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Effects of Anti-Histamine Treatment on Liver Injury Triggered by Small Intestinal Ischemia Reperfusion in Rats

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Abstract

Mast cell (MC) degranulation has been implicated in small intestinal ischemia reperfusion (IIR) injury, therein, inhibiting overproduction of histamine released from activated MC may provide promising strategies against IIR-mediated liver injuries. The aim of the present study was to explore whether anti-histamine treatment contribute to attenuating IIR-mediated liver injury. Adult SD rats were randomized into sham-operated group (S group), sole IIR group (IIR group), and IIR treated with Ketotifen, a histamine antagonist (IIR+K group), Cromolyn Sodium, a MC stabilizer (IIR+C group), and Compound 48/80, a MC degranulator (IIR+CP group), respectively. IIR was induced by superior mesenteric artery occlusion for 75 min followed by 4 h of reperfusion. The agents were intravenously administrated 5 min before reperfusion to induce different levels of histamine. Subsequently, serum concentrations of ALT, AST and histamine; levels of LDH,TNF-α, IL-8 and MDA as well as SOD activities in the liver were assessed. Histopathologic changes were also evaluated. IIR resulted in severe liver injury as demonstrated by significant increases in injury scores, with concomitant significant increases in serum ALT, AST and histamine levels, as well as LDH, TNF-α, IL-8, and MDA levels in the liver, accompanied by reduction in SOD activities (all $P < 0.05$, IIR *vs.* S). Treatments by Ketotifen and Cromolyn Sodium similarly markedly alleviated IIR-mediated liver injury as confirmed by significant reduction of the above biomedical changes whereas Compound 48/80 further aggravated IIR-mediated liver injury by dramatically enhancing the above biomedical changes. Data of our study suggest that anti-histamine treatments may provide promising benefits in alleviating liver injury triggered by IIR.

Key Words: histamine, intestinal, ischemia reperfusion, liver injury, mast cell degranulation

Introduction

Small intestinal ischemia reperfusion (IIR) leads not only to local damage, but also to injuries in remote organs, contributing to high mortality in the clinic (10, 18). Of the remote organs, liver is particularly vulnerable to injuries subsequent to IIR, presumably because of the washing out of toxic substances from the reperfused intestine (27, 31). Therefore, protection of the gut-liver axis may provide promising benefits against IIR-mediated dysfunctions to multiple organs (23). The development of oxidative injuries with a subsequent systemic inflammatory response have been shown to play a pivotal role in the liver injury induced by IIR (5, 26, 27), but owing to numerous toxic substances rooted in

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the intestine, it is imperative to define the underlying mechanisms of IIR induced liver injury and to explore promising therapeutics.

Mast cells, as shown in many experiments, are implicated in ischemia reperfusion injury in the intestine, heart, and lungs by releasing a group of mediators, including histamine, TNF-α and tryptase (7, 13, 28). Our previous studies have also demonstrated that mast cell activation contributed to small intestinal ischemia reperfusion injury and the secondary lung injury (12, 14). Vural *et al*. further revealed that pretreatment with the mast cell stabilizer, cromolyn sodium, could attenuate the inflammatory response induced by pulmonary ischemia-reperfusion injury in a rat model (28). Histamine, mostly released from mast cell degranulation, contributed to the genesis and development of various disorders (22). Our preliminary *in vitro* study has shown that histamine at slightly elevated concentrations aggravated hypoxia/reoxygenation-induced rat liver BRL-3A cell injury (29). It is, therefore, reasonable to speculate that IIR may mediate liver injury primarily *via* mast cell activation and the subsequently elevated release of histamine. Another study showed that treatment with Ketotifen, a histamine H1 receptor antagonist, significantly enhanced survival rate in rats subjected to IIR injury (15). Hence, stabilizing mast cell or antagonizing histamine may provide promising strategies in alleviating IIR injury.

