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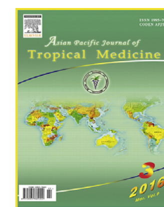

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Protective effect of apoptosis signal-regulating kinase 1 inhibitor against mice liver injury

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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; $IL-1\beta$ 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.

3. Results

3.1. Effects of APAP-induced liver injury on ASK1 expression

As shown in Table 1, through western blot test, expression level of ASK1 and P-ASK1 in APAP-induced liver injury group increased; at the same time, GS-459679 significantly decreased the protein levels of ASK1 and P-ASK1, which was consistent with the previous report [9]. Hence, GS-459679 could be used as an effective ASK1 inhibitor.

3.2. Effects of ASK1 inhibitor on serums of ALT and AST

Compared to control group, after 12 h of APAP-induced liver injury, levels of ALT and AST increased significantly, while those in GS combined with APAP-induced group and APAP-induced group after 12 h of administration significantly decreased (ALT: $P < 0.05$; AST: $P < 0.05$) (Table 2).

Table 1

Inhibition of ASK1 and P-ASK1 levels by addition of GS-459679 [*n* (repeat count) = 3].

Group	ASK1 (normalized to GAPDH)	P-ASK1 (normalized to GAPDH)
Control group	0.233 ± 0.080	0.199 ± 0.056
GS group	0.145 ± 0.058	0.153 ± 0.047
APAP-induced group	0.611 ± 0.095	0.546 ± 0.078
GS + APAP-induced group	0.349 ± 0.070	0.374 ± 0.066
P-value[1]	0.002	0.003
P-value[2]	0.018	0.043

P-value[1]: APAP-induced group compared with control; *P*-value[2]: GS + APAP-induced group compared with APAP-induced group.

Table 2

Effects of ASK1 inhibitor on serum ALT and AST levels in the mice of APAP-induced liver injury [*n* (repeat count) = 3].

Group	ALT (U/L)	AST (U/L)
Control group	75.00 ± 15.00	70.67 ± 9.50
GS group	71.67 ± 14.57	61.33 ± 11.02
APAP-induced group	1860.00 ± 263.62	2313.34 ± 339.38
GS + APAP-induced group	1016.00 ± 218.58	1632.33 ± 213.89
P-value[1]	0.001	0.000
P-value[2]	0.013	0.042

P-value[1]: APAP-induced group compared with control; *P*-value[2]: GS + APAP-induced group compared with APAP-induced group.

Table 4

Effects of ASK1 inhibitor on the mRNA levels of *TNF-α*, *IL-6* and *IL-1β* in the mice of APAP-induced liver injury [*n* (Repeat Count) = 3].

Group	*TNF-α*	*IL-6*	*IL-1β*
Control group	1.029 ± 0.135	0.674 ± 0.147	1.062 ± 0.171
GS group	0.923 ± 0.065	0.940 ± 0.162	0.932 ± 0.264
APAP-induced group	3.941 ± 0.503	2.091 ± 0.416	7.281 ± 1.047
GS + APAP-induced group	2.654 ± 0.462	1.325 ± 0.247	3.262 ± 0.403
P-value[1]	0.001	0.005	0.001
P-value[2]	0.031	0.052	0.003

P-value[1]: APAP-induced group compared with control; *P*-value[2]: GS + APAP-induced group compared with APAP-induced group.

3.3. Effects of ASK1 inhibitor on the contents of GSH, GSSG and MAD

As shown in Table 3, compared to normal control group, after 12 h of APAP induction, GSH level changed, GSSG level increased ($P < 0.05$), GSSG/GSH ratio increased significantly ($P < 0.05$), and MAD level increased ($P < 0.05$). In GS combined with APAP-induced group, compared to APAP-induced group, there were no significant difference in terms of GSH, GSSG and MAD levels; at the same time, GSSG/GSH ratio recovered significantly ($P < 0.05$). The results showed that ASK1 decreased the APAP-induced oxidative stress level.

3.4. Effects of ASK1 inhibitor on the mRNA level of inflammatory factors

Q-PCR detection showed that compared to control group (Table 4), the mRNA levels of *TNF-α*, *IL-6* and *IL-1β* were significantly increased after 6 h of APAP induction (*TNF-α*: $P < 0.01$; *IL-6*: $P < 0.01$; *IL-1β*: $P < 0.01$). Compared to APAP-induced group, the mRNA levels of *TNF-α*and *IL-1β* in GS combined with APAP-induced group were significantly decreased (*TNF-α*: $P < 0.05$; *IL-1β*: $P < 0.01$), but were higher than those in control group. But, there was no significant differences in mRNA levels of *IL-6* ($P = 0.052$). Therefore, the decrease of ASK1 inhibitor was produced by APAP-induced inflammatory factors.

3.5. Effects of ASK1 inhibitor on JNK expression level

Table 5 showed that P-JNK level in GS group was slightly lower than that in control group, and it was higher in APAP-induced group compared to that in control group. However, P-JNK level in GS combined with APAP-induced group was significantly lower than that in APAP-induced group, but was

Table 3

Effects of ASK1 inhibitor on the levels of GSH, GSSG and MAD in the mice of APAP-induced liver injury [*n* (repeat count) = 3].

