Original Article

Fasudil alleviates acetaminophen-induced liver injury via targeting Rhoa/ROCK signal pathway

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ABSTRACT — Fasudil is an inhibitor of Rhoa/ROCK signaling, which is involved in anti-inflammatory and anti-injury effects. The purpose of this study was to explore the effects of Fasudil on acetaminophen (APAP)-induced liver injury and reveal its potential molecular mechanism. In this study, C57BL/6 J mice were divided into different groups and treated with APAP and specified dose of Fasudil. HE staining was used to detect the changes of liver pathological tissues induced by APAP. ELISA assay was performed to detect the level of related factors. Western blot was used to detect the expressions of Rhoa, ROCK1, ROCK2. CD86 and CD6 were determined by RT-PCR and immunohistochemical staining detected the difference in CD86 expression. Rhoa/ROCK expression was increased in APAP-induced liver injury, and Fasudil targeted the expression of Rhoa/ROCK. Fasudil inhibits APAP-induced hepatic pathological changes and liver function injury. Fasudil inhibits the release of APAP-induced systemic inflammatory factors in liver tissue. Fasudil inhibits the activity of antioxidant enzymes, lipid peroxidation and macrophage infiltration induced by APAP in liver tissues. Fasudil alleviates APAP-induced liver injury via targeting Rhoa/ROCK signal pathway, indicating the possibility for clinical use of Fasudil in APAP-induced liver injury.

Key words: Fasudil, Acetaminophen, Liver injury, Rhoa/ROCK

INTRODUCTION

The liver is an important metabolic and detoxifying organ of the body, which is vulnerable to damage by chemicals such as alcohol, carbon tetrachloride and acetaminophen (APAP) (Wang *et al.*, 2020; Gatsou Djibersou *et al.*, 2020), leading to hepatocyte necrosis and liver dysfunction. The study of drug-induced liver injury has increased dramatically over the past decade and has become a hot topic among clinicians, researchers, drug companies and regulators (García-Román and Francés, 2020; Monroe *et al.*, 2020; Zhang *et al.*, 2020; Kang *et al.*, 2020). Acetaminophen (APAP) is a commonly used antipyretic and analgesic drug in the clinic, and the hepatotoxicity of APAP is the main factor causing acute liver failure (Bunchorntavakul and Reddy, 2018).

Fasudil is a new type of selective inhibitor of isoquinoline sulfonamide derivative Rho kinase, which is mainly used in clinical practice (Guo *et al.*, 2020). Many clinical trials have demonstrated its safety and efficacy in the treatment of pulmonary hypertension and other cardiovascular and cerebrovascular diseases (Yan *et al.*, 2020; Han *et al.*, 2020; Wang *et al.*, 2019). Studies have shown that Fasudil can slow down or even block the fibrosis process of heart and kidney (Ruan *et al.*, 2019; Xia *et al.*, 2019). Fasudil for hyperlipidemia rat heart and the liver has anti-inflammatory and antioxidant properties, can improve the diabetic rats cardiac function, myocardial injury, decrease collagen deposition (Li *et al.*, 2014, 2018; Huang *et al.*, 2018), clinical mainly used for the prevention of cerebral vasospasm after subarachnoid hemorrhage, improve cerebral ischemia caused by symptoms, drug quickly shifts to

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the group after the treatment, in the liver, kidney, spleen and intestine of content is higher (Fukuta *et al.*, 2016; Murakami *et al.*, 2019). By activating the downstream rho-associated coiled-protein kinase (ROCK), Rho regulates various biological behaviors such as cell adhesion, proliferation and migration, and the role of Rhoa/ROCK signal transduction pathways in chronic inflammatory fibrosis of various organs and tissues has attracted wide attention (Wei *et al.*, 2017; Zhang *et al.*, 2019). Recent studies have suggested that Fasudil therapy can protect the liver from ischemia/reperfusion injury by promoting M2 macrophage polarization (Xie *et al.*, 2018b), but its role in APAP-induced liver injury has not been studied. Therefore, this paper aims to explore the effect of Fasudil (Fasudil) on Rhoa/ROCK signal expression and reducing inflammatory response and macrophage infiltration when Fasudil (Fasudil) is targeted to inhibit APAP injury.

MATERIALS AND METHODS

Animals and treatments

SPF male C57BL/6 J mice weighing 16–20 g were purchased from the Shanghai SLAC Animal Center (Shanghai, China). All animals were housed in temperature-controlled cages (temperature 20–25°C, humidity 20–30%) with free access to water and food. All animals received the human care and experimental procedures. All experimental operations were approved by Qingdao University. Measures were taken to minimize animal suffering and the animal work took place in the animal laboratory of Qingdao University.

