Protective Effect of Baicalin Against Carbon Tetrachloride–Induced Acute Hepatic Injury in Mice

Sang-Won Park¹, Chan-Ho Lee¹, Yeong Shik Kim², Sam Sik Kang², Su Jin Jeon³, Kun Ho Son³, and Sun-Mee Lee¹,*

¹College of Pharmacy, Sungkyunkwan University, 300 Cheoncheon-dong, Jangan-gu, Suwon-si, Gyeonggi-do 440-746, Korea
²College of Pharmacy, Seoul National University, Seoul 110-460, Korea
³Department of Food Science and Nutrition, Andong National University, Gyeongbuk 760-749, Korea

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Abstract. This study examined the effects of baicalin, a bioactive flavonoid isolated from Scutellariae Radix, on carbon tetrachloride (CCl₄)-induced liver injury. Mice were treated intraperitoneally with 0.5 ml/kg CCl₄ and different groups of animals received 25, 50, 100, and 200 mg/kg baicalin. At 24 h after the CCl₄ treatment, the level of serum aminotransferases and lipid peroxidation was significantly elevated, whereas the hepatic glutathione content was decreased. These changes were attenuated by baicalin. The histological studies showed that baicalin inhibited the portal inflammation, centrilobular necrosis, and Kupffer cell hyperplasia, which are the three most common characteristics of CCl₄-induced liver damage. The serum level and mRNA expression of tumor necrosis factor-α were markedly increased by the CCl₄ treatment but suppressed by baicalin. The mRNA and protein expression levels of inducible nitric oxide synthase and heme oxygenase-1 increased significantly at 24 h after the CCl₄ treatment. Baicalin attenuated the increase in the protein and gene expression of inducible nitric oxide synthase but augmented the increase in those of heme oxygenase-1. These findings suggest that baicalin protects hepatocytes from the oxidative damage caused by CCl₄, and this protection is likely due to the induction of HO-1 expression and the inhibition of the proinflammatory mediators.

Keywords: baicalin, carbon tetrachloride (CCl₄), heme oxygenase-1, oxidative stress, proinflammatory mediator

Introduction

Liver diseases are a major problem throughout the world. Many environmental toxins cause liver injury to humans, and despite new advances in hepatology, the treatment for liver diseases does not resolve the problems caused by these toxins. Furthermore, despite the increasing need for agents to protect the liver from damage, modern medicine lacks a reliable liver protective drug. Therefore, there has been considerable interest in the role of complementary and alternative medicines for the treatment of liver diseases (1).

The radix of Scutellariae baicalensis Georgi is an eastern traditional medicine that is used as an anti-inflammatory agent and smooth muscle relaxant, and used as a component of some hepatoprotective herb mixtures in China, Japan, and Korea (2). Baicalin is the major active constituent of the isolated root of Scutellaria baicalensis Georgi that scavenges reactive oxygen species (ROS) and has an anti-inflammatory activity (3). A recent report suggested that baicalin suppresses the hemin-nitrite-H₂O₂–induced liver injury caused by inhibitory oxidation and nitration in HepG2 cells (4) and inhibits the activation of redox-sensitive nuclear factor-κB (NF-κB) in kidney tissue from old rats (5). However, there is limited information on the in vivo hepatoprotective effects of baicalin.

Carbon tetrachloride (CCl₄) is commonly used as a chemical inducer of experimental liver injury (6). It has been suggested that the hepatic necrosis caused by CCl₄ involves bioactivation by a microsomal cytochrome P450–dependent monooxygenase system, resulting in...
the formation of trichloromethyl free radicals and ROS, which initiate lipid peroxidation and protein oxidation leading to hepatocellular membrane damage (7). This process is followed by the release of inflammatory mediators from the activated hepatic macrophages, which are believed to potentiate the CCl₄-induced hepatic injury.

This study evaluated the role of the inhibition of oxidative stress and inflammation as a possible molecular mechanism for the protective effect of baicalin in a CCl₄-induced liver injury.

