Protective Effects of Salidroside against Acetaminophen-Induced Toxicity in Mice

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Received February 4, 2008; accepted May 28, 2008; published online May 30, 2008

The protective effect of salidroside (SDS) isolated from Rhodiola sachalinensis A. Bor. (Crassulaceae), was investigated in acetaminophen (APAP)-induced hepatic toxicity mouse model in comparison to N-acetylcysteine (NAC). Drug-induced hepatotoxicity was induced by an intraperitoneal (i.p.) injection of 300 mg/kg (sub-lethal dose) of APAP. SDS was given orally to mice at a dose of 50 or 100 mg/kg 2 h before the APAP administration in parallel with NAC. Mice were sacrificed 12 h after the APAP injection to determine aspartate aminotransferase, alanine aminotransferase (ALT), and tumor necrosis factor-alpha (TNF-α) levels in serum and glutathione (GSH) depletion, malondialdehyde (MDA) accumulation, and caspase-3 expression in liver tissues. SDS significantly protected APAP-induced hepatotoxicity for SDS improved mouse survival rates better than NAC against a lethal dose of APAP and significantly blocked not only APAP-induced increases of AST, ALT, and TNF-α but also APAP-induced GSH depletion and MDA accumulation. Histopathological and immunohistochemical analyses also demonstrated that SDS could reduce the appearance of necrosis regions as well as caspase-3 and hypoxia inducible factor-1α (HIF-1α) expression in liver tissues. Our results indicated that SDS protected liver tissue from the APAP-induced oxidative damage via preventing or alleviating intracellular GSH depletion and oxidation damage, which suggested that SDS would be a potential antidote against APAP-induced hepatotoxicity.

Key words salidroside; acetaminophen; hepatotoxicity; hypoxia inducible factor-1α

Acetaminophen (APAP) is a widely used analgesic and antipyretic agent. APAP is metabolized by a cytochrome P450 system to N-acetyl-p-benzoquinoneimine (NAPQI). Overdose of APAP causes a highly reactive metabolite that depletes the intracellular pool of glutathione (GSH),1 for NAPQI reacts rapidly with GSH. The abrupt decrease in liver GSH is particularly harmful because it is a basic cytosolic oxidant scavenger and redox regulation capacity,2 which would exacerbate oxidation stress in conjunction with mitochondrial dysfunction, especially lead to massive hepatocyte necrosis, liver damage or death. Much evidence has pointed to the importance of GSH defense mechanism against the hepatic damage induced by free radicals in many pathophysiological situations involving lipid peroxidation reaction.3

Oxidative damage, mediated by reactive oxygen species (ROS) which can be generated following cell lysis, oxidative burst, or the presence of an excess of transition metals, can attack proteins, deoxynucleic acid, and lipid membranes, thereby disrupting cellular function and integrity.4 Malondialdehyde (MDA) is a secondary product of oxidative stress formed during lipid peroxidation processes. Increase in the production of ROS during APAP metabolism leads to increase tumor necrosis factor-alpha (TNF-α) production.5 In many forms of liver injury, including ischemia/reperfusion and fulminant hepatic failure, TNF-α signaling appears to play an important role.6 In the APAP-induced liver damage, TNF-α also participates in the processes causing liver failure, because the administration of anti-TNF-α antibody to APAP-treated animals ameliorated the enzyme leakage during the early phase of the intoxication.7 Executioner caspases proteolytically cleave a number of cytoskeletal and nuclear structural proteins. This effect is responsible for the characteristic morphological changes of cells undergoing apoptosis such as cell shrinkage, membrane blebbing and chromatin condensation.8 Because of the number of parameters that affect hypoxia inducible factor (HIF) induction and the many downstream targets of this signal transduction factor, HIF is considered to be a generalized stress response gene regulator. In addition to hypoxia, oxidative stress may promote HIF-1α induction. In the following study, we examined the induction of MDA, TNF-α, caspase-3 and HIF-1α in the livers of APAP-induced mice.

