatic I/R injury

Serum ALT in the I/R group (8351.583 ± 1310.280 IU/L) increased significantly compared with the Sham group (25.833 ± 8.747 IU/L) as shown in Fig. 2A. HCH significantly decreased I/R induced increases in serum ALT (1739.583 ± 419.826 IU/L). LY294002, an inhibitor of PI3K, significantly increased serum ALT after HCH treatment (7099.417 ± 973.170 IU/L). Moreover, there was a significant difference in the serum ALT between I/R group and LY + I/R + HCH group. However, there was no significant difference in serum ALT between I/R group and LY + I/R group. The change in the ratio of injured area was the same as serum ALT (Fig. 2B and C). The ratio of injured area was 0% in the Sham group and 71.3% in the I/R

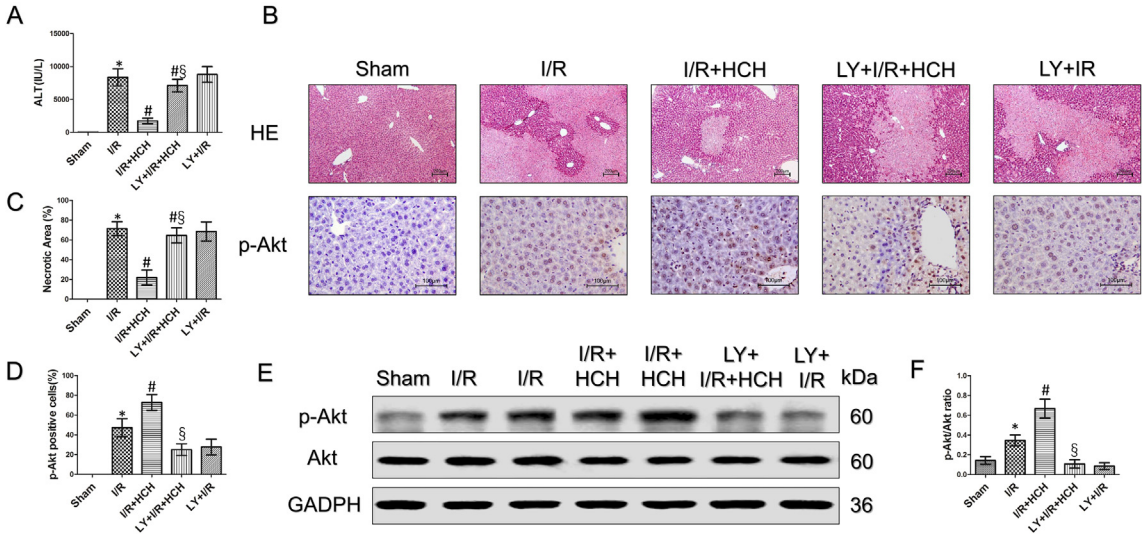

Fig. 2. Serum ALT levels, necrosis area and p-Akt expression after I/R in experiment 1. Six hours after I/R, the animals were sacrificed and the livers were collected for the detection of the necrosis area (B, C), immunohistological staining (B, D) and Western Blot (E, F), and blood was drawn for ALT detection (A). According to the result, HCH reduced ALT levels (A) and necrosis area (B, C) after I/R, and promoted p-Akt expression (B, D–F). These effects were attenuated by LY294002. Data are expressed as mean ± SD. $^{*}p < 0.05$ vs. Sham group; $^{\#}p < 0.05$ vs. I/R group; $^{\S}p < 0.05$ vs. I/R + HCH group. Magnification = 100×.

group, but HCH significantly reduced the ratio of injured area (21.9%) compared with the I/R group. In addition, the ratio of injured area in LY + I/R + HCH group (64.5%) was significantly higher than in the I/R + HCH group (Fig. 2B and C), and there was a significant difference in the ratio of injured area between the IR group and LY + I/R + HCH group. There was no significant difference in the ratio of injured area between I/R and LY + I/R groups.

3.2. HCH increases Akt phosphorylation

The liver tissues were harvested after 6-h reperfusion and processed for Western blotting. Total Akt expression was comparable among groups, as shown in Fig. 2E. p-Akt expression increased in the I/R group compared with the Sham group. In the I/R + HCH group, p-Akt expression further increased and was significantly higher than in the I/R group. However, LY294002 inhibited HCH-induced phosphorylation of Akt (Fig. 2F).

p-Akt protein expression was further detected by immunohistochemistry (Fig. 2B). In the Sham group, there were no p-Akt positive cells in the liver (0%), as shown in Fig. 2D. In the I/R group, the percentage of p-Akt positive cells increased significantly in the liver (47.2%), and HCH further enhanced p-Akt positive cells after I/R (72.8%). The increased p-Akt positive cells were reduced by LY294002 (25.0%) compared with the I/R and I/R + HCH groups.