However, whether mast cell activation and the subsequently increased release of histamine are implicated in liver injury induced by IIR has not been well documented. In particular, it remains largely unknown whether histamine is implicated in the process of inflammation- and oxidative stress-mediated liver injury triggered by IIR. The present study was designed to investigate the role of histamine released from activated mast cells in liver injury induced by IIR, and to define the protective roles of anti-histamine treatments achieved by stabilizing mast cells, or antagonizing histamine, in combating IIR mediated liver injury.

Materials and Methods

Experimental Model

This study was approved by the Institutional Animal Care and Use Committee of Sun Yat-Sen University in Guangzhou, PRC, and followed the national guidelines for the treatment of animals. Forty healthy adult Sprague-Dawley rats weighing 180-230 g were raised by basic diet for a week at a stable room temperature of 25-27°C, illuminated from 8:00 am to 8:00 pm. All rats were anesthetized with 10% choloral hydrate at a dose of 3.5 ml/kg intraperitoneal injection after they were fasted for 16 h. In the IIR group, the abdomen was opened and the superior mesenteric artery (SMA) was identified and clamped for 75 min. The clamp was then released and reperfusion of the splanchnic region was maintained for another 4 h. In sham-operated group (group S), the abdomen was opened and the SMA was isolated without clamping. In the other three groups, the rats were subjected to IIR and were respectively treated with cromolyn sodium (ICN, USA; 25 mg/kg, IIR+C group), Compound 48/80, a mast cell degranulator (Sigma, St. Louis, MO, USA; 0.75 mg/kg, IIR+CP group), and Ketotifen (Sigma; 1 mg/kg, IIR+K group), as previously reported (12, 14, 18). Because IIR injury was mostly critical and clinically an emergency, the agents were injected intravenously through the caudal vein 5 min before releasing the clamp in accordance with clinical protocol. The same volume of normal saline was similarly administrated to the S and IIR groups. One-third dosage of first-dosage Choloral hydrate was administrated when the anesthesia was weakened. The narcosis was lasted during the procedure until 4 h after the reperfusion.

Collection of Samples and Measurements

Four h after the reperfusion, rats were sacrificed by an overdose of anesthetics (10% Choloral hydrate at a dose of 10 ml/kg intraperitoneal injection) and the liver was excised. The isolated left-inside leaf of the liver was fixed in 10% formaldehyde and embedded in paraffin. The right lobe of the liver was flushed with cold physiological saline, and was cut into two pieces, then frozen in liquid nitrogen and stored at -80°C for further measurements of lactic dehydrogenase (LDH) and superoxide dismutase (SOD) activities, tumor necrosis factor-α (TNF-α) and interleukin-8 (IL-8) levels and maleic dialdchyde (MDA) contents.

Measuring Serum Liver Enzymes and Histamine Levels

Two mL blood was obtained from the inferior vena cava, frozen at -20°C for 5 min and centrifuged for 15 min at 4,000 rpm. Supernatants were transferred into clean tubes for the following evaluations. The activities of alanine aminotransferase (ALT, a specific marker for hepatic parenchymal injury), and aspartate aminotransferase (AST, a nonspecific marker for hepatic injury) in the serum were determined using an automatic biochemistry analyzer (Abbott Laboratories, Abbott Park, IL, USA); the activities were expressed as units per liter. The histamine levels in the serum were measured by a commercial ELISA kit (R&D Systems China Co. Ltd, Shanghai, PRC) following the manufacturer's instructions.

Histopathological Evaluation

Paraffin-embedded samples were sectioned at 4-μm thickness and were stained with hematoxylin and eosin for light microscopic examination. Hepatic histopathological changes were assessed under light microscopy by two pathologists who were initially blinded to the experimental groups. Five areas were randomly selected to evaluate liver damages induced by IIR using the following standard (9): Grade 0, normal structure of hepatic lobule, hepatocyte, hepatic cords, central vein and hepatic sinus; Grade 1, some degeneration of liver cells and some inflammatory cells infiltrated in the portal area; Grade 2, blood stasis in the central vein and hepatic sinus, scattered degeneration or necrosis of liver cells, and more inflammatory cells infiltrated in the liver tissues, and the hepatic lobule was in good integrity; Grade 3, blood stasis in the central vein and hepatic sinus, extensive degeneration or necrosis of liver cells, and a large number of inflammatory cells infiltrated in the liver tissues, and parts of the hepatic lobule were broken.