Salidroside, (SDS, (4-hydroxy-phenethyl)-β-D-glucopyranoside, C14 H20 O7: 300.30) isolated from Rhodiola sachalinensis A. Bor. (Gao-shan-hong-jing-tian in Chinese) is one of the most popular traditional Chinese medicines.9 Rhodiola sachalinensis has a reputation for stimulating the nervous system, decreasing depression, enhancing work performance, resisting anoxia, preventing high altitude sickness, and is especially famous in the treatment of mountain malhypoxia in China. Rhodiola sachalinensis A. Bor. showed effectively protected against carbon tetrachloride-induced liver injury, resulting in reduced lipid peroxidation in the liver and improved serum biochemical parameters.10 SDS could significantly ameliorate effect on experimental liver fibrosis and have inhibitory action on experimental liver fibrosis to some extent.11 The neuroprotective effects of SDS on hydrogen peroxide (H2O2)-induced apoptosis in SH-SY5Y cells12 could markedly attenuate H2O2-induced cell viability loss and apoptotic cell death in a dose-dependent manner. The mechanisms by which SDS protected neuron cells from oxidative stress included the induction of several antioxidant enzymes, thioredoxin, heme oxygenase-1, and peroxiredoxin-1; the downregulation of pro-apoptotic gene Bax and the upregulation of anti-apoptotic genes Bcl-2 and Bcl-X(L). SDS could play a sedative and hypnotic activity in mice by using synergism with pentobarbital as an index for the hypnotic effect.13 As for SDS, most researches were focused on...
hepatic apoptosis and less about drug-induced liver disease, which accounts for one-half of all cases of acute hepatic failure. This study just worked on protective effect of SDS on APAP-induced toxicity for the first time. And we also firstly discuss the relationship between SDS and hypoxias induced factor, HIF-1α, and hope to explore the mechanism of SDS against APAP-induced toxicity. SDS might be a potential therapeutic agent for preventing neurodegenerative diseases implicated with oxidative stress.

The objective of the present paper is to examine the mechanism of SDS against APAP-induced toxicity, and investigate whether SDS affects the production of oxidative stress, inflammatory mediators or apoptosis to inhibit liver damage. Our results suggest that SDS suppresses the depletion of GSH, which might effect by oxidation stress, lipid peroxidation, inflammatory conditions, apoptosis, and hypoxia.

MATERIALS AND METHODS

Materials Salidroside (Purity 99%) control article was purchased from National institute for the control of pharmaceutical and biological products (Beijing, China), lot number was 818-9401. APAP and N-acetyl-cysteine (NAC) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, U.S.A.). Detection kits for glutathione and malondialdehyde were purchased from Oxis International, Inc. (Portland, OR, U.S.A.). Mouse TNF ELISA Kit was purchased from BD Biosciences (San Diego, CA, U.S.A.). Caspase-3 p20 (N-19) (sc-1226) and HIF-1α (H1667) (sc-53546) monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). DAB (3,3’-diaminobenzidine) Plus Substrate System was purchased from Lab Vision Corporation (Fremont, CA, U.S.A.). The alamine aminotransferase (ALT) and aspartate aminotransferase (AST) Reagent Strips were purchased from Arkaray InCorporated (Kyoto, Japan).

Animals Six-week-old male C57BL/6 mice weighing 21—25 g were kept in an environmentally controlled room with temperature 24±1°C and relative humidity 55±1%. The animals were acclimated for 7 d prior to initiation of any procedures and allowed access to normal chow and water ad libitum prior to initiation of any treatment. However, mice were fasted prior to treatment with APAP as indicated below. Animal experiments were carried out under the “Guiding Principles in the Use of Animals in Toxicology” adopted by the Society of Toxicology (U.S.A.) in July 1989 and revised in March 1999. The animal care committee of our institution approved the present study.

