

Full Paper

Hepatoprotective Effect of Pinoresinol on Carbon Tetrachloride–Induced Hepatic Damage in Mice

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Abstract. *Forsythiae Fructus* is known to have diuretic, anti-bacterial, and anti-inflammatory activities. This study examined the hepatoprotective effects of pinoresinol, a lignan isolated from *Forsythiae Fructus*, against carbon tetrachloride (CCl₄)–induced liver injury. Mice were treated intraperitoneally with vehicle or pinoresinol (25, 50, 100, and 200 mg/kg) 30 min before and 2 h after CCl₄ (20 μl/kg) injection. In the vehicle-treated CCl₄ group, serum aminotransferase activities were significantly increased 24 h after CCl₄ injection, and these increases were attenuated by pinoresinol at all doses. Hepatic glutathione contents were significantly decreased and lipid peroxidation was increased after CCl₄ treatment. These changes were attenuated by 50 and 100 mg/kg of pinoresinol. The levels of protein and mRNA expression of inflammatory mediators, including tumor necrosis factor-α, inducible nitric oxide synthase, and cyclooxygenase-2, were significantly increased after CCl₄ injection; and these increases were attenuated by pinoresinol. Nuclear translocation of nuclear factor-κB (NF-κB) and phosphorylation of c-Jun, one of the components of activating protein 1 (AP-1), were inhibited by pinoresinol. Our results suggest that pinoresinol ameliorates CCl₄-induced acute liver injury, and this protection is likely due to anti-oxidative activity and down-regulation of inflammatory mediators through inhibition of NF-κB and AP-1.

Keywords: carbon tetrachloride, hepatoprotective activity, inflammation, oxidative stress, pinoresinol

Introduction

Liver injury can be caused by viral infection, auto-immune disorders, ischemia, and several xenobiotics, including drugs, alcohol, or toxins (1). Carbon tetrachloride (CCl₄)–induced hepatic injury is an experimental model that is widely used in hepatoprotective drug screening. CCl₄ injury results from direct damage to tissue after injection and metabolism. CCl₄ is converted to trichloromethyl radical (CCl₃·) by cytochrome P450 (CYP), especially CYP 2E1 (2). CCl₃· can react with oxygen to form another reactive oxygen species (ROS), trichloromethylperoxy radical (CCl₃OO·), which triggers

lipid peroxidation (3). Parenchymal and nonparenchymal cells, especially activated Kupffer cells, mediate the hepatic inflammation process by producing tumor necrosis factor-α (TNF-α) and other cytotoxic cytokines (4). A previous study showed that the production of these inflammatory factors is associated with the nuclear factor-κB (NF-κB) pathway and increased activating protein 1 (AP-1) expression in liver after CCl₄ treatment (5).

Forsythiae Fructus has traditionally been used to treat dysuria, edema, or urinary tract infection (6). The extract of *Forsythiae Fructus* inhibits the allergic reaction, secretion of regulated on activation, normally T cell expressed and secreted (RANTES) in virus-infected human bronchial epithelial cells and 5-lipoxygenase (7–9). A previous study reported that *Forsythiae Fructus* protects liver from oxidative damage by enhancing the anti-oxidative defense system (10). Pinoresinol (Fig. 1),

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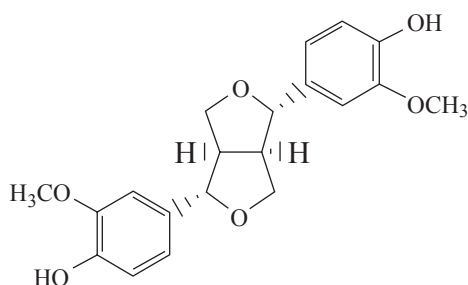


Fig. 1. The structure of pinoresinol ($C_{20}H_{22}O_6$, molecular weight: 358).

one of the lignan constituents from *Forsythiae Fructus*, exerts anti-oxidative and anti-inflammatory activities by inhibition of TNF- α production (11, 12). Pinoresinol also inhibits tumor growth by up-regulation of the ATM-p53 cascade (13).

