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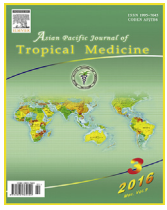

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Protective effect of apoptosis signal-regulating kinase 1 inhibitor against mice liver injury

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ABSTRACT

Objective: To explore the protective effect and its molecular mechanism of apoptosis signal-regulating kinase 1 (ASK1) inhibitor (GS-459679) on acetaminophen-induced liver injury in mice.

Methods: The model of liver injury was established by administration of acetaminophen (APAP) (300 mg/kg, i.p.) on C57BL/6 mice. Forty-eight male C57BL/6 mice were randomly divided into four groups, consisting of control group, GS group (GS-459679, 30 mg/kg, i.p.), APAP-induced group, and GS combined with APAP-induced group. For GS combined with APAP-induced group, mice were treated with GS 30 min prior to administration of APAP. After mice were euthanized at 6 h or 12 h, respectively, serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed, and mRNA levels of *TNF-α*, *IL-6* and *IL-1β* were tested. The activity of glutathione (GSH), oxidized GSH (GSSG) and malondialdehyde were quantified. In addition, ASK1, P-ASK1, JNK and P-JNK protein levels were tested in all groups.

Results: The ASK1 and P-ASK1 levels were up-regulated in APAP-induced group. Compared to the control group, serum levels of ALT and AST, and mRNA levels of *TNF-α*, *IL-6* and *IL-1β* were increased in APAP-induced group. Meanwhile, the levels of MAD and GSSG, and the ratio of GSSG/GSH were higher and the JNK was activatedin APAP-induced group compared with that in control group. However, compared to APAP-induced group, GS combined with APAP-induced group displayed a decrease of protein expression levels of ASK1, P-ASK1 and P-JNK, a reduction of serum levels of ALT and AST, a decrease in *TNF-α*, *IL-6* and *IL-1β* mRNA levels, and a low ration of GSSG/GSH.

Conclusions: GS-459679 treatment effectively down-regulates ASK1 and P-ASK1 expression. Addition of GS-459679 decreases the generation of liver metabolites and inflammatory factors, reduces oxidative stress reaction, inhibits JNK activation, and then protects the responsiveness to APAP-induced liver injury.

1. Introduction

In recent years, drug-induced liver injury has become an important factor affecting the treatment effect and prognosis of patients in clinic, with paracetamol [acetaminophen (APAP)] induced liver injury as a typical representative [1–4]. High doses or frequent cumulative use of APAP can induce acute and severe liver tissue necrosis, and even cause liver failure and death. APAP-induced liver injury is mainly due to that metabolism of cytochrome P450 enzyme system produces excessive *N*-acetyl-*p*-benzo-quinone imine (NAPQI), leading to peroxidatic reaction of hepatic cellular mitochondrion eventually inducing the activation of MAP and JNK signal path [5,6], thus causing cell apoptosis or necrosis. Meanwhile, APAP activates the body's immune cells to produce inflammatory factors, thus activating immune system [2,7–10]. Therefore, to explore the molecular mechanism of APAP-induced liver injury and find inhibiting effect of APAP-induced liver injury will provide an

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important basis for development of drugs to prevent and control APAP-induced liver injury.

Apoptosis signal-regulating kinase 1 (ASK1) is one of the family members of mitogen-activated protein kinase. Multiple inflammatory factors and oxidative stress can activate ASK1 and its Ser-83 and Thr-845 phosphorylation. Activated ASK1 induces the activation of JKN downstream signaling pathways through MKK3 and MKK4 [11], and finally leads to cell apoptosis by caspase 3 pathway [12,13]. Nakagawa *et al.* found that the APAP-induced liver injury was decreased in ASK1 knockout mice through inhibiting JNK pathway activation, reducing serum level of alanine aminotransferase (ALT) and oxidative stress level and decreasing the numbers of APAP-induced liver cell apoptosis [9]. Xie *et al.* reported that pretreatment of ASK1 inhibitor (GS-459679) lowered APAP-induced peroxide stress of cellular mitochondria and inhibited mitochondrial JNK activation [14]. However, the defensive function of ASK1 inhibitor on APAP-induced liver injury is still unclear. The present study aimed to discuss the protective effect of GS-459679 on APAP-induced liver injury and the preliminary research of its mechanism, which will provide new drug molecules for the exploitation of treatment of APAP-induced liver injury.

2. Materials and methods

2.1. Materials

Forty-eight male C57/BL6 mice of clean grade, aged 8 weeks and weighted 20–25 g were purchased from Laboratory Animal Centre of Nanjing University. GS-459679 (ASK1 inhibitor) was bought from Gilead Sciences, Inc. Anti-bodies of ASK1, P-ASK1, JNK1 and P-JNK were all purchased from Abcam. cDNA reverse transcription kit (PrimeScript 1st Strand cDNA Synthesis Kit) and fluorescence quantitative PCR kit (SYBR Green PCR Master Mix Kit) were bought from Takara Bio. Protein lysis buffer (RIPA) and protein quantitative kit were purchased from Thermo Fish. Glutathione (GSH) and malondialdehyde (MAD) assay kit were bought from Beyotime.

