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RESEARCH ARTICLE

Effect of sinapic acid against dimethylnitrosamine-induced hepatic fibrosis in rats

Dong-Su Shin · Kung Wook Kim · Hae Young Chung · Sik Yoon · Jeon-Ok Moon

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Abstract Sinapic acid is a member of the phenylpropanoid family and is abundant in cereals, nuts, oil seeds, and berries. It exhibits a wide range of pharmacological properties. In this study, we investigated the hepatoprotective and antifibrotic effects of sinapic acid on dimethylnitrosamine (DMN)-induced chronic liver injury in rats. Sinapic acid remarkably prevented DMN-induced loss of body weight. This was accompanied by a significant increase in levels of serum alanine transaminase, aspartate transaminase, and liver malondialdehyde content. Furthermore, sinapic acid reduced hepatic hydroxyproline content, which correlated with a reduction in the expression of type I collagen mRNA and histological analysis of collagen in liver tissue. Additionally, the expression of hepatic fibrosis-related factors such as α-smooth muscle actin and transforming growth factor-β1 (TGF-β1), were reduced in rats treated with sinapic acid. Sinapic acid exhibited strong scavenging activity. In conclusion, we find that sinapic acid exhibits hepatoprotective and antifibrotic effects against DMN-induced liver injury, most likely due to its antioxidant activities of scavenging radicals, its capacity to suppress TGF-β1 and its ability to attenuate activation of hepatic stellate cells. This suggests that sinapic acid is a potentially useful agent for the protection against liver fibrosis and cirrhosis.

Keywords Sinapic acid · Hepatoprotective effect · Antifibrogenic effect · Antioxidant effect · Liver fibrosis · Hepatic stellate cell · NF-κB p65 · TGF-β1

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Introduction

Hepatic fibrosis is the wound-healing or scarring process that occurs in response to a variety of chronic stimuli. It involves excessive accumulation of extracellular matrix (ECM) components including mainly collagen in liver. Advanced fibrosis, which is the result of an imbalance between production and dissolution of ECM, can ultimately lead to cirrhosis. Excessive deposition of ECM disrupts the normal architecture of the liver, resulting in pathophysiological damages to the organ (Gabel et al. 2003). The most common causes of liver fibrosis and cirrhosis include alcoholic liver disease (60–70 %) in western countries (Crawford 1999) and chronic hepatitis B (70 %) and hepatitis C (10 %) in Korea. Other known stimuli for liver fibrosis include helminthic infection, metabolic diseases due to overload of drug, iron or copper, autoimmune attack of hepatocytes or bile duct epithelium, or congenital abnormalities (Friedman 1998). If the liver is constantly responding to such types of stress, the associated formation of scar tissue increases the risk of morbidity and mortality if left untreated (Gabel et al. 2003). According to updated projections of global mortality and burden of disease published by the World Health Organization, by 2020 liver cirrhosis and cancer will be the ninth leading cause of death in western countries (Mathers and

Loncar 2006). Furthermore, liver cancer is expected to have the highest mortality rates in Korea men up to 50 years of age (Jung et al. 2011) (Fig. 1).

Hepatic stellate cells (HSCs) are regarded as the primary target cells that are responsible for liver fibrosis. HSCs comprise 15 % of the total number of resident liver cells. In normal liver, they are the principal storage site for retinol (Wang 1999) and are nonparenchymal, quiescent cells with physiological functions such as production of ECM in the space of Disse and a role in the regulation of the sinusoidal microcirculatory flow (Geerts 2001; Pinzani 1995). In response to liver injury, injured hepatocytes, and neighboring endothelial and Kupffer cells, play roles in early stellate cell activation (Friedman 2000). Hepatocytes and Kupffer cells are a potent source of reactive oxygen intermediates (Maher 1999), which exert paracrine stimulation of stellate cells (Friedman 2000). Endothelial cells convert latent transforming growth factor-β1 (TGF-β1) to the active, fibrogenic form through the activation of plasmin. Subsequently, HSCs undergo an 'activation' process characterized by proliferation and myofibroblastic transformation (Pinzani 1995; Hautekeete and Geerts 1997; Safadi and Friedman 2002). Activated HSCs increase production of fibrillar collagen (collagen type I and type III) and alpha-smooth muscle actin (α-SMA) which are the major cell types for matrix production in damaged liver tissue, leading to fibrosis (Safadi and Friedman 2002). Cirrhosis, the end stage of progressive fibrosis, is strongly associated with oxidative stress, chronic inflammation and increased activity of TGF-β1 (Muriel 2007; Meyer et al. 2010; Gressner and Bachem 1995).