Materials and Methods

Chemicals
Unless stated otherwise, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of baicalin from Scutellariae Radix
The dried roots of Scutellaria baicalensis GEORGI (Labiatae) were purchased in Sun-Chon and were authenticated by Dr. J.H. Lee, an Oriental medicine specialist. A voucher specimen was deposited at the Department of Food and Nutrition, Andong University, Korea. The dried roots of Scutellaria baicalensis Georgi (20 kg) were percolated with 70% ethanol (EtOH) three times and concentrated in vacuo, and a residue (6 kg) was suspended in H₂O and partitioned successively with CH₂Cl₂, EtOAc, and butanol (BuOH), to give CH₂Cl₂ (300 g), EtOAc (120 g), and BuOH (2.2 kg) soluble fractions, respectively. A portion of the BuOH fraction (50 g) was subjected to silica gel column chromatography eluted with a stepwise gradient of CH₂Cl₂:MeOH to yield ten fractions (C₁ – C₁₀) based on their polarities. Also fraction C7 was rechromatographed on a silica gel column eluted with CHCl₃:MeOH:H₂O = 7:3:1 – 52:28:8 to give five subfractions (C₇.1 – C₇.5). The C₇.5 fraction was re-crystallized in methanol to obtain pure baicalin, which was subjected to analytical HPLC (3.9 × 300 mm, µBondapak C-18 column) with elution by MeOH : 5% acetic acid [7:3 (v/v), 0.5 ml /min]; the obtained baicalin, with a purity >95%, had a retention time of 5.9 min. The structure of baicalin was verified by comparison of the NMR data with those reported in the literature (Fig. 1) (8).

Animals and experimental treatments
Male ICR mice (25 – 30 g) were given access to water and food throughout the experiments ad libitum. The animals were fasted for 16 h before the CCl₄ treatment. The use and care of animals were carried out according to the Sungkyunkwan University Animal Care Committee guidelines. The mice were randomly divided into 7 groups with 8 animals each. Group 1 was used as the normal control and was given the respective vehicles. Groups 2 – 7 were administered CCl₄ (0.5 ml /kg in olive oil) intraperitoneally. The mice in groups 3 – 7 were treated intraperitoneally with baicalin (25, 50, 100, and 200 mg/kg) and silymarin (positive control for hepatoprotective properties, 200 mg/kg) twice 30 min before and 2 h after the CCl₄ injection, respectively. These baicalin doses were selected because they had been previously evaluated in a lung injury model of rats induced by paraquat poisoning (9). Twenty four hours after injecting the CCl₄, blood was taken from the abdominal aorta and the liver was isolated and stored at −75°C for later analysis.

Determination of serum aminotransferase activities
The serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined by standard spectrophotometric procedures using a ChemiLab ALT and AST assay kit (IVDLab Co., Ltd., Korea), respectively.

Measurements of hepatic lipid peroxidation and glutathione content
The steady-state level of malondialdehyde (MDA), which is the end product of lipid peroxidation, was measured in the liver homogenates by measuring the level of thiobarbituric acid-reactive substances spectrophotometrically at 535 nm, as described by Buege and Aust (10). The hepatic glutathione content was determined in the liver homogenates after precipitation with 1% picric acid using yeast-glutathione reductase, 5,5'-dithio-bis(2-nitrobenzoic acid), and NADPH, at 412 nm (11). The hepatic glutathione values are expressed as mmol/mg protein.

Histological analysis of tissues
The liver slices were made from a part of the left lobe and fixed immediately in a 10% buffered formalin phosphate solution, embedded in paraffin, and sectioned at 5 µm. The serial sections were stained with hematoxylin
and eosin (H&E) to evaluate the portal inflammation, hepatocellular necrosis, and Kupffer cell hyperplasia. The sections were examined in a blind manner under an Olympus CKX 41 microscope (Olympus Optical Co., Ltd., Tokyo) (12).

**Measurement of serum TNF-α**

The tumor necrosis factor-α (TNF-α) concentrations were quantified using a commercial TNF-α ELISA assay kit (eBiosciences Co., San Diego, CA, USA).

**Western blot immunoassay**

Freshly isolated liver tissue was homogenized in a lysis buffer. A 20-µg sample of protein from the liver homogenates was loaded per lane on 7.5% or 10.0% polyacrylamide gels. Electrophoresis was then performed. The proteins were then transferred to nitrocellulose membranes. Western blot analysis was performed using the polyclonal antibodies against mouse inducible nitric oxide synthase (iNOS; Transduction Laboratories, San Jose, CA, USA), cyclooxygenase-2 (COX-2; Cayman, Ann Arbor, MI, USA), heme oxygenase-1 (HO-1; Stressgen Bioreagents Corp., Ann Arbor, MI, USA), and the monoclonal antibody against mouse β-actin. The binding of all the antibodies was detected using an ECL detection system (iNtRON Biotechnology Co., Ltd., Korea), according to the manufacturer’s instructions. The intensity of the immunoreactive bands was determined using a densitometric analysis program (Image Gauge V3.12; Fuji Photo Film Co., Ltd., Tokyo).

