Protective Mechanisms of PF2401-SF, Standardized Fraction of Salvia miltiorrhiza, Against Carbon Tetrachloride–induced Hepatotoxicity

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We previously prepared a standardized and purified extract of Salvia miltiorrhiza, PF2401-SF, and showed that it protected against hepatic injury more effectively than ethanol based extraction. In this study, we determined the hepatoprotective mechanisms of PF2401-SF in vivo. Hepatic injury was induced in mice by using carbon tetrachloride (CCl4). Treatment with PF2401-SF (1 or 10 mg/kg, p.o.) significantly reduced the levels of alanine transaminase (ALT) and aspartate transaminase (AST) in the plasma. PF2401-SF treatment resulted in further elevation of the CCl4-induced heme oxygenase-1 (HO-1) expression, which contributed to the PF2401-SF-mediated liver protection. Additionally, PF2401-SF treatment significantly reduced the c-Jun NH2-terminal kinase (JNK) phosphorylation induced by CCl4. Taken together, these results suggest that the protective effect of PF2401-SF, a standardized fraction of S. miltiorrhiza, against CCl4-induced hepatic injury in mice arises from its induction effect on HO-1 and inhibitory effect on JNK phosphorylation.

Key words: PF2401-SF, Heme oxygenase-1, c-Jun NH2-terminal kinase, Salvia miltiorrhiza, Hepatoprotection, Carbon tetrachloride

I. INTRODUCTION

Salvia miltiorrhiza Bunge (Labiatae) is traditionally used to treat liver disease in Asia1,2. Despite its popularity for treating liver disease3,4, it is rarely incorporated into conventional medicine as a hepatoprotective agent, partly because of the lack of standardization of S. miltiorrhiza herbal preparations5. We previously prepared a standardized and purified extract, PF2401-SF, enriched with tanshinone I, tanshinone IIA, and cryptotanshinone by using a simple, economical procedure that can be industrialized, and showed it to protect against hepatocyte injury more effectively than ethanol based extraction5,6.

Heme oxygenase-1 (HO-1) is emerging as an important cellular protective protein against oxidative injury7,8. HO-1 deficiency develops severe chronic hepatic inflammation, iron deposition, and oxidative damage in the liver9,10. In contrast, protective effects of enhanced HO-1 activity have been reported in liver cells under stress conditions in cases of various liver diseases, suggesting a role of HO-1 in anti-oxidation and the cytoprotective defense mechanism of the liver11-13. Mitogen-activated protein kinases (MAPKs) are important...
mediators of signal transduction processes that serve to regulate diverse cellular responses to extracellular stimuli\textsuperscript{14,15}). There are 3 major subclasses of MAPKs: extracellular signal-regulated kinase (ERK), c-Jun NH\textsubscript{2}-terminal kinase (JNK), and p38 MAPK. Carbon tetrachloride (CCl\textsubscript{4}) causes severe liver injury by inducing oxidative stress, a process that involves the activation of MAPKs\textsuperscript{16}). MAPKs have been investigated extensively in cultured cells, but studies of MAPKs in animal tissue have been limited\textsuperscript{17,18}). In this study, we investigated the mechanisms underlying the protective effect of PF2401-SF against CCl\textsubscript{4}-induced hepatotoxicity in vivo. Our data indicate that the protective effect of PF2401-SF may result from its induction effect on HO-1 and inhibitory effect on JNK activation.

II. MATERIALS and METHODS

1. Chemicals and Reagents

CCl\textsubscript{4}, hemin, and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tin mesoporphyrin (SnMP) was purchased Frontier Scientific, Inc. (Logan, UT, USA). SP600125 (JNK inhibitor) was purchased Calbiochem (La Jolla, CA, USA).

2. Preparation of PF2401-SF

The roots of \textit{S. miltiorrhiza} were purchased from the University Oriental Herbal Drugstore, Iksan, Korea, and were identified by Dr. Kyu-Kwan Jang, Botanical Gardens, Wonkwang University, Korea. A voucher specimen (No. WP05-87) was deposited at the Herbarium of the College of Pharmacy, Wonkwang University, Korea. A voucher specimen was deposited at the Herbarium of the College of Pharmacy, Wonkwang University. Dried and pulverized roots of \textit{S. miltiorrhiza} (2 kg) were soaked in 1.6 L distilled water for 12 h at room temperature, extracted with hot ethanol for 2 h, and filtered with filter paper; the filtrate was evaporated \textit{in vacuo} to produce an ethanol extract of 277 g. The ethanol extract was suspended in distilled water (500 ml), followed by filtration. The residue derived from the filtration was dissolved in hot ethanol and filtered again. The filtrate was then evaporated \textit{in vacuo} to obtain a standardized fraction of \textit{S. miltiorrhiza} (PF2401-SF, 20 g, 1.0 w/w\%). HPLC was used to determine the content of tanshinone I, tanshinone IIA, and cryptotanshinone in the standardized fraction \textsuperscript{5,6}).