Transforming growth factor-β1 is major fibroblast growth factor that increased collagen deposition and α-SMA via activation of HSCs, and present in both normal and fibrotic liver, but increased in cirrhosis and experimental hepatic fibrosis (Reeves and Friedman 2002).

Numerous antifibrotic therapeutic strategies have been suggested, and are generally focused on suppressing hepatic inflammation, downregulating HSC activation and promoting matrix degradation (Li and Friedman 1999). In recent years, flavonoids have attracted the interest of researchers as their powerful antioxidant properties can protect the body from free radicals and against oxidative stress (Bors et al. 1996). Similarly, recent studies have documented inhibition of stellate cell activation by natural antioxidants, such as vitamin E, resveratrol, quercetin and N-acetyl cysteine (Li and Friedman 1999). Thus, flavonoids are likely also to play a vital biological role, particularly due to their ability to scavenge reactive oxygen species (Pietta and Simonetti 1998).

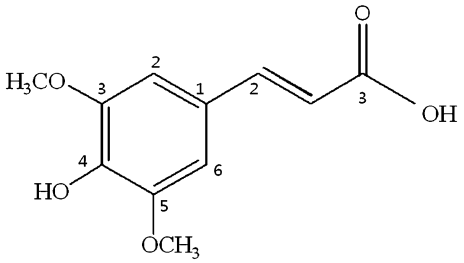

Fig. 1 Chemical structures of sinapic acid [3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid]

Sinapinic acid or sinapic acid (4-hydroxy-3,5-dimethoxy cinnamic acid), is a small naturally occurring member of the phenylpropanoid family and is abundant in cereals, nuts, oil seeds, and berries (Shahidi and Naczk 2004). To date, several studies for sinapic acid have shown that it possesses potent antioxidant (Kikuzaki et al. 2002), anti-inflammatory (Yun et al. 2008), anxiolytic (Yoon et al. 2007), peroxynitrite scavenging effects (Zou et al. 2002) and neuroprotective effects (Kim et al. 2010). However, the efficacy of sinapic acid in prevention of liver inflammation and fibrosis has not yet been studied.

Dimethylnitrosamine (DMN) is a potent hepatotoxin, carcinogen and mutagen (Haggerty and Holsapple 1990). The toxicity of DMN is mediated by reactive metabolites produced in the process of metabolism via cytochrome P450 2E1 (CYP2E1) and not by the parent compound (George et al. 2001). In the rat, a repeated low dose of DMN administration causes subacute and chronic liver injury with varying degrees of necrosis, fibrosis and nodular regeneration (Terracini et al. 1967) and also deposition of extracellular matrix proteins in the liver, particularly collagen (Ala-Kokko et al. 1987; Savolainen et al. 1988). The DMN-induced fibrosis model has various benefits, such as progressive and remarkable pathological alteration, a high fibrosis reproduction rate and a low mortality rate in experimental animals (Jezequel et al. 1989). This reproducible animal model recapitulates the biochemical and pathological alterations associated with development of liver fibrosis and cirrhosis in humans (George 2003). We therefore chose an experimental DMN-induced liver fibrosis model for the current study.

We evaluated whether sinapic acid exerts hepatoprotective and antifibrotic effects in DMN-induced liver fibrosis in vivo models by histopathological analysis and measurement of liver injury parameters. Possible mechanisms of the antifibrogenic effect were explored by analyzing inflammatory protein, NF-κB p65 and fibrogenic factors, including TGF-β1, collagen type1 (COL1) and α-SMA.

Materials and methods

Chemicals

Bicinchoninic acid solution, bovine serum albumin, butylated hydroxytoluene (BHT), copper(II) sulfate solution,

DMN, 1,1-diphenyl-2-picrylhydrazyl (DPPH), formalin, sinapic acid, sodium carboxy methylcellulose (CMC), phosphoric acid, potassium hydroxide, potassium monobasic phosphate (anhydrous), 1,1,3,3-tetraethoxypropane (TEP), vitamin C (Vit. C), were obtained from Sigma-Aldrich Co. *n*-Butanol and methanol were obtained from Merck Co., and 2-thiobarbituric acid (TBA) was obtained from Tokyo Chemical Industry Co.