Measurement of LDH Activities in Liver Tissues

Liver tissue was weighed and homogenized in 9-time volumes of cooled normal saline at 4°C, and then spun at 4000 rpm for 15 min. Supernatants were transferred into clean tubes for measurements of LDH activities following the manufacturer's protocols (Jiancheng Bioengineering Ltd, Nanjing, PRC), and the results were expressed in units per liter. The total protein content of the liver was measured by the BCA Protein Assay Kit (KenGen Biotech Company, Nanjing, PRC), and the hepatic protein concentration was expressed as μg/ml. The final LDH activity in the liver was normalized by the tissue protein content.

Determination of TNF-α and IL-8 Levels in Liver Tissues

The contents of TNF-α and IL-8 were measured by ELISA kits (R&D Systems China Co. Ltd) respectively according to the manufacturer's instructions. The absorbance was read at 450 nm by a Biokinetics microplate reader Model EL340 (Biotek Instruments, VT, USA). The TNF-α and IL-8 levels in the liver tissues were normalized by the tissue protein content.

Determination of SOD Activities and MDA Contents in Liver Tissues

Liver tissue was harvested and homogenized using a teflon homogenizer. Detection of MDA in cell lysis was performed according to the instruction of the MDA kit (Jiancheng Bioengineering Ltd). SOD activity was determined following the instruction of the SOD Assay Kit-WST (Jiancheng Bioengineering Ltd).

Statistical Analyses

All measurement data were expressed as mean ± SD. Ranked data were expressed as median, and (md). One way analysis of variance (one-way ANOVA) was used to compare values among multiple groups, the Student-Newman-Keuls (SNK) test was used for pairwise comparison and Wilcoxon rank test was used for multiple comparisons. Pearson correlation analysis was used between the serum level of histamine and other parameters using the SPSS 12.0 software. A P value of less than 0.05 was considered statistically significant.

Results

Changes of ALT and AST Levels in the Serum and LDH Activities in the Liver

The serum ALT and AST levels represent the severity of liver injury. IIR resulted in substantial increases in serum ALT and AST levels as compared with that in the sham-operated group ($P < 0.05$, IIR *vs.* S) (Fig. 1, A and B). Moreover, mast cell degranulation with Compound 48/80 further increased ALT and AST serum levels as compared with the IIR group ($P < 0.05$, IIR+CP *vs.* IIR). However, treatments with Cromolyn sodium or Ketotifen significantly attenuated the increases in the serum levels of ALT and AST resulted by IIR ($P < 0.05$, *vs.* IIR), but the ALT and AST levels in the IIR+C and IIR+K groups were higher than those in the S group (both $P < 0.05$).

LDH activities in the liver reflect the severity of hepatic cell injury. The present study also found that IIR triggered dramatic increases in LDH activities in the liver by comparison with the S group. Inducing anti-histamine effects by stabilizing mast cells or inhibiting histamine significantly lowered the LDH activities in the IIR+C and IIR+K groups, respectively, while mast cell activation by Compound 48/80 further increased the LDH activities in the liver when compared with the IIR group (all $P < 0.05$) (Fig. 1C).