3.3. HCH protects hepatocytes against apoptosis by modulating downstream effectors of Akt

Fas and Fas-L are apoptosis inducers and their expressions are related to the PI3K-Akt pathway [23,24]. Western blotting showed that protein expression of Fas and Fas-L increased significantly in the I/R group compared with the Sham group (Fig. 3A). HCH reduced the expression of Fas and Fas-L; however, this effect was attenuated by LY294002.

Caspase-3 is an executive factor in apoptosis. Western blotting showed that cleaved caspase-3 increased significantly after I/R compared with the Sham group (Fig. 3A and C) but decreased markedly with HCH. However, the reduction in cleaved caspase-3 was inhibited in the presence of LY294002. Similar findings were also observed in the immunohistochemistry (Fig. 3B and D). Almost no cleaved caspase-3 positive hepatocytes were observed in the liver of the Sham group, but they increased significantly after hepatic I/R (from 0.6% to 22.8%, $P < 0.05$). After HCH treatment, the percentage of cleaved caspase-3 positive hepatocytes decreased markedly compared with the I/R group (from 22.8% to 8.4%, $P < 0.05$). In the presence of LY294002, the percentage of cleaved caspase-3 positive hepatocytes increased significantly compared with the I/R + HCH group (from 8.4% to 23.8%, $P < 0.05$) and was even higher than in the I/R group.

JNK is an important molecule related to apoptosis. Phosphorylation of JNK may lead to apoptosis, and Akt activation is able to reduce JNK phosphorylation [25]. In the I/R group, p-JNK increased significantly but decreased after HCH treatment. In addition, LY294002 abolished HCH-induced reduction in p-JNK (Fig. 3A and C).

Other Akt downstream molecules, such as Bad and GSK-3β, are known to possess anti-apoptotic activity. After I/R, GSK-3β and Bad were slightly phosphorylated, but phosphorylated GSK-3β and Bad increased significantly in the I/R+ HCH group compared with the I/R group. In addition, the enhanced phosphorylation of GSK-3β and Bad was attenuated by LY294002, and it was even lower than in the I/R group (Fig. 3A and C).

3.4. HCH reduces I/R induced hepatic inflammation

TNF-α, IL-6, and IL-1β are important cytokines related to inflammation [26,27]. ELISA was used to measure the contents of TNF-α (Fig. 4A), IL-6 (Fig. 4B), and IL-1β (Fig. 4C) in the serum. In the I/R group, the contents of these cytokines were significantly higher than in the Sham group. HCH treatment significantly reduced these pro-inflammatory cytokines compared with the I/R group, but the HCH-induced reduction in these cytokines was attenuated by LY294002.

NF-κB p65 is a classic inflammatory molecule, and inflammation can induce the nuclear translocation of NF-κB p65 [28]. As shown in Western blotting (Fig. 4F), the nuclear NF-κB p65 in the I/R group was markedly higher than in the Sham group, but HCH treatment significantly reduced the nuclear NF-κB p65 after I/R. In addition, HCH-induced reduction in nuclear NF-κB p65 was abolished by LY294002.