The mice were randomly assigned into the following treatment groups ($n = 3$ mice/group): (1) control group; (2) APAP group; (3) APAP+ Fasudil (low, 20 mg/kg); (4) APAP+ Fasudil (high, 40 mg/kg). Mice were fasted for 16 hr prior to APAP administration. The mice in the control group were injected intraperitoneally with saline, mice in the APAP group were injected intraperitoneally with a single dose of 300 mg/kg APAP (15 mL/kg in saline). Mice of the APAP+ Fasudil (low) and APAP+ Fasudil (high) group were injected the same dose of APAP 1 hr after the specified dose of Fasudil, then were given with Fasudil orally again 6 hr later. At 24 hr after APAP and Fasudil administration, the mice were sacrificed. Liver tissues were collected from each animal for biochemical and histopathological analyses.

Histopathological analysis

Appropriate weight liver tissues were conventionally fixed in 10% buffered formalin overnight and the tissues were embedded in paraffin. Then, all the sections were

stained with hematoxylin and eosin (HE). The stained slides were observed and evaluated under a light microscope (Olympus Corp., Tokyo, Japan) using 200X magnification (100 fields per section).

Enzyme-linked immunosorbent assay (ELISA)

Levels of IL-1 β (#PI305; Beyotime Biotechnology, Shanghai, China), IL-6 (#PI330; Beyotime Biotechnology), TNF- α (#PT518; Beyotime Biotechnology) and MCP-1 (#PC130; Beyotime Biotechnology) in serum with commercially available standard sandwich enzyme-linked kits in accordance with the manufacturer's instructions. The samples of each group were quantified by the Automatic Microplate Reader (Syngene, Frederick, MD, USA). Each sample was measured in triplicate.

Determination of GSH, GST, GPx, SOD and MDA in liver tissue

The liver tissue homogenate was added with PBS buffer, and the supernatant was taken after centrifugation. The levels of glutathione (GSH), glutathione transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD) and malondialdehyde (MDA) were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocols.

Immunohistochemistry (IHC)

The paraffin-embedded sections were dewaxed and rehydrated, and the antigen was recovered by microwave treatment with 1 mM ediamine teacetic acid buffer (pH = 9.0) for 3 min. Follow the instructions of the tissue staining TM-Plus and diaminobenzidine substrate kit. Briefly, 3% H₂O₂ blocks endogenous peroxidase activity. Nonspecific protein binding is blocked by normal goat serum. CD86 is used as the main antibody. The slides were then incubated for 1 hr with biotin-labeled IgG and HRP-conjugated streptomycin. The immune reaction was observed with diaminobenzidine and restained with hematoxylin. The images are taken with a light microscope.

Quantitative real-time polymerase chain reaction (qRT-PCR)

After different treatments, total RNA was isolated from liver tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, total RNA was further reverse transcribed into cDNA using the Prime Script RT Reagent Kit (Takara, Shanghai, China). The cDNA then served as the template for SYBR Green quantitative real-time polymerase chain reaction (qRT-PCR) (Takara) anal-

ysis to detect mRNA levels of every genes. The reactions were processed using a 7500 Realtime PCR System (Applied Biosystems, Foster, CA, USA) with SYBR Premix Ex Taq Kit (Takara). mRNA expression was normalized to GAPDH level. The specific primers of target mRNA and internal control were designed as following CD68 forward, 5'-TGTTGCGGAAAT-ACAAGCA-3', CD68 reverse, 3'-GGCAGCAA-GAGAGATTGGTC-5'; CD86 forward, 5'-AGC-CTTATCGGAAATGATCCAGT-3', CD86 reverse, 3'-TTGAGCCTTTGTAAATGGGCA-5'; GAPDH forward, 5'-AACGACCCCTTCATTGAC-3', GAPDH reverse, 3'-GGCCTTGTAGACACCTTGGT-5'. Results were shown in form of relative expression calculated by $2^{-\Delta\Delta CT}$ method. Each experiment was independently performed three times.

