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Research Paper

Hepatoprotective effects of salidroside on fulminant hepatic failure induced by D-galactosamine and lipopolysaccharide in mice

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Abstract

Objectives The aim was to investigate the protective effect of salidroside isolated from *Rhodiola sachalinensis* A. Bor. (Crassulaceae) on D-galactosamine/lipopolysaccharide-induced fulminant hepatic failure.

Methods Hepatotoxicity was induced by an intraperitoneal injection of D-galactosamine (700 mg/kg) and lipopolysaccharide (10 μg/kg); salidroside (20, 50 and 100 mg/kg) was administered intraperitoneally 1 h before induction of hepatoxicity. Liver injury was assessed biochemically and histologically.

Key findings Salidroside attenuated the induced acute increase in serum aspartate aminotransferase and alanine aminotransferase activities, and levels of tumour necrosis factor-alpha levels and serum nitric oxide. It restored depleted hepatic glutathione, superoxide dismutase, catalase and glutathione peroxidase activities, decreased malondialdehyde levels and considerably reduced histopathological changes. Histopathological, immunohistochemical and Western blot analyses also demonstrated that salidroside could reduce the appearance of necrotic regions and expression of caspase-3 and hypoxia-inducible factor-1α in liver tissue.

Conclusions Salidroside protected liver tissue from the oxidative stress elicited by D-galactosamine and lipopolysaccharide. The hepatoprotective mechanism of salidroside appear to be related to antioxidant activity and inhibition of hypoxia-inducible factor-1α.

Keywords antioxidation; D-galactosamine; hypoxia-inducible factor-1α; lipopolysaccharide; nitric oxide; salidroside

Introduction

D-Galactosamine/lipopolysaccharide (LPS)-induced hepatic injury in animals mimics the sequences of events in human hepatitis and is widely used in the screening of hepatoprotective drugs. The advantage of this model is that D-galactosamine potentiates the toxic effects of LPS and produces fulminate hepatitis within a few hours. D-Galactosamine is an amino sugar selectively metabolised by hepatocytes, which depletes the uridine triphosphate pool, resulting in the inhibition of mRNA and protein synthesis.[1] These phenomena may lead to cellular damage and inflammation, resulting in a histological and biochemical picture that closely resembles viral hepatitis. Upon stimulation with LPS, liver macrophages secrete various pro-inflammatory cytokines, leading to hepatic necrosis and decreased levels of antioxidant enzymes and the scavenging of free radicals.[2,3] This model provides a practical tool for the evaluation of drugs and compounds that interfere with hepatic apoptosis and inflammatory liver injury.

Salidroside (Figure 1) is isolated from *Rhodiola sachalinensis* A. Bor. (Gao-shan-hong-jing-tian in Chinese). *R. sachalinensis* is used in China to treat depression, enhance work performance, resist anoxia and prevent altitude sickness. Salidroside has been shown to ameliorate the effects of experimental liver fibrosis and partially inhibits experimental liver fibrosis.[4] The neuroprotective effects of salidroside on hydrogen peroxide (H_2O_2)-induced apoptosis in SH-SY5Y cells markedly attenuated H_2O_2-induced loss of cell viability and apoptotic cell death in a dose-dependent manner.[5] The effect of salidroside on oxidative stress in rat hepatic stellate cells may be related to inhibition of lipid peroxidation.[6] Most studies of salidroside have focused on hepatic apoptosis but less on drug-induced liver

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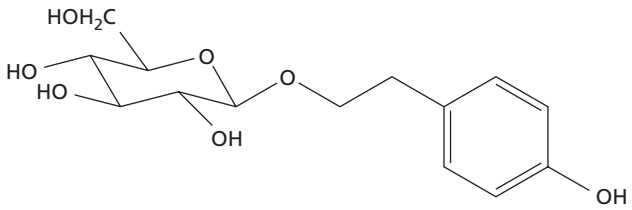

Figure 1 The chemical structure of salidroside (4-hydroxy-phenethyl)-D-glucopyranoside; $C_{14}H_{20}O_7$; molecular weight 300.30)

disease, which accounts for one-half of all cases of acute hepatic failure. Our previous work has indicated that salidroside could protect liver tissue from paracetamol-induced oxidative damage by suppressing the depletion of glutathione (GSH), which suggests that salidroside would be a potential antidote against paracetamol-induced hepatotoxicity.[7]

Liver damage results in free-radical-mediated oxidant processes, even apoptosis and necrosis. LPS produces protein inflammatory mediators, such as tumour necrosis factor (TNF) and interleukin.

The aim of the present study was to evaluate the protective effect of salidroside against D-galactosamine/LPS-induced liver damage. The hepatoprotective activity was evaluated by measuring the activities of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT); the activity of intracellular antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and GSH peroxidase (GSH-Px); the levels of the reactive oxygen scavengers and reduced GSH; and the levels of inflammatory mediators such as TNF-α, which is a critical mediator of apoptotic liver damage, and malondialdehyde (MDA). The expression of caspase-3 was also investigated to determine the pivotal role of apoptosis in D-galactosamine/LPS-induced acute liver failure. The caspase family play a part in apoptosis activated by various apoptotic pathways.[8] Cytotoxicity is usually correlated with nitric oxide (NO) produced by inducible NO synthase (iNOS), which has been shown to play an important part in the survival or death of liver cells. Paradoxically it causes cytotoxicity and is cytoprotective.[9] In addition, expression and activity of hypoxia-inducible factor (HIF)-1α are tightly regulated by cellular oxygen concentration and it is a physiological marker of hypoxia in response to D-galactosamine/LPS poisoning.[10] *N*-Acetyl-L-cysteine (NAC), a potent antioxidant, may serve as a precursor for glutathione synthesis,[11] and showed some protective effect against liver damage induced by drugs such as paracetamol and ciclosporin.[12] NAC was therefore used as a positive control in this study. We anticipated that D-galactosamine/LPS toxicity would increase the expression of HIF-1α, possibly as a result of induction of intracellular hypoxia by drug metabolism, and that salidroside would reduce the expression of HIF-1α and associated liver damage.