Pathomorphological Changes in the Liver

To further confirm the anti-histamine effects against hepatic injury induced by IIR, liver sections of the liver were obtained for histopathological assessment. IIR resulted in obvious damages to the liver in the IIR group, manifested as swollen hepa-

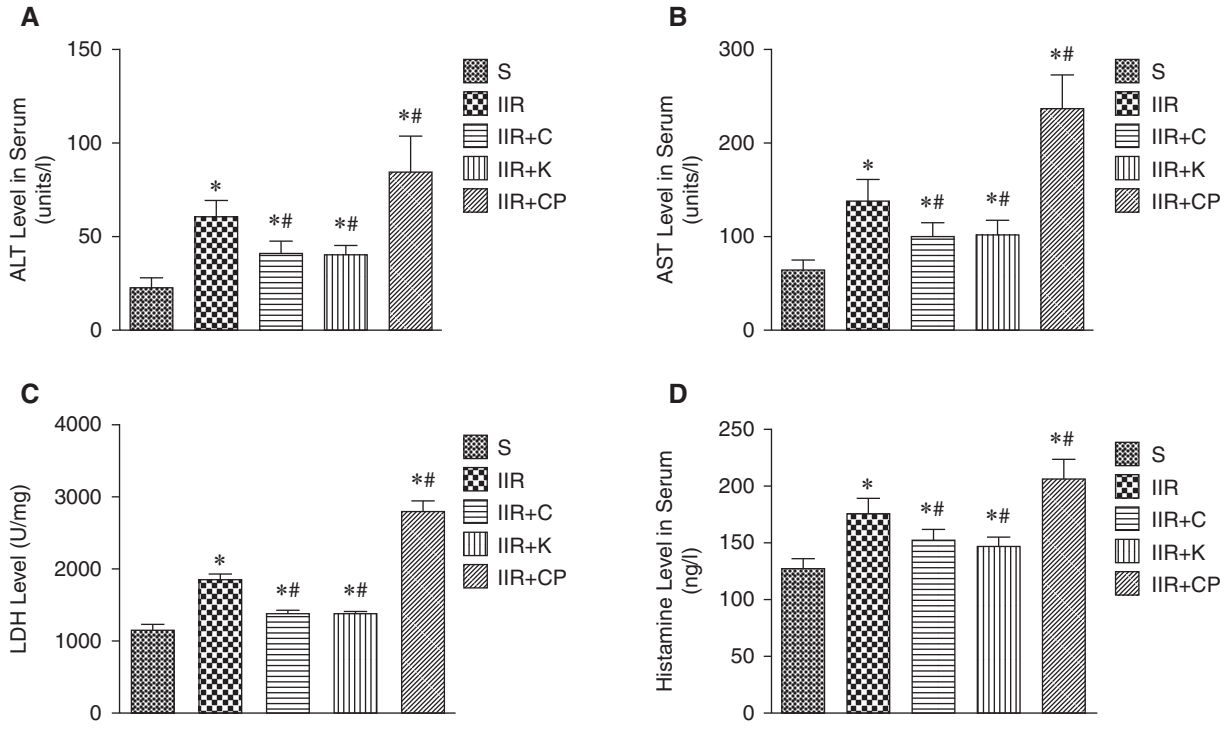

Fig. 1. Levels of ALT, AST and histamine in the serum and hepatic LDH activities after IIR injury. S group (Control, sham-operated group); IIR group (75 min intestinal ischemia and 4 h reperfusion); IIR+C group (25 mg/kg Cromolyn Sodium-treated IIR group); IIR+K group (1.0 mg/kg Ketotifen-treated IIR group); IIR+CP group (0.75 mg/kg Compound 48/80-treated IIR group). Results are expressed as mean ± SD. n = 8 per group. Compared with the S group, $^{*}P < 0.05$; compared with the IIR group, $^{\#}P < 0.05$.

tocytes with cytoplasmic vacuolization and larger nucleus that resulted in great increases in hepatic injury scores when compared to S group ($P < 0.05$) (Fig. 2B). Furthermore, Compound 48/80 led to deleterious changes in hepatocyte structures evidenced as even higher hepatic injury scores by comparing to that in the IIR group ($P < 0.05$), *e.g.*, liver tissues in the IIR+CP group showed significant cytoplasmic vacuolization, sinusoidal congestion, extensive hepatic cellular necrosis and massive cellular infiltration (Fig. 2E). By contrast, normal findings were observed in the sham-operated group (Fig. 2A). As expected, treatments with Cromolyn sodium and Ketotifen significantly attenuated liver pathological changes such that the parenchymal appearance in the IIR+C and IIR+K groups was better than that in the IIR group (Fig. 2, C and D), with moderate cytoplasmic vacuolization, and mild necrosis as well as comparatively preserved lobular structure, and consequently the hepatic injury scores in the IIR+C and IIR+K groups were dramatically lower as compared with the IIR group (both $P < 0.05$).