Western blot assay

Total protein was extracted from liver tissues using a RIPA kit (Beyotime). Harvested cells were lysed on ice. Protein concentrations of the cell supernatants were determined using a BCA Protein Assay kit (Beyotime). The samples were then spiked into loading buffer, and heated in boiling water for 5 min. Equal amounts of proteins (40 μ g) were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Massachusetts, MA, USA). After blocking with 5% non-fat milk in TBST for 2 hr at room temperature, membranes were incubated at 4°C overnight with specific primary antibodies. After washing in TBST, membranes were incubated in secondary antibodies (Rabbit or Mouse) at room temperature for 2 hr and then were washed again. Specific bands of proteins were visualized using an ECL Plus kit (Millipore, WBKLS0500) with bio-imaging system (Quantity One, version 4.6.2). All experiments were independently performed three times.

Statistical analysis

All experiments were independently performed three times and data are presented as mean $\pm$ standard deviation (SD). Quantile-quantile plots were used to test for normal distribution of data. Statistical analysis was performed using GraphPad Prism 8.0.1 software and performed with one-way ANOVA followed by Tukey's post hoc test. The value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Fasudil inhibits Rhoa/ROCK expression

Fasudil, as Rhoa/ROCK signal inhibitor, is involved in anti-inflammatory and anti-damage effects. Under the condition of APAP treatment, the expression of Rhoa, ROCK1 and ROCK2 in tissue extracts of injured liver were upregulated remarkably (Fig. 1A). Rhoa, ROCK1 and ROCK2 expression was inhibited remarkably by pretreatment of Fasudil in a concentration-dependent manner (Fig. 1B).

Fasudil inhibits APAP-induced hepatic pathological changes and liver function injury

To explore the protective effect of Fasudil on APAP-induced hepatic, C57BL/6 J mice were injected Fasudil or APAP to observe liver injury. The histopathological analysis was used to confirm the protective effect of Fasudil. As shown in Fig. 2A, lipid droplets formation, centrilobular necrosis, hepatocellular degeneration, sinusoidal congestion, hemorrhage and lymphocyte infiltration were seen in the APAP group. The severity of liver injury was reversed after administration of Fasudil. Meanwhile, after 16 hr of administration, serum ALT and AST were analyzed. The notable increase of serum ALT and AST levels in the APAP-administered group compared to the normal control group and Fasudil administration significantly reduced serum ALT and AST levels (Fig. 2B-C). All these results indicate that Fasudil can protect the liver from APAP-induced hepatic.

Fasudil was effective in suppression of inflammation

We next measured the serum levels of TNF- α , IL-1 β and IL-6 in the different treatment groups. The levels of TNF- α , IL-1 β and IL-6 were significantly increased in the APAP group, whereas pretreatment of mice with Fasudil significantly inhibited the levels of TNF- α , IL-1 β and IL-6 (Fig. 2D). The results demonstrated that Fasudil inhibits the release of APAP-induced systemic inflammatory factors in liver tissue and Fasudil was effective in suppression of inflammation.

Fasudil was effective in suppression activity of antioxidant enzymes and lipid peroxidation

Determination of glutathione level (GSH), glutathione transferase (GST) and glutathione peroxidase (GPx) in liver tissue supernatant from liver tissue homogenate. As shown in Fig. 3A, the expression of GSH, GST and GPx was markedly decreased in the APAP group. As expected, the expression of GSH, GST and GPx was significantly

Fig. 1. Fasudil inhibits RhoA/ROCK expression. (A) Western blot analyses of RhoA, ROCK1 and ROCK2 protein in tissue extracts of injured liver. (B) Western blot analyses of RhoA, ROCK1 and ROCK2 protein in different group treatment with Fasudil (low or high) or APAP. *p < 0.05, **p < 0.01 and ***p < 0.001 vs. Control; #p < 0.05, ##p < 0.01 and ###p < 0.001 vs. APAP.

Fig. 2. Effect of Fasudil on APAP-induced hepatic cells. (A) Images of hepatic cells stained with HE. (B, C) The expression of serum ALT (B) and AST (C) was detected by biochemical reagent. (D) The serum levels of TNF- α , IL-1 β and IL-6 were measured by ELISA. *p < 0.05, **p < 0.01 and ***p < 0.001 vs. Control; ###p < 0.01 and ###p < 0.001 vs. APAP.

Fasudil alleviates liver injury

Fig. 3. Fasudil was effective in suppression activity of antioxidant enzymes and lipid peroxidation. (A) ELISA assay was performed to detect the level of GSH, GST and GPx in liver tissues. (B) The expression of SOD and MDA was detected by ELISA. (C) The serum MCP-1 expression was detected by ELISA. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. Control; ## $p < 0.01$ and ### $p < 0.001$ vs. APAP.

increased after treatment with Fasudil.

