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Antioxidative and Hepatoprotective Effects of Magnolol on Acetaminophen-induced Liver Damage in Rats

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Acute liver failure (ALF), an often fatal condition characterized by massive hepatocyte necrosis, is frequently caused by drug poisoning, particularly with acetaminophen (*N*-acetyl-p-aminophenol/APAP). Hepatocyte necrosis is consecutive to glutathione (GSH) depletion and mitochondrial damage caused by reactive oxygen species (ROS) overproduction. Magnolol, one major phenolic constituent of *Magnolia officinalis*, have been known to exhibit potent antioxidative activity. In this study, the anti-hepatotoxic activity of magnolol on APAP-induced toxicity in the Sprague-Dawley rat liver was examined. After evaluating the changes of several biochemical parameters in serum, the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) were elevated by APAP (500 mg/kg) intraperitoneal administration (8 and 24 h) and reduced by treatment with magnolol (0.5 h after APAP administration; 0.01, 0.1, and 1 μg/kg). Histological changes around the hepatic central vein, lipid peroxidation (thiobarbituric acid-reactive substance/TBARS), and GSH depletion in liver tissue induced by APAP were also recovered by magnolol treatment. The data show that oxidative stress followed by lipid peroxidation may play a very important role in the pathogenesis of APAP-induced hepatic injury; treatment with lipid-soluble antioxidant, magnolol, exerts anti-hepatotoxic activity. Our study points out the potential interest of magnolol in the treatment of toxic ALF.

Key words: Magnolol, Antioxidant, Acetaminophen, Hepatotoxicity, Lipid peroxidation

INTRODUCTION

An overdose of the analgesic drug acetaminophen (*N*-acetyl-p-aminophenol/APAP) can lead to severe liver injury in humans and in experimental animals. The biochemical mechanism by which APAP induces liver injury has been elucidated in detail and is believed to be the result of the metabolic conversion of APAP to a highly reactive intermediate, namely, *N*-acetyl-p-benzoquinonimine (NAPQI), by cytochrome P-450-mediated mix function oxidases; this metabolite is known to be detoxified by glutathione (GSH) (Jaw and Jeffery, 1993), a nonprotein thiol with both oxidant-scavenger and redox-regulating capacities. At sufficiently high doses of APAP, GSH becomes depleted, leaving NAPQI free to bind to possibly critical cellular proteins and cause hepatic necrosis. Therefore, the toxicity of APAP is a function of the amount of NAPQI formed and the insufficient availability of hepatic GSH for detoxification of this toxic metabolite (Banerjee et al., 1998, Dimova et al., 2000, Jaw and Jeffery, 1993). Lipid peroxidation mediated by oxygen free radicals is

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believed to be an important cause of destruction and damage to cell membranes, and attention has been focused on the role of reactive oxygen species (ROS) in mediating the microvascular disturbances that precede tissue damage induced by various chemicals (Tan et al., 2000, Youn et al., 1992). Lipid peroxidation in cell membranes is devastating to the functional integrity of these structures, and if the damage is severe, cell death is inevitable. It has recently been shown that, during APAP intoxication in the mouse, toxic ROS are generated and actively participate in the pathophysiological process leading to hepatocyte apoptosis (Bedda et al., 2003, Ferret et al., 2001). *N*-acetylcysteine (NAC), which augments intracellular GSH and can be used to treat APAP-induced ALF, is known to be protective against APAP hepatotoxicity but is not constantly effective (Jones, 1998, Smilkstein et al., 1988).

Magnolia officinalis is a commonly used Chinese medicinal herb for the treatment of fever, headache, anxiety, abdominal fullness, constipation, and thrombotic stroke. Magnolol, an antioxidant purified from *M. officinalis,* is approximately 1,000 times more potent than α-tocopherol (Hong et al., 1996, Lo et al., 1994) in inhibiting lipid peroxidation in heart mitochondria and 340 times more potent in rat liver mitochondria (Chiu et al., 1999). In addition, magnolol has been demonstrated to suppress polymorphonuclear leukocyte infiltration in a mouse model of ionophore A23187-induced pleurisy and to reduce leukotriene B formation in rat-isolated peripheral neutrophil suspension (Wang et al., 1992) and in rat basophilic leukemia-2H3 cells (Hamasaki et al., 1999). Magnolol also inhibits platelet-activating factor PAF production in human polymorphonuclear leukocytes (Yamazaki et al., 1994). Recently, magnolol was found to be a potential therapeutic agent for neurodegenerative diseases (Lin et al., 2006). Our previous studies reported that magnolol reduced infarct size and suppressed both ischemia and reperfusion-induced arrhythmias in a rat model (Hong et al., 1996), and attenuated intimal thickening and monocyte chemotactic protein-1 (MCP-1) expression after balloon injury of the aorta in rabbits (Chen et al., 2001). These beneficial effects of magnolol could contribute to the suppression of inflammatory processes (Chen et al., 2006a, Tse et al., 2007) by its antioxidative properties (Chen et al., 2006b).