Animal Treatments The C57BL/6 male mice were fasted overnight (16—18 h) prior to administration of a single intraperitoneal (i.p.) dose (300 mg/kg) of APAP dissolved in sterile phosphate buffered saline (PBS, pH 7.4) warmed to 40°C. The mice were randomly assigned into six experimental groups, each group contained 8 to 10 mice, including saline group, APAP group, APAP+NAC groups with doses 20 mg/kg, 50 mg/kg, 100 mg/kg of SDS respectively and APAP+NAC group with dose 300 mg/kg of NAC. Saline group received sterile saline only. APAP group was achieved by an intraperitoneal injection of 300 mg/kg (sub-lethal dose) of APAP which prepared as described previously. APAP+NAC groups were designed to administrate mice with SDS (20 mg/kg, 50 mg/kg, 100 mg/kg, respectively, p.o.), 2 h before APAP treatment. APAP+NAC group was administrated with NAC (300 mg/kg, p.o.) 2 h before APAP treatment. And according to reference and our previous research, we selected 20 mg/kg, 50 mg/kg, 100 mg/kg of SDS and 300 mg/kg of NAC to discuss its protection. All mice were survival after 12 h APAP administration. At 12 h after APAP treatment, the mice were sacrificed to collect blood from arteria carotis communis. Plasma was separated and stored at −70°C until analysis the serum levels of ALT, AST, TNF-α. The liver was quickly excised and divided into portions. These were immediately utilized for metabolic analysis, snap-frozen in liquid nitrogen and stored at −70°C for biochemical analyses, or immersion-fixed in 15% neutral buffered formalin for histological examination. In our previous research, we have confirmed that ALT and AST levels presented highest about 12 h after the APAP treatment. Therefore, in this study, mice were sacrificed after 12 h APAP injection.

During survival experiments, mice were intoxicated with 500 mg/kg (lethal dose) of APAP to observe the mortality. In each group, 16 mice were treated with SDS 20 mg/kg, 50 mg/kg, 100 mg/kg, or NAC 300 mg/kg, respectively, before 2 h APAP treatment.

Serum Enzyme and TNF-α Assay Twelve hours after APAP treatment, blood was collected and separated to measure ALT, AST, and TNF-α levels. Plasma was collected following centrifugation of blood for 30 min at 3000 rpm. Sera were stored at −20°C to measure serum biochemical parameters. The levels of ALT and AST were measured using an Autodory chemistry analyzer (Spotchem SP4340, Arkaray, Kyoto, Japan). TNF-α level was measured by ELISA using a Mouse TNF (Mono/Mono) ELISA Set (BD Bioscience, San Diego, CA, U.S.A.) according to manufacturer’s instructions.

Glutathione and Malondialdehyde Determination The frozen liver slices were washed in ice-cold EDTA solution (0.02 mol/l), blotted, dissected to remove connective tissues, weighed, and homogenized with 10% saline and then absorbance was measured at 412 nm (GSH) and 532 nm (MDA), respectively. GSH and MDA contents were determined according to the manufacturer’s instructions using Bioxytech GSH-400 (cat # 21011) colorimetric assay kit and Bioxytech MDA-586 (cat # 21044) spectrometric assay kit (Oxis International, Inc., Portland, OR, U.S.A.), respectively. Triplicate assays were performed in each measurement and the average counts were obtained from each individual sample. Protein in the tissues was determined by a Bradford method using Commassie Blue Solution according to the instructions. Results were expressed in mg GSH/g protein and nmol MDA/mg protein.

Histopathology and Immunohistochemistry For light microscopic investigations, samples from the liver were placed in 15% neutral buffered formalin and processed by routinely for embedding in paraffin. Tissue sections (4 μm) were stained with hematoxylin and eosin (H&E) and examined under an Olympus BH2 photomicroscope.

Immunohistochemistry for caspase-3 and HIF-1α were performed in frozen sections which prepared with 4 μm poly-l-lysine sections, using an immunohistology staining kit and anti-caspase-3 and HIF-1α monoclonal antibody respectively.

