Protective Effect of Salvianic Acid A on Acute Liver Injury Induced by Carbon Tetrachloride in Rats

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Received March 2, 2006; accepted August 28, 2006

Previous research has shown that salvianic acid A [2-(3,4-dihydroxyphenyl)-2-hydroxy-propanoic acid, SA] extracted from Salvia miltiorrhiza BGE (Danshen) markedly inhibits lipid peroxidation of mitochondrial membrane of hepatic cells in vitro. This study was conducted to examine protective effect of SA on liver injury induced by carbon tetrachloride (CCL4) and its mechanism in vivo. Male Sprague-Dawley rats weighing 180—200 g were used in the experiments. Five mmol/kg CCL4 in olive oil was given to rats i.p. Spectrophotometrical method was used to measure activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum, activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) as well as malondialdehyde (MDA) level in hepatic tissue and the rate of superoxide anion (O2•−) generation in hepatic submitochondrial particles. Hepatic histological structure was observed under light microscopy. CCL4 caused significant changes of activities of the enzymes, MDA level, and the rate of O2•− generation and histopathological changes of acute hepatic injury were noted. SA reversed the significant changes induced by CCL4. These results demonstrate that SA produces protective action on acute hepatic injury induced by CCL4 via an antioxidative mechanism.

Key words salvianic acid A; aspartate aminotransferase; alanine aminotransferase; antioxidative enzyme; malondialdehyde; superoxide anion

Oxidative stress is implicated in the pathogenesis of age-related liver diseases. Hepatic damage caused by chronic ethanol ingestion,1 iron overload,2 and carbon tetrachloride (CCL4)3,4 is attributed to heightened production of reactive oxygen species. Normally, the free radical level in the body is low because healthy organisms can neutralize, metabolize, or subtract the toxic effects by free radical scavengers such as superoxide dismutase (SOD), and catalase (CAT).5 Salvianic acid A [2-(3,4-dihydroxyphenyl)-2-hydroxy-propanoic acid, SA] is one of the main active components of Salvia miltiorrhiza BGE. Previous studies have shown that SA is effectively able to protect myocardial mitochondrial membrane from ischemia-reperfusion injury,6 inhibit lipid peroxidation of mitochondrial membrane of hepatic cells,7 and prevent apoptosis of human neuroblastoma SH-SY5Y cells by antioxidant mechanism in vitro.8 So far, however, there has been little research reported on the hepatoprotective effect of SA. The present study was undertaken to evaluate whether SA elicits protective action against acute hepatic injury induced by CCL4 and its mechanism in rats.

MATERIALS AND METHODS

Extraction and Isolation of SA  Salvia miltiorrhiza BGE (Bozhou Crude Drug Market, Anhui, P. R. China) was identified by the authors and a voucher specimen (No. 045214) was kept at Shandong Engineering Research Center for Nature Drug. Fifty grams of Salvia miltiorrhiza BGE was extracted twice with 600 ml of boiling water containing 0.4% sodium hydroxide. The total water extract was adjusted to pH 3—4 with hydrogen chloride and was applied to macroporous adsorption resin columns D301 and AB-8 (Anhui Tianxing Resin Co., Ltd, P. R. China) and eluted with water containing 0.4% sodium hydroxide. Finally, 0.5 g of SA was obtained by condensing and evaporating of the collected extract. The active compound was identified by instrumental analysis with the following physical and chemical properties: white needle powder (purity >98% by HPLC), [α]D25 = +35° (H2O), mp 253—255 °C, MFC7H5O4, MW 198.173 (Fig. 1).12

Animals  Experiments were carried out according to the National Institutes of Health Guidelines for the care and use of laboratory animals and were approved by the local authority. Sprague-Dawley rats weighing 180—200 g (Animal Center of Shandong Engineering Research Center for Nature Drug, Yantai, P. R. China) were housed at 22±1 °C in a 12 h/12 h light/dark cycle with pellet food and tap water.}

Acute CCL4-Induced Liver Injury In Rats  A total of 50 rats were divided into 5 groups: normal control group with i.g. saline, model control group with CCL4 treatment and i.g. saline, silymarin (Madaus AG, German) group with CCL4 treatment and i.g. 25 mg/kg silymarin and SA groups with CCL4 treatment and i.g. 10 or 20 mg/kg SA. All rats were treated once daily for 3 d. At 60 min after the last pretreatment, 20% CCL4 diluted with olive oil (5 mmol/kg) was given to rats i.p. (olive oil only for normal control group). Sixteen hours after administration of CCL4, rats were anaesthetized with 20% urethane (i.p., 1 ml/kg) to collect blood for measuring activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum in accordance with


methods provided by the diagnostic kits (Nanjing Jiancheng Bioengineering Institute, P. R. China), and liver for measuring activities of SOD, CAT, glutathione peroxidase (GPx), and malondialdehyde (MDA) and superoxide anion (O$_2^-$) levels with the following methods.