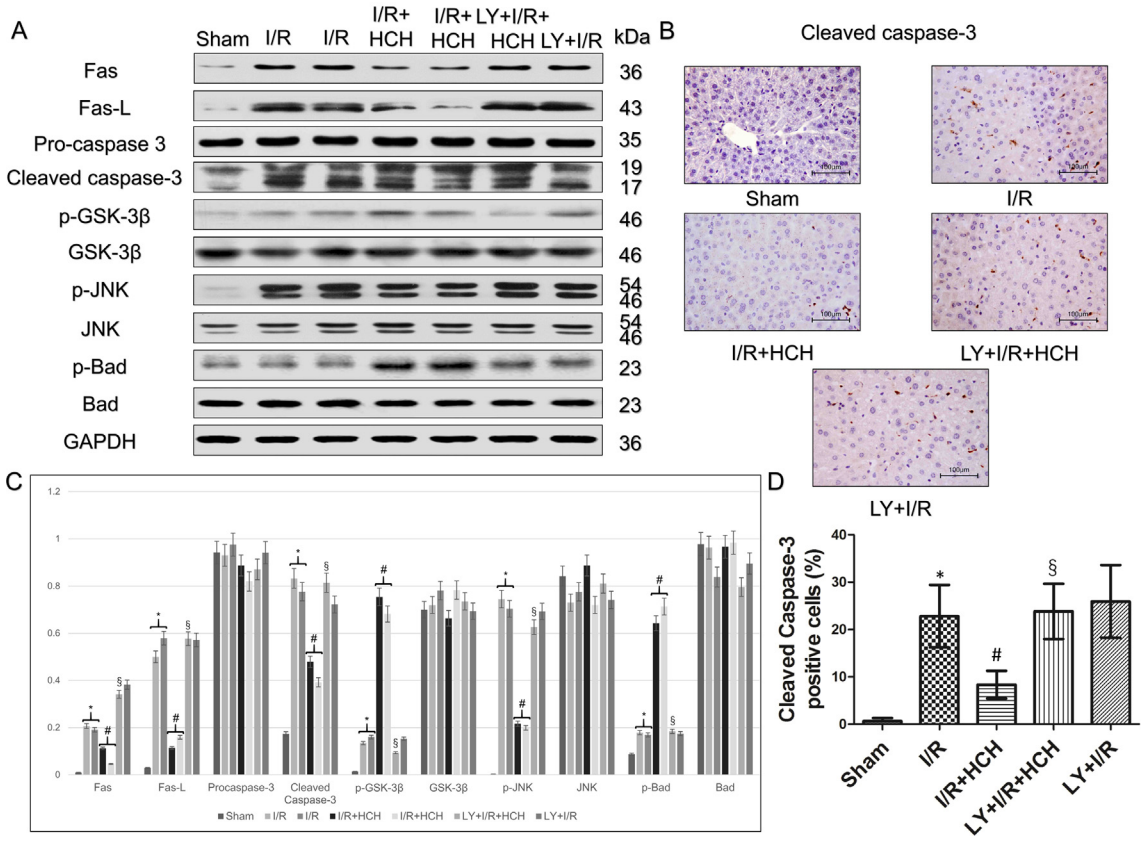

Fig. 3. Western blot of apoptosis factors and Akt downstream molecules, and immunohistology of liver tissue by cleaved caspase-3 after I/R in experiment 1. Six hours after I/R, the animals were sacrificed and the livers were collected for western blot (A, C) and immunohistological staining (B, D). According to the result, apoptosis factors decreased after HCH treatment and Akt downstream anti-apoptosis molecules were induced (A, C). Immunohistological staining reflexed the decrease of cleaved caspase-3 after HCH treatment at tissular level (B, D). These effect could be attenuated by LY294002. Data are expressed as mean ± SD. $^{*}p < 0.05$ vs. Sham group; $^{\#}p < 0.05$ vs. I/R group; $^{\S}p < 0.05$ vs. I/R + HCH group. Magnification = 400×.

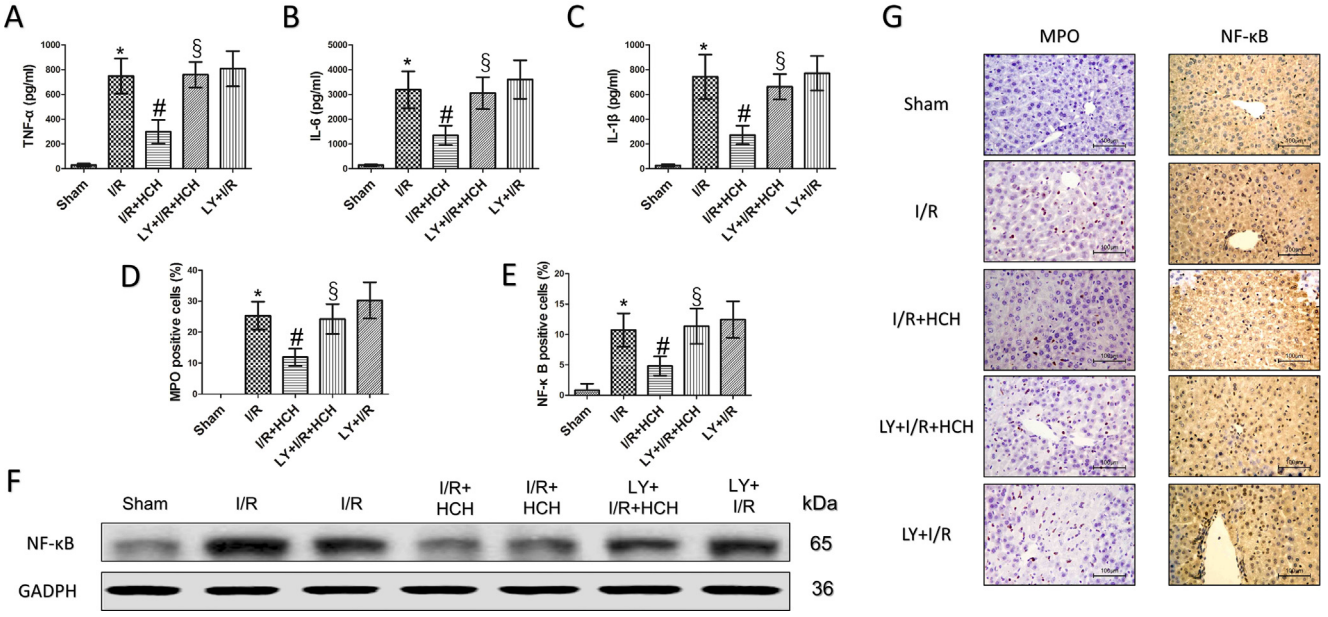

Fig. 4. Serum cytokine levels, western blot of NF-κB p65 and immunohistological staining of MPO and NF-κB p65 after I/R in experiment 1. Six hours after I/R, the animals were sacrificed, the serum was drawn for cytokine analysis (A–C) and the livers were collected for western blot (F) and immunohistological staining (D, E, G). According to the result, HCH treatment reduced the cytokine levels (A–C) and NF-κB p65 levels (F) after I/R. Immunohistological staining by MPO and NF-κB p65 antibodies showed the positive rate of MPO was decreased and NF-κB p65 was reduced after HCH treatment (D, E, G). These effects could be attenuated by LY294002. Data are expressed as mean ± SD. $^{*}p < 0.05$ vs. Sham group; $^{\#}p < 0.05$ vs. I/R group; $^{\S}p < 0.05$ vs. I/R + HCH group. Magnification = 400×.