Statistical Analysis All values were expressed as
plasma ALT and AST levels were determined as a measure of hepatic function. Both ALT and AST levels were increased significantly in the APAP group after 12 h (p<0.001). SDS or NAC pretreatment suppressed the plasma ALT and AST activity increases 12 h after APAP administration significantly. As shown in Fig. 2, the SDS pretreatment groups showed dose-dependent plasma ALT activity reductions, and the effects of 20, 50 and 100 mg/kg were significant (p<0.001, 20 mg/kg; p<0.001, 50, 100 mg/kg). The percentages of inhibition of plasma ALT and AST activities were calculated using the following equation:

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100 \times \frac{(ALT/AST in APAP group - ALT/AST in SDS group)}{(ALT/AST in APAP group - normal ALT/AST level)}
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For the groups pretreated with 20, 50, 100 mg/kg SDS and 300 mg/kg NAC, the percentages of inhibition of ALT were 51.78%, 57.02%, 68.01% and 92.33%, respectively. The percentages of inhibition of AST were 53.18%, 68.29, 76.96% and 78.89%, respectively (Fig. 2).

The Effect of SDS on Glutathione and Malondialdehyde Contents

As GSH plays an important role in the detoxification of APAP, the hepatic GSH content was examined. The hepatic GSH content of APAP group decreased rapidly to 57.23% of the saline group (p<0.01). This reduction in GSH was partly attenuated in the SDS preventive group. In the preventive treatments with SDS 50 and 100 mg/kg significantly recovered the APAP-induced GSH depletion to the normal level (425.0±171.9 and 489.0±142.8 mg/g protein, respectively) (Fig. 3). However, treatment with SDS 20 mg/kg prevented GSH depletion statistically insignificantly.

Liver MDA content, an end-product of lipid peroxidation, were increased in the APAP group (8.90±2.16 nmol/mg protein) than that in the saline group (1.95±1.05 nmol/mg protein). The MDA levels of SDS 20, 50 and 100 mg/kg group were suppressed significantly compared with APAP group (5.58±1.74, 4.61±2.06, 4.23±1.90 nmol/mg protein, p<0.05—0.001) just like NAC group (4.73±1.98 nmol/mg protein, p<0.001), but only SDS 50 and 100 mg/kg group were similar with those of the saline group (p>0.05, compared with saline group) (Fig. 4).

The Effect of SDS on TNF-α Level

APAP-induced in-

RESULTS

The Effect of SDS in the APAP-Induced Lethality in Mice

When mice were intoxicated with 500 mg/kg (lethal dose) of APAP, 16.000% of animals were survived after 24 h. However, treatments with SDS 20 mg/kg, 50 mg/kg and 100 mg/kg administered 2 h before APAP (500 mg/kg) injection the survival rate was increased to 44.196%, 67.692%, and 75.524% at 24 h, respectively. While treatment with NAC 300 mg/kg administered 2 h before the APAP (500 mg/kg) injection resulted in survival rate of 72.692% at 24 h (Fig. 1). These results indicated that SDS could inhibit the lethality of APAP, and this inhibition presented a dose-effect relationship, higher dose of SDS with higher survival rate. No death was observed in the group treated with SDS alone up to 1000 mg/kg, indicating that SDS was safe to mice.

The Effect of SDS on Plasma AST and ALT Levels

Plasma ALT and AST levels were determined as a means of hepatic function. Both ALT and AST levels were increased significantly in the APAP group after 12 h (p<0.001). SDS or NAC pretreatment suppressed the plasma ALT and AST activity increases 12 h after APAP administration significantly. As shown in Fig. 2, the SDS pretreatment groups showed dose-dependent plasma ALT activity reductions, and the effects of 20, 50 and 100 mg/kg were significant (p<0.001, 20 mg/kg; p<0.001, 50, 100 mg/kg). The percentages of inhibition of plasma ALT and AST activities were calculated using the following equation:

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The Effect of SDS on TNF-α Level

APAP-induced in-
crease in the TNF-α levels (225.0±71.5 pg/ml), as compared to saline group (13.2±4.2 pg/ml, \( p<0.001 \)). Both SDS groups with 20, 50, 100 mg/kg and NAC group significantly inhibited TNF-α level (Fig. 5), compared with APAP group \( (p<0.001) \). Especially, 20, 50 and 100 mg/kg SDS showed 69%, 71% and 72% inhibition for TNF-α. At the same time, NAC showed 61% inhibition for TNF-α. These results suggest that SDS could modulate TNF-α similar to NAC to protect liver.