Serum Levels of Histamine

The histamine released by activated mast cells can be absorbed into the circulatory system and acts on distant organs and tissues (18). IIR resulted in significant increases in the histamine levels as compared with the S group; stabilizing mast cells with Cromolyn sodium significantly decreased the histamine levels in the serum whereas mast cell activation with Compound 48/80 markedly increased the serum histamine levels as compared to the IIR group (both $P < 0.05$). Not surprisingly, treatment with Ketotifen dramatically lowered the serum histamine levels ($P < 0.05$) (Fig. 1D). The findings suggest that Cromolyn Sodium and Ketotifen could reduce the histamine released from the activated mast cells, while Compound 48/80 did the opposite.

TNF-α and IL-8 Levels in the Liver

Liver injury is characterized by inflammatory stress; therefore, cytokines and chemokines were measured in the current study. TNF-α and IL-8 are main mediators leading to inflammatory responses. TNF-α and IL-8 levels in the liver were statistically higher in the IIR group than those in the S group. Treatments with Cromolyn sodium and Ketotifen markedly attenuated the increases in the levels of TNF-α and IL-8, respectively, in the liver in the

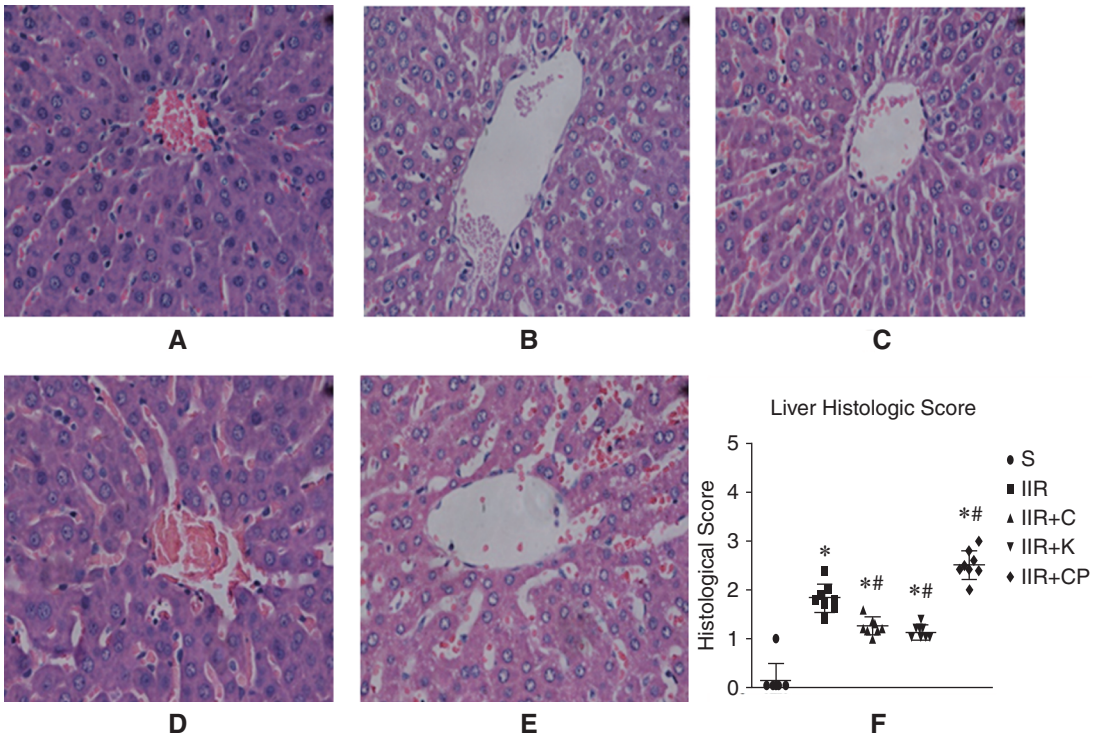

Fig. 2. Morphological changes of the liver and liver histological scores under light a microscope after IIR injury. A, B, C, D and E are respectively representative images of sham-operated group (S group); intestinal ischemia-reperfusion group (IIR group); IIR Cromolyn Sodium-treated group (IIR+C group); IIR Ketotifen-treated group (IIR+K group); IIR Compound 48/80-treated group (IIR+CP group). HE staining × 400. Bar graph in F quantified the liver histological scores. Results are expressed as median. n = 8 per group. Compared with the S group, $*P < 0.05$; compared with the IIR group, $^{\#}P < 0.05$.

IIR+C and IIR+K groups as compared with IIR group (Fig. 3, A and B); however, treatment with Compound 48/80 further aggravated the increases of TNF-α and IL-8 (both $P < 0.05$). Histamine released from mast cell degranulation is, thus, implicated in inflammatory stress of liver injury induced by IIR.