Group	GSH (μmol/g)	GSSG (μmol/g)	GSSG/GSH (%)	MAD (μmol/g)
Control group	7.912 ± 1.901	2.504 ± 0.657	0.316 ± 0.031	0.077 ± 0.075
GS group	7.946 ± 1.723	1.611 ± 0.313	0.203 ± 0.036	0.071 ± 0.082
APAP-induced group	7.185 ± 1.185	3.576 ± 0.778	0.498 ± 0.091	0.924 ± 0.523
GS + APAP-induced group	7.812 ± 1.126	2.875 ± 0.692	0.338 ± 0.031	0.323 ± 0.231
P-value[1]	0.544	0.046	0.031	0.050
P-value[2]	0.515	0.171	0.045	0.143

P-value[1]: APAP-induced group compared with control; *P*-value[2]: GS + APAP-induced group compared with APAP-induced group.

Table 5

Effects of ASK1 inhibitor on protein levels of JNK and P-JNK in the mice of APAP-induced liver injury [*n* (repeat count) = 3].

Group	P-JNK (normalized to GAPDH)	JNK (normalized to GAPDH)
Control group	0.160 ± 0.029	0.930 ± 0.105
GS group	0.079 ± 0.062	0.943 ± 0.095
APAP-induced group	0.945 ± 0.110	0.878 ± 0.058
GS + APAP-induced group	0.608 ± 0.116	0.896 ± 0.076
P-value[1]	0.000	0.495
P-value[2]	0.016	0.761

P-value[1]: APAP-induced group compared with control; *P*-value[2]: GS + APAP-induced group compared with APAP-induced group.

higher than that in control and GS groups. There were no significant differences in JNK expression levels among control, APAP-induced and GS combined with APAP-induced groups. The results showed that ASK1 inhibitor decreased the phosphorylation of JNK. What's more, ASK1 inhibitor significantly decreased the JNK activation which was caused by APAP induction.

4. Discussion

ASK1 is serine/threonine-protein kinase, which activates P38 and JNK molecule through MAPK pathway activation [15]. Under resting state, thioredoxin (Trx) bonded the N-terminal domains of ASK1 to form Trx-ASK1 compound, thus inhibiting ASK1 activation. Under the external stimulation (such as oxidative stress and inflammatory factors), Trx and ASK1 were separated to cause ASK1 activation, thus inducing the MAP2 phosphorylation and downstream JNK activation [9,16]. Study of Nakagawa *et al.* showed that ASK1−/− mice effectively protect APAP-induced liver injury through inhibiting JNK activation [9]. Xie *et al.* found that pretreatment of ASK1 inhibitor (GS-459679) lowered APAP-induced peroxide stress of cellular mitochondria and inhibited mitochondrial JNK activation [14]. On the basis of the result, the present study further illuminated the mechanism of effect of ASK1 inhibitor on APAP-induced liver injury. Our study showed that GS-459679 can decrease the APAP-induced serum transaminase levels (ALT and AST), reduce oxidative stress product (GSSG), inhibit the expressions of inflammatory factors (TNF-α, IL-6 and IL-1β), and JNK activation, and thus protect APAP-induced liver injury.

When excessive APAP is ingested, most of the drugs are combine with glucuronic acid and expelled from the body, while a small part of drugs generate NAPQI by oxidation reaction of cytochrome P450 enzyme system; after exhausting GSH, NAPQI is combined with sulfhydryl-containing proteins, thus causing oxidative stress and dysfunction of mitochondria and finally inducing a function of liver cells [7,8,17,18]. Hence, inhibition of oxidative stress is the key to protect APAP-induced liver injury. It was found that the oxidant and antioxidant balance (GSSG/GSH) of liver cells was broken in APAP-induced liver injury. After the pretreatment of ASK1 inhibitor, GSSG level and GSSG/GSH ratio were decreased, that is to say, the balance of the redox reaction of liver cells got certain recovery. Hyun also reported that ASK1 inhibitor can decrease the ROS level of liver cells, reduce the output of lipid oxidation products and inhibit oxidative stress [19].

Many literatures reported that APAP-induced liver cell apoptosis and necrosis can further activate immune cells such as NK and neutrophile granulocyte which secrete a variety of inflammatory factors, and then accelerate the failure of liver function [9,10,20]. Nakagawa *et al.* reported that in APAP-induced liver injury model of ASK1−/− mice, expression levels of IL-1α and IL-1β were all reduced [9]. It is found in our study that compared to APAP-induced group, *TNF-α*, *IL-6* and *IL-1β* in GS combined with APAP-induced group decreased by different degrees, which indicated that ASK1 inhibitor can also reduce products of inflammatory factors and lower the body's immune response.

Mitochondrial oxidative stress in APAP-induced liver injury eventually activates JNK pathway induced liver cell apoptosis or necrosis [5]. ASK1 is the upstream signal molecule of JNK, which can activate JNK into the nucleus by the activation of MKK3 and MKK4, thus causing apoptosis [11]. Hayakawa *et al.* reported that ASK1 inhibitor (K188) decreased the proliferation and tumor size of gastric carcinoma cells through inhibiting JNK phosphorylation [12]. Xie *et al.* found that GS-459679 reduced the JNK activator positioning of mitochondria, and GS-459679 protected APAP-induced liver injury through inhibiting JNK pathway [14]. Our study also showed that GS-459679 inhibited JNK phosphorylation, which further illustrates that the protective effect of ASK1 inhibitor on APAP-induced liver injury depends on activation inhibition of JNK.

In conclusion, ASK1 inhibitor (GS-459679) can protect the APAP-induced liver injury through eliminating toxic metabolite of liver, inhibiting oxidative stress, removing immune response and inhibiting JNK activation at the molecular level. However, the therapeutical effect of GS-459679 still needs to be further confirmed, and therapeutic window of GS-459679 use needs to be intensively studied.

Conflict of interest statement

We declare that we have no conflict of interest.

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