The protective effects of HCH on liver inflammation after I/R were further investigated by immunohistochemistry for NF-κB p65 and MPO. As shown in Fig. 4E and G, the percentage of NF-κB p65 positive cells was low in the Sham group (0.6%), but increased significantly after hepatic I/R injury (11.5%). After HCH treatment, the percentage of NF-κB p65 positive cells was reduced significantly (4.1%). This finding was consistent with that from Western blotting. In addition, LY294002 abolished the HCH-induced reduction in NF-κB p65 positive cells (10.5%). MPO is a marker of neutrophil activation [29]. As shown in Fig. 4D and G, the percentage of MPO positive cells was significantly higher in the I/R group than in the Sham group (22.8% vs 0%, $P < 0.05$), but HCH treatment significantly reduced the percentage of MPO positive cells (11.3%). However, LY294002 abolished the HCH-induced reduction in MPO positive cells compared with the LY + I/R + HCH group (21.9%).

3.5. A_{2A} receptor blocker attenuates hepatoprotection of HCH

Studies have shown the therapeutic effects of A_{2A} receptor activation on liver I/R injury [19]. ZM241385 is commonly used as an antagonist of the A_{2A} receptor [30]. As shown in Fig. 5A, ZM241385 administered 10 min before I/R abolished the HCH-induced reduction in serum ALT (1863.083 ± 707.785 IU/L vs 8381.083 ± 1459.775 IU/L, $P < 0.05$). In the presence of both ZM241385 and bpv(HOpic) before I/R, serum ALT decreased significantly compared with ZM241385 pretreatment alone (8381.083 ± 1459.775 IU/L vs 1542.417 ± 309.939 IU/L, $P < 0.05$). In addition, in the ZM + I/R group, serum ALT was increased slightly compared with the I/R group (8570.500 ± 1424.906 IU/L vs 9688.083 ± 1330.451 IU/L, $P = 0.0596$). In bpv(HOpic) + I/R group, bpv(HOpic) significantly reduced the serum ALT (8570.500 ± 1424.906 IU/L vs 1853.167 ± 516.409 IU/L, $P < 0.05$).

As shown above, HCH treatment decreased the ratio of the injured area, but this effect was attenuated by ZM241385. (13.9% vs 76.6%, $P < 0.05$). In addition, bpv(HOpic) reduced the ratio of the injured area in the presence of ZM241385 compared with ZM241385 pretreatment alone (Fig. 5B and F) (76.6% vs 19.4%, $P < 0.05$). There was no significant difference in injured area between the I/R group and ZM + I/R group. The injured area was significantly reduced in the bpv(HOpic) + I/R group compared with the I/R group (68.2% vs 21.3%, $P < 0.05$).

3.6. HCH induced Akt phosphorylation is blocked by A_{2A} receptor antagonism, but restored by additional bpv(HOpic)

Western blotting showed that HCH-induced increases in p-Akt were attenuated by ZM241385, but were restored by additional bpv(HOpic) (Fig. 5C and E). This was further confirmed using immunohistochemistry. As shown in Fig. 5D and F, the percentage of p-Akt positive cells in the ZM + I/R + HCH group was significantly lower than in the I/R + HCH group, but additional bpv (HOpic) pre-treatment restored the HCH-induced increase in p-Akt positive cells.