In the current study, we examined the putative protective effects of magnolol in comparison with NAC against APAP-induced hepatotoxicity. We hypothesized that this molecule could also exert an antioxidant activity and be possibly used as a treatment for APAP-induced ALF. We then investigated the therapeutic activity of magnolol *in vivo*, in a rat model of APAP-induced ALF.

MATERIALS AND METHODS

Reagents

Magnolol was obtained from Nacalai Tesque Inc. (Kyoto, Japan); the chemical structure of magnolol is shown in Fig. 1. APAP and NAC were purchased from Sigma (MD, USA). Carboxymethycellulose (CMC) and dimethyl sulfoxide (DMSO) were purchased from Merck (NJ, USA). Other chemicals of analytical grade were from Sigma. Magnolol was dissolved in DMSO as stock (100 mg/mL) and diluted with sterile saline; APAP was dissolved in 2% CMC. The dose of solvent used in the study did not affect the biochemical parameters in rats.

Animals

Male Sprague-Dawley (SD) rat weighting 200-250 g were obtained from the Experimental Animal Center, National Yang-Ming University (Taipei, Taiwan), and housed in a 12-h light/dark cycle room at 22±3ºC, 55±5% humidity, and fed with a standard laboratory diet and tap water. All experimental animal protocols were evaluated by the Institutional Animal Care and Use Committee of National Yang-Ming University, which complies with the European Community Guidelines for the use of experimental animals.

Drug administration protocols and tissue collection

The method of acetaminophen-induced acute hepatotoxicity was modified from a previous study (Muriel et al., 1992). Rats were fasted for 16 h, and then divided into 11 groups (n=7 for each group). Group 1 served as a control group, receiving vehicle (normal saline) only (i.p.). APAP (500 mg/kg, i.p.) was administered to animals of the other 10 groups. Magnolol (0.01, 0.1, and 1 μg/kg, i.p.) and the reference drug NAC (1 g/kg, i.p.) were administered to groups 3-6 and 8-11, 0.5 h after APAP administration (APAP-intoxicated for 8 h and 24 h, respectively). The remaining three groups, control and APAP group 2 and 7 were given no additional antioxidant treatment. All animals were sacrificed 8 or 24 h after APAP administration, whole blood was drawn from the heart and plasma was separated for the different assays. Liver tissues were removed, and then liver sections were taken from each lobe of the liver and fixed in 10% neutral formalin. The remaining liver tissues were cut into approximately 50-

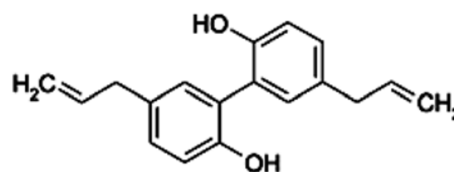

Fig. 1. The chemical structure of magnolol

100 mg pieces, placed on ice and stored separately at -70ºC in plastic vials for lipid peroxidation assay.

Serum analysis

After blood collection, serum was separated by centrifugation at 3,000 rpm and 4ºC for 20 min. The serum aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), and creatinine (CRE) values were measured using standard clinical semi-automatic analyzer (SPOTCHEM, Model SP-4410, Kagaku Co., Ltd., Kyoto, Japan).

Histopathological observation

After 12 h of fixing in 10% neutral formalin solution, liver tissues were dehydrated with a sequence of ethanol solutions, embedded in paraffin, cut into 5 μm sections, stained with haematoxylin-eosin dye (H&E stain) and then observed under a photomicroscope. The morphological changes observed included hepatocyte cell piecemeal necrosis, fatty change, cell fibrosis and the infiltration of lymphocytes and Kupffer cells. The histological diagnosis was based on accepted criteria and histological activity index (HAI) (Knodell et al., 1981). Briefly, HAI represents the sum of the scores attributed to the necro-inflammatory lesions, that is, periportal±bridging necrosis (0-10), intralobular degeneration and focal necrosis (0-4), portal inflammation (0-4), and fibrosis (0-4). All biopsies were simultaneously reviewed by two pathologists in a blinded fashion, obtaining a consensus diagnosis and histological score.