2.2. Methods

2.2.1. Acute liver injury model

Forty-eight male C57/BL6 mice were randomly divided into four groups (12 mice in each group), consisting of control group, GS group (GS-459679), APAP-induced group, and GS combined with APAP-induced group. Animals were fasting for 12 h before experiment and water *ad libitum*. Mice in control group were given intraperitoneal injection of PBS. GS group was treated with 30 mg/kg GS-459679 dissolved in PBS. In APAP-induced group, 300 mg/kg APAP was injected intraperitoneally. For GS combined with APAP-induced group, mice were treated with GS 30 min prior to administration of APAP. After administration of 6 h or 12 h, all the animals were sacrificed (3 mice each time). Blood was collected and livers were separated. Various indexes were detected to estimate the liver injury status. All the animal experiments complied with the animal ethics standards.

2.2.2. Effects on biochemical index

Blood was collected from eyeball of mice in all groups. After coagulation and keeping for 30 min, it was centrifuged at 3 000 rpm/min, 4 °C for 15–20 min. Precipitation was discarded and supernatant was taken. Contents of ALT and aspartate aminotransferase (AST) were determined through 7170A automatic biochemical analyzer. Liver tissues were taken to make into homogenate, and contents of GSH, oxidized GSH (GSSG) and MDA were tested according to kit instructions. MDA content was detected according to the MAD kit operational procedure.

2.2.3. Q-PCR method

Mice in all the groups were sacrificed under anesthesia. Livers were separated, and 50–100 mg liver issues were made into homogenate. Total RNA was extracted by phenol chloroform extracting method as follows: 1 mL Trizol was added in liver issue and kept for 50 min, and then 200 μL chloroform was added and mixed to stand for 10 min; after centrifugation at 10 000 rpm/min, 4 °C for 10 min, upper water phase was taken to add 1-fold volume of isopropyl alcohol and keep for 10 min; after centrifugation at 4 °C for 10 min, supernatant was discarded, and precipitation was washed with 75% ethanol and dried at room temperature. The extracted RNA was reversed to cDNA by two steps method according to the kit instruction, and the concentration of cDNA was detected by UV spectrophotometer. *TNF-α* primer sequence: forward primer: GACGTGGAACTGGCAGAAGAG, reverse primer: TTGGTGGTTTGTGAGTGTGAG; *IL-6* primer sequence: forward primer: CCAAGAGGTGAGTGCTTCCC, reverse primer: CTGTTGTTCAGACTCTCTCCCT; *IL – 1β* primer sequence: Forward primer: GCAACTGTTCCTGAACTCAACT, reverse primer: ATCTTTTGGGGTCCGTCAACT. *GAPDH* was considered as internal reference. *GAPDH* primer sequence: forward primer: AGGTCGGTGTGAACGGATTTG, reverse primer: TGTAGACCATGTAGTTGAGGTCA. After standardization of cDNA, Q-PCR reaction system amplification was as follow: pre degeneration at 94 °C for 5 min, degeneration at 94 °C for 30 s, anneal at 65 °C for 50 s, extension at 72 °C for 1 min, 30 cycles; extension at 70 °C for 10 min. mRNA levels of *TNF-α*, *IL-6* and *IL-1β* were detected by ABI 7900 HT Fast software.

2.2.4. Western blot method

About 100–200 g liver issue was taken and pyrolyzed by RIPA. After centrifugation at 12 000 rpm/min, 4 °C for 10 min, supernatant was obtained. Total protein content was acquired according to protein quantitative kit steps. A total of 40–60 μg protein was taken to perform SDS-PAGE electrophoresis, transmembrane, sealing, hatching primary antibodies (anti-ASK1, 1:1 500; anti-P-ASK1, 1:1 000; anti-JNK, 1:2 000; anti-P-JNK, 1:1 000; anti-ASK1, 1:1 500; anti-P-ASK1, 1:1 000; anti-JNK, 1:2 000; anti-P-JNK, 1:1 000), washing, hatching second antibodies and developing, expression levels of ASK1, P-ASK1, JNK and P-JNK were detected. GAPDH was the internal reference.

2.3. Statistical analysis

All the data were processed with Graphpad prism 5.0. Data were expressed as mean ± SEM. *t*-test was used for the comparisons of ALT, AST, GSH, GSSD levels and mRNA levels of *TNF-α*, *IL-6* and *IL-1β* between APAP-induced group and GS combined with APAP-induced group. $P < 0.05$ was considered as statistically significant.