Materials and Methods

Materials

Salidroside (purity 99%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China; lot 818-9401). D-Galactosamine, LPS (≥98.0% HPLC) and NAC (≥99.0% TLC) were purchased from Sigma-Aldrich (St Louis, MO, USA). Detection kits for GSH and MDA were purchased from Oxis International, Inc. (Portland, OR, USA). SOD, CAT, GSH-Px, NO and NOS kits were from Nanjing Jiancheng Bioengineering Institute. Mouse TNF ELISA kit was purchased from BD Biosciences (San Diego, CA, USA). Caspase-3 (sc-1226) and HIF-1α (sc-53546) monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ALT and AST reagent strips were purchased form Arkray Incorporated (Kyoto, Japan).

Animals

Male C57BL/6 mice (6–8 weeks old; weight 18–23 g) were kept in an environmentally controlled room at temperature 23 ± 2°C and relative humidity 55 ± 1%. All mice received care according to the guidelines of the Guiding Principles in the Use of Animals in Toxicology adopted by the Society of Toxicology (USA) in July 1989 and revised in March 1999. The animal care committee of the local institution approved the study.

Treatments

Mice were fasted overnight (16–18 h) prior to administration of a single intraperitoneal dose of D-galactosamine (700 mg/kg) and LPS (10 μg/kg) dissolved in sterile phosphate-buffered saline (pH 7.4). Mice were randomly assigned to six experimental groups of 10 mice. The normal group was given sterile saline only. The salidroside100 group was given salidroside (100 mg/kg) only. The remaining four groups were all given D-galactosamine/LPS to induce liver failure and were treated with salidroside 20, 50 or 100 mg/kg or NAC (300 mg/kg). Dosages were determined from our previous work.[7] Mice were injected with D-galactosamine/LPS 1 h after salidroside or NAC.

Blood was collected 2 h after D-galactosamine/LPS injection for measurement of TNF-α levels, and after 6 h for measurement of serum ALT, AST and NOS activities and NO levels. Blood samples were allowed to coagulate at 4°C for 30 min. Serum was then separated by centrifugation at 2000*g* at 4°C.

Mice were killed 6 h after D-galactosamine/LPS injection and the same liver lobe excised from each animal. Tissue samples were immersed in neutral buffered formaldehyde for histopathological and immunohistochemical examinations. The remainder was kept at −80°C for subsequent analysis of MDA level and GSH, SOD, CAT and GSH-Px activities.

Measurement of serum enzymes, TNF-α, NO and NOS

TNF-α levels were determined 2 h after D-galactosamine/LPS injection using a TNF-α ELISA kit (BD Bioscience, San Diego, CA, USA) according to the manufacturer's instructions. Serum ALT and AST activities were measured using an Autodry chemistry analyser (Spotchem SP4430, Arkray, Kyoto, Japan). NO, total (tNOS) and iNOS were assayed by colorimetric assay kits (Nanjing Jiancheng Biotechnology Institute, China) according to the manufacturers' protocols. In brief, NO is chemically active and is quickly converted to

NO^{2-} and NO^{3-} *in vivo*; NO^{2-} is converted into NO^{3-}. The NO kit use nitrate reducatase to reduce NO^{3-} to NO^{2-}, then determines the content by colorimetry. NOS catalyses synthesis of NO from L-arginine and oxygen. NOS can be divided into two types: the constitutive form, which is present in neurones and endotheliocytes and is dependent on Ca^{2+}, and iNOS, which is present in macrophages and does not depend on Ca^{2+}. NOS kits quantify the activities of tNOS and iNOS using colorimetry to measure the amount of NO synthesised.

Measurement of antioxidant activity

The frozen liver slices were washed in ice-cold EDTA solution (0.02 mol/l), blotted, dissected to remove connective tissue, weighed and then homogenised with 10% saline. MDA level, GSH, total SOD, CAT and GSH-Px activities were measured according to the manufacturers' instructions. Liver protein was determined by Coomassie brilliant blue. The MDA assay kit detects MDA activity by measuring a red product formed when MDA reacts with thiobarbituric acid. The GSH kit detects GSH activity through yellow tetramethyl-benzidine and oxidised glutathione produced by the combination of GSH and dithio-nitrobenzene. The SOD kit detects SOD activity through nitroblue tetrazolium coloration. CAT can catalyse oxidation of H_2O_2 to form a red chromogenic substrate that is detected by the CAT kit. The GSH-Px kit detects GSH-Px activity through detecting selenium cysteine, the active centre of GSH-Px.

Histopathology and immunohistochemistry

Consecutive sections (4 μm) from frozen section were prepared for haematoxylin and eosin (H&E) staining and immunohistochemistry. For immunohistochemistry staining, briefly, paraffin sections were deparaffinised and hydrated, followed by antigen preparation. After blocking of endogenous peroxidase with 3% hydrogen dioxide, sections were incubated with anti-caspase-3 (1 : 50) or anti-HIF-1α monoclonal antibodies (1 : 200), and then treated with the Max Vision TM DAB kit, re-stained with haematoxylin, mounted and assessed by light microscopy.