Therefore, this study was designed to investigate the protective effects of pinoresinol against CCl_4 -induced acute hepatic injury, particularly in the oxidative stress and inflammatory pathways.

Materials and Methods

Isolation and purification of pinoresinol

The dried *Forsythiae Fructus* (10 kg) was extracted three times in MeOH at room temperature for five days each. After filtration, the MeOH extraction was concentrated under reduced pressure to give a residue, 750 g. It was suspended in water and partitioned between EtOAc and H_2O . A portion of the EtOAc fraction (201 g) was subjected to silica gel column chromatography, eluting with a gradient of *n*-hexane and acetone (100:0 to 0:100) to afford 26 fractions. Among them, fraction 18 was subjected to silica gel column chromatography, using gradient of methylene chloride and acetone (100:0 to 50:50) to obtain pinoresinol, subfraction 18 – 5 (200 mg) (14).

Animals and treatment regimens

Male ICR mice weighing 25 – 30 g (Daehan Biolink Co., Eum-seong, Korea) were fasted overnight but given tap water ad libitum. All animals were treated humanely under the Sungkyunkwan University Animal Care Committee Guidelines. The animals were randomly assigned to 7 groups of 8 animals per group. The mice in group 1 (control) received only olive oil (10 ml/kg, i.p.). In groups 2 to 7, CCl_4 was dissolved in olive oil (1:499, v/v) and administered intraperitoneally (final concentration of 20 μ l/kg). Groups 1 and 2 (vehicle) were treated intraperitoneally with Tween-80 in saline (1:9, v/v). The animals in groups 3 to 6 were treated intraperitoneally

with pinoresinol (25, 50, 100, 200 mg/kg), and group 7 was treated with silymarin (positive control, 800 mg/kg, i.p.), 30 min before and 2 h after administering CCl_4 . The timing of the pinoresinol treatment was selected based on the previous report (15). Blood was collected 24 h after CCl_4 administration. Each liver was isolated and stored at $-75^\circ C$ for analysis, except for the left lobe, which was used for histological studies.

Serum aminotransferase activities

The serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined using ChemiLab ALT and AST assay kits (IVDLab Co., Ltd., Uiwang, Korea), respectively.

Lipid peroxidation and hepatic glutathione contents

The steady-state level of malondialdehyde (MDA), a lipid peroxidation end product, was analyzed by measuring the level of thiobarbituric acid reactive substances (TBARS) spectrophotometrically at 535 nm using 1,1,3,3-tetraethoxypropane (Sigma, St. Louis, MO, USA) as the standard (16). The level of total glutathione was measured spectrophotometrically at 412 nm, with yeast glutathione reductase, 5,5'-dithio-bis(2-nitrobenzoic acid) and NADPH (17). The oxidized glutathione (GSSG) level was measured using the same method in the presence of 2-vinylpyridine (18); the reduced glutathione (GSH) level was calculated as the difference between the levels of total glutathione and GSSG, and the ratio of GSH to GSSH was determined.

Histological analysis

Twenty-four hours after CCl_4 injection, the anterior portion of the left lateral lobe of the liver was sectioned and used for histological analysis. The tissue was fixed by immersion in 10% neutral-buffered formalin. The sample was then embedded in paraffin, sliced into 5- μ m sections and stained with hematoxylin-eosin, followed by blinded histological assessment. The degree of portal inflammation, hepatocellular necrosis, and inflammatory cell infiltration was evaluated (19). The histological changes were evaluated in non-consecutive, randomly chosen $\times 200$ histological fields.

