Protective Mechanism of Andrographolide against Carbon Tetrachloride-Induced Acute Liver Injury in Mice

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The aim of this study was to investigate the protective effects of andrographolide (AP), a bioactive component isolated from Andrographis paniculata, on carbon tetrachloride (CCl4)-induced liver injury as well as the possible mechanisms involved in this protection in mice. Acute liver injury was induced by CCl4 intoxication in mice. Serum biological analysis, lipid peroxides and antioxidant estimation, histopathological studies, reverse transcription polymerase chain reaction (RT-PCR) and Western blot assay were carried out. CCl4 treatment resulted in severe hepatic injury, as evidenced by significant elevation of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels and typical histopathological changes, such as hepatocyte necrosis. Additionally, CCl4 administration led to oxidative stress in mice, as indicated by a remarkable increase in the hepatic malondialdehyde (MDA) level, together with a significant decrease in liver reduced glutathione (GSH) content. However, CCl4-induced hepatotoxicity was significantly attenuated by pretreatment with AP, as demonstrated by significant reduction of serum ALT, AST levels and hepatic MDA activity, along with a remarkable increase in hepatic GSH content. Histopathological changes induced by CCl4 were also ameliorated by AP pretreatment. The marked increase of tumor necrosis factor-α (TNF-α) induced by CCl4 was attenuated by AP, and the dramatic elevation of heme oxygenase-1 (HO-1) at transcriptional and protein levels was augmented following AP pretreatment. AP can effectively prevent liver injury induced by CCl4, which may be due to inhibition of oxidative stress and inflammatory responses.

Key words carbon tetrachloride; andrographolide; liver injury; inflammation; heme oxygenase-1

Liver diseases are a serious health problem throughout the world. Many hepatotoxins are known to cause liver injury in humans, such as viruses, fungal products, bacterial metabolites, minerals, environmental pollutants and chemotherapeutic agents.1) Despite new advances in hepatology, there is a lack of effective therapeutic strategies or specific medicines for the treatment of hepatic disorders.2) Therefore, herbal medicines that possess hepatoprotective effects have attracted the attention of many researchers in recent years.3,4)

Carbon tetrachloride (CCl4), a potent hepatotoxin, is widely used as a chemical inducer of experimental liver injury.5) CCl4 metabolism begins with the highly reactive trichloromethyl free radicals (CCl3·) by the action of the liver reduced nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P450 enzyme system.6) These free radicals are believed to cause lipid peroxidation, and the breakdown of cellular membranes.7)

Andrographolide (AP; C20H30O5; molecular weight (Mw) 350.44), a labdane diterpene lactone isolated from the leaves of the Andrographis (A.) paniculata plant,8) has the chemical structure 3-[2-[1-decylhydro-6-hydroxy-5-(hydroxymethyl)-5,8a-dimethyl-2-methylene-1-naphthalenyl]ethylidene]dihydro-4-hydroxy-2(3H)-furanone. Pharmacokinetic studies proved that andrographolide was quickly absorbed after the drug was orally administered to rats and humans.9) Many studies have shown that A. paniculata has a variety of pharmacological activities, including anti-inflammatory,10) anti-diarrhoeal,11) antiviral,12) anti-malarial,13) cardiovascular,14) anticancer and immunostimulatory15) activities. AP has also been reported to possess anti-inflammatory,16) anticancer,17) and hepatocyte-protective18) activities. In addition, AP was effective in preventing CCl4-induced liver damage in rats and mice.19,20) However, the exact molecular mechanism of its hepatoprotective effects has not been reported so far. Therefore, the aims of this study were to evaluate the hepatoprotective effect of AP on CCl4-induced acute hepatic injury, and to evaluate the specific molecular mechanisms of protection in mice.

MATERIALS AND METHODS

Drugs and Chemical Test Agents AP and Silymarin were purchased from Aldrich (Milwaukee, WI, U.S.A.). Assay kits for alanine aminotransferase (ALT), aspartate aminotransferase (AST), malonaldehyde (MDA) and reduced glutathione (GSH) activity were purchased from the Jiancheng Institute of Biotechnology (Nanjing, China). CCl4 was purchased from Changjiang Chemical Co., Ltd. (Shanghai, China). All other chemicals were of analytical grade.