Western blotting

The liver was homogenised in lysis buffer containing 0.5% Nonidet P-40, 10% glycerol, 137 mmol/l NaCl, 2 mmol/l ethylendiamine-tetraacetic acid, and 50 mmol/l Tris-HCl buffer (pH 8.0). After centrifugation at 3000*g* for 10 min, the supernatant was separated and stored at −80°C. The stored specimens were subjected to 12% polyacrylamide gel electrophoresis in the presence of 0.1% salidroside. The electrophoresed proteins in the gel were transferred to a PVDF membrane (Millipore, Bedford, MA, USA). The membranes were blocked overnight with 5% skimmed milk, and incubated with caspase-3 primary antibody (Santa Cruz, 1 : 1000) and mouse anti-goat IgG secondary antibody (Santa Cruz, 1 : 2000). Equal loading was confirmed by stripping the immunoblot and reprobing it for α-tubulin.

Statistical analysis

All values are expressed as means ± SD. The results were evaluated by one-way analysis of variance and Tukey's multiple comparison tests. Statistically significant differences between groups were defined as P values less than 0.05. Calculations were performed with the GraphPad Prism program (Graphpad Software, Inc., San Diego, CA, USA).

Results

Effect of salidroside on plasma AST and ALT levels

Plasma ALT and AST levels were determined as a measure of hepatic function. Both ALT and AST levels were increased significantly in the D-galactosamine/LPS group after 6 h (2238 ± 293 and 1558 ± 319 IU/l, respectively) compared with the normal group (40 ± 11 and 35 ± 8 IU/l, respectively; $P < 0.001$). Levels in the salidroside100 group were 37 ± 5 and 39 ± 7 IU/l, respectively. Salidroside or NAC pretreatment suppressed the increase in plasma ALT and AST activity at 6 h induced by D-galactosamine/LPS. As shown in Figure 2, the salidroside pretreatment groups showed dose-dependent reductions in these enzyme activities. ALT activities were reduced to 1743 ± 261, 1027 ± 240 and 416 ± 183 IU/l in the groups treated with 20, 50 and 100 mg/kg salidroside, respectively, and to 552 ± 101 IU/l in the group treated with NAC. Serum AST activities were reduced to 1109 ± 304, 647 ± 357, 213 ± 79 and 338 ± 148 IU/l, respectively ($P < 0.01$ or $P < 0.001$ vs D-galactosamine/LPS group) (Figure 2a).

Antioxidation effect of salidroside

Mice treated with D-galactosamine/LPS alone (Table 1) showed significant decreases in liver levels of enzymatic (SOD, CAT, GSH-Px) and non-enzymatic antioxidants (GSH) compared with the normal group. There was no difference between the normal group and salidroside 100 group. The antioxidant defence system was protected by pretreatment with salidroside or NAC. The activities of enzymatic antioxidants in the mice pretreated with 20 mg/kg salidroside increased significantly compared with the D-galactosamine/LPS group, whereas changes in GSH were not significant. MDA produces oxygen free radicals through enzymatic antioxidant and non-enzymatic antioxidants. Liver MDA content, an end-product of lipid peroxidation, was increased in the D-galactosamine/LPS group compared with the normal group. The MDA levels were significantly suppressed in the groups pretreated with 50 or 100 mg/kg salidroside or NAC compared with the D-galactosamine/LPS group, but not in mice pretreated with 20 mg/kg salidroside (Table 1).

Effect of salidroside on TNF-α production

TNF-α is a critical mediator of liver injury induced by D-galactosamine/LPS. We postulated that salidroside protected against D-galactosamine/LPS-induced liver injury through inhibiting elevation of TNF-α levels. Two hours after D-galactosamine/LPS administration, the level of TNF-α was markedly induced compared with the normal group (764.72 ± 89.73 vs 21.25 ± 5.14 pg/ml; $P < 0.001$). Levels in the salidroside 100 group were 25.73 ± 4.16 pg/ml. Salidroside-pretreated mice showed significant and dose-dependent lower TNF-α levels than D-galactosamine/LPS-treated