Preparation of protein extracts

Freshly isolated liver tissue was homogenized in lysis buffer for preparation of whole protein extracts. NE-PER[®] (Pierce Biotechnology, Rockford, IL, USA) was used to extract nuclear proteins and cytosolic proteins according to the manufacturer's instructions. Protein concentrations were determined using the BCA Protein Assay kit (Pierce Biotechnology).

Table 1. PCR primers used in this study and the amplified products

Gene	Primer sequences (5'-3')	Product length (bp)
<i>TNF-α</i>	Sense: AGCCCACGTCGTAGCAAACCACCAA	446
	Antisense: AACACCCATTCCCTTCACAGAGCAAT	
<i>iNOS</i>	Sense: AAGCTGCATGTGACATCGACCCGT	598
	Antisense: GCATCTGGTAGCCAGCGTACCGG	
<i>COX-2</i>	Sense: ACTCACTCAGTTTGTGAGTCATTC	582
	Antisense: TTTGATTAGTACTGTAGGGTTAATG	
β -Actin	Sense: TGGAATCCTGTGGCATCCATGAAA	348
	Antisense: TAAAACGCAGCTCAGTAACAGTCCG	

Immunoblots

Protein samples were loaded on 10% – 15% polyacrylamide gels. The protein samples were then separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) using the Semi-Dry Trans-Blot Cell (Bio-Rad Laboratories, Hercules, CA, USA). After transfer, the membranes were washed with 0.1% Tween-20 in 1 × Tris-buffered saline (TBS/T) and blocked for 1 h at room temperature with 5% (w/v) skim milk powder in TBS/T. The blots were then incubated overnight at 4°C with primary antibodies. The next day, the blots were incubated in appropriate secondary antibodies and detected using an ECL detection system (iNtRON Biotechnology Co., Ltd., Seongnam, Korea), according to the manufacturer's instructions. The visualized immunoreactive bands were evaluated densitometrically with ImageQuant™ TL software (Amersham Biosciences/GE Healthcare, Piscataway, NJ, USA). Primary antibodies against TNF- α (Cell Signaling Technology, Beverly, MA, USA; 1:1,000 dilution), inducible nitric oxide synthase (iNOS) (Transduction Laboratories, San Jose, CA, USA; 1:1,000 dilution), cyclooxygenase-2 (COX-2) (Cayman, Ann Arbor, MI, USA; 1:1,000 dilution), I κ B- α (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:500), NF- κ B/p65 (Santa Cruz Biotechnology, 1:1,000), and c-Jun p39 phosphorylated on serine-63 (Santa Cruz Biotechnology, 1:500) were used; and the signals were normalized to that of β -actin (Sigma, 1:2,500 dilution) or lamin B1 (Abcam, Cambridge, UK; 1:2,500).

Total RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted (20) and the first strand cDNA was synthesized by reverse transcription of the total RNA using the oligo(dT)_{12–18} primer and SuperScript™ II RNase H⁻ Reverse Transcriptase (Invitrogen Tech-Line™, Carlsbad, CA, USA). The PCR reaction

was carried out in a 20- μ l reaction volume with a diluted cDNA sample. The final reaction concentrations were as follows: sense and antisense primers, 10 pmol; dNTP mix, 250 μ M; ×10 PCR buffer; and Ex Taq DNA polymerase, 0.5 U/reaction. The gene-specific primers are listed in Table 1. PCR was carried out with an initial denaturation step at 94°C for 5 min and a final extension step at 72°C for 7 min in the GeneAmp 2700 thermocycler (Applied Biosystems, Foster City, CA, USA). The amplification cycling conditions are as follows: for *TNF- α* , 28 cycles at 94°C for 30 s, 65°C for 30 s, and 72°C for 60 s; for *iNOS*, 35 cycles at 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s; for *COX-2*, 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and for β -actin, 25 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s. After PCR, 10- μ l samples of the PCR products were electrophoresed through 1.5% agarose gel, stained with ethidium bromide, and visualized by ultraviolet illumination. The intensity of each PCR product was analyzed semiquantitatively using SLB Mylmager (UVP Inc., Upland, CA, USA) and ImageQuant™ TL (Amersham Biosciences/GE Healthcare).