Homogenate preparation

The frozen liver samples were homogenized in Tris-HCl or phosphate buffer solution to give a 20% homogenate. To measure the lipid peroxidation levels, homogenates were centrifuged at 14,000 rpm in 4ºC for 10 min. The protein content of liver tissue was measured by the Bio-Rad protein assay reagent.

Measurements of tissue lipid peroxidation and GSH levels

Lipid peroxidation was quantified by measuring TBARS by spectrophotometric assay (Beckman Coulter, DU 640 spectrophotometer, Germany) according to a previous study (Ohkawa et al., 1979). The level of lipid peroxides, expressed as nanomoles of malondialdehyde (MDA) per gram of protein, was calculated from the absorbance at 532 nm using tetraethoxypropane (TEP) as an external standard (Wong et al., 1987). GSH and oxidized GSH (GSSG) concentrations (μmol/g protein) in liver were determined by commercial colorimetric GSH and GSSG assay kits (OxisResearch, Portland, OR, USA).

Statistical evaluation

Results were expressed as mean±S.E.M., and data were analyzed using ANOVA followed by *post-hoc* Fisher's LSD (Least Significant Difference) test for significant difference. Statistical significance was defined as $p < 0.05$. All statistical procedures were performed with SigmaStat version 3.1 (Jandel, USA).

RESULTS

Changes of plasma biochemical parameters in rats

The results from measuring the effects of magnolol or NAC on this liver injury model are summarized in Table I

Table I. Effects of antioxidant drug treatment on body weight and plasma biochemical parameters in APAP intoxicated (8 h and 24 h) rats

No.	Treatment group	Dose	BW (g)	AST (U/L)	ALT (U/L)	LDH (U/L)	BUN (mg/dL)	CRE (mg/dL)
1	Control (NaCl)	-	208±3	94±5	38±4	302±19	14.5±0.8	0.5±0.1
	8-h intoxicated							
2	APAP (mg/kg)	500	214±5	354±62*	232±18*	921±78*	15.1±1.5	0.5±0.1
3	+ Magnolol (μg/kg)	0.01	217±2	418±33	202±28	712±87#	14.2±0.6	0.5±0.1
4		0.1	217±3	352±48	161±24	578±64#	14.3±0.7	0.4±0.1
5		1	216±5	267±27#	121±29#	546±66#	14.5±0.8	0.5±0.1
6	+ NAC (g/kg)	1	204±4	271±33#	123±19#	700±61#	13.8±1.8	0.5±0.1
	24-h intoxicated							
7	APAP (mg/kg)	500	215±5	642±62*	374±31*	974±86*	15.0±1.4	0.4±0.1
8	+ Magnolol (μg/kg)	0.01	219±5	501±43#	161±19#	821±96	14.1±1.6	0.5±0.1
9		0.1	220±4	399±38#	126±27#	599±46#	14.7±1.7	0.5±0.1
10		1	220±6	372±37#	87±18#	635±81#	14.4±1.8	0.5±0.1
11	+ NAC (g/kg)	1	209±3	271±23#	87±11#	622±54#	13.8±1.1	0.6±0.1

Data are expressed as the mean±S.E.M. (n=7).
*$p < 0.05$ compared with control group.
#$p < 0.05$ compared with APAP group.

(8 and 24 h APAP administration, respectively). Data show that 8 or 24 h after APAP injection, there were significant increases of serum AST, ALT, and LDH compared with the control group. Treated with magnolol or NAC, the serum AST, ALT, LDH levels were significantly decreased compared with the APAP groups after 8 or 24 h intoxication. Body weight (BW), blood urea nitrogen (BUN), and creatinine (CRE) did not significantly change in all groups compared with control.