Animals and Treatments Male C57BL/6J mice (25—30 g), purchased from Southern Medical University Laboratory Animal Co., Ltd. (Guangzhou, China), were housed at a controlled temperature of 25—28 °C and a 12 h light/dark cycle with free access to standard diet and water. Experiments on animals were performed according to the animal ethics guidelines of the Institutional Animal Care and Use Committee (IACUC).

The animals were randomly divided into 5 groups of 8 mice each. Group I and II served as normal and CCl4 control, where the animals received only the vehicle (physiological saline) orally. AP and silymarin were suspended in physiological saline, group III to group IV were treated orally with
the suspensions of AP (50, 100 mg/kg), and the animals in group V were oral administered the suspensions of silymarin (positive control, 100 mg/kg) once daily for 8 consecutive days.

Eight hours after the last treatment, group II—V animals were administered intraperitoneally a single dose of CCl\textsubscript{4} (0.5 ml/kg of 1 : 19 mixture in olive oil). The dose and timing of the AP treatment have been reported previously.\textsuperscript{23} Group I received an equal amount of olive oil instead of CCl\textsubscript{4}. All animals were sacrificed 24 h after CCl\textsubscript{4} administration. Blood was collected by puncturing the retro-orbital venous sinus (in heparinized tubes). Serum was separated by centrifugation at 2500 rpm at 4 °C for 15 min, and then stored at −20 °C until analysis. The livers were removed rapidly, and cut into separate portions. One portion of liver was fixed for histopathological examination, another portion was used for hepatic glutathione and lipid peroxidation estimation, while the remaining tissue was stored at −80 °C until it was used for reverse transcription polymerase chain reaction (RT-PCR) and Western blotting. Liver tissues were homogenized on ice in 0.9% saline. The homogenates were then centrifuged at 3000 rpm for 10 min at 4 °C, and the supernatants were taken to assay for hepatic glutathione and lipid peroxidation by spectrophotometry.

**Serum ALT and AST Assay** The enzymatic activities of ALT and AST were determined using a RA1000 Automatic Biochemical Analyzer (Japan).

**Estimation of Lipid Peroxides** Lipid peroxidation in the liver was measured by the formation of malondialdehyde (MDA). The liver MDA content was assayed by spectrophotometrically measuring the thiobarbituric acid-reactive substances (TBARS) levels at 532 nm. Results are expressed as nmol mg\(^{-1}\) protein.

**Estimation of Reduced Glutathione (GSH)** Reduced glutathione (GSH) was estimated by its reaction with dithiobis-2-nitrobenzoic acid (DTNB) that produces a yellow coloured complex with an absorption maximum at 412 nm.\textsuperscript{22}

Results are expressed as mg g\(^{-1}\) protein.

**Histopathological Studies** Small pieces of liver, fixed in 10% buffered formalin, were processed for embedding in paraffin. Sections of 5—6 \(\mu\)m were cut for histopathological evaluation. Liver sections were stained with hematoxylin and eosin using a standard protocol, and then analyzed by light microscopy.

**Total RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)** Total RNA was extracted by Trizol Reagent (Invitrogen, Groningen, the Netherlands). Reverse transcription of the total RNA to cDNA using MMLV Reverse transcriptase Reagent kits (Fermentas, Lithuania) with Oligo(dT) primer transcriptase. The gene-specific primers tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) (forward: 5′-AGCCCACTGGTAGCAAAACCAAA-3′; reverse: 5′-AACACCATTCCCTTACAGAGGAAT-3′), heme oxygenase-1 (HO-1) (forward: 5′-AACAAGCAGAACCAGCT-3′; reverse: 5′-TGCTACTCAGAAGTGTCC-3′) and \(\beta\)-actin (forward: 5′-TGGATACCTGCTGCGCATCATT-3′; reverse: 5′-TAAAAGCCAGCTCAGTAAACAGTCCG-3′) were used to amplify TNF-\(\alpha\) and HO-1 target-gene cDNA. The PCR reaction was performed with an initial denaturation step at 94 °C for 5 min and a final extension at 72 °C for 7 min using a GeneAmp 2700 thermocycler (Applied Biosystems, Foster City, CA, U.S.A.). The PCR amplification cycling conditions were as follows: for TNF-\(\alpha\), 28 cycles of 94 °C (30 s), 65 °C (30 s), and 72 °C (30 s); for HO-1, 30 cycles, and for \(\beta\)-actin, 25 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. PCR products were analyzed via electrophoresis on a 1.5% agarose gel in a Tris-acetate-ethylenediaminetetraacetic acid (EDTA) buffer supplemented with 0.005% ethidium bromide. The intensity of each PCR product was analyzed semiquantitatively using SLB Mylmager (UVP Inc., Upland, CA, U.S.A.) and ImageQuant\textsuperscript{TM} TL (Amersham Biosciences/GE Healthcare).