**Measurement of Lipid Peroxidation** MDA, production of lipid peroxidation (LPO), was assayed by the method of Buege et al.$^{15}$ Reaction mixture corresponding to a total volume of 10 ml of tissue homogenate was heated at 95 °C for 60 min. The pink-colored chromogen formed by the reaction of 2-thiobarbituric acid with MDA was spectrophotometrically measured at 535 nm. The results were expressed as MDA nmol/mg protein.

**Isolation of Mitochondria and Preparation of Submitochondrial Particles** All steps in the isolation of mitochondria and preparation of submitochondrial particles were performed at ice-cold temperature. Hepatic mitochondria were isolated according to the method of Sohal et al.$^{16}$ Liver was homogenized in 10 volumes (w/v) of isolation buffer containing 0.22 m mannitol, 0.07 m sucrose, 3 mm EDTA, and 1 m Tris–HCl (pH 7.4). The homogenate was centrifuged at 2800 g for 20 min. Then, the supernatant was recentrifuged at 12000 g for 20 min and at 30000 g for 10 min. Finally, the pellet was resuspended in 30 mm phosphate buffer (pH 7.0).

Hepatic submitochondrial particles were isolated by resuspending the mitochondrial pellet in 4 volumes of phosphate buffer. The suspended solution was sonicated 3 times for 30 s each at 1 min intervals. The sonicated mitochondria were centrifuged at 8000 g for 10 min to sediment the unfragmented mitochondria, and the supernatant was recentrifuged at 80000 g for 40 min to pellet the submitochondrial particles. The collected pellet was resuspended in phosphate buffer for further assay or storage at −80 °C.$^{15}$

**Determination of Superoxide Generation** The rate of superoxide anion (O$_2^-$) generation by submitochondrial particles was measured according to the method of Boveris.$^{16}$ Both the test and reference cuvettes contained 20 to 40 μl of the submitochondrial suspension, 0.1 m phosphate buffer (pH 7.4), 7.2 μM cytochrome C, 0.6 μM antimycin A, and 7 mm succinate. Two hundred U/ml SOD (Sigma, U.S.A.) was added into the reference cuvette. The reduction of cytochrome C was spectrophotometrically monitored at 550 nm. Since both the test and reference cuvettes contained identical ingredients except SOD, the measured rate of cytochrome C reduction was specific because of its interaction with O$_2^-$.

**Detection of SOD, CAT, and GPx Activities** Hepatic tissue was homogenized in 10 volumes (w/v) of 0.1% Triton X. The homogenate was centrifuged at 3000 g for 5 min, then the supernatant recentrifuged at 50000 g for 30 min. The remaining supernatant was used for measurement of activities of SOD, CAT, and GPx. The method for the SOD assay was a slight modification of the indirect inhibition assay developed by Sun$^{7}$ in the method, xanthine–xanthine oxidase was used to generate superoxide, which catalyzed the reduction of nitrotetrazolium blue tetrazolium (NBT) to blue formazon. The absorbance was determined at 560 nm. SOD in the sample competed for superoxide and inhibited the reaction rate of superoxide with NBT. One unit of SOD was defined as the amount of protein that inhibited the rate of NBT reduction by 50%. The results were given in U/mg protein.

CAT activity was assayed by the method of Takahara et al.$^{19}$ After 0.2 ml of the homogenate was added to 1.2 ml of 50 mm phosphate buffer (pH 7.0), the reaction was started by addition of 1.0 ml of 30 mm H$_2$O$_2$ solution. The decrease in absorbance was measured at 240 nm in 30 s intervals for 3 min. The reaction in the blank was run simultaneously with distilled water instead of H$_2$O$_2$. The enzyme activity was expressed as μmol of decomposed H$_2$O$_2$/min/mg protein.

GPx activity was assayed by the method of Rotruck et al.$^{19}$ The reaction mixture consisted of 0.2 ml of 0.8 mm EDTA, 0.1 ml of 10 mm sodium azide, 0.1 ml of 2.5 mm H$_2$O$_2$, 0.2 ml of the reduced glutathione, 0.4 ml of 0.4 m phosphate buffer (pH 7.0), and 0.2 ml of homogenate, and was incubated at 37 °C for 10 min. The reaction was arrested by addition of 0.5 ml of 10% trichloroacetic acid and tubes were centrifuged at 2000 rpm for 15 min. Three ml of 0.3 mm disodium hydrogen phosphate and 1.0 ml of 0.04% DTNB [5,5-dithiobis(2-nitrobenzoic acid)] were added to the supernatant and the developed colour was detected at 420 nm immediately. GPx activity was expressed as μmol of the oxidized glutathione/min/mg protein.