Histopathological observation

The appearance of liver tissues after various treatments (24-h APAP intoxicated) was confirmed by histological observation (Fig. 2). Gross necrosis in the centribular area (zone 3), sinusoidal congestion, infiltration of the lymphocytes and Kupffer cells around the central vein, loss of cell boundaries and ballooning degeneration were observed after administration of acetaminophen (Fig. 2B). Rats treated with magnolol (0.01, 0.1, and 1 μg/kg body weight) presented less damage than the acetaminophen group (Figs. 2C, D, and E), however, administration of low-dose magnolol (0.01 μg/kg body weight) resulted in less effect. The reference antioxidant, NAC (1 g/kg body weight), also presented hepatoprotective effect (Fig. 2F).

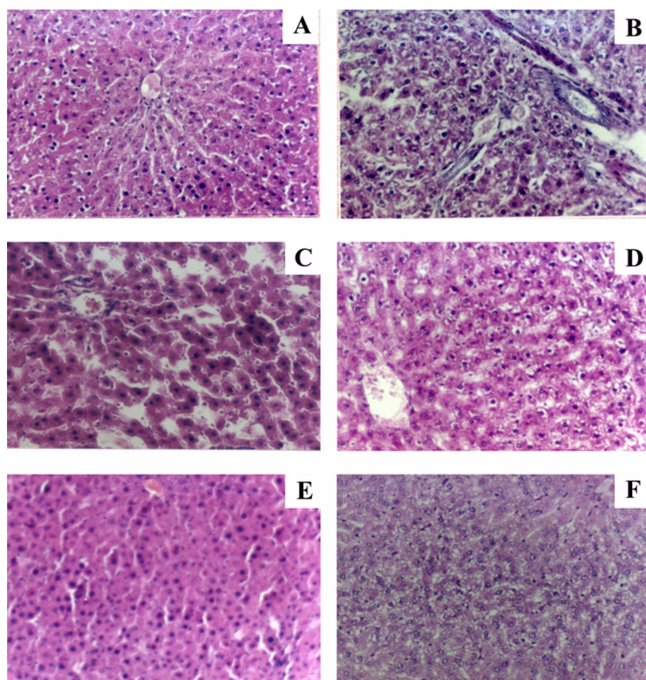

Fig. 2. Photomicrographs of liver sections taken from rats. A: vehicle (NaCl) control; B: APAP (500 mg/kg) intoxicated for 24 h. Note that gross necrosis, sinusoidal congestion, Kupffer cell, ballooning degeneration and infiltrating lymphocytes are observed; C: magnolol treatment (0.01 μg/kg); D: mgnolol treatment (0.1 μg/kg); E: magnolol treatment (1 μg/kg); F: NAC treatment (1 g/kg).

The appearance of liver tissues in 8-h APAP intoxicated rats was similar (data not shown). The histological activity index (HAI) scoring of liver specimens was calculated and is shown in Fig. 3 (8 h and 24 h APAP intoxicated, respectively).

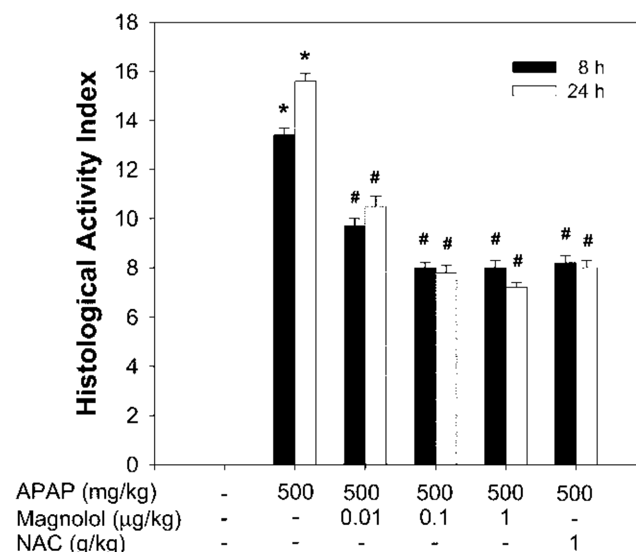

Fig. 3. The histological activity index (HAI) scoring of liver specimens. HAI was recorded in control, APAP administration (500 mg/kg for 8 and 24 h), magnolol treatment (0.01, 0.1, and 1 μg/kg), and NAC treatment (1 g/kg) groups. Data are expressed as the mean±S.E.M. (n=7). $^{*}p < 0.05$ compared with control group. $^{\#}p < 0.05$ compared with APAP group.