**Total RNA extraction and reverse transcription-polymerase chain reaction**

The total RNA was isolated using the method described by Chomczynski and Sacchi (13). Reverse transcription of the total RNA was carried out to synthesize the first strand cDNA using oligo(dT)12-18 primer and SuperScript™ II RNase H+ Reverse Transcriptase (Invitrogen Tech-Line™, Carlsbad, CA, USA). The PCR reaction was performed with a diluted cDNA sample and amplified in each 2-µl reaction volume. The final reaction concentrations are as follows: primers, 10 µM; dNTP mix, 250 mM; 10 × PCR buffer; Ex Taq DNA polymerase, and 0.5 U/reaction. The gene-specific primers are listed in Table 1. All the PCR reactions had 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, USA). The PCR amplification cycling conditions were as follows: 28 cycles of 94°C (30 s), 65°C (30 s), and 72°C (30 s) for TNF-α; 35 cycles of 94°C (30 s), 60°C (30 s), and 72°C (30 s) for iNOS; and 35, 30, and 25 cycles of 94°C (30 s), 56°C (30 s), and 72°C (30 s) for COX-2, HO-1, and β-actin, respectively. After RT-PCR, 10-µl samples of the amplified products were resolved by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. The intensity of each PCR product was evaluated semiquantitatively using a digital camera (DC120; Eastman Kodak, New Haven, CT, USA) and a densitometric scanning analysis program (1D Main; Advanced American Biotechnology, Fullerton, CA, USA).

**Statistics**

All the results are reported as the means ± S.E.M. The overall significance of the data was examined by one-way analysis of variance (ANOVA). The differences between the groups were considered significant at P<0.05 with the appropriate Bonferronic correction made for multiple comparisons.

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**Table 1.** PCR primers used in this study and the amplified product length

<table>
<thead>
<tr>
<th>Gene (accession number)</th>
<th>Primer sequences (5'-3’)</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (M11731)</td>
<td>Sense: AGCCCACGTCGTAAGCAAACCACAAA</td>
<td>446</td>
</tr>
<tr>
<td></td>
<td>Antisense: AACACCATCCTCCCCATCACGAGCAAT</td>
<td></td>
</tr>
<tr>
<td>iNOS (NM_010927)</td>
<td>Sense: AAGCTGCATGTGACATCGACCCGT</td>
<td>598</td>
</tr>
<tr>
<td></td>
<td>Antisense: GCATCTGGTAGCCAGGCGTACCGG</td>
<td></td>
</tr>
<tr>
<td>COX-2 (NM_011198)</td>
<td>Sense: ACTCACCTAGTTTGGAGTCATTC</td>
<td>582</td>
</tr>
<tr>
<td></td>
<td>Antisense: TTTGATTAGTACTGAGGTAAATG</td>
<td></td>
</tr>
<tr>
<td>HO-1 (NM_010442)</td>
<td>Sense: ACAACGAGAACCAGGTCT</td>
<td>374</td>
</tr>
<tr>
<td></td>
<td>Antisense: TGTCATCTCCAGAGTGTTC</td>
<td></td>
</tr>
<tr>
<td>β-Actin (X03672)</td>
<td>Sense: TGGAAATCCTGGGCAATCCATGAAA</td>
<td>348</td>
</tr>
<tr>
<td></td>
<td>Antisense: TAAACGCAGCTCAGTAACGTCGCC</td>
<td></td>
</tr>
</tbody>
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with baicalin at the dose of 100 mg/kg.

**iNOS, COX-2, and HO-1 protein expression**

Similar to the serum TNF-α level, the level of iNOS and COX-2 protein expression was significantly higher in the CCl₄-treated mice than in the controls. The increase in the level of iNOS protein expression was significantly suppressed by the 100 mg/kg baicalin treatment, but baicalin had little effect on the level of COX-2 protein expression. The level of HO-1 protein expression in the CCl₄ group was significantly higher than that in the control group. This increase was augmented by the baicalin (100 mg/kg) treatment (Fig. 4).

**TNF-α, iNOS, COX-2, and HO-1 mRNA expression**

The TNF-α, iNOS, COX-2, and HO-1 mRNA expression was quite low in the control group. However, the level of TNF-α, iNOS, COX-2, and HO-1 mRNA expression increased markedly 24 h after the CCl₄ injection. The increase in the level of TNF-α and iNOS mRNA expression was suppressed by the baicalin treatment at a dose of 100 mg/kg, whereas the level of COX-2 mRNA expression was not affected by baicalin. The level of HO-1 mRNA expression was further elevated by the baicalin treatment (Fig. 5).