Moreover, lipid peroxidation has been reported to be closely related to APAP-induced toxicity. A significant increase in Malondialdehyde (MDA) and superoxide dismutase (SOD) activity was observed in the APAP group compared with the control group. However, pretreatment of mice with Fasudil induced a significant decrease in the activity of MDA and SOD (Fig. 3B).

Fasudil inhibits APAP-induced infiltration of macrophages in liver tissue

To explore the effect of Fasudil on APAP-induced infiltration of macrophages in liver tissue. The serum monocyte chemotactic protein 1 (MCP-1) expression was detected by ELISA and the expression of CD86 and CD68 was detected by RT-PCR. As shown in Fig. 3C, the expression of MCP-1 was upregulated in the APAP group, and we observed that Fasudil treatment led

to the decreased MCP-1 expression. Next, we explored the expression of CD86 and CD68 in the liver tissues. According the results (Fig. 4A and 4B), the expression of CD86 and CD68 was upregulated in the APAP group, and downregulated after treatment with Fasudil. Further, immunohistochemical staining detected the expression of CD86, and we found that CD86 had a certain amount of macrophage infiltration in the APAP group and the infiltration of macrophages was significantly reduced after Fasudil treatment (Fig. 4C). These results implied that the protective effect of Fasudil may also be due to its anti-macrophages infiltration ability.

DISCUSSION

As a widely used antipyretic, analgesic and anti-inflammatory drug, APAP may cause liver damage if taken in large doses or for a long time (Mizrahi *et al.*, 2018).

Fig. 4. Fasudil inhibits APAP-induced infiltration of macrophages in liver tissue. qRT-PCR analysis was conducted to determine the expression of CD86 (A) and CD68 (B) in liver tissues. (C) Images of hepatic cells stained with immunohistochemical. ** $p < 0.01$ and *** $p < 0.001$ vs. Control; ### $p < 0.001$ vs. APAP.

In this study, the acute liver injury model of mice induced by APAP was used to observe the effect of Fasudil intervention on the damaged liver tissue and speculate the possible mechanism of action.

APAP is mainly metabolized in the liver. Under normal conditions, most APAP is rapidly metabolized to a glucuronic acid or sulfuric acid conjugation and converted into non-toxic metabolites (Chen *et al.*, 2015; Ni *et al.*, 2016; Jaeschke *et al.*, 2012). The results of this study showed that Rhoa, ROCK1 and ROCK2 expression levels were significantly increased after liver injury induced by APAP. As an inhibitor of Rhoa/ROCK signaling, Fasudil reduces the expression levels of Rhoa, ROCK1, and ROCK2 in a concentration-dependent manner after Fasudil treatment. These results are consistent with previous reports (Xie *et al.*, 2018a; Wang *et al.*, 2011).

In this study, excessive APAP action in model group mice resulted in significant oxidative stress damage in liver tissues. In addition to oxidative stress, the hepatotoxicity of APAP was also closely related to inflammatory response. APAP metabolites also lead to liver Kupffer cell activation, inflammatory cell infiltration and the production of a large number of inflammatory cytokines, such as TNF- α and IL-6, which trigger an inflammatory response (Kubes and Mehal, 2012). Inflammation leads to the production of a large number of toxic ROS, which aggravates oxidative stress injury (Seo *et al.*, 2020). The levels of TNF- α , IL-6 and IL-1 β in the liver tissues of mice in the APAP model group were significantly higher than

those in the control group, and Fasudil intervention significantly reduced the levels of inflammation-related factors, suggesting that the protective effect of Fasudil on the liver was not only related to the antioxidant effect, but also related to its anti-inflammatory effect.

Macrophages are essential cells in the immune response. They play an important role in both innate and acquired immunity and are involved in a variety of pathophysiological processes, such as acute kidney injury (Lee *et al.*, 2020). We found that Fasudil inhibits APAP-induced infiltration of macrophages in liver tissue, providing evidence for the potential mechanism of Fasudil in the prevention and treatment of drug-induced liver injury. However, due to the limitation by time and funds, we have only explored the role of Fasudil on mice models. Further studies are needed to focus on animal models, clinical samples and other signaling pathway to further support the findings in our study.

In conclusion, taken together, our study demonstrated that Fasudil alleviates APAP-induced liver injury via targeting Rhoa/ROCK signal pathway. These findings suggested that Fasudil might be a novel therapeutic target for the prevention of APAP-induced liver injury. The results of this study provide experimental basis for the prevention and treatment of drug-induced liver injury by Fasudil and its related products.

Conflict of interest---- The authors declare that there is no conflict of interest.

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