Induction of liver fibrosis with DMN

Animal care and all experimental procedures were conducted in accordance with the Guide for Animal Experiments of the Korean Academy of Medical Sciences. Male Sprague–Dawley rats were obtained from Samtako. Animals were provided standard rat chow with free access to tap water and were maintained in a temperature (23 ± 3 °C)—and humidity (50 ± 20 %)—controlled facility under a 12 h light–dark. Twenty-four rats weighing 140–160 g and aged 6–7 weeks were assigned to 4 groups (n = 6/group) designated as control, DMN, SA10, and SA20 groups. Animals in the control group received saline (DMN vehicle) by intraperitoneal injection and CMC (sinapic acid vehicle) by oral gavage; the DMN group received DMN and CMC; the SA10 group received DMN and sinapic acid at 10 mg/kg/day; and the SA20 group received DMN and sinapic acid at 20 mg/kg/day. Liver injury in rats was induced by i.p. injection of 1 % (w/v) DMN (10 mg/kg body weight) in saline once a day for 3 consecutive days per week for 4 weeks (Jezequel et al. 1989; Lee et al. 2003). Sinapic acid was suspended in 0.1 % CMC solution to a concentration of 10 mg/ml and administered once every day for 4 weeks by gavage (10 mg/kg or 20 mg/kg body weight). At the end of the fourth week, all rats were sacrificed under ether anesthesia and their livers were excised and weighed (wet weight). Blood samples for biochemical analyses were obtained from the abdominal inferior vena cava. The liver specimens were either immediately frozen for hydroxyproline measurements or fixed in 10 % neutral buffered for histological studies. The remaining liver tissue was rapidly cut into small pieces and homogenized with 2 volumes (w/v) of ice-cold potassium phosphate buffer (pH 7.4) using a IKA® T10 basic Ultra-Tur Rax homogenizer. Debris and nuclei were removed from the homogenate by centrifugation at 700×*g* at 4 °C for 10 min and stored at −80 °C for further analysis. The protein concentrations of the supernatants were determined by the bicinchoninic acid assay using bovine serum albumin as the reference standard.

DPPH assay

The antioxidant activity was determined on the basis of the scavenging of the stable DPPH free radical (Brand-Williams et al. 1995) and slightly modified. The reaction mixture contained 50 mM phosphate buffer, pH 7.4 (80 µl), 100 µM DPPH dissolved in ethanol (100 µl), and the indicated concentrations of sinapic acid and Vit. C (20 µl). Their action tubes, in triplicates, were wrapped in aluminum foil and kept at 30 °C for 30 min in dark. Spectrophotometric measurements were done at 517 nm by a microplate (TECAN). The percentage inhibition activity was calculated from ([A0 − (A − Ab)]/A0) × 100, where A0 is the absorbance of the control, and A is the absorbance of the control, A is the absorbance of the sample and Ab is the absorbance of the blank sample (containing all reagents except DPPH). The inhibition curves were prepared and IC_{50} values were obtained.

Biochemical analysis of liver enzymes

Activities of alanine transaminase (ALT) and aspartate transaminase (AST) in serum were measured by the method described Reitman and Frankel (1957) using an ALT/AST Shin Yang Chemical Co.

Histology and immunohistochemistry

Liver specimens were routinelyp fixed by immersion in 10 % NBF for 8 h and washed overnight. The sample were dehydrated in graded ethanol solutions, cleared in xylene and embedded in paraffin. Tissue sections (6 µm thick) were mounted on slides and stained with hematoxylin-eosin (H&E) and Sirius red (SR) (Junqueira et al. 1979), and were examined immunohistochemically for α-SMA using routine indirect avidin-biotin immunolabeling. Non-immune isotype-matched immunoglobulin was used in place of the primary antibody in the negative control.