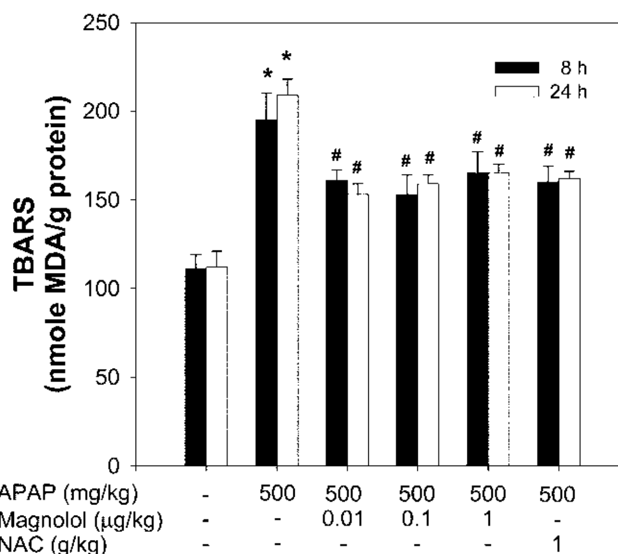

Fig. 4. The thiobarbituric acid reactive substance (TBARS) in rat liver tissues. Hepatic MDA content were determined in control, APAP administration (500 mg/kg for 8 and 24 h), magnolol treatment (0.01, 0.1, and 1 μg/kg), and NAC treatment (1 g/kg) groups. Data are expressed as the mean±S.E.M. (n=7). $^{*}p < 0.05$ compared with control group. $^{\#}p < 0.05$ compared with APAP-intoxicated group.

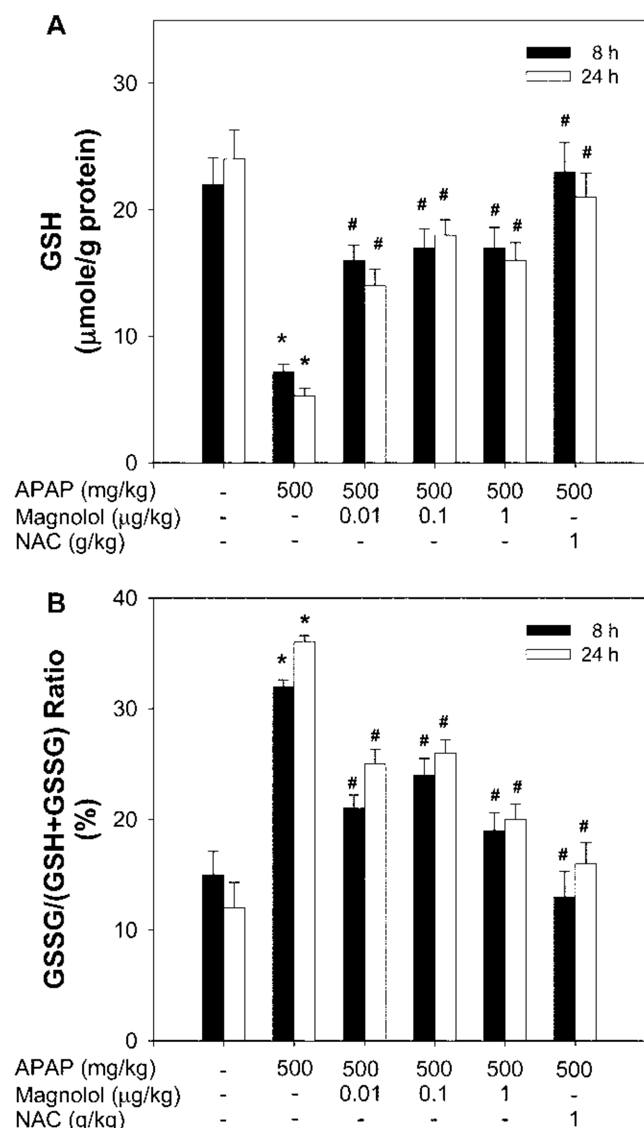

Fig. 5. The GSH and GSSG/(GSH+GSSG) ratio in rat liver tissues. Hepatic GSH and GSSG contents were determined in control, APAP administration (500 mg/kg for 8 and 24 h), magnolol treatment (0.01, 0.1, and 1 μg/kg), and NAC treatment (1 g/kg) groups. Data are expressed as the mean±S.E.M. (n=3). $^{*}p < 0.05$ compared with control group. $^{\#}p < 0.05$ compared with APAP-intoxicated group.