**Western Blot Assay** Tissue samples were homogenized in complete radio immunoprecipitation assay (RIPA) lysis buffer. A 20-\(\mu\)l sample of protein from the liver homogenates was loaded per lane and electrophoretically separated on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred to nitrocellulose membranes. Western blot analysis was carried out using the polyclonal antibodies against mouse TNF-\(\alpha\) (Santa Cruz, U.S.A.), HO-1 (Santa Cruz, U.S.A.) and the respective secondary antibody. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Monoclonal; Santa Cruz, U.S.A.) was used as internal control. The bands were visualized with an ECL\textsuperscript{TM} detection system (Amersham, NJ, U.S.A.) and expressed as the ratio represented by arbitrary units in the figures.

**Statistical Analysis** Results were expressed as means± standard errors (S.E.M.) and all statistical comparisons were made by means of one-way analysis of variance (ANOVA) test followed by Student–Newman–Keuls post hoc tests using SPSS 13.0 software. Differences were considered significant at \(p<0.05\).

**RESULTS**

**Serum Aminotransferase Activities** Compared with the normal control group, the levels of ALT and AST in Group II were significantly elevated by CCl\textsubscript{4} administration. Pretreatment with 100 mg/kg AP resulted in significant reversal of the elevated serum enzymes induced by CCl\textsubscript{4}. The ALT and AST activities were also decreased in the silymarin-pretreated group in contrast to the vehicle-pretreated CCl\textsubscript{4} control group (Table 1).

**Lipid Peroxidation and Hepatic Glutathione Contents** Similar to the aminotransferase activities, the MDA level was significantly reduced in the CCl\textsubscript{4} control group, which was lowered significantly by pretreatment with AP (100 mg/kg) or silymarin at a dose of 100 mg/kg. The GSH level was significantly reduced in CCl\textsubscript{4} treated mice when compared to the normal control group. The pretreatment of AP (100 mg/kg) or silymarin showed significant increases in GSH levels in liver compared with CCl\textsubscript{4} treated mice (Table 1).

**Histological Analysis** Histologically, in contrast to the control group, CCl\textsubscript{4} administration resulted in extensive hepatic cellular damage, including inflammatory cell inflammation and hepatocyte necrosis. However, compared to the CCl\textsubscript{4} control group, the mice pretreated with AP (100 mg/kg) or silymarin (100 mg/kg) showed obvious improvement indi-
cated by mild to moderate infiltration of lymphocytes and less hepatocyte necrosis in liver morphology (Fig. 1).

**TNF-α and HO-1 mRNA Expression**

In this study, the TNF-α and HO-1 mRNA expression levels were found to be significantly increased in the CCl4 control group. However, CCl4-induced elevation of TNF-α mRNA expression was significantly reduced by pretreatment with AP at a dose of 100 mg/kg. On the other hand, pretreatment with 100 mg/kg AP further augmented the increased level of HO-1 mRNA expression induced by CCl4 (Fig. 2).

**TNF-α and HO-1 Protein Expression**

Similar to the mRNA expression, the levels of TNF-α and HO-1 protein expression were significantly elevated by CCl4 administration. Pretreatment with 100 mg/kg AP resulted in significant reversal of the elevated TNF-α protein expression induced by CCl4. However, the level of HO-1 protein expression was further elevated by the AP pretreatment (Fig. 3).