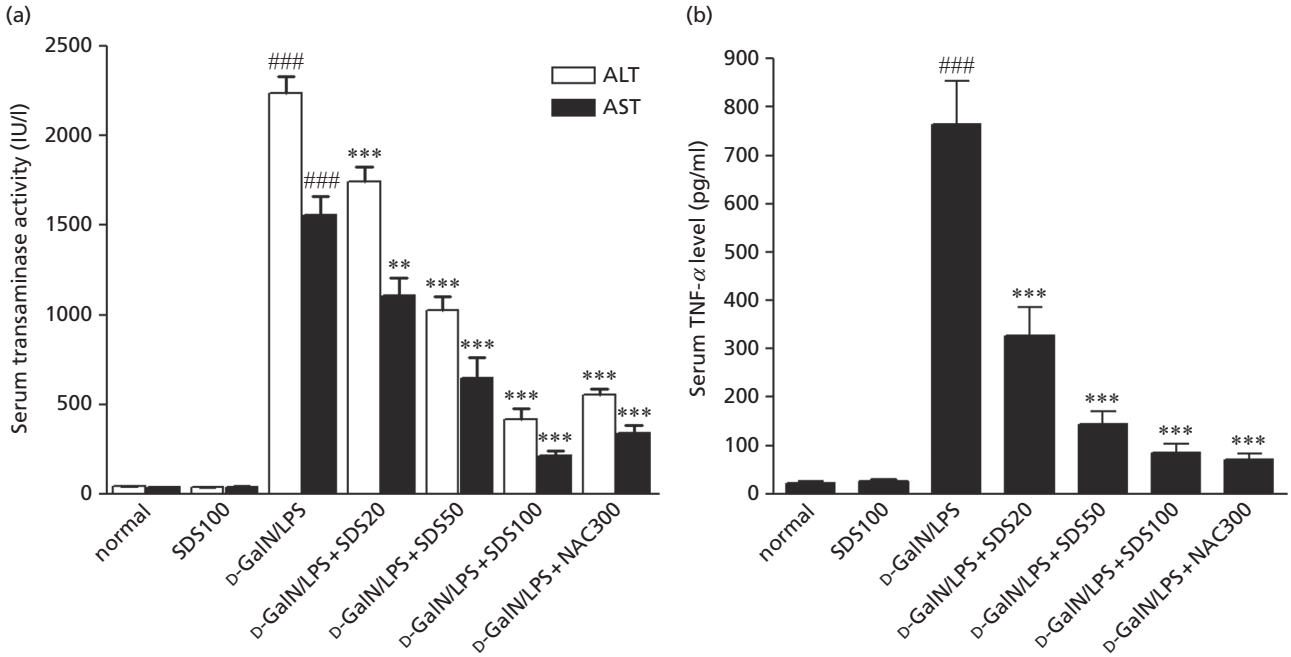

Figure 2 Effect of salidroside on serum transaminase enzymes and tumour necrosis factor α levels. Effect of salidroside treatment on (a) serum alanine aminotransferase (ALT)/aspartate aminotransferase (AST) levels and (b) serum tumour necrosis factor (TNF)-α levels in mice with D-galactosamine/lipopolysaccharide (D-GalN/LPS)-induced liver injury pretreated with salidroside (SDS; 20, 50, 100 mg/kg) or *N*-acetyl-L-cysteine (300 mg/kg) or salidroside (100 mg/kg) without liver injury. Bars represents means ± SD (n = 10). $^{###}P < 0.001$ vs normal group; $^{**}P < 0.01$, $^{***}P < 0.001$ vs D-GalN/LPS group.

mice: 327.35 ± 58.36, 143.75 ± 27.48 and 84.36 ± 18.04 pg/ml in the groups treated with 20, 50 and 100 mg/kg salidroside, respectively, and 69.71 ± 13.89 pg/ml in the mice pretreated with NAC ($P < 0.001$ vs D-galactosamine/LPS group; Figure 2b).

Effect of salidroside on the production of NO, and expression of tNOS and iNOS

NO production was investigated by measuring nitrite accumulation in serum. Six hours after treatment with D-galactosamine/LPS, nitrite concentration in serum reached 59.31 ± 12.15 μmol/ml, which was significantly higher than that of the normal group (20.43 ± 5.74 μmol/ml, $P < 0.001$). Administration of salidroside or NAC 1 h before D-galactosamine/LPS injection produced a significant dose-dependent inhibition of NO. The NO contents were 50.39 ± 11.35, 47.45 ± 8.63 and 30.10 ± 4.45 μmol/ml at salidroside doses of 20, 50 and 100 mg/kg, and 23.24 ± 2.70 μmol/ml with NAC. NO production with 50 and 100 mg/kg salidroside and NAC were significantly lower than in the D-galactosamine/LPS group. To investigate whether the inhibition of NO production is due to the reduction of iNOS and tNOS activities, we assessed the effect of salidroside on expression of these enzymes. Results were similar for tNOS and iNOS with salidroside 50 and 100 mg/kg and NAC. Levels of tNOS were 30.08 ± 6.55 and 26.26 ± 5.66 U/ml with salidroside 50 and 100 mg/kg, respectively, and 24.19 ± 4.13 U/ml with NAC. Levels of iNOS were 18.46 ± 3.86, 18.02 ± 5.82 and 16.57 ± 4.87 U/ml, respectively. The expressions of NO, tNOS and iNOS in

Table 1 Protective effect of salidroside against hepatotoxicity

Treatments	MDA (nmol/mg)	GSH (mg/g)	SOD (U/mg)	CAT (U/mg)	GSH-Px (U/l)
Normal	4.59 ± 0.82	588.04 ± 62.15	78.37 ± 4.79	60.48 ± 8.07	813.71 ± 70.37
Salidroside 100 mg/kg	5.01 ± 0.67	557.86 ± 54.37	79.35 ± 5.60	61.37 ± 6.25	827.45 ± 59.64
D-galactosamine/LPS	12.83 ± 4.96†	320.00 ± 72.94†	35.26 ± 4.83†	38.24 ± 4.02†	401.81 ± 54.57†
D-galactosamine/LPS + salidroside 20 mg/kg	9.45 ± 3.12	365.33 ± 58.46	47.95 ± 5.58***	46.38 ± 3.48*	524.70 ± 70.48**
D-galactosamine/LPS + salidroside 50 mg/kg	6.16 ± 1.30***	412.00 ± 67.38*	67.55 ± 7.18***	53.67 ± 5.82***	657.59 ± 64.32***
D-galactosamine/LPS + salidroside 100 mg/kg	4.99 ± 1.49***	521.00 ± 77.35***	69.55 ± 6.38***	58.03 ± 4.31***	739.96 ± 69.62***
D-galactosamine/LPS + NAC 300 mg/kg	4.84 ± 0.89***	467.00 ± 67.40***	72.61 ± 4.21***	59.34 ± 4.95***	717.86 ± 101.22***

CAT, catalase; GSH, glutathione; GSH-Px, glutathione peroxidase; LPS, lipopolysaccharide; MDA, malondialdehyde; NAC, *N*-acetyl-L-cysteine, SOD, superoxide dismutase. Values are means ± SD (n = 10), per mg of protein. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs D-galactosamine/LPS group; $^{\dagger}P < 0.001$, significantly different from normal group.