Hydroxyproline determination

Hydroxyproline was determined according to the method of Woessner (Woessner 1961). Approximately 100 mg of liver tissue was hydrolyzed in 2 ml of 6 M HCl in a sealed tube at 110 °C for 24 h. The hydrolysates were dried in a water bath set at 60 °C. The residues were dissolved in 10 ml of distilled water and filtered through 0.45 µm Millipore filter. For the determination of hydroxyproline content, 1 ml of the clear filtrate was mixed with 1 ml of freshly prepared 1.4 % chloramine-T solution and allowed to stand at room temperature for 20 min. Then, it was mixed with 1 ml of 19 % perchloric acid and incubated at room temperature for 5 min. Finally, 1 ml of freshly prepared 20 % *p*-dimethylaminobenzaldehyde in CMC was added, mixed well and placed in a water bath at 60 °C for 20 min. After cooling, absorbance was measured at 560 nm in a UV–VIS spectrophotometer.

Determination of malondialdehyde (MDA) content

MDA levels were determined by a published method, with modifications (Agarwal and Chase 2002). Standards were prepared through serial dilution of a stock solution of 10 mM TEP in 40 % ethanol. A 100 µl homogenate of the liver or TEP was heated with 1.25 ml of solution of 42 mM TBA in a buffer comprised of 0.44 M phosphoric acid and 0.05 % BHT (80:20, v/v) for 1 h in a boiling water bath. After cooling, a 1.25 ml *n*-Butanol subsequently was added to each tube for extraction of the MDA-TBA complex. The tubes were vortexed for 5 min and then centrifuged 15 min at 4,000 rpm to separate the two phases. Aliquots of 100 µl were removed from the *n*-Butanol layer of each sample and placed in HPLC vial for analysis. The MDA were quantified using a reverse-phase HPLC on a Capcell Pak 5 µ UG 120 150 × 4.6 mm column (Shisheido) placed in a column warmer set to 37 °C and the fluorescence detection at an excitation wavelength of 515 nm and emission wavelength of 553 nm with flow rate of 1.2 ml/min. with a buffered mobile phase, comprised of methanol and 50 mM potassium monobasic phosphate (anhydrous) (40:60, v/v) and adjusted to pH 6.8 with 5 M potassium hydroxide.

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

Total RNA was extracted from frozen 100 mg liver tissue using homogenizer in Trizol reagent (Invitrogen) and underwent the purification process until the ratio between the absorbance values at 260 and 280 nm gave an estimate of RNA purity. After adding to 1 µg of RNA to each PCR tube and mixing with RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas Life Science) according to the protocol provided by the manufacturer. Reverse transcription was carried out as follows: 42 °C for 60 min, 70 °C for 5 min (one cycle). cDNA was stored at −20 °C for RT-PCR. TGF-β1, COL1 and α-SMA of primers were synthesized by Bioneer (Table 1). RT-PCR was performed in 20 µl of reaction solution containing 1 µg of DNA and template the corresponding primers with Qiagen Multi PCR kit. The cycles for PCR were as follows: 1 cycle of 95 °C for 10 min for initial denaturation, 35 cycles of 30 s at 95 °C, 90 s at 60 °C, 60 s at 72 °C and a final 5 min at 72 °C. GAPDH was used as an internal control. After the PCR, the amplified products were analyzed by electrophoresis in 1.5 % agarose gel (Tebu) and visualized by ethidium bromide staining under UV light illumination. All tests were done in triplicate to ensure reproducibility.

Table 1 Oligonucleotide sequences used in RT-PCR analysis

mRNA	Sequence
TGF-β1	sense 5′-TATAGCAACAATTCCTGGCG-3′
	(antisense) 5′-TGCTGTCACAGGAGCAGTG-3′
COL1	Sense 5′-AAGAAGGCGGCAAAGGTC-3’
	(antisense) 5′-GGACCTTGTTTGCCAGGT-3′
α-SMA	Sense 5′-CCGAGATCTCACCGACTACC-3′
	(antisense) 5′-TCCAGAGCGACATAGCACAG-3′
GAPDH	Sense 5′-GACAACTTTGGCATCGTGGA-3′
	(antisense) 5′-ATGCAGGGATGATGTTCTGG-3′

TGF-β1 transforming growth factor-β1, *COL1* collagen type I, *α-SMA* α-smooth muscle actin, *GAPDH* glyceraldehy-de-3-phosphate dehydrogenas