3. Animals and treatment

Male ICR mice (18-20 g) were supplied by Dae Han Laboratory Animal Research and Co. (Chungbuk, Korea), and fed a standard chow diet (Jae Il Chow, Korea) with tap water \textit{ad libitum}. Animal experiments were performed under the latest edition of “Guiding Principles in the Use of Animals in Toxicology” adopted by the Society of Toxicology (USA). The study was approved by Wonkwang University animal care committee. Liver injury was induced by a single administration of CCl\textsubscript{4} (20 μl/kg, \textit{i.p.}, diluted in corn oil). PF2401-SF (1 or 10 mg/kg, \textit{p.o.}) was administered to mice for four consecutive days. CCl\textsubscript{4} was given once to mice 2 h after the final dose of PF2401-SF. Mice were euthanized 20 h later under ether anesthesia and plasma was obtained. The hepatoprotective effect was assessed by measuring alanine transaminase (ALT) and aspartate transaminase (AST) levels using the Autodry chemistry Analyzer (SPOTCHEM SP4410; Arkray, Japan).

4. Western blot analysis

Frozen liver tissues were homogenized in lysis buffer, containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 10 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml Nonidet P-40, and
Fig. 1. The effect of PF2401-SF on alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in carbon tetrachloride (CCl₄)-treated mice. (A) PF2401-SF (1 or 10 mg/kg) or (B) 1 mg/kg PF2401-SF was administered to mice for 4 consecutive days. Hepatic injury was induced by administering carbon tetrachloride (CCl₄; 20 μl/kg, i.p.) once to mice 2 h after the last dose of PF2401-SF. Hepatotoxicity was determined (A) 20 h later or (B) 2, 4, or 8 h later by quantifying the levels of ALT and AST. Data are expressed as mean ± SD. The number of mice in each group was at least 5, ***p < 0.001 vs. the CCl₄-treated control group; *p < 0.05 vs. the 8-h vehicle-treated group.
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CCl₄ administration. As shown in Fig. 1B, plasma ALT and AST levels increased significantly in a time-dependent manner in the CCl₄-treated mice than in the normal control mice. Plasma ALT and AST levels started to decrease 4 h after CCl₄ administration in the PF2401-SF-treated mice, and these mice showed significantly reduced levels of ALT and AST 8 h after CCl₄ administration than those shown by the vehicle-treated mice.

2. Effect of PF2401-SF on hepatic HO-1 expression

HO-1 is an important component of the cellular defense system against oxidative stress. Because the hepatoprotective activity of PF2401-SF was evident even 8 h after CCl₄ administration (Fig. 1B), we examined the effect of 1 mg/kg PF2401-SF treatment on HO-1 protein expression up to 8 h after CCl₄ administration in vivo. As shown in Fig. 2A, hepatic HO-1 protein expression was upregulated in a time-dependent manner after CCl₄ administration relative to that in the control group at all time points (2, 4, and 8 h). Moreover, PF2401-SF treatment resulted in further elevation of the CCl₄-induced HO-1 protein expression, which was significant 8 h after CCl₄ administration.

To determine whether this augmented HO-1 expression contributed to the hepatic protective effect of PF2401-SF, we treated mice with tin-mesoporphyrin IX (SnMP), a specific competitive inhibitor of HO-1. As shown in Fig. 2B, SnMP treatment significantly abolished the protective effect of PF2401-SF on CCl₄-induced hepatic injury. These results suggest that the PF2401-SF-mediated increase in HO-1 expression contributed to the PF2401-SF-mediated liver protection.

Fig. 2. The effect of PF2401-SF treatment on heme oxygenase-1 (HO-1) protein expression in the livers of carbon tetrachloride (CCl₄)-treated mice. Liver samples were collected at the indicated time points (2, 4, and 8 h) after CCl₄ treatment. (A) Protein was extracted from the liver samples and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting analysis of the HO-1 protein. The HO-1 protein densitometry values were normalized to their respective β-actin densitometry values. Data are expressed as mean ± SD of 3 mice; *p < 0.05 vs. the 8 h vehicle-treated group. (B) The effect of tin-mesoporphyrin IX (SnMP) pretreatment on the hepatoprotective activity of PF2401-SF in mice with CCl₄-induced liver injury. Hepatotoxicity was determined 24 h later by quantifying the levels of ALT and AST. Values are expressed as mean ± SD. The number of mice in each group was at least 6; *p < 0.05 vs. the CCl₄-treated control group (column 2); #p < 0.05 vs. the PF2401-SF-treated group (column 4).
3. Effect of PF2401-SF on JNK phosphorylation

To investigate the effect of PF2401-SF on MAPKs during CCl₄ intoxication, the phosphorylation of ERK, JNK, and p38 MAPK in liver was examined by immunoblotting. Phosphorylated JNK was significantly, but transiently, increased 2 h after CCl₄ administration, but PF2401-SF treatment markedly reduced the level of JNK phosphorylation (Fig. 3A). PF2401-SF did not, however, affect CCl₄-induced ERK and p38 phosphorylation (data not shown).