**DISCUSSION**

It has been reported that the CCl4-induced hepatotoxicity

<table>
<thead>
<tr>
<th>Group</th>
<th>ALT (IU/l)</th>
<th>AST (IU/l)</th>
<th>MDA (nmol/mg protein)</th>
<th>GSH (mg/g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>16.09±1.08</td>
<td>30.27±0.65</td>
<td>1.07±0.01</td>
<td>4.17±0.09</td>
</tr>
<tr>
<td>CCl4 control</td>
<td>121.38±0.72*</td>
<td>61.65±1.11*</td>
<td>2.64±0.10*</td>
<td>2.14±0.08*</td>
</tr>
<tr>
<td>AP (50 mg/kg)+CCl4</td>
<td>117.62±0.82</td>
<td>58.97±0.78</td>
<td>2.43±0.05</td>
<td>2.35±0.07</td>
</tr>
<tr>
<td>AP (100 mg/kg)+CCl4</td>
<td>45.93±0.69*</td>
<td>38.86±0.64*</td>
<td>1.31±0.04*</td>
<td>3.51±0.09*</td>
</tr>
<tr>
<td>Silymarin (100 mg/kg)+CCl4</td>
<td>43.59±0.98*</td>
<td>37.30±0.86*</td>
<td>1.26±0.03*</td>
<td>3.60±0.13*</td>
</tr>
</tbody>
</table>

Data are presented as means±S.E.M. (n=8). Means of each parameter observed between different treatments were compared using one-way analysis of variance (ANOVA).

$^*$p<0.05, compared with the normal control group. $^p$<0.05, compared with the CCl4 control group.

Fig. 1. Histopathological Features of Liver in the Experiments

Liver tissues were stained with H&E. (A) Normal control; (B) CCl4 control; (C) AP (100 mg/kg)+CCl4; (D) silymarin (100 mg/kg)+CCl4. The liver of vehicle-treated mice (A) displayed normal morphology, while necrotic areas and large amounts of inflammatory cell infiltration could be observed in CCl4-treated mice (B). Pretreatment with AP (C) or silymarin (D) significantly reduced the necrotic area, and less inflammatory cell infiltration was observed. CV denotes central vein. Arrows indicated necrotic area and inflammatory cell infiltration. Original magnification, 100×.

Fig. 2. Effect of AP on TNF-α and HO-1 mRNA Levels

The mRNA expression levels of TNF-α and HO-1 were determined by RT-PCR. The levels of mRNA were expressed as a ratio of signal intensity for the target genes relative to that for β-actin, and their values were considered arbitrary units that represented the relative values among all samples. Values are mean±S.E.M., n=8. $^*$p<0.05, compared with the normal control group. $^p$<0.05, compared with the CCl4 control group.

Fig. 3. Effect of AP on TNF-α and HO-1 Protein Expression Levels

The protein expression levels of TNF-α and HO-1 were determined by Western blot, and quantitated by densitometric scanning. The levels of protein were expressed as a ratio of signal intensity for the target proteins relative to that for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and their values were considered arbitrary units that represented the relative values among all samples. Values are mean±S.E.M., n=8. $^*$p<0.05, compared with the normal control group. $^p$<0.05, compared with the CCl4 control group.
model has been widely used for decades for the screening of the antithetapotic/hepatoprotective activity of drugs.\textsuperscript{3,23} Previous reports have showed that AP has a protective effect against CCl\textsubscript{4}-induced hepatotoxicity,\textsuperscript{9,20}\textsuperscript{29} but the choice of the parameters tested (liver enzymes, GSH) was inadequate. This study was undertaken to elucidate the possible mechanism that mediates the hepatoprotective effect of AP on CCl\textsubscript{4}-induced acute liver injury. The increased serum levels of AST and ALT are due to the damage to the structural integrity of the liver, since these enzymes are normally located in the cytoplasm and released into the circulation after cellular injury.\textsuperscript{24} Generally, MDA is used as an index of lipid peroxidation, and lipid peroxidation is postulated as the mechanism of free radical induced tissue injury, hence free radical scavenging is established as the means by which antioxidants inhibit lipid peroxidation. The results showed that AP (100 mg/kg) significantly reduced the serum AST and ALT levels and hepatic MDA content (Table 1). This result is consistent with previous reports that indicated AP showed free radical scavenging properties and protective effects on hepatotoxicity induced in mice by CCl\textsubscript{4}.\textsuperscript{28,29} This phenomenon was also demonstrated by histopathological changes (Fig. 1). Furthermore, the hepatoprotective activity of AP (100 mg/kg) seemed to be similar to that of silymarin (100 mg/kg), which is considered to be a potent hepatoprotective agent.