Protein was detected according to the method described by Lowry et al.$^{20}$

**Histopathological Observation** Few millimeter-thick midsection of the left lobe of liver from each animal was processed for observation by light microscopy. The process involved fixing the tissue specimen in 10% neutral buffered formalin solution, preparing the block in paraffin, cutting into 5–6 μm thick sections, and staining the sections with haematoxylin–eosin stain. The sections were scanned and analyzed by pathologist who was blinded to the different treatments in the experiment.

**Statistical Analysis** Data from experiments were expressed as mean±S.E.M. with 10 rats in each group. Statistical analysis was conducted by one-way ANOVA with Newman–Keuls multiple comparison test. *p<0.05 was considered statistically significant.

**RESULTS**

Table 1 shows levels of AST and ALT in serum. AST and ALT levels were elevated from 132.28 and 51.02 U/ml in the normal control group to 323.89 and 270.89 U/ml in model control group, respectively, and significantly reduced to 226.10 and 196.09 U/ml, respectively, in the SA-treated group (20 mg/kg) and to 281.78 and 199.10 U/ml, respectively, in the silymarin-treated group compared with model control group (p<0.05 or 0.01). These findings suggest that...
SA and silymarin protect the liver from injury induced by CCl₄.

Figure 2 shows extent of MDA in liver. MDA was increased from 2.93 nmol/mg protein in the normal control group to 8.00 nmol/mg protein in the model control group. SA (10, 20 mg/kg) decreased MDA level in liver to 5.33 and 5.15 nmol/mg protein, respectively, and silymarin to 5.69 nmol/mg protein from 8.00 nmol/mg protein in the model control group (*p<0.01). The results indicate that SA and silymarin are effectively able to inhibit production of MDA in CCl₄-injured liver.

Figures 3—6 show the effects of SA on rate of O₂·⁻ generation by submitochondrial particles, and total SOD, CAT, and GPx activities in liver. The rate of O₂·⁻ generation in submitochondrial particles was increased and SOD, CAT, and GPx activities were decreased in livers of model control group animals in comparison with the normal control group (*p<0.05 or 0.01). SA (20 mg/kg) and silymarin remarkably increased SOD, CAT, and GPx activities in liver and decreased the rate of O₂·⁻ generation in submitochondrial particles of liver (*p<0.05 or 0.01).

Histological observation of the liver provided further evidence to support the hepatoprotective role of SA and silymarin. Figure 7B shows necrosis of hepatocytes with collapse of hepatic architecture, hemorrhage, and so on in CCl₄-treated rats alone. Figures 7C and 7D indicate that SA (20 mg/kg) and silymarin were effectively able to relieve the histopathological changes of hepatic cells injured by CCl₄.
DISCUSSION

CCL₄ hepatotoxicity depends on its reductive dehalogenation catalyzed by Cyt P450 in the endoplasmic reticulum of hepatic cells leading to the generation of an unstable complex trichloromethyl radical. The superoxide anion O₂⁻, H₂O₂, and the hydroxyl radical (OH⁻) are reactive oxygen species (ROS) mainly produced in mitochondria. Cells have a number of mechanisms to protect themselves from the toxic effects of ROS including free radical scavengers and chain reaction terminators such as SOD, CAT, and GPXs systems.²² SOD removes O₂⁻ by converting it to H₂O₂ that can be rapidly converted to water by CAT and GPX.²³ Cellular injury occurs when ROS generation exceeds the cellular capacity of removal.

In the present study, increased production of MDA and O₂⁻ as well as decreased activities of SOD, CAT, and GPX occurred in liver injury induced by CCL₄, implying downregulation of numerous enzymatic oxidation reactions in the cytosolic compartments and mitochondria. Furthermore, liver damage via ROS pathway caused a remarkable increase of ALT and AST levels in serum and histological changes of hepatic cells. SA had a protective effect on liver injury in response to CCL₄ by producing significant elevation of SOD, CAT, and GPX activities to increase the scavenging of ROS in mitochondria, leading to decreased activities of AST and ALT in serum and relieved changes of hepatic histopathology. These results are consistent with the report from Wang et al. (2005a) that SA inhibits lipid peroxidation of mitochondrial membrane of hepatic cells in vitro. In conclusion, SA elicits a protective effect on hepatic injury by antioxidative action in vivo.

Acknowledgements The study was financially supported by Shandong Engineering Research Center for Nature Drug. We also thank Mr Guiwu Qu for his generous assistance.

REFERENCES