Statistical analyses

The overall significance of the results was examined using one-way analysis of variance (ANOVA). The differences between the groups were considered statistically significant at $P < 0.05$ with the appropriate Bonferroni correction made for multiple comparisons. The results are presented as the mean \pm S.E.M.

Results

Serum aminotransferase activities

In the control group, serum levels of ALT and AST were 34.5 ± 5.9 and 38.9 ± 6.1 U/L, respectively. Serum ALT and AST activities significantly increased 24 h after CCl₄ injection. The increase in the serum ALT level was attenuated by 50, 100, and 200 mg/kg of pinoresinol,

Table 2. Effects of pinoselinol on serum aminotransferase activities, lipid peroxidation, and hepatic glutathione contents

Group	Dose (mg/kg)	ALT (U/L)	AST (U/L)	MDA (nmol/mg protein)	GSH (μ mol/g liver)	GSH/GSSG ratio	
Control		35 \pm 6	39 \pm 6	0.35 \pm 0.04	6.8 \pm 0.4	4.6 \pm 0.5	
CCl ₄	Vehicle	9180 \pm 448**	6460 \pm 413**	0.72 \pm 0.04**	3.6 \pm 0.3**	1.3 \pm 0.2**	
	Pinoselinol	25	8284 \pm 632**	3179 \pm 304**,+	0.64 \pm 0.08	4.9 \pm 0.4*	2.2 \pm 0.1**
		50	5691 \pm 312**,+	3374 \pm 259**,+	0.51 \pm 0.05 ⁺	5.1 \pm 0.3	2.7 \pm 0.3**,+
		100	5476 \pm 176**,+	3250 \pm 282**,+	0.40 \pm 0.05 ⁺⁺	6.2 \pm 0.7 ⁺⁺	2.7 \pm 0.3**,+
		200	6742 \pm 702**,+	2808 \pm 300**,+	0.57 \pm 0.08	5.9 \pm 0.4 ⁺⁺	2.0 \pm 0.3**
Silymarin	800	4066 \pm 687**,+	1853 \pm 387**,+	0.42 \pm 0.04 ⁺⁺	5.6 \pm 0.4 ⁺	1.8 \pm 0.2**	

Mice were treated intraperitoneally with vehicle, pinoselinol (25, 50, 100, and 200 mg/kg) or silymarin (800 mg/kg) 30 min before and 2 h after CCl₄ injection. Serum aminotransferase activities, MDA, and hepatic glutathione contents were measured in liver tissue 24 h after CCl₄ injection. The results are presented as the mean \pm S.E.M. * P < 0.05, ** P < 0.01: significant differences vs. the control group; ⁺ P < 0.05, ⁺⁺ P < 0.01: significant differences vs. the vehicle-treated CCl₄ group.

and the increase in the serum AST level was attenuated by all doses of pinoselinol (Table 2). Pinoselinol alone did not affect serum aminotransferase activities (data not shown).

Lipid peroxidation and hepatic glutathione contents

As shown in Table 2, the level of MDA increased significantly by 2.1-fold compared with that of the control group after CCl₄ injection. In mice treated with 50 and 100 mg/kg of pinoselinol, MDA levels decreased significantly to 70.5% and 55.2% of the vehicle-treated CCl₄ group, respectively. The hepatic GSH concentration decreased significantly after CCl₄ treatment and this decrease was attenuated by pinoselinol (100 and 200 mg/kg) and silymarin. The ratio of GSH to GSSG decreased by 28.3% compared with the control group after CCl₄ injection, and pinoselinol significantly restored this decrease at doses of 50 and 100 mg/kg. Pinoselinol alone did not affect lipid peroxidation and hepatic glutathione contents (data not shown).