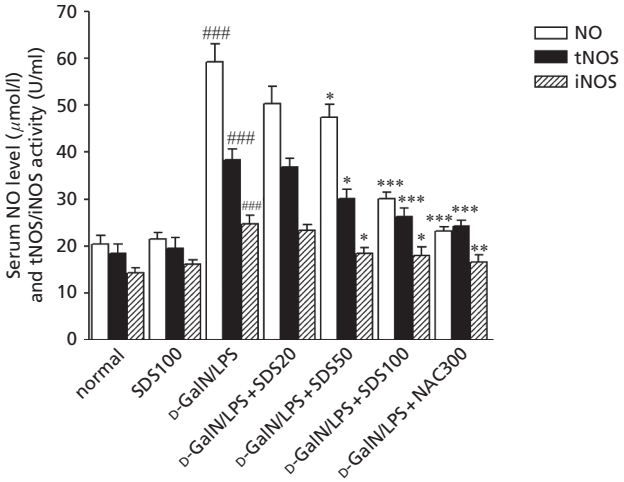

Figure 3 Effect of salidroside on nitric oxide production and nitric oxide synthase expression. Effect of salidroside on the production of nitric oxide (NO) and the expressions of total and inducible nitric oxide synthase (tNOS and iNOS) 6 h after induction of liver injury. Mice were pretreated with salidroside, 20, 50 or 100 mg/kg or *N*-acetyl-L-cysteine (NAC) 300 mg/kg 1 h before administration of D-galactosamine/lipopolysaccharide (LPS). Bars represent means ± SD ($n = 10$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs D-galactosamine/LPS group; $^{\#\#\#}P < 0.001$ vs normal group.

the salidroside 100 group were 21.47 ± 4.35, 19.51 ± 7.26 and 16.16 ± 2.96 U/ml respectively, where were not significantly difference from the normal group (Figure 3).

Effect of salidroside on histopathology, immunohistochemistry and Western blotting

All mice treated with D-galactosamine/LPS showed histopathological signs of hepatotoxicity. Liver sections from these mice showed severe confluent and focal necrosis, apoptosis, inflammatory cell infiltrate around the central zone, periportal vacuolation and haemorrhage (Figure 4a). Many apoptotic bodies and nuclei with condensed chromatin appeared in these mice. The normal group did not show any abnormal changes in liver architecture (Figure 4b). The degree of tissue damage was obviously less in the mice pretreated with salidroside 20 and 50 mg/kg than in the D-galactosamine/LPS group: only central necrosis, cell infiltration and periportal vacuolation were observed, and apoptotic cell death was significantly reduced (Figures 4c and d). Prereatment with salidroside 100 mg/kg reduced degeneration: regular morphology of the liver parenchyma with well-defined hepatic cells and sinusoids was observed (Figure 4e). Mice treated with NAC also showed well-preserved hepatocytes and