Western blotting analyses of NF-κB p65 and α-SMA

Nuclear extracts of liver tissue were prepared as a modification of previously published methods (Ejarque-Ortiz et al. 2007). The protein concentration was determined using BCA protein assay reagent. A liquots of nuclear protein (50 µg) were denatured at 95 °C for 5 min, and proteins were resolved in 10 % SDS-PAGE, and successively transferred to a polyvinylidene fluoride membrane (Millipore). The membranes were stained with 5 % Ponceau S in 1 % acetic acid solution acid to verify efficient protein transfer. The membrane was blocked with 5 % nonfat milk solution for 1 h at room temperature and then incubated with a 1:10,000 dilution of primary antibodies against NF-κB p65, α-SMA and β-actin (Santa Cruz Biotechnology) in Tris-buffered saline Tween-20 (TBST) at 4 °C overnight, followed by 1 h at room temperature. After washing with TBST buffer solution, a 1:100,000 dilution of horseradish peroxidase-conjugated rabbit or mouse secondary antibodies (Santa Cruz Biotechnology) was applied to the membrane and then incubated with for 1 h at room temperature. After washing with TBST buffer solution, the membrane was developed with an enhanced chemiluminescence agent (GE Healthcare) for 3 min at a room temperature, and then exposed to film (KODAK BIOMAX Light). β-actin was used to confirm that an equal amount of protein was loaded in each lane. Band intensities were determined using a Gel Doc/ChemiDoc imager (Bio-Rad) and processed with Adobe Photoshop CS4 software (Adobe).

Statistical analyses

All values were expressed as the mean ± SE. Significant differences were statistically analyzed using a one-way

analysis of variance (ANOVA), followed by a non-parametric post hoc test. A p value of 0.05 or less was considered statistically significant.

Results

Free radical scavenging activity

Based on the scavenging activity of the DPPH, antioxidant activity of sinapic acid was measured (Table 2). As a positive control, Vit. C was also tested. Sinapic acid showed good DPPH free radical scavenging activities (IC_{50} = 20.6 μM) compared with Vit. C (IC_{50} = 17.8 μM)

Table 2 Effects of sinapic acid on radical scavenging activity

Substances	Concentration (μM)	Inhibition (%)	IC_{50}(μM)
Sinapic acid	5	14.8 ± 0.1	20.6
	10	24.5 ± 0.5	
	20	44.1 ± 0.4	
	40	73.1 ± 0.3	
Vit. C	5	16.6 ± 0.6	17.8
	10	35.5 ± 1.2	
	20	52.8 ± 0.8	
	40	68.9 ± 2.0	

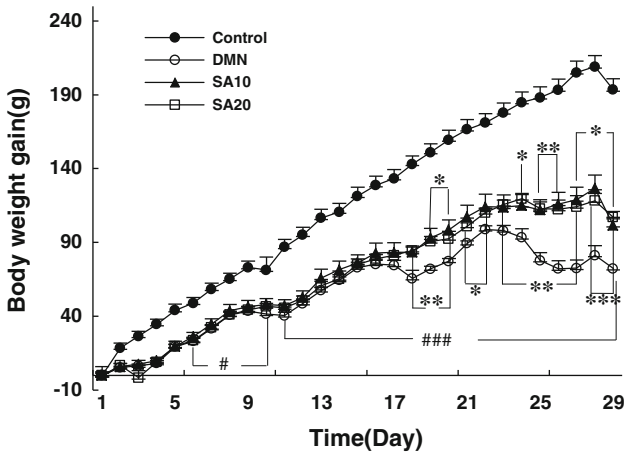

Fig. 2 Effect of sinapic acid on body weight. DMN was intraperitoneally given at a dose of 10 mg/kg on 3 consecutive days a week for 4 weeks to each group except the control group. DMN, DMN alone treated group; SA10 group, sinapic acid (10 mg/kg/day) with DMN; SA20 group, sinapic acid (20 mg/kg/day) with DMN. Values are mean ± SE of n = 6 rats/group. $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$, compared with the DMN group. $^{\#\#\#}p < 0.01$, and $^{\#}p < 0.05$ compared with the control group

Body weights

Treatment with DMN caused a significant decrease in body weights compared with the control group (Fig. 2). However, treatment of rats with sinapic acid at 10 or 20 mg/kg/day for 4 weeks prevented this decrease. In the DMN group, body weight gain for 4 weeks was only 37 % of the control group. In the SA10 and SA20 groups, the body weight loss was decreased by 53 and 55 % of the control group, respectively. There were no significant differences between doses. Thus, oral administration of sinapic acid can prevent DMN-induced loss of body weights.