Histological analysis

The histological features shown in Fig. 2 demonstrate the normal liver lobular architecture and cell structure in the control group. The liver exposed to CCl₄ showed multiple and extensive areas of portal inflammation and hepatocellular necrosis, randomly distributed throughout the parenchyma, as well as moderate increase in inflammatory cell infiltration. These pathological changes were attenuated by 100 mg/kg of pinoselinol.

TNF- α , iNOS, and COX-2 protein and mRNA expression

The levels of TNF- α , iNOS, and COX-2 protein expression significantly increased 24 h after CCl₄ injection. These increases were significantly attenuated by

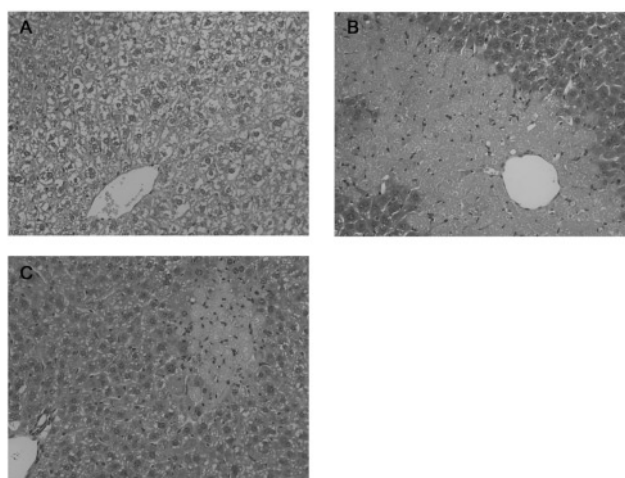


Fig. 2. Liver sections stained with hematoxylin and eosin. Mice were treated intraperitoneally with vehicle or pinoselinol (100 mg/kg) 30 min before and 2 h after CCl₄ injection. A: The control group, showing normal hepatic architecture; B: the vehicle-treated CCl₄ group, showing hepatocellular degeneration and necrosis with inflammatory infiltration; C: pinoselinol (100 mg/kg) + CCl₄ group, showing mild hepatocellular necrosis and inflammatory infiltration. Original magnification \times 200.

pinoselinol (Fig. 3). After CCl₄ treatment, the level of TNF- α mRNA expression increased by 3.6-fold over that of the control group. The levels of iNOS and COX-2 mRNA expression also significantly increased 3.3-fold and 12.0-fold over the control group, respectively. The increases in TNF- α , iNOS, and COX-2 mRNA expression were significantly attenuated by 100 mg/kg of pinoselinol (Fig. 4).

Nuclear translocation of NF- κ B, degradation of cytosolic I κ B- α and activation of nuclear AP-1

The nuclear level of p65, a subunit of NF- κ B, was significantly increased 24 h after CCl₄ injection and this