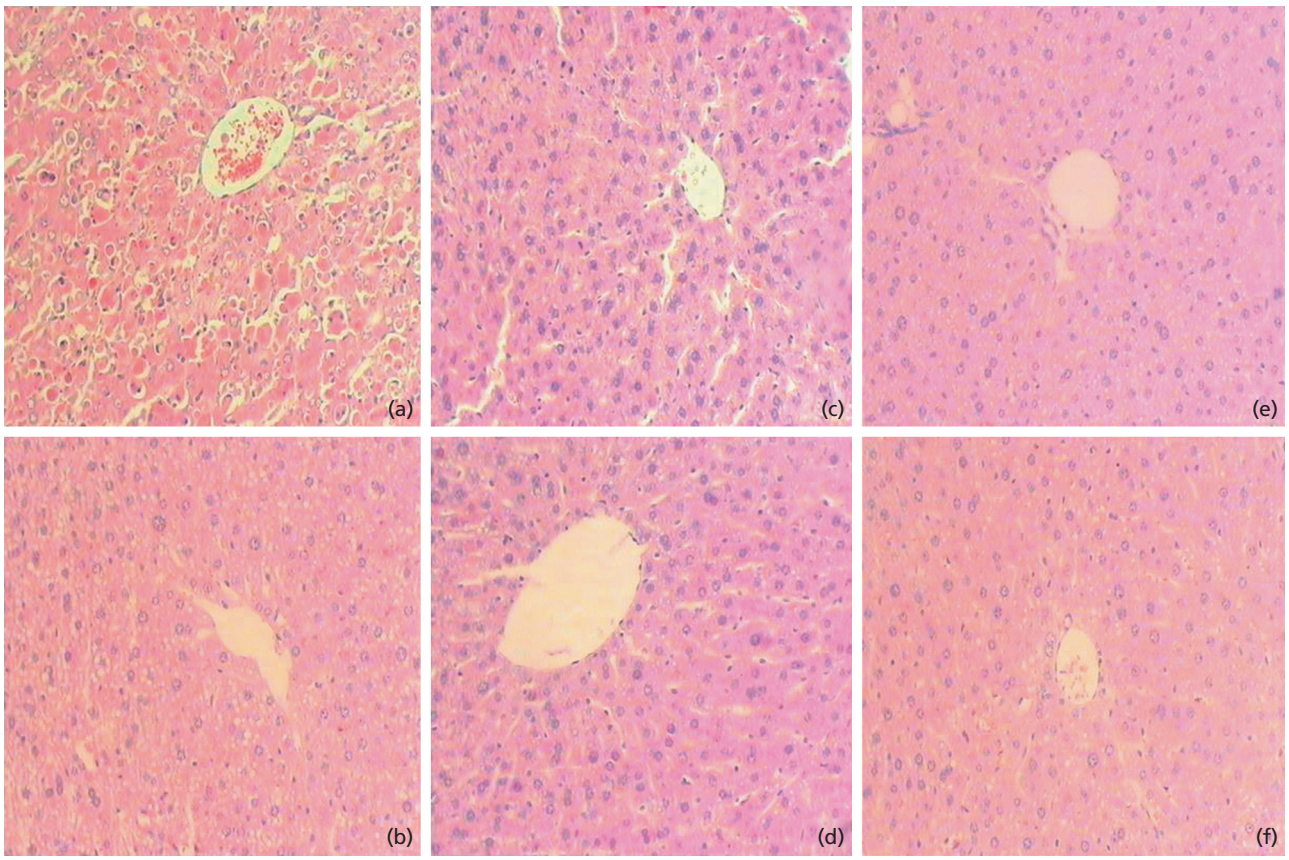

Figure 4 Effect of salidroside on histopathology of liver sections. H&E stained sections from livers 6 h after induction of liver injury by D-galactosamine/lipopolysaccharide (LPS). Mice were treated with salidroside, 20, 50 or 100 mg/kg or *N*-acetyl-L-cysteine (NAC) 300 mg/kg 1 h before D-galactosamine/LPS injection: (a) D-galactosamine/LPS group; (b) normal group (given saline only); (c) D-galactosamine/LPS + salidroside 20 mg/kg; (d) D-galactosamine/LPS + salidroside 50 mg/kg; (e) D-galactosamine/LPS + salidroside 100 mg/kg; (f) D-galactosamine/LPS + NAC 300 mg/kg. All slides are ×100 magnification.

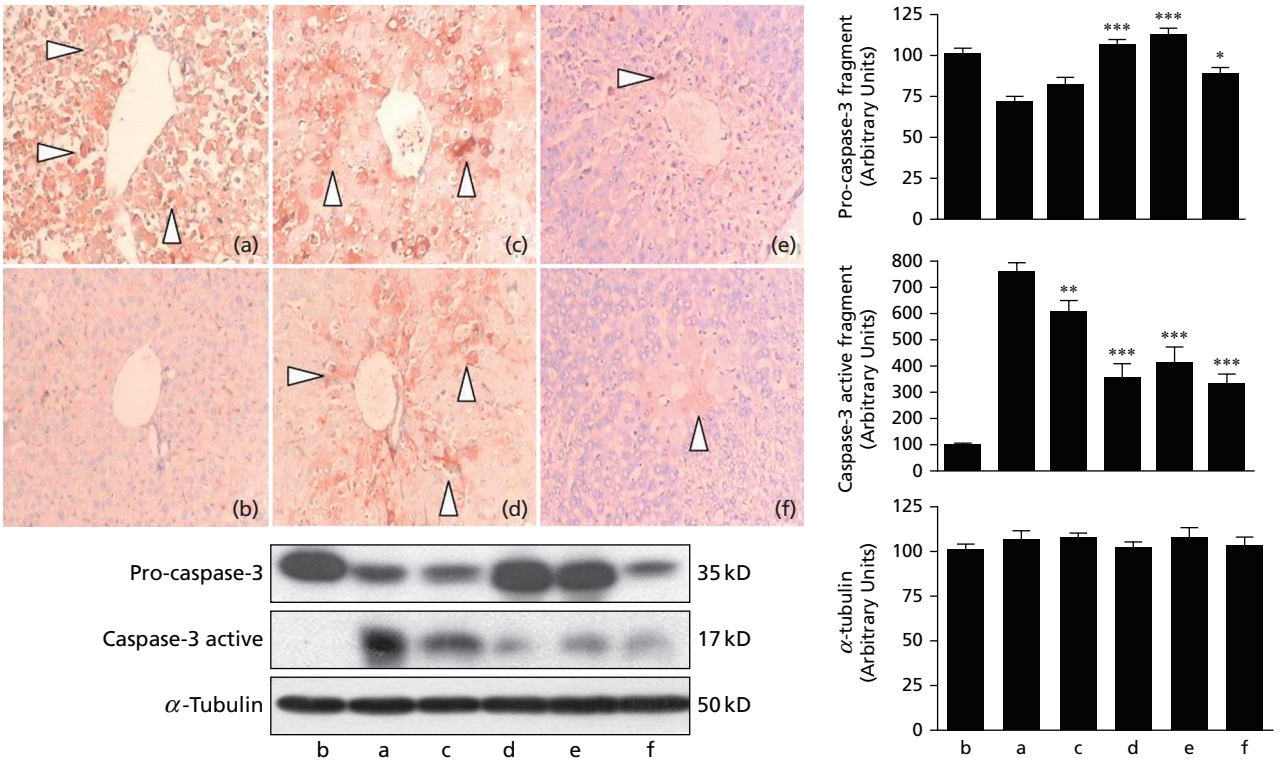

Figure 5 Effect of salidroside on expression of caspase-3. Immunohistochemical staining and Western blotting with an anti-caspase-3 antibody after induction of liver injury by D-galactosamine/lipopolysaccharide (LPS): (a) D-galactosamine/LPS group; (b) normal group; (c) D-galactosamine/LPS + salidroside 20 mg/kg; (d) D-galactosamine/LPS + salidroside 50 mg/kg; (e) D-galactosamine/LPS + salidroside 100 mg/kg; (f) D-galactosamine/LPS + *N*-acetyl-L-cysteine (NAC) 300 mg/kg. All slides are ×100 magnification. Areas stained in pale brown or brown indicate positive caspase-3 expression. Arrowheads point to areas of positive staining for caspase-3. Western blotting shows bands at 35 kD and 17 kD, which indicate the pro-form and active forms of caspase-3, respectively.

tissue architecture, with less necrosis and inflammatory cell infiltration (Figure 4f).