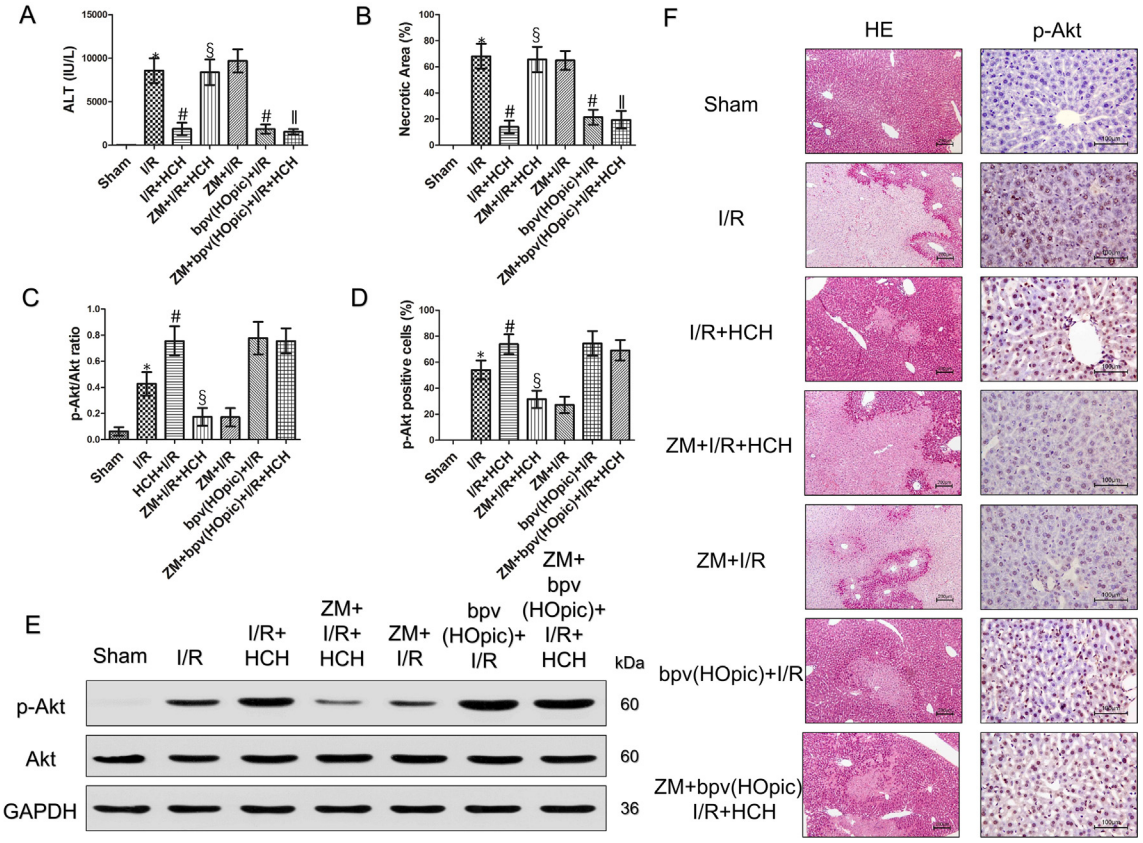

Fig. 5. Serum ALT levels, necrosis area after I/R and p-Akt expression in experiment 2. Six hours after I/R, the animals were sacrificed and the livers were collected for the detection of the necrosis area (F), Western Blot (C, E) and immunohistological staining (D, F). Blood was drawn for ALT detection (A). According to the result, application of PTEN abolished the reduction of serum ALT levels (A) and necrosis area (F), which was induced by HCH treatment. The protective effect could be re-established by the use of bpv(HOpic). In addition, Akt activation induced by HCH treatment was abolished by PTEN and could be re-established by bpv(HOpic) (C–F). Data are expressed as mean ± SD. $^{*}p < 0.05$ vs. Sham group; $^{\#}p < 0.05$ vs. I/R group; $^{\S}p < 0.05$ vs. I/R + HCH group; $^{\|}p < 0.05$ vs. I/R group. Magnification = 400×.

3.7. HCH induced anti-apoptotic and anti-inflammatory effects are attenuated by A_{2A} blocker, but restored by additional bpv(HOpic)

As shown above, HCH reduced significantly the expression of cleaved caspase-3, Fas, Fas-L, and p-JNK, and increased the expression of p-GSK-3β and p-Bad in the liver after I/R injury. However, these effects were abolished by ZM241385 pre-treatment (Fig. 6). In addition, co-administration of ZM241385 and bpv(HOpic) restored the effects of HCH, as shown by reduced expression of cleaved caspase-3, Fas, Fas-L, and p-JNK, and also enhanced the expression of p-GSK-3β and p-Bad (Fig. 6A and B). Inhibition of apoptosis was further confirmed by immunohistochemistry for cleaved caspase-3 (Fig. 6C and D).

As shown in Fig. 7A–C, the HCH-induced reduction in serum pro-inflammatory cytokines was abolished by ZM241385 and restored by additional bpv(HOpic). In addition, Western blotting showed that HCH-induced reduction in NF-κB p65 after liver I/R was abolished by ZM241385 and was re-stored by additional bpv (HOpic) before I/R (Fig. 7F). Immunohistochemistry for NF-κB p65 and MPO also confirmed the above findings (Fig. 7G and H).

4. Discussion

In this study, we investigated the hepatoprotective effects of HCH in a mouse model. Our results showed that, after liver I/R injury, inhalation of hydrogen at a high concentration (66.7% H_2) improved the liver pathology and liver function, reduced the oxidative stress and inflammation in the liver, and inhibited the apoptosis of hepatocytes after I/R injury, which were, at least partially, related to the activation of A_{2A} receptor mediated PI3K/Akt pathway because inhibition of this pathway partially abolished the hepatoprotective effects of HCH.