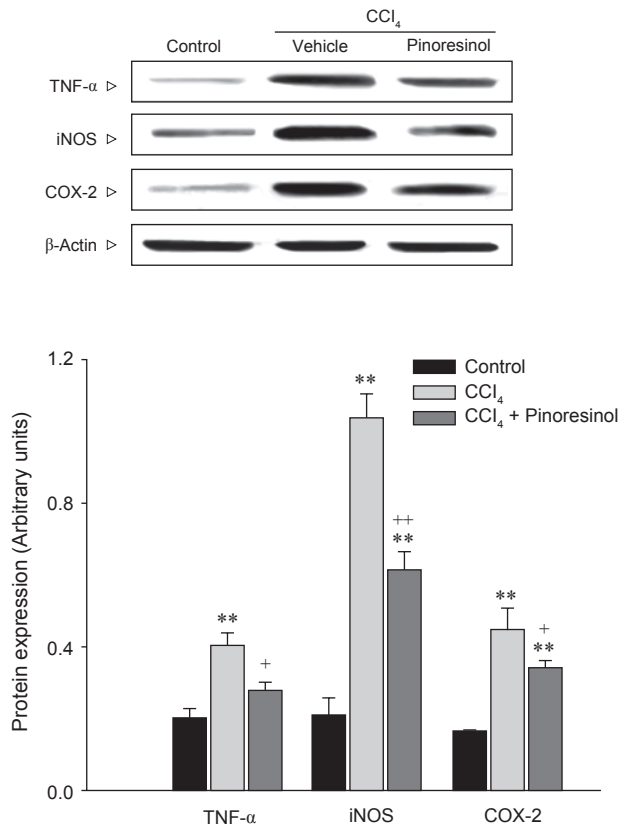


Fig. 3. Effects of pinoresinol (100 mg/kg) on protein expression of inflammatory mediators. Whole hepatic proteins were extracted from mice 24 h after CCl₄ injection. TNF-α, iNOS, and COX-2 were detected by Western blotting as described in Materials and Methods, and β-actin was used as a loading control. The results are presented as the mean ± S.E.M. ***P* < 0.01: denotes significant differences vs. the control group; +*P* < 0.05, ++*P* < 0.01: denote significant differences vs. the vehicle-treated CCl₄ group.

increase was attenuated by pinoresinol. Consistent with this, the cytosolic level of IκB-α decreased after CCl₄ and pinoresinol significantly attenuated this decrease. The level of phosphorylated c-Jun (p-c-Jun) in the nucleus was markedly increased compared with that of the control group. The increase in p-c-Jun was attenuated by pinoresinol (Fig. 5).

Discussion

CCl₄ causes acute hepatotoxicity with necrotic and apoptotic hepatocellular injury and impairment of liver function (21). Recently, we screened the 70% ethanol extract of *Forsythiae Fructus* and its active components for hepatoprotective agents. Among them, lariciresinol and pinoresinol inhibited CCl₄- and galactosamine-induced hepatotoxicity in primary hepatocyte cultures (data not shown). In the present study, we investigated

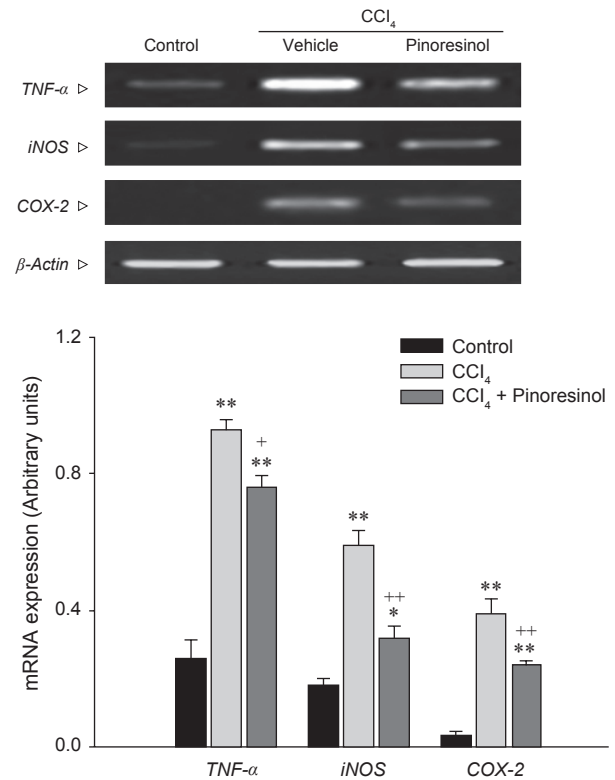


Fig. 4. Effects of pinoresinol (100 mg/kg) on mRNA expressions of inflammatory mediators. The levels of TNF-α, iNOS, and COX-2 mRNA expression were measured by using RT-PCR as described in Materials and Methods. β-Actin was used as a loading control. The results are presented as the mean ± S.E.M. **P* < 0.05, ***P* < 0.01: denote significant differences vs. the control group; +*P* < 0.05, ++*P* < 0.01: denote significant differences vs. the vehicle-treated CCl₄ group.

the hepatoprotective effects of pinoresinol against CCl₄-induced liver injury in vivo.