**Discussion**

CCl₄-induced hepatic injury is an experimental model widely used in hepatoprotective drug screening. This study shows for the first time that baicalin can prevent the acute hepatic damage induced by CCl₄. CCl₄-induced hepatotoxicity is believed to involve two phases. The initial phase involves the metabolism of CCl₄ by cytochrome P450, which leads to the formation of free radicals and lipid peroxidation (14). The second step involves the activation of Kupffer cells, probably by free radicals and lipid peroxidation.
The activation of Kupffer cells is accompanied by the production of proinflammatory mediators (15). As a result of the hepatic injury, the altered permeability of the membrane causes the enzymes from the cells to be released into the bloodstream.

Fig. 2. Effect of baicalin on the histological changes in the liver of the CCl₄-treated mice (original magnification ×100). A: Control group: normal lobular architecture and cell structure; B: vehicle-treated CCl₄ group: extensive hepatocellular damage with the presence of portal inflammation, centrizonal necrosis, and Kupffer cell hyperplasia; C: CCl₄ and baicalin (100 mg/kg)-treated group: mild portal inflammation and minimal hepatocellular necrosis and Kupffer cell hyperplasia. D: CCl₄ and silymarin (200 mg/kg)-treated group: mild portal inflammation and minimal hepatocellular necrosis and Kupffer cell hyperplasia.

Fig. 3. Effect of baicalin (100 mg/kg) on the serum TNF-α level in the liver of the CCl₄-treated mice. The values are reported as the means ± S.E.M. of 8 mice per group. **Significantly different (P<0.01) from the control group. †Significantly different (P<0.01) from the CCl₄ group.

Fig. 4. Effect of baicalin (100 mg/kg) on the iNOS, COX-2, and HO-1 protein expression in the liver of the CCl₄-treated mice. The values are reported as the means ± S.E.M. of 8 mice per group. **Significantly different (P<0.01) from the control group. †Significantly different (P<0.01) from the CCl₄ group.
Significantly different \((P < 0.01)\) from the control group.

**Significantly different \((P < 0.01)\) from the control group. **

**Significantly different \((P < 0.01)\) from the CCl\(_4\) group.

**Fig. 5.** Effect of baicalin (100 mg/kg) on the TNF-\(\alpha\), iNOS, COX-2, and HO-1 mRNA expression in the liver of the CCl\(_4\)-treated mice. The values are reported as the means ± S.E.M. of 8 mice per group. **Significantly different \((P < 0.01)\) from the control group. **

Significantly different \((P < 0.01)\) from the control group.

released into circulation (16), which damages the hepatic cells, as shown by the abnormally high level of serum hepatospecific enzymes. In this study, the serum ALT and AST levels increased markedly 24 h after the CCl\(_4\) injection, but these increases were attenuated by treatment with baicalin at 50 and 100 mg/kg. These results indicate that baicalin preserves the structural integrity of the hepatocellular membrane and protects the mice against CCl\(_4\)-induced hepatotoxicity. This phenomenon was also supported by histological examinations. CCl\(_4\) caused a variety of histological changes to the liver, including centrilobular necrosis, portal inflammation, and Kupffer cell hyperplasia. These changes were significantly attenuated by baicalin. Furthermore, the hepatoprotective effect of baicalin seemed to be the same as that of silymarin, which has been used as a potent hepatoprotective agent. The present results suggest that baicalin may have potential clinical application for treating liver diseases.

It is generally accepted that the hepatotoxicity of CCl\(_4\) is the result of reductive dehalogenation. The trichloroethyl methyl free radical is capable of binding to lipids, which initiates lipid peroxidation and liver damage (17). GSH constitutes the first line of defense against free radicals and is a critical determinant of the tissue susceptibility to oxidative damage. Previous studies on the mechanism of CCl\(_4\)-induced hepatotoxicity reported that GSH plays a key role in detoxifying the reactive toxic metabolites of CCl\(_4\) and that liver necrosis begins when the GSH stores are depleted (18). In this study, baicalin exhibited protective effects by impairing the CCl\(_4\)-mediated oxidative stress through the decreased production of free radical derivatives, as evidenced by the decreased MDA level. Furthermore, baicalin attenuated the hepatic glutathione depletion 24 h after the CCl\(_4\) injection. The increase in the hepatic glutathione level in the baicalintreated mice could be due either to its effect on the \textit{de novo} synthesis of glutathione, its regeneration, or both. As a consequence, the hepatic glutathione level could be maintained at levels sufficient to counteract the increased formation of free radicals, as in the case of CCl\(_4\) toxicity. These results suggest that the antioxidant properties may be one mechanism through which baicalin protects against the liver damage mediated by CCl\(_4\). Baicalin at 100 mg/kg was selected as the optimal effective dose for evaluating the molecular mechanisms of baicalin against CCl\(_4\)-induced hepatotoxicity.