Immunohistochemistry staining of caspase-3 was used to depict apoptosis degree in liver sections taken 6 h after treatment with D-galactosamine/LPS (Figure 5). Positive caspase-3 showed pale brown or brown in the endochylema after DAB coloration and was clearly identified. The D-galactosamine/LPS group presented large numbers of chocolate-brown areas (Figure 5a), with fewer in the normal group (Figure 5b). In livers from mice treated with salidroside (20 and 50 mg/kg), the positive staining appeared as mild centrilobular staining, the positive cell population decreased and staining intensity gradually decreased with higher doses of salidroside – 50 mg/kg of salidroside showed less positive staining than 20 mg/kg (Figure 5c and d). There was virtually no positive staining in the mice pretreated with 100 mg/kg salidroside, similar to that with NAC (Figure 5e and f). Similar results were seen with Western blotting: degradation of 32 kDa procaspase-3 which generated the 17 kDa active fragment was observed. It is clear that most of the caspase-3 pro-form changed into active form in mice treated with D-galactosamine/LPS and there was less change in the salidroside-pretreated groups, and this change showed dose dependence. Salidroside administration resulted in further downregulation of activated caspase-3 levels induced by D-galactosamine/LPS.

In immunohistochemical sections, HIF-1α expression was focused in nuclei and particularly in the endochylema; positive staining is shown as tan. Positive HIF-1α expression was clearly evident in the D-galactosamine/LPS group (Figure 6a) compared with the normal group (Figure 6b). Positive HIF-1α staining intensity decreased with salidroside prereatment in a dose-dependent manner (Figures 6c–e), similar to that with NAC (Figure 6f).

Discussion

Serum ALT and AST levels increased markedly 6 h after D-galactosamine/LPS injection, indicating that liver damage was induced. This damage was attenuated by treatment with salidroside at 20, 50, and 100 mg/kg. These results indicate that salidroside preserves the structural integrity of the hepatocellular membrane and protects the mice against D-galactosamine/LPS-induced hepatotoxicity. Histological examination of liver sections showed severe confluents and focal necrosis, apoptosis and focal inflammation. Liver damage and histological changes were significantly less severe in mice pretreated with salidroside (Figure 4). ALT and AST activities, TNF-α level and antioxidative parameters in mice treated only with salidroside were similar to values in the normal group. None of the mice treated with salidroside died within 72 h (data not shown). The LD50 of