In the vehicle-treated CCl₄ group, the ALT and AST activities were dramatically increased compared with the control group, indicating severe hepatocellular damage. In contrast, pinoresinol markedly decreased the release of ALT and AST. These results were also strongly supported by the histological observations. The hematoxylin-eosin stained liver sections showed significantly fewer histological changes in the pinoresinol-treated CCl₄ group than the vehicle-treated CCl₄ group. These results suggest that pinoresinol may be clinically applied to treat liver diseases.

The mechanism of CCl₄ injury involves oxidative damage by metabolism of CCl₄ to CCl₃· in hepatocytes; this causes cell death with accumulation of lipid peroxidation and intracellular calcium ions and triggers secondary damage from the inflammatory process (22). In our study, a significant increase in lipid peroxidation was observed after CCl₄ injection and treatment with

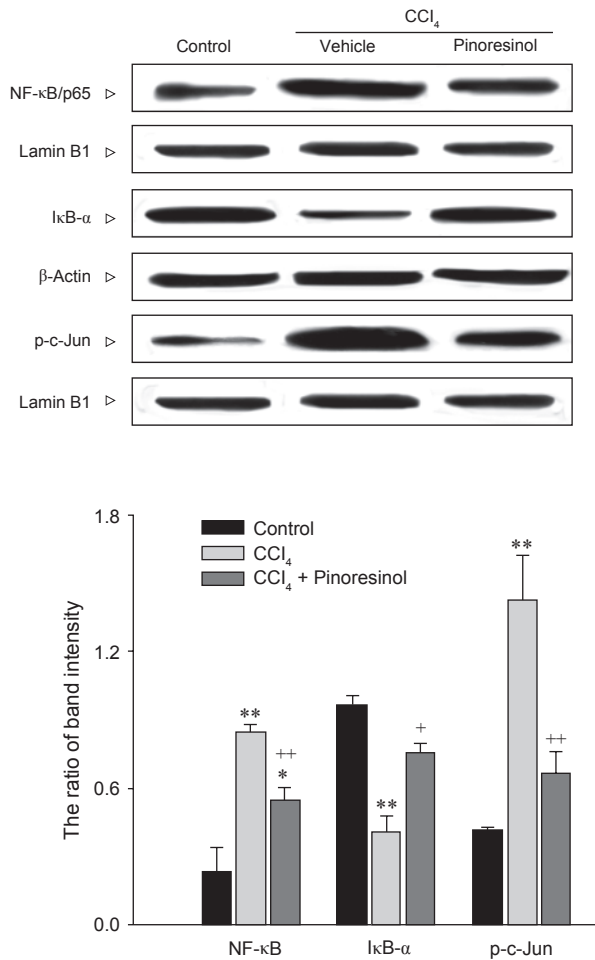


Fig. 5. Effects of pinoresinol (100 mg/kg) on transcription factors. NF- κ B/p65 and phosphorylated c-Jun (p-c-Jun) were detected in nuclear extract and I κ B- α was detected in cytosolic extract. Signals were quantified using lamin B1 and β -actin as loading controls. The ratio of band intensity corresponded to NF- κ B / lamin B1, I κ B- α / β -actin, and p-c-Jun / lamin B1. The results are presented as the mean \pm S.E.M. * P < 0.05, ** P < 0.01: denote significant differences vs. the control group; + P < 0.05, ++ P < 0.01: denote significant differences vs. the vehicle-treated CCl₄ group.

pinoresinol attenuated this increase. GSH is the most abundant redox system and the GSH/GSSG ratio represents the cellular ability to prevent oxidative damage caused by most hepatotoxins. Anti-oxidative defense systems such as uncoupling protein have been reported to prevent CCl₄ damage and GSH has beneficial effects through its restoration of the Ca²⁺-pump disorder caused by CCl₄ (23). Accordingly, we demonstrated that hepatic GSH content and the GSH/GSSG ratio decreased after CCl₄ injection and these decreases were attenuated by pinoresinol. Our findings indicate that pinoresinol may protect against CCl₄-induced oxidative damage by building up anti-oxidant systems.