MDA Content and SOD Activity in the Liver

Tissue MDA content represents the severity of tissue oxidative stress, while SOD activity reflects the anti-oxidative ability in the tissue given that SOD is the major endogenous antioxidant enzyme. The hepatic MDA content in the rats subjected to IIR was substantially higher, and the hepatic SOD activities were lower in the IIR group in comparison with the S group ($P < 0.05$). Treatments with cromolyn sodium and ketotifen markedly attenuated the reduction in tissue SOD activity and reduced tissue levels of MDA as compared with the IIR group. The SOD activities in the IIR+CP group were lower while the MDA content was higher than those in the IIR group (all $P < 0.05$) (Fig. 3, C and D). Histamine released from mast cell degranulation is implicated in oxidative stress of liver injury induced by IIR.

Correlation Analysis

The results of Pearson correlation analysis showed that there were positive correlations between the serum histamine level, the serum levels of ALT and AST, and LDH activities in the liver. There were also positive correlations between serum histamine level, TNF-α level and the MDA content in the liver (all $P < 0.01$) (Table 1). The results collectively indicated that histamine released from mast cell degranulation was implicated in liver injury induced by IIR.

Discussion

Mast cell granules store a diverse range of preformed mediators, including histamine and proteases that are released from mast cells activated by many signal pathways. Studies proved that these mediators play different roles in the progression of various diseases or injuries, such as increasing vascular permeability, promoting accumulation of inflammatory cells and aggravating inflammatory reactions (6, 25). Histamine, a well-known bioactive diamine in mast cells, plays an important role in inflammatory and allergic responses (4). Cromolyn sodium, a mast cell