The Effect of SDS on Histopathology and Immunohistochemistry

According to the light microscopic evaluation of saline group and SDS or NAC treated groups, a regular morphology of the liver parenchyma, with well-designated hepatic cells and sinusoids, was evident (Fig. 6A). In the APAP group, severe sinusoidal congestion and hemorrhage, dilated central vein, severe inflammatory cell infiltration, and degenerated hepatocytes showing perinuclear vacuolization were observed (Fig. 6B). In the SDS groups, mild sinusoidal congestion, inflammatory cell infiltration, and mostly normal hepatocytes were observed in most areas (Figs. 6C, D, E). SDS groups showed well preserved hepatocytes as well as the architecture with less area of necrosis and inflammatory cells infiltration when compared with that of NAC group (Fig. 6F).

Histological analysis of tissue sections of liver 12 h after the treatment with APAP (Fig. 7A) revealed more cells immunohistochemically stained for caspase-3, a specific marker of apoptosis, whereas in contrast, very few cells stained positively for this marker in saline group (Fig. 7B). In immunohistochemical sections, caspase-3 expression was focused in endochylema and positive staining was presented in pale brown or brown. Compared APAP with SDS treated-groups resulted in decreased caspase-3 expression. The animal pretreated with SDS 20, 50, 100 mg/kg (Figs. 7F, C, E) and NAC 300 mg/kg (Fig. 7D), presented mild centrilobular staining for caspase-3. Although NAC groups showed less positive expression than SDS groups and was similar to normal. SDS showed...
groups even decreased caspase-3 expression significantly than APAP group.

In immunohistochemical sections, HIF-1α expression was focused in nucleus and endochylema, especially in endochylema, and positive staining was presented in tan. HIF-1α positive expression was clearly present in after induction of APAP (Fig. 8A), and its expression was further increased in response to saline group (Fig. 8B). Treatment with SDS and NAC decreased the immunohistochemical labeling of HIF-1α (Figs. 8C, D, E, F) and this decrease presented dose-effect relationship. This expressions present less positive staining similar to saline group.

For NAC is a antioxidant which could scavenge free radicals. NAC was selected as a positive control to demonstrate SDS the same protective effect in the GSH, MDA and TNF-α with NAC. All treatment groups with SDS showed no significance compared with NAC (p>0.05).

DISCUSSION

The major objective of this study was to determine the protective effects of SDS in APAP-induced toxicity. To this end, this study has demonstrated that SDS pretreatment suppresses the plasma ALT and AST activity increases caused by APAP administration (Fig. 2). We suggest that SDS pretreatment has a strong protective effect against APAP-induced liver injury in mice. The aim of our study was to examine the mechanism underlying this protective effect. In the
preliminary experiment, APAP was administered to mice, and the time course for the plasma ALT, AST and cytokine production was determined relative to toxicity. Subsequently, SDS was administered at early times following APAP, and the effect of each of the above parameters was determined at 12 h relative to toxicity. Twelve hours was chosen as a time point because of previous data showing that highest ALT and AST level and maximal toxicity at 12 h after APAP administration. Therefore, in the present study, we principally approach protective effect of SDS on APAP-induced toxicity 12 h after APAP administration.