It is widely believed that the hepatotoxicity of CCl\textsubscript{4} is due to reductive dehalogenation. The trichloromethyl free radicals bind to lips, which results in lipid peroxidation and liver injury.\textsuperscript{27} GSH constitutes the first line of defense against free radicals.\textsuperscript{28} Previous studies on the mechanism of CCl\textsubscript{4}-induced hepatotoxicity have shown that GSH plays a vital role in detoxifying the reactive toxic metabolites of CCl\textsubscript{4}. In particular, depletion of the GSH pool can cause liver necrosis. Moreover, the depletion of mitochondrial GSH is a key determinant for cell death under oxidative stress conditions.\textsuperscript{29,30} Therefore, GSH plays an important role in reducing the toxic effect of CCl\textsubscript{4}. Indeed, our results showed that the CCl\textsubscript{4}-induced depletion of hepatic GSH was significantly inhibited by AP (100 mg/kg) pretreatment (Table 1).

Previous studies reported that hepatic injury originates from the overproduction of trichloromethyl-free radicals, followed by inflammatory processes that are initiated by the activation of Kupffer cells. Activated Kupffer cells are capable of releasing a variety of inflammatory mediators or cytokines, which eventually lead to liver injuries.\textsuperscript{31,32} TNF-\textalpha, a pleiotropic proinflammatory cytokine, is rapidly induced by macrophages when tissue damage occurs.\textsuperscript{33} Overproduction of TNF-\textalpha has been directly related to hepatic necrosis and apoptosis.\textsuperscript{34} In this study, TNF-\textalpha was dramatically induced in CCl\textsubscript{4}-treated mice. However, pretreatment with AP significantly inhibited the hepatic TNF-\textalpha expression at both the transcriptional and protein levels, compared with CCl\textsubscript{4}-treated mice (Figs. 2, 3).

Heme oxygenase-1 (HO-1) is not only a rate-limiting enzyme in the catabolism of heme but is also a heat shock protein (HSP32). HO-1 expression is induced by a wide variety of stimuli including heme, heavy metals, cytokines and chemical carcinogens. Moreover, HO-1 has been considered to be a cytoprotective enzyme and plays an important protective role against oxidative stress.\textsuperscript{35,36} It was reported that CCl\textsubscript{4} administration caused a significant increase in hepatic HO-1 protein expression, which reached a maximum at 24 h.\textsuperscript{37} In our study, the levels of hepatic HO-1 mRNA and protein increased significantly after CCl\textsubscript{4} treatment. Additionally, pretreatment with AP markedly increased the increase in the levels of HO-1 gene and protein expression after CCl\textsubscript{4} injection (Figs. 2, 3). Interestingly, when animals were pretreated with ZnPP (an inhibitor of HO), the histology of AP-pretreated CCl\textsubscript{4}-exposed liver tissue did not markedly differ from that of the CCl\textsubscript{4}-exposed ones (data not shown). These results suggest that the protective mechanism of AP against CCl\textsubscript{4}-induced hepatic injury might be closely connected with overexpression of HO-1. It is well-known that endogenously induced HO-1 can degrade heme into biliverdin, carbon monoxide (CO) and free iron. Biliverdin is subsequently converted to bilirubin by an NAD(P)H-dependent reductase.\textsuperscript{38,39} Recent work has demonstrated that each of these reaction products has important biological effects. For instance, bilirubin acts as a scavenger of reactive oxygen species (ROS) \textit{in vivo} and has been shown to protect cells from oxidative injury.\textsuperscript{40} CO has numerous biological functions, such as anti-inflammatory and anti-apoptotic effects.\textsuperscript{41} It is now widely accepted that CO, an intriguing signaling molecule, affects numerous critical cellular functions and plays a vital role in mediating the cytoprotection against oxidant-induced injury during inflammatory processes.\textsuperscript{42,43} The iron released by HO-1 activity increases the synthesis of ferritin, which acts not only as an effective anti-oxidant but also as a defense against lipid peroxidation.\textsuperscript{41,44} As it is unclear as to whether the HO-1 increase is CCl\textsubscript{4}-dependent or not, more studies will be needed to examine the effect of AP alone on the induction of HO-1.

CONCLUSION

We report here for the first time that AP effectively prevents acute liver injury induced by CCl\textsubscript{4} \textit{via} induction of HO-1 and inhibition of an inflammatory response. Because AP is one of the principal constituents of a famous traditional Chinese herbal medicine, \textit{A. paniculata}, and shows good intestinal absorption, the actions of AP are of significant clinical importance, and may be a potential agent for preventing CCl\textsubscript{4}-induced hepatotoxicity.

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