To investigate the potential role of HO-1 induction and/or inhibition of JNK phosphorylation in CCl₄-induced hepatotoxicity, mice were treated with hemin, a potent HO-1 inducer, and/or SP600125, a specific JNK inhibitor, before CCl₄ administration and their effects on liver injury were examined. As shown in Fig. 3B (left panel), phosphorylated JNK was barely detectable in SP600125-pretreated mice and strong and dense HO-1 expression was observed in hemin-pretreated mice compared to CCl₄ alone treated mice. The degree of hepatic injury was assessed by assessing plasma ALT activity. As shown in Fig. 3B (right panel), the ALT content of the plasma was decreased moderately in SP600125-pretreated mice, but significantly decreased in hemin-pretreated mice compared to mice treated with CCl₄ alone. Additionally, the combination of hemin and SP600125 had no additive effect on the reduction of ALT levels.

IV. DISCUSSION

To develop natural products with good quality control, it is necessary to establish new methods to standardize the
extract quality and optimize its content of active components\textsuperscript{23).} The standardization of processing methods for natural herbs, such as \textit{S. miltiorrhiza} root, is as important as their authentication to maintain their quality and ensure their safe use\textsuperscript{24).} We previously developed a simple and uniform method for preparing PF2401-SF, the standardized fraction of \textit{S. miltiorrhiza} (Korean patent registration no. 1008279380000\textsuperscript{5}) that was enriched with tanshinone I, tanshinone IIA, and cryptotanshinone, and showed that it protected against hepatocyte injury more effectively than ethanol based extraction\textsuperscript{5,6).} In the current study, we determined the mechanisms by which PF2401 protects against CCl\textsubscript{4}-induced hepatotoxicity \textit{in vivo}.

CCl\textsubscript{4} is a typical hepatotoxin that is metabolized by cytochrome P450 to trichloromethyl radical, which induces extensive lipid peroxidation in the liver\textsuperscript{25,26). Therefore, CCl\textsubscript{4} induces massive necrosis of the liver via oxidative stress, HO-1 is induced in cells and tissues by oxidative stress and is, therefore, thought to play an important protective role in oxidative injury\textsuperscript{27,28). It is believed that all products of heme degradation produced by HO-1 are involved in this cytoprotective effect both \textit{in vivo} and \textit{in vitro}\textsuperscript{11-13). Therefore, we investigated whether PF2401-SF could upregulate hepatic HO-1 expression in the CCl\textsubscript{4}-treated liver and, if so, whether HO-1 could mediate the hepatoprotective effect of PF2401-SF. Hepatic HO-1 expression is upregulated by CCl\textsubscript{4} administration and confers protection against oxidative injuries in liver tissue\textsuperscript{20,22). We found that PF2401-SF treatment resulted in further elevation of the CCl\textsubscript{4}-induced HO-1 protein expression, This indicated that the protective effect of PF2401-SF against CCl\textsubscript{4}-induced hepatotoxicity may be related to the augmented HO-1 expression, Therefore, we confirmed whether the hepatoprotective effect of PF2401-SF was related to its ability to upregulate HO-1 expression in CCl\textsubscript{4}-induced liver injury. Our results showed that an inhibitor of HO-1, SnMP, significantly abolished the protective effect of PF2401-SF in CCl\textsubscript{4}-induced liver injury (Fig. 2B). Moreover, we showed that HO-1 induction by hemin, a potent HO-1 inducer, protects mice from CCl\textsubscript{4}-induced liver injury (Fig. 3B). Thus, we suggest that the upregulation of HO-1 by PF2401-SF could partially underlie its protective effect that enables resistance to oxidative injury induced by CCl\textsubscript{4}.

MAPKs are important mediators of signal transduction processes that regulate diverse cellular responses to extracellular stimuli, such as oxidative stress\textsuperscript{14,15). It is widely accepted that MAPKs are activated by oxidative stress in cultured cells\textsuperscript{17,18). In this study, we determined the effect of PF2401-SF on MAPK activation in the CCl\textsubscript{4}-intoxicated liver. Phosphorylated JNK increased significantly as early as 2 h after CCl\textsubscript{4} administration, when liver injury had not yet developed, based on plasma ALT and AST levels (Fig. 1B). However, PF2401-SF pretreatment markedly reduced the level of JNK phosphorylation. This result suggests that the inhibition of JNK phosphorylation by PF2401-SF might play an important role in its protective effect against CCl\textsubscript{4}-induced hepatic injury. This finding is consistent with our previous observation that PF2401-SF protects hepatocytes from apoptosis by inhibiting JNK phosphorylation\textsuperscript{5). Moreover, through our experiments using a specific HO-1 inducer and/or specific kinase inhibitor, we found that CCl\textsubscript{4}-induced hepatic injury was strongly inhibited by the HO-1 inducer, hemin, while the JNK inhibitor, SP600125, moderately inhibited hepatic injury. More interestingly, the combination of hemin and SP600125 did not show a synergistic effect.

In summary, the present study demonstrated that the protective effect of PF2401-SF, a standardized fraction of \textit{S. miltiorrhiza}, against CCl\textsubscript{4}-induced hepatic injury in mice arises from its induction effect on HO-1 and inhibitory effect on JNK phosphorylation. This study provides a molecular basis that supports the use of PF2401-SF in the treatment of various oxidative stress-induced disorders,
VI. REFERENCES