Kupffer cells release a number of inflammatory mediators with cytotoxic potential. The two mediators of particular interest are TNF-\(\alpha\) and nitric oxide (NO). TNF-\(\alpha\) is a pleiotropic proinflammatory cytokine that is produced rapidly by macrophages in response to tissue injury. TNF-\(\alpha\) stimulates the release of cytokine from macrophages and induces a phagocyte oxidative metabolism and NO production (19). NO is a highly reactive oxidant that is produced by parenchymal and nonparenchymal liver cells from L-arginine through the action of NOS. NO participates in a variety of physiologic processes, including vasodilation, neurotransmission, and nonspecific host defense. When released by macrophages against infectious agents, NO can also react with the superoxide anion to form a potent and versatile oxidant, peroxynitrite, which stimulates the production of TNF-\(\alpha\) in Kupffer cells through the activation of the oxidant-sensitive transcription factor NF-\(\kappa\)B (20). The notion that NO is involved in acute liver injury is based on several observations that toxin-induced hepatic damage is associated with increased NO production by the liver (21). However, it remains to be determined whether the augmented production of NO has a protective or deleterious role in the liver. In this study, the serum level of TNF-\(\alpha\) and iNOS protein expression increased after CCl\(_4\) administration with a concomitant increase in their gene expression, and this increase was prevented by baicalin. These results suggest that baicalin largely regulates the CCl\(_4\)-induced production of TNF-\(\alpha\) and iNOS at the transcriptional level.

Previous studies reported that the induction of COX
in the inflammatory response is the secondary effect of CCl\textsubscript{4}-induced hepatotoxicity (22). COX-2 is the mitogen-inducible isoform of COX, and it is induced in macrophages by several proinflammatory stimuli, such as cytokines and growth factors, leading to COX-2 expression and the subsequent release of prostaglandins (23). In this study, the levels of COX-2 protein and gene expression increased significantly after the CCl\textsubscript{4} treatment. However, the baicalin treatment did not affect this increase. Kim et al. (24) also reported that baicalin inhibited iNOS gene expression and NO production but does not affect the production of prostaglandin E\textsubscript{2} and the expression of the COX-2 gene in lipopolysaccharide-treated Raw 264.7 macrophages.

HO-1 is not only a key enzyme in the heme catabolism but is also a heat shock protein (HSP 32) in rats. HO-1 is induced by its substrate heme, as well as by various oxidative stresses, and is believed to play an important protective role against oxidative injury (25). Wen et al. (26) reported that the administration of CCl\textsubscript{4} resulted in a time-dependent increase in hepatic HO-1 activity, which reached a maximum at 24 h. This increase was accompanied by the rapid and significant induction of HO-1 protein expression, which occurred in the same time-dependent manner following CCl\textsubscript{4} administration. In this study, the levels of HO-1 protein and gene expression also increased markedly 24 h after the CCl\textsubscript{4} injection. Interestingly, the baicalin treatment augmented the increase in the levels of HO-1 protein and gene expression. When animals were pretreated zinc-protoporphyrin IX for inhibition of HO-1 expression, the histology of baicalin-treated CCl\textsubscript{4}-exposed liver tissue did not markedly differ from that of the CCl\textsubscript{4}-exposed ones (data not shown). This finding suggests that the protective mechanism of baicalin against CCl\textsubscript{4}-induced hepatic injury might be closely associated with overexpression of HO-1. HO-1 oxidatively cleaves heme yielding CO, iron, and biliverdin IX\textalpha, which is then reduced to bilirubin IX\textalpha by biliverdin reductase (27). Recently, it was reported that CO has anti-inflammatory and antipapoptotic properties (28). CO, a newly identified signaling molecule, has long been believed to participate in many biological events and play a key role in mediating the cytoprotection against oxidant-induced injury (29). The cellular functions of overproduced CO in the liver cells are unclear but the CO generated through the HO-1 pathway during CCl\textsubscript{4}-induced tissue injury might be important in the antioxidant defense as well as in the anti-inflammatory response (30). More studies will be needed to examine this effect in detail.

In conclusion, baicalin protects the liver from CCl\textsubscript{4}-induced oxidative stress, which might be due to the induction of HO-1 and the inhibition of the inflammatory response. Because baicalin can be consumed over long periods of time without any known side effects, its possible role as a promising therapeutic in human oxidative stress and inflammatory liver disease deserves consideration. The potential for using baicalin in experimental and practical applications should be examined further.

Acknowledgment

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