Hydrogen is the smallest molecule in nature and is non-toxic; in recent years, several studies confirmed its organ protection in animal studies and clinical trials [31,32]. As a new, potent antioxidant, hydrogen has some advantages over other commonly used antioxidants; for example, it is sufficiently mild as not to disturb metabolic redox reactions or affect ROS because it mainly reacts with highly toxic ROS hydroxyl radicals and peroxynitrite [10]. In addition, it has favorable distribution characteristics in its own physical ability to penetrate biomembranes and diffuse through barriers

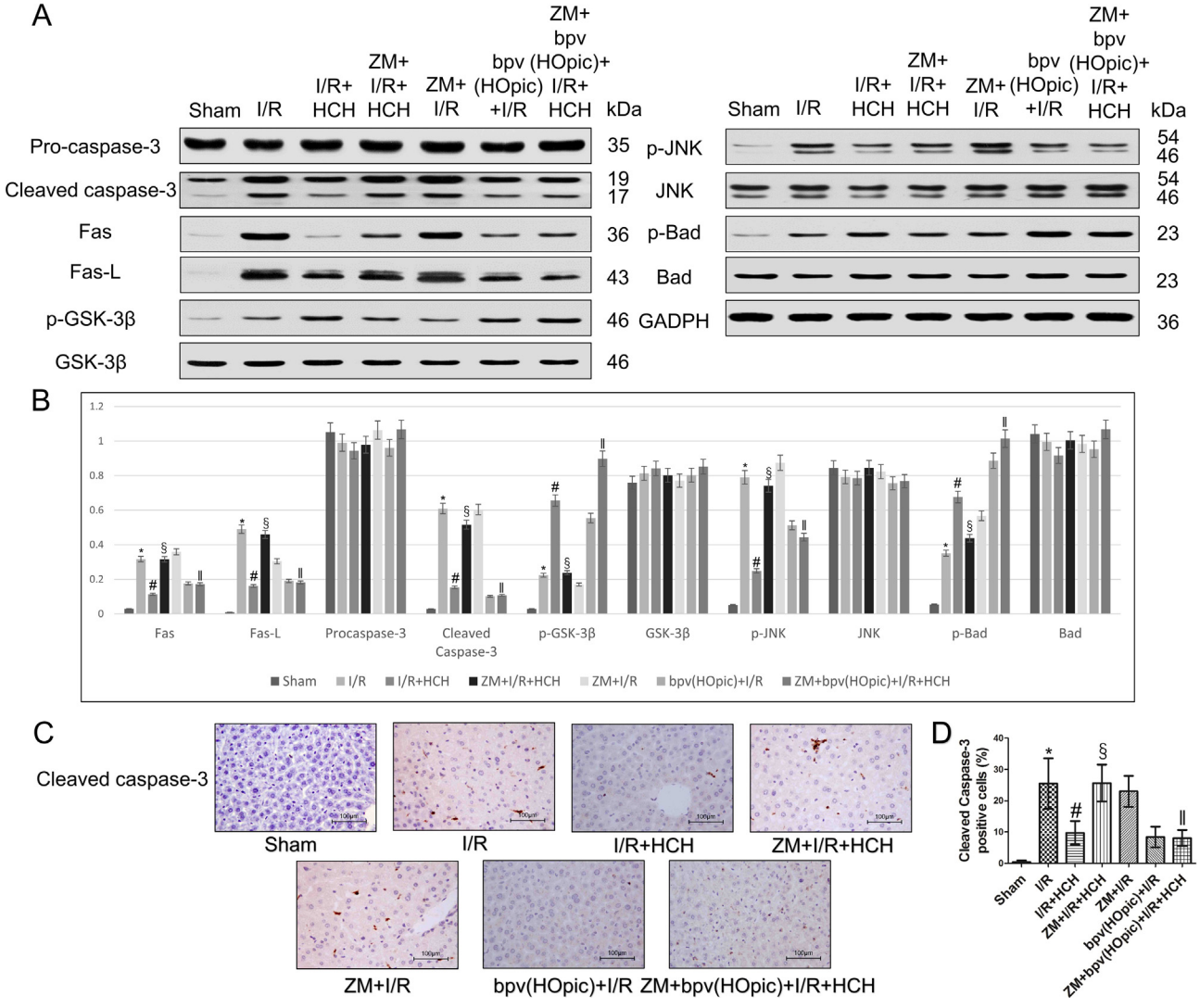

Fig. 6. Western blot of apoptosis associated molecules, Akt downstream molecules and immunohistology of liver tissue by cleaved caspase-3 after I/R in experiment 2. Six hours after I/R, the animals were sacrificed and the liver was collected for western blot (A, B) and immunohistological staining (C, D). According to the result, HCH treatment induced decrease of apoptosis factors and increase of Akt downstream anti-apoptosis molecules could be abolished by PTEN and re-established by bpv(HOpic) (A, B). Immunohistological staining by cleaved caspase-3 antibodies showed PTEN abolished the decrease of cleaved caspase-3 expression which could be re-established by bpv (HOpic) (C, D). Data are expressed as mean ± SD. $^{*}p < 0.05$ vs. Sham group; $^{\#}p < 0.05$ vs. I/R group; $^{\S}p < 0.05$ vs. I/R + HCH group; $^{\|}p < 0.05$ vs. ZM + I/R + HCH group. Magnification = 400×.