Many studies on the biochemical mechanisms of APAP-induced hepatotoxicity have been elucidated in detail and believed to involve a change in cellular redox status toward a state of oxidative stress, was due to its metabolic conversion to the highly reactive intermediate NAPQI by cytochrome P-450 mediated oxidases. APAP-induced hepatotoxicity has demonstrated that GSH plays an important role in the detoxification of NAPQI. Toxic metabolites of APAP are known to be eliminated or scavenged by the antioxidant action of GSH, a reactive and toxic metabolite of APAP. GSH becomes almost exhausted, leaving NAPQI free to bind covalently and irreversibly to, possibly, critical cellular proteins and to cause centrilobular hepatic necrosis. In both in vivo and in vitro studies it has been found that this reactive metabolite forms adduct with cytosolic mitochondrial and microsomal proteins. For the concentration of intracellular GSH is the key determinant of the extent of APAP-induced hepatic injury, the inhibition of APAP-induced toxicity should be emphasized on how to reduce thiol agents and to cause centrilobular hepatic necrosis. In both in vivo and in vitro studies it has been found that this reactive metabolite forms adduct with cytosolic mitochondrial and microsomal proteins.

In the present study, we investigated the effects of pretreatment with NAC on APAP-induced liver injury and found that pretreatment with NAC significantly reduced serum ALT and AST levels in APAP-treated mice. In parallel, NAC pretreatment significantly attenuated APAP-induced hepatic necrosis and congestion. So we selected NAC as a positive comparison when we investigated the effects of pretreatment with NAC on APAP-induced toxicity. Treatment of mice with APAP attenuated organ dysfunction and prevented the depletion of antioxidants and hepatotoxicity induced by APAP. NAC also abrogated APAP-induced increases in expression of KC/gro. The finding that SDS could inhibit MDA content, suggesting a minimization in oxidative stress and lipid peroxidation. This finding suggests that SDS had a protection on APAP-induced oxidative damage. Results in vitro also showed SDS suppressed the expression of inflammatory gene products, such as TNF-α. TNF-α is a proinflammatory cytokine produced predominantly by liver macrophages, the primary mediator of liver damage and systemic toxicity. Following administration of APAP, hepatic expression of TNF-α is increased abnormality. Therefore, low levels of TNF-α may play a protective role in this model by inducing hepatocyte proliferation and the release of mediators involved in tissue repair. SDS pretreatment inhibited the elevation of TNF-α production after APAP-induced, which would indicate that suppression of the production of TNF-α might be useful for the treatment of inflammatory diseases.

Histological sections of liver showed centrilobular necrosis with inflammatory cell infiltration in APAP-intoxicated mice (Fig. 6). Centrilobular necrosis, the pathogenomonic feature of APAP-induced hepatotoxicity, was significantly reduced in AA treated mice. Further, the congestion and inflammatory cell infiltration evoked by APAP were considerably ameliorated by SDS treatment. Moreover, APAP administration significantly increased caspase-3 activity in the mouse liver. Caspase-3 plays a un-sucessedaneous effect in apoptosis, activated by mult Cache, such as Fas/FasL, granzyme B. The present study showed that SDS pretreatment significantly inhibited hepatic caspase-3 activity in APAP-induced mice, which indicate that the protective effect of SDS pretreatment against APAP-induced apoptosis liver injury might be mediated by its anti-apoptosis effects. HIF-1α is a transcriptional activator that functions as a master regulator of O2 homeostasis. In our research, administration of APAP commonly caused induction of hepatic HIF-1α (Fig. 8). APAP causes pooling of blood in the livers of treated mice and this could lead to tissue hypoxia. Since HIF-1α induction occurs before toxicity, our results showed SDS pretreatment attenuated the positive expression of HIF-1α. The finding that SDS blocked HIF-1α induction would show that HIF-1α induction occurred as a result of the toxic metabolite NAPQI, which detoxified by GSH leading to its depletion, might be a result of oxidative stress. According to all those, we thought that the enzymatic properties of SDS could neutralize the deleterious effects generated by APAP.

In summary, the present results illustrate that supplementation SDS, as an antioxidant, alleviates GSH depletion which consequently suppress oxidation stress, lipid peroxidation, inflammatory conditions, apoptosis, hypoxia and protected liver against APAP-induced toxicity.

Acknowledgement This work was supported in part our research grant (No. 30660225 and No. 30711140382 to Ji-Xing NAN) from National Natural Science Foundation of China.
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