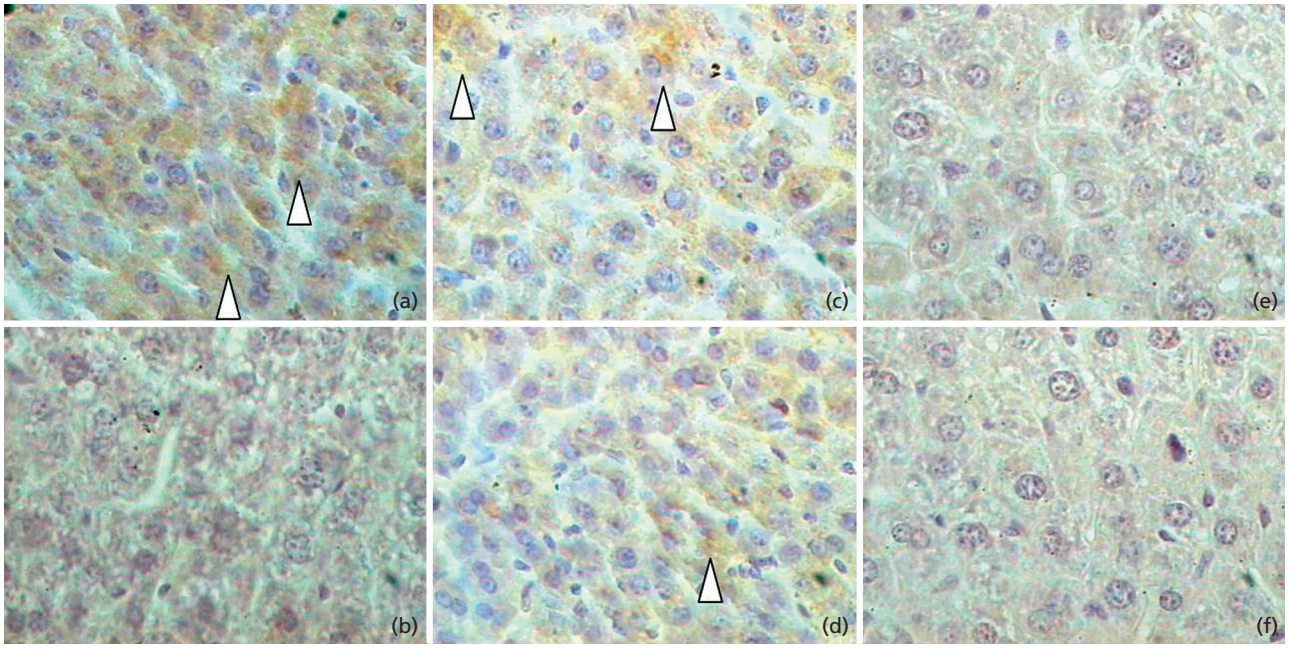

Figure 6 Effect of salidroside on expression of hypoxia-inducible factor. Immunohistochemical staining with antibodies against hypoxia-inducible factor (HIF)-1α after induction of liver injury by D-galactosamine/lipopolysaccharide (LPS): (a) D-galactosamine/LPS group; (b) normal group; (c) D-galactosamine/LPS + salidroside 20 mg/kg; (d) D-galactosamine/LPS + salidroside 50 mg/kg; (e) D-galactosamine/LPS + salidroside 100 mg/kg; (f) D-galactosamine/LPS + *N*-acetyl-L-cysteine (NAC) 300 mg/kg. All slides are ×400 magnification. Areas stained tan indicate HIF-1α-positive expression. Arrowheads point to positive staining for HIF-1α.

salidroside following intravenous injection in mice is 8.6 g/kg, and no deaths or toxic reactions were found within 5 days after salidroside administration.[13]

Oxidative stress due to increased levels of reactive oxygen species (ROS), coupled with deficiency of the host antioxidant defence mechanism, might be a determining factor contributing to the development of liver damage.[14] MDA produces oxygen free radicals through enzymatic and non-enzymatic systems. GSH is found in particularly high concentrations where there is oxidative stress, where it protects cells from damage. Cells are also protected from damage by radical and non-radical reactive species, including peroxides and superoxides, through the enzymatic inactivation by CAT, SOD and GPx, antioxidant enzymes that provide cellular defence against the intermediates of dioxygen reduction. There was a significant increase in MDA and decrease in the activities of GSH, SOD, GPx and CAT after D-galactosamine/LPS administration, indicative of oxidative stress. The administration of salidroside, perhaps working as a precursor of GSH and free-radical scavenger, reduced cellular GSH depletion and balanced ROS to ameliorate peroxide and superoxide activities. The protection provided by salidroside against D-galactosamine/LPS-induced hepatotoxicity may be by scavenging of superoxide and peroxyl radicals.

LPS activates Kupffer cells to release pro-inflammatory mediators, such as TNF-α, which mediate liver apoptosis.[15] Macrophages produce TNF-α rapidly in response to tissue injury. TNF-α stimulates the release of cytokines from macrophages and production of NO, which is a highly reactive oxidant produced by parenchymal and non-parenchymal liver cells through the action of NOS.[16] NO exacerbates oxidative stress by reacting with ROS.[17] NO can react with the superoxide anion to form a potent and versatile oxidant, peroxynitrite, which stimulates the production of TNF-α in Kupffer cells through the activation of the oxidant-sensitive transcription factor nuclear factor-κB.[18] NO has been shown to play an important part in the survival or death of liver cells and to exhibit paradoxical functions: cytotoxicity and cytoprotection.[9,19] The development of this double-edged role depends on the production of different NO synthases: endothelial NOS (eNOS) represents activities in normal tissues and produces NO to maintain normal angiostasis, blood pressure and blood flow to ischaemic regions. While iNOS shows no activities in physiological states, it increases significantly 3–4 h after LPS-induced damage, thus producing large amounts of NO, resulting in cytotoxicity. Cytotoxicity is usually correlated with NO produced by iNOS. Our study showed that the content of NO increased significantly in D-galactosamine/LPS-induced liver damage, due to LPS-induced damage in Kupffer cells and the transcription of iNOS in sinus hepatic endotheliocytes. Serum levels of TNF-α, NO, cNOS and iNOS expression increased after D-galactosamine/LPS administration, and this increase was prevented by salidroside.

Activation of caspase-3 is important in the process of apoptosis. TNF-α can induce caspase-3 expression and result in apoptosis and necrosis of different types of cells, which might be mediated by NO. HIF-1α was increased after D-galactosamine/LPS administration, which might cause a reduction in available oxygen in the cell. Decreased oxygen would prevent breakdown of HIF-1α, resulting in a net

increase in HIF-1α levels. Major mechanisms for HIF-1α induction are growth factor-stimulated signal transduction pathways and hypoxia.[20] HIF-1α promotes adaptive responses to hypoxia and has been implicated in pathological inflammatory processes. HIF-1α is a transcriptional activator that functions as a master regulator of oxygen homeostasis. HIF-1α target genes include those that encode proteins that increase oxygen delivery and mediate adaptive responses in cells. Our data showed that oxidative stress emerged and the levels of NO and TNF-α in the serum increased with the high expression of HIF-1α, which indicated there were some relationships between these parameters. Oxidative stress,[21] TNF-α and NO mediate oxygen delivery and affect the stabilisation of HIF-1α. NO and TNF-α affect HIF-1α protein levels in a concentration-dependent manner *in vitro*.[22] As mentioned above, D-galactosamine/LPS caused oxidative stress, since it induced increases in MDA and NO and depletion of GSH, SOD, CAT and GPX, which release oxygen free radicals. All these are important mechanism in oxidative stress. These data suggest the that oxygen free radicals may directly affect HIF-1α expression and function. D-Galactosamine/LPS toxicity may also lead to tissue hypoxia. Our research indicates that salidroside prevents oxidative stress induced by D-galactosamine/LPS and simultaneously attenuates HIF-1α expression in liver tissue. It is clear that D-galactosamine/LPS may increase HIF-1α via oxidative stress, and regulation of NO and TNF-α activity would affect hepatic injury induced by D-galactosamine/LPS. Further studies are needed to clarify these mechanisms.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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