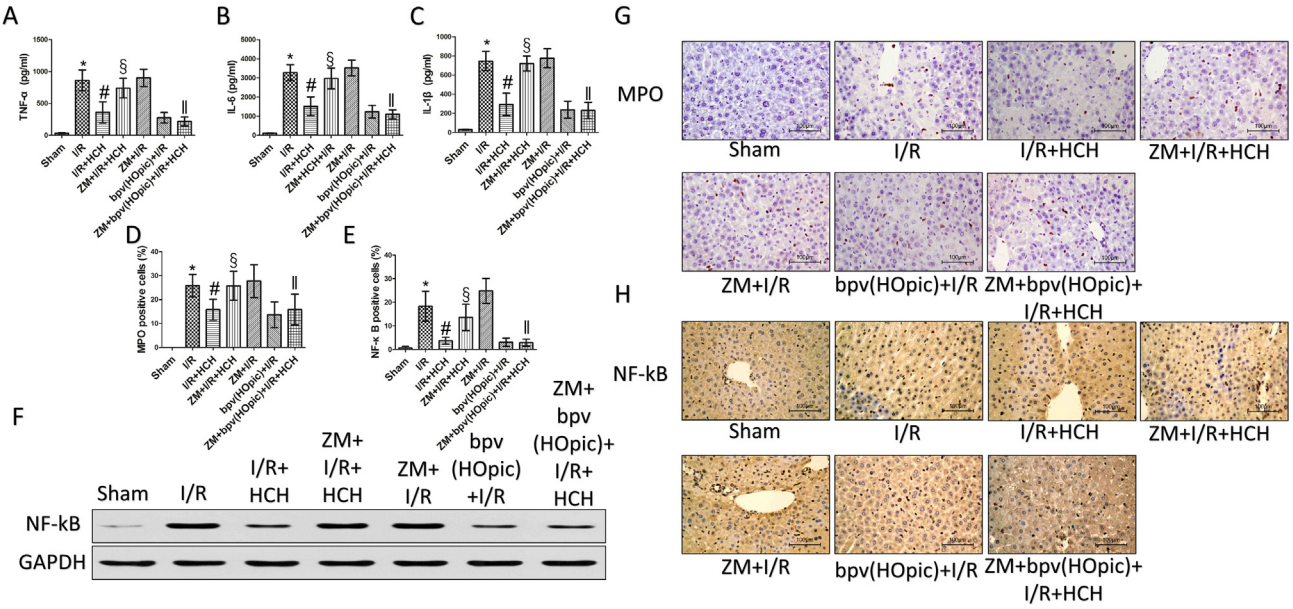

Fig. 7. Serum cytokine levels, western blotting of NF-κB and immunohistological staining of MPO and NF-κB after I/R in experiment 2. Six hours after I/R, the animals were sacrificed, the serum was drawn for cytokine analysis (A, B, C) and the liver was collected for western blot (F) and immunohistological staining (D, E, G, H). According to the result, HCH treatment reduced cytokines and this effect was abolished by the use of PTEN and re-established by bpv(HOpic) (A, B, C). Decrease of NF-κB p65 was abolished by PTEN and it was re-established by bpv(HOpic) (F). Immunohistological staining showed that the decrease of MPO positive rate and NF-κB levels was abolished by PTEN and re-established by bpv(HOpic) (D, E, G, H). Data are expressed as mean ± SD. $^{*}p < 0.05$ vs. Sham group; $^{\#}p < 0.05$ vs. I/R group; $^{\S}p < 0.05$ vs. I/R + HCH group; $^{\|}p < 0.05$ vs. ZM + I/R + HCH group. Magnification = 400×.

into cellular components; and its metabolite is water, which is not harmful to cells. Currently, hydrogen can be administered via inhalation, intraperitoneal injection, and drinking [33]. For inhalation, hydrogen is typically administered at a low concentration (≤4%) due to safety concerns, because it is highly flammable when its concentration is higher than 4%. Compared with hydrogen inhalation, intraperitoneal injection may ensure that the proper dose of hydrogen administered, but it is an invasive technique. In addition, although drinking hydrogen water has been used in previous studies, the amount of hydrogen in water may not be accurate and the hydrogen concentration in the water may decrease gradually over time. In recent years, a new hydrogen generator was developed by the Asclepius company, which can produce 66.7% hydrogen and 33.3% oxygen by electrolyzing water [15,16]. With a specific technique, it may avoid the risk for explosion of hydrogen at this high concentration. Our group has attempted to use this generator for the treatment of diseases. Wang et al. found that inhalation of HCH for 1 h after retinal I/R injury could attenuate the injury [15], and Peng et al. found that this treatment was also able to limit glyoxylate-induced calcium oxalate deposition in mice [16]; these outcomes were ascribed to the anti-inflammatory, anti-oxidative, and anti-apoptotic effects of hydrogen. Although the organ protective effects of low and high concentrations of hydrogen gas have been confirmed in numerous studies, the specific molecular mechanism of HCH remains unclear.