Serum parameters of liver function

Biochemical analyses of ALT and AST in serum were performed to verify the role of sinapic acid in protection of the liver from injury (Fig. 3a, b). Compared with those in the control group (14.8 ± 1.1 U/l and 58.1 ± 9.0 U/l), the activities of serum ALT and AST were significantly higher in the DMN group (116.6 ± 29.1 U/l and 174.1 ± 34.6 U/l). The activities of serum ALT and AST were significantly reduced by oral administration of sinapic acid; those in the SA10 group (47.8 ± 5.6 U/l and 95.1 ± 15.5 U/l); in the SA20 group (43.9 ± 5.7 U/l and 92.8 ± 12.8 U/l), respectively. There were no significant differences between doses.

Histopathology and immunohistochemistry

The effect of sinapic acid on DMN-induced hepatic fibrosis was evaluated by histopathological examination of the liver sections with H&E staining. No histological abnormalities were observed in the control group (Fig. 4A1). The administration of DMN for 4 weeks disrupted normal tissue architecture and caused centrilobular necrosis in the hepatic sinusoid (Fig. 4A2). In contrast, these alterations, and specially the necrotic area, were reduced in the liver sections of the SA10 and SA20 groups in the presence of sinapic acid (Fig. 4A3, A4). SR stain revealed a marked presence of collagen fibers (stained in red) in the portal triad of the liver. With the exception of the portal area, there were almost no collagen fibers in the liver sections taken from the control group (Fig. 4B1). The liver of the DMN group exhibited an increase in collagen content (Fig. 4B2). However, in the sinapic acid groups (SA10, SA20), the increase in collagen content was markedly reduced (Fig. 4B3, B4). The expression of α-SMA, a specific marker for activated HSCs, was detected by immunohistochemistry. Compared with the control group (Fig. 4C1), more α-SMA-positive cells were detected around the periportal fibrotic band areas and were scattered in the regions of connective tissue septa in the DMN-treated rats (Fig. 3C2). However, sinapic acid prevented the activation of most HSCs. As a result, very few

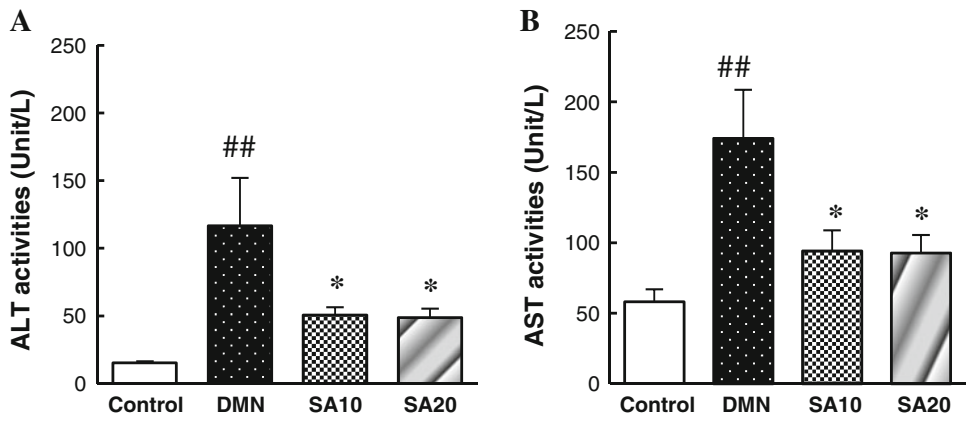

Fig. 3 Measurements of serum **a** ALT and **b** AST levels. Groups are as described in "Methods". Values are mean ± SE of n = 6 rats/group. $^{*}p < 0.05$ compared with the DMN group, and $^{\#\#}p < 0.01$ compared with the control group