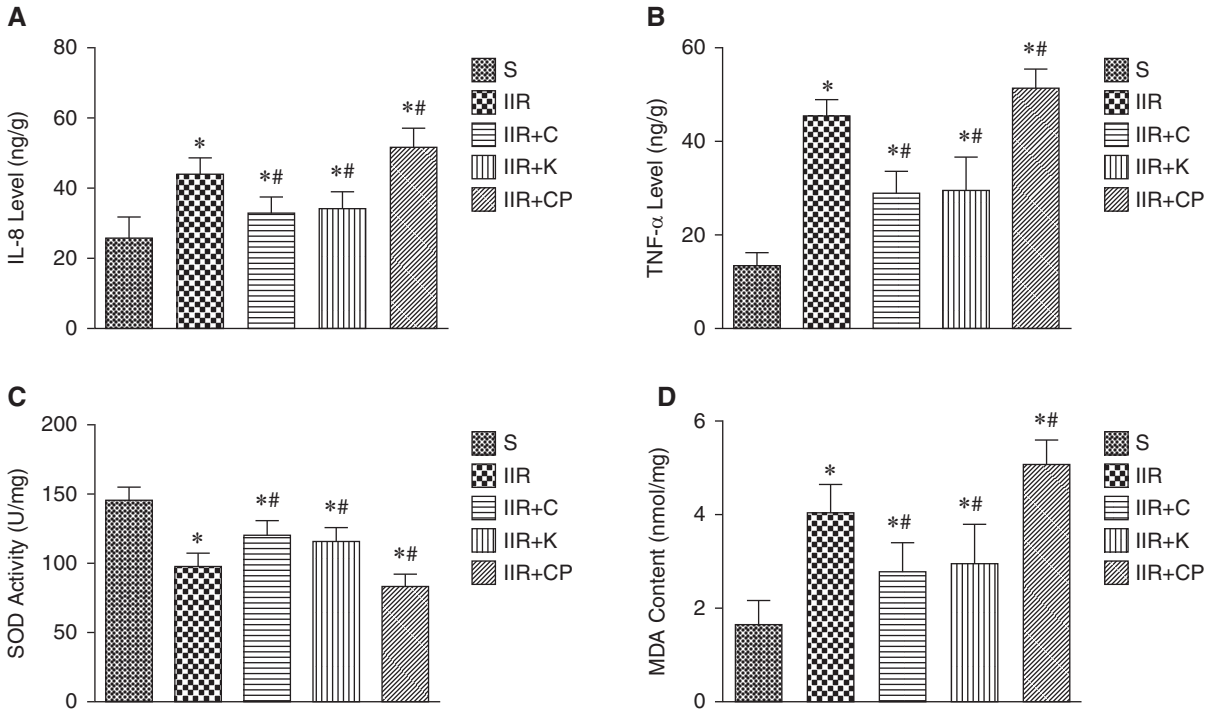

Fig. 3. Levels of IL-8, TNF-α and MDA, and SOD activities in the liver after IIR injury. S group (Control, sham-operated group); IIR group (75 min intestinal ischemia and 4 h reperfusion); IIR+C group (25 mg/kg Cromolyn Sodium-treated IIR group); IIR+K group (1.0 mg/kg Ketotifen-treated IIR group); IIR+CP group (0.75 mg/kg Compound 48/80-treated IIR group). Results are expressed as mean ± SD. n = 8 per group. Compared with the S group, $^{*}P < 0.05$; Compared with the IIR group, $^{\#}P < 0.05$.

Table 1. Correlation analysis between serum histamine levels and liver function tests, TNF-α, MDA levels in the liver

Correlations	n	r	*P*
His and ALT	40	0.787	< 0.01
His and AST	40	0.757	< 0.01
His and LDH	40	0.784	< 0.01
His and TNF-α	40	0.742	< 0.01
His and MDA	40	0.747	< 0.01

His, serum histamine level; ALT, serum ALT level; AST, serum AST level; LDH, hepatic LDH activity; TNF-α, hepatic TNF-α level; MDA, hepatic MDA level. r, correlation index.

inhibitor, acts by stabilizing the membrane of mast cells thus preventing mast cell degranulation from releasing media (14). Correspondingly, Compound 48/80, a classical activator to mast cells, facilitates the degranulation and then promotes the release of mediators (20). To identify the adverse effects of histamine, ketotifen, a well-known histamine receptor antagonist (8), was chosen in this study. In the current study, IIR resulted in liver injuries, manifested as higher pathological scores, elevated ALT, AST and LDH levels, and accompanied with increasing amount of cytokines and more severe oxidative stress. Mast cell activation was also manifested as the increase in serum histamine levels. Treatments with cromolyn sodium to stabilize mast cells and ketotifen to inhibit histamine both greatly attenuated the liver injury triggered by IIR, alleviated oxidative stress and inflammatory response, and reduced serum histamine levels. By contrast, Compound 48/80 treatment conferred increased serum histamine levels and exacerbated the above indicators. Correlation analysis showed that there were positive correlations between serum histamine levels, serum ALT and AST levels, and LDH activity in the liver. The same results of correlation analysis were found in the IIR group. All of the findings from the current study suggest that mast cell degranulation and histamine released from activated mast cell participated in the IIR-mediated liver injury and that anti-histamine treatment would be a promising therapeutic strategy against IIR-mediated liver injury.