Effects on rat liver lipid peroxide and GSH levels

Lipid peroxidation induced by APAP in the rat liver homogenate is shown in Fig. 4. The effects of administration of the drugs were determined by the assay of MDA-TBA adduct at 532 nm. Data show that a significant increase of the level of thiobarbituric acid-reactive substance (TBARS) may be induced by APAP injection. The TBARS levels of rats administered with magnolol or NAC were significantly decreased.

After APAP treatment, the GSH content in the liver dramatically decreased. The proportion of GSSG to GSH +GSSG was significantly increased after APAP treatment. Magnolol (0.01 to 1 μg/kg) and NAC (1 g/kg) treatment reduced the depletion in hepatic GSH content and decreased the proportion of GSSG in total GSH induced bt APAP treatment (Fig. 5A and 5B).

DISCUSSION

The results of the present study demonstrate that treatment of rats with magnolol had a markedly protective effect against APAP-induced hepatotoxicity. Previous studies on the mechanisms of APAP-induced hepatotoxicity have shown that lipid peroxidation was increased in APAP-induced hepatotoxicity and GSH played a key role in the detoxification of the reactive toxic metabolites of APAP (Zwingmann and Bilodeau, 2006). Our results showed that treatment with magnolol significantly reduced APAP-induced hepatotoxicity and the increase of lipid peroxidation index, TBARS. These results are probably due to the inhibition of the bioactivation of APAP by magnolol resulting in decreased formation of ROS.

APAP is a widely used analgesic and antipyretic drug, which is considered to be safe and nontoxic when used at ordinary doses. When taken in larger doses, it becomes a potent hepatoxin, generating fulminated hepatic and renal tubular necrosis, which is lethal in humans and many species of animals (Pessayre et al., 1980). The laboratory features of hepatotoxicity induced by APAP resemble other kinds of acute necro-inflammatory liver disease, with prominent increases of serum AST and ALT levels. The histopathological appearances of the liver biopsy or autopsy revealed a variably extensive centrizonal necrosis without steatosis and with a relatively light inflammatory infiltration (Davidson and Eastham, 1966). APAP is primarily metabolized by sulphation and glucuronidation to unreactive metabolites, and then activated by the cytochrome P-450 system to result in liver injury (Moldeus, 1978). The characteristic zone 3 necrosis of APAP appears to be produced by an electrophilic metabolite of the drug NAPQI. NAPQI is initially detoxified by conjugation with reduced GSH to form mercapturic acid (Whitehouse et al., 1985). However, when the rate of NAPQI formation exceeds the rate of detoxication by GSH, NAPQI will oxidize tissue macromolecules, such as lipids or protein thiols, and alter the homeostasis of calcium after depleting GSH. Lipid peroxidation has been postulated to be the destructive process in liver injury due to APAP administration (Muriel et al., 1992). The coincidence of antioxidant activity and liver tissue protective effects after APAP administration suggest that both free radical generation and lipid peroxidation may be involved in this kind of drug injury process.

Depletion of GSH and an increased GSSG/(GSH+

GSSG) ratio are characteristic features of APAP overdose (Knight et al., 2002). The effects of NAC against APAP toxicity depend on its ability to be a precursor of GSH or to directly trap electrophilic intermediates. NAC can effectively eliminate electrophiles and free radicals through conjugation and reduction reactions (Flanagan, 1987). Improvement in GSH levels is considered the most significant mechanism of NAC against APAP toxicity (Atkuri et al., 2007). Our present study indicate enhanced baseline GSH levels and decreased GSSG/(GSH+GSSG) ratios as a result of NAC pretreatment, which resulted in reduced liver injury subsequent to APAP treatment. Our data also found that magnolol could restore hepatic GSH level in APAP-treated rat liver. The finding suggested that this herbal compound might be rich in GSH. Therefore, the observed antioxidant protection from magnolol against APAP-induced hepatotoxicity might be partially due to the increased GSH. Further study is necessary to examine how magnolol inhibits GSH depletion or how magnolol influences the enzymes involved in GSH biosynthetic pathway are up-regulated (Saito et al., 2008). Since this herbal compound could provide anti-oxidative protection via increasing GSH level, this herbal compound might be also applied to prevent and/or several deviate other diseases with oxidative stress.