The PI3K/Akt signaling pathway is involved in the regulation of critical cellular functions including survival, proliferation, cell cycle, and metabolism [34,35]. PI3K activation can phosphorylate and activate Akt, localizing it in the plasma membrane. Upon activation, p-Akt phosphorylates a large number of downstream substrates, offering the following effects: regulation of microtubule dynamics and organization via GSK-3β and tau, regulation of cell growth and protein synthesis by controlling the activity of the mTOR/Raptor complex 1 (mTORC1), and promotion of cellular survival via either direct inactivation by phosphorylation of multiple proapoptotic proteins or inhibition of Forkhead box transcription factors [34,36,37]. The PI3K/Akt pathway is negatively regulated by phosphatase and tensin homolog (PTEN), a lipid phosphatase that dephosphorylates PIP3 [38]. Akt is a downstream effector of PTEN, and PTEN activation may inhibit Akt activation. In this study, bpv(HOpic), a PTEN inhibitor, was used to inhibit PTEN, activating Akt.

Our results confirmed that hydrogen at a high concentration was protective against hepatic ischemia, characterized by reductions in ALT and ischemic area, attenuation of hepatic inflammation, and alleviation of hepatocyte apoptosis. In the presence of HCH treatment, additional LY294002 significantly increased ALT and ischemic area, suggesting that the hepatoprotection of HCH is associated with PI3K activation. As a downstream factor of the PI3K pathway, Akt phosphorylation increased significantly after I/R, which further increased after HCH treatment. However, LY294002 inhibited the HCH-induced phosphorylation of Akt. In addition, there were no significant differences in serum ALT and ischemic area between I/R group and I/R + LY group, indicating that LY294002 has no hepatoprotective effects. These results indicate that the hepatoprotective effects of HCH are related to activation of the PI3K/Akt signaling pathway; the protective effects were also confirmed by the treatment of bpv(HOpic), a drug that can lead to Akt activation. Of note, additional LY294002 only partially inhibited the hepatoprotection of HCH because significant differences were observed in ALT and ischemic area between the I/R group and I/R + HCH + LY group, suggesting that the PI3K signaling pathway is not the only pathway affected by hydrogen.

We further explored whether HCH-induced activation of PI3K/Akt was dependent on A_{2A} receptor activation. During A_{2A} receptor inhibition with ZM241385, the hepatoprotection of HCH was significantly compromised, but additional bpv(HOpic) for Akt activation restored the hepatoprotection of HCH, which were further confirmed by the detection of Akt phosphorylation. These results indicate that HCH-induced Akt phosphorylation is mediated, at least partially, by A_{2A} receptor activation.

Of note, 33% oxygen was used in this study, while air contains only about 21% oxygen. In our pilot study, we also explored whether hyperoxia protected the liver against I/R injury. Mice were

exposed to 33% oxygen and 66.7% nitrogen for 1 h, but the ratio of injured area and ALT remained comparable to those in the I/R group. Moreover, there is evidence that hyperoxia may worsen the hepatic I/R injury in mice [39]. Thus, this group was not included in this study.

This study had several limitations. First, the hydrogen concentration of the liver was not detected after 1-h exposure to HCH. In addition, because hydrogen can be administered via intraperitoneal injection, inhalation, and drinking, it is better to compare the hepatoprotective effects of hydrogen administered via different routes and investigate whether hydrogen concentration is related to its protective effects, as Ohsa et al. found that neuroprotection differed between 2% hydrogen and 4% hydrogen [10]. In our study, animals received HCH treatment for only 1 h; whether prolonged exposure to HCH or intermittent exposure to HCH may further improve the hepatoprotection of HCH remains unclear.

Taken together, our findings indicate that hydrogen inhalation at a high concentration can ameliorate liver I/R injury, which is at least partially related to the A_{2A} receptor mediated PI3K-Akt pathway activation. Further studies using gene knock-out animals are required to confirm our results. Hydrogen is non-toxic and can permeate cell membranes, and it is easy and safe to produce the stable HCH; therefore, hydrogen might be a promising gas for the clinical treatment of liver ischemia/reperfusion injury.

Conflict of interest

There is no conflict of interest in this paper.

Author contribution statement

Li H., Che O. and Liu W. wrote the main manuscript. Ye Z. prepared Figs. 1 and 2. Li H. finished Figs. 5 and 6. Sun X. designed all figures. Zhang R. revised the whole manuscript. Zhang N. finished Figs. 3 and 4. Huang J. designed the supplements 1.

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