The liver is the first injured 'distance' organ affected by IIR because the vasculature of this tissue is coupled in series with the intestinal circulation (1). Several lines of evidence have shown that IIR-induced releases of toxic substances contribute to post-ischemic

liver injury through portal circulation (16, 21, 23, 32). Mast cells are widely distributed throughout the gastrointestinal tract; therefore, the released media from mast cells would impact the liver through portal circulation (3). As the major medium from activated mast cells, the role of histamine in IIR-induced liver injury is still unknown. A study has suggested that elevated plasma histamine level is associated with lipopolysaccharide-induced liver injury (19). In our previous study, we have found that histamine, ranging from 10^{-7} M to 10^{-9} M, exacerbated hepatic injury induced by hypoxia/reoxygenation (29). In the current study, the results showed that the serum histamine levels markedly increased during the IIR period, which was further enhanced by Compound 48/80. The trend of liver pathological injury was in accordance with the serum histamine levels. On the contrary, stabilizing mast cells and anti-histamine treatment conferred similar protective effects on relative liver injuries. That indicated under the circumstance of IIR, histamine mainly served as a pro-inflammatory mediator. Previous studies have demonstrated that histamine, acting as a pro-inflammatory mediator, could induce increases in vasopermeability resulting in following edema or even more severe injuries such as microhemorrhage, microthrombosis and infiltration of leukocytes, mostly neutrophils (2, 11, 30). In addition, a recent study also showed that histamine could induce up-regulation of P-selectin and recruitment of leukocytes to inflammation sites (24). Taking together, multiple reports support that histamine released from activated mast cells may participate in IIR-induced liver injury through the above mechanisms.

The mechanisms of IIR mediated liver injury are highly complicated; oxidative stress and inflammatory responses have been proven to be the major mechanisms involved (15, 17, 26, 27). Toxic substances released after IIR , including LPS, oxygen free radicals, neutrophils, pro-inflammatory cytokines and adhesion molecules, contribute to liver injury through portal circulation and induce oxidative stress and inflammatory responses in the liver. Our previous *in vitro* research has found that histamine may enhance oxidative stress and inflammatory response while exacerbate hepatocyte injury was induced by hypoxia/reoxygenation (29). In the present study, we also found IIR resulted in substantial elevations in the MDA contents, the levels of IL-8, TNF-α and concomitant reduction in SOD activities in the liver, suggesting that oxidative stress and pro-inflammatory cytokines may play important roles in IIR-induced liver injury. Oxidative stress and inflammatory response were significantly attenuated either by cromolyn sodium or Ketotifen, while Compound 48/80 aggravated both. The trend of aggravating or alleviating liver injury induced by IIR was in accordance with changes in serum histamine levels. Moreover, positive correlations were found between serum histamine levels and the TNF-α level and MDA content in the liver. And the same results of correlation analysis were found in the IIR group. All these findings suggest that increased serum histamine released from activated mast cells induced by IIR may aggravate oxidative stress and inflammatory responses and finally leading to liver injury.

In conclusion, data from our study suggest that histamine released from activated mast cells participate in liver injury induced by IIR, and anti-histamine by stabilizing mast cell or histamine receptor antagonist may be the promising strategies. It should be noted that mast cell activation can release various mediators, such as histamine and tryptase. Potential contributions of other mediators in IIR-induced liver injury require further studies.

Acknowledgments

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