Magnolol, 1,000 times more potent than a-tocopherol in inhibiting lipid peroxidation (Hong et al., 1996; Lo et al., 1994), is an active component purified from *Magnolia officinalis.* It is a commonly used Chinese medical herb and has anti-inflammatory and analgesic effects (Wang et al., 1992), anti-asthmatic (Homma et al., 1993), anti-arrhythmia (Hong et al., 1996) and antimicrobial effects (Clark et al., 1981), anti-platelet activity (Teng et al., 1990), anti-cerebral ischemic injury (Chang et al., 2003), anti-atherosclerosis (Chen et al., 2001), anti-lipid peroxidation (Hong et al., 1996, Lo et al., 1994) and anti-xanthine oxidase effects (Chang et al., 1994), and a vascular smooth muscle relaxation effect (Teng et al., 1990). Furthermore, it is an effective scavenger of free radicals such as hydroxyl radicals (Taira et al., 1993), is able to inhibit UV-induced mutation (Fujita and Taira, 1994) and attenuates peroxidative damage and improves survival of rats with sepsis (Kong et al., 2000). *In vitro,* magnolol is proved to be an effective anti-oxidant and suppresses lipid peroxidation in rat liver mitochondria (Chiu et al., 1999). Our results showed that after treatment with different doses of magnolol, the increased ALT, AST, LDH, and TBARS levels induced by APAP were attenuated. Magnolol also decreased the ballooning degeneration, sinusoidal congestion and cell necrosis (centribular, zone 3). Parallel study showed similar results that either pre- (data not shown) or post-treatment (0.5 h before or after APAP intoxication, respectively) with magnlol could attenuate APAP-induced hepatotoxicity. Recently, Park et al. found that magnolol had a protective effect against D-galactosamine (GalN)-induced hepatotoxicity acting by inhibiting intracellular GSH depletion in rat primary hepatocytes (Park et al., 2003), suggesting the hepatoprotective effects of magnolol were probably by their antioxidant activity. It deserves mention that in our pilot study, high dose (1 and 10 mg/kg) of magnolol was lethal and caused hematuria in our experimental rat model. Magnolol is one of components in the Kampo (Chinese/Japanese herbal) medicine, Saiboku-to, and it has been well demonstrated that several potential side effects such as allergic reactions, cramps, diarrhea, fever, gastrointestinal disturbances, headaches, hematuria, and vomiting may be experienced when taking Kampo medicine (Ikegami et al., 2004). Therefore, the use of this kind of drug should be carefully monitored for its possible toxicity when used at a larger dose.

However, several limitations of the present study should be noted. The activities of cytochrome P-450-related oxidases and/or antioxidant enzymes (such as GSH peroxidase, superoxide dismutase/SOD, or catalase) have not been measured in our rat experimental model. Previous study showed that magnolol strongly and competitively suppressed CYP1A1 and CYP1A2 enzyme activities, suggesting it inhibited mutation induced by indirect mutagens through suppression of these enzymes (Saito et al., 2006). On the other hand, it has been shown that magnolol suppressed the lipid peroxidation but not the SOD activity (Shih et al., 2004). However, little is known whether magnolol affects other antioxidant enzymes (such as GSH peroxidase or catalase); further studies are needed to clarify the precise mechanisms by which magnolol can modulate redox-balance in liver.

It has been shown that magnolol has an anti-apoptotic effect and protects the liver against warm ischemia-reperfusion injury of rat liver through upregulation of the anti-apoptotic Bcl-XL gene and suppression of the Bcl-xS gene (Jawan et al., 2003). In addition, the anti-inflammatory effect of magnolol was proposed to be dependent on reducing the level of eicosanoid mediators (Wang et al., 1992). Our previous study also showed that magnolol has anti-inflammatory effects by inhibiting MCP-1 (Chen et al., 2001) and VCAM-1, and by attenuating intracellular H_2O_2 generation (Chen et al., 2002). Therefore, further studies investigating the protective effect of magnolol in terms of anti-apoptosis and anti-inflammation should be conducted.

In summary, the present study demonstrated that magnolol has protective effects against APAP-induced hepatotoxicity. The protective effect was most likely mediated through its inhibitory effect on APAP bioactivation. The protective activity of magnolol against APAP-induced hepatotoxicity appears to be related to its antioxidative activity and

inhibitory effects on lipid peroxidation. Our study points out the potential interest of magnolol in the treatment of toxic acute liver failure.

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