CCl₄ produces ROS that not only directly damage

tissues, but also initiate inflammation. When tissue is damaged, TNF- α is released and binds to its receptors. TNF- α relates to necrosis and apoptosis in the liver; and release of TNF- α from activated Kupffer cells not only up-regulates adhesion molecule and iNOS, but also stimulates other macrophages to release cytokines (24). Nitric oxide (NO) is involved in various processes including vasodilation and neurotransmission; however, it can react with ROS to form cytotoxic oxidants such as peroxynitrite (25). Moreover, overproduction of NO by iNOS may mediate CCl₄-induced acute hepatotoxicity through up-regulation of inflammatory responses (26, 27). However, NO produced by iNOS after CCl₄ treatment is reported to reduce hepatic injury by inhibition of TNF- α (28). It remains to be determined whether the augmented production of NO has a protective or deleterious role in the liver. In the present study, the levels of TNF- α and iNOS protein and mRNA expression increased after CCl₄ injection, and pinoresinol decreased both TNF- α and iNOS protein and mRNA expression. COX is another key molecule in the inflammatory pathway and is induced by several stimuli such as cytokines and mitogens. COX-2 expression results in the release of prostaglandin at the site of inflammation. Recently, it has been verified that COX-2 mediates nitrooxidative stress (29). In the present study, the levels of COX-2 protein and mRNA expression increased after CCl₄ injection and pinoresinol markedly attenuated these increases. Taken together, our results suggest that pinoresinol largely regulates production of TNF- α , iNOS, and COX-2 at the transcriptional level.

The two transcription factors, NF- κ B and AP-1, are sensitive to redox status in abnormal physiological conditions such as CCl₄-induced acute liver injury (30). NF- κ B is an early response transcription factor, and nuclear translocation of NF- κ B leads to gene expression of pro-inflammatory cytokines, chemokines, adhesion molecules, matrix metalloproteinases, COX-2, and iNOS (31). An oxidative stress in liver activates NF- κ B, triggering expression of oxidative stress responsive genes (32). AP-1 is a dimer consisting of JUN, FOS, ATF (activating transcription factor), and MAF (musculoaponeurotic fibrosarcoma); and the combination in the AP-1 complex affects its DNA binding activity (33). One of the main mechanisms of AP-1 activation involves mitogen-activated protein kinases (MAPKs) of the extracellular-signal-regulated kinase (ERK), p38, and JUN amino-terminal kinase (JNK) families. MAPKs increase the number of AP-1 and phosphorylate the AP-1 complex, thus increasing the AP-1 activity. Two potent domains of AP-1, c-Jun and c-Fos, are reported to have oncogenic effects, and c-Jun is reported to play a major role in the liver (34). A previous study showed that

oxidative damage induced by depletion of GSH activates JNK, which causes phosphorylation of the N-terminal transcription activation domain in c-Jun at Ser63 and Ser73 (35). In this study, pinoresinol attenuated increase in nuclear accumulation of NF- κ B/p65 and decrease in cytosolic I κ B- α . In addition, pinoresinol significantly decreased the nuclear level of p-c-Jun induced by CCl₄. Our results provide evidence that pinoresinol inhibited the transactivation of NF- κ B and AP-1.

In summary, our study demonstrates that pinoresinol can protect against CCl₄-induced acute hepatotoxicity through restoration of the anti-oxidative defense system and down-regulation of the pro-inflammatory pathway. This study provides evidence that pinoresinol may be an alternative treatment for liver diseases caused by xenobiotics.

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