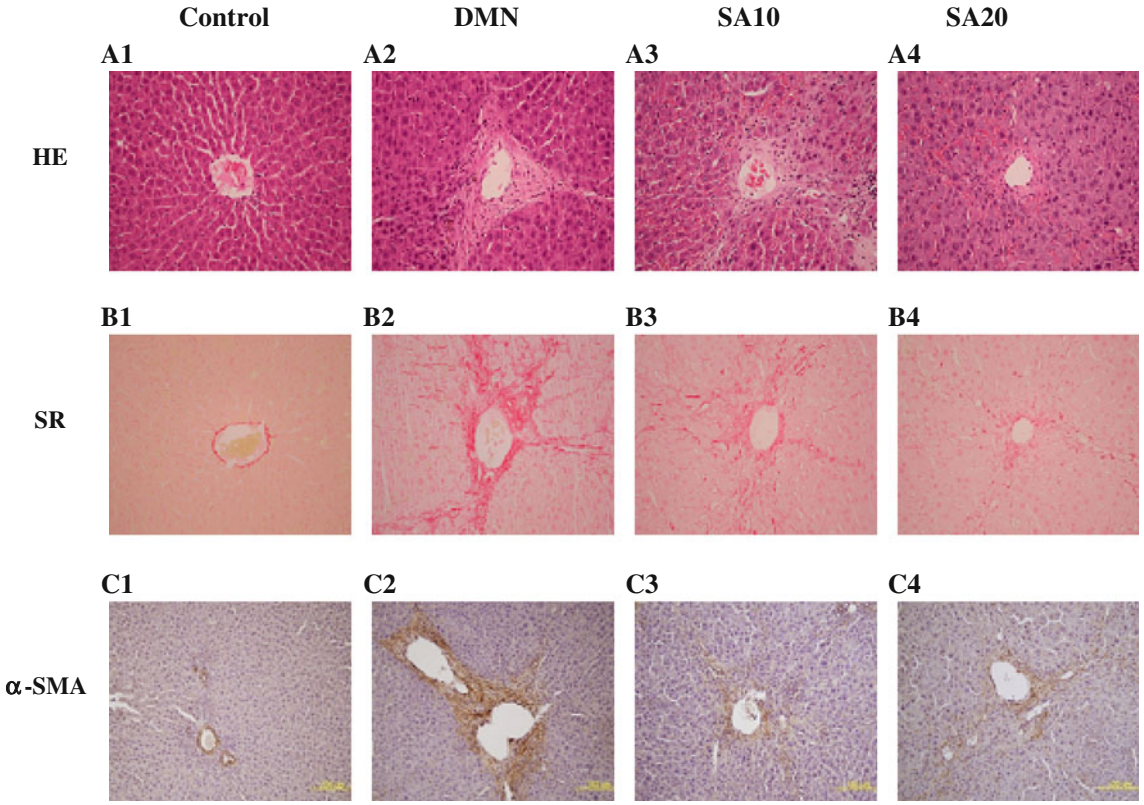

Fig. 4 Histopathological analyses of rat liver sections. Liver samples were taken from the non-treated rats (control group), the rats treated with DMN and rats treated with DMN and sinapic acid. Groups are as described in "Methods". The sections were stained using hematoxylin-eosin, sirius red (SR) and α-SMA. (**A***1*) The control livers show normal lobular architecture with central veins and radiating hepatic cords; (*2*) DMN alone-induced group; (*3*, *4*) DMN with sinapic acid-treated group (10 mg/kg; 20 mg/kg p.o.). Activated HSC were detected by immunohistochemistry with α-SMA antibody (α-SMA). All images are original magnification X400

α-SMA positive cells were detected. The level of α-SMA expression was almost as the same as in the liver of the control group (Fig. 4C3, C4).

Hepatic hydroxyproline and lipid peroxidation

Liver fibrosis was evaluated by measurement of hepatic hydroxyproline (Fig. 5a). DMN administration increased hepatic hydroxyproline content (101.66 ± 8.23 μg/100 mg liver, 195 % of control) compared with the control group (55.64 ± 3.32 μg/100 mg liver), whereas sinapic acid treatment attenuated the DMN-induced rise in hepatic hydroxyproline content (64.35 ± 4.88 and 58.32 ± 5.51 μg/100 mg, 116 and 105 % of the control group) in the SA10 and SA20 groups, respectively. Administration of sinapic acid inhibited the production of hydroxyproline, resulting in 84 and 95 % protection of the DMN group in the SA10 and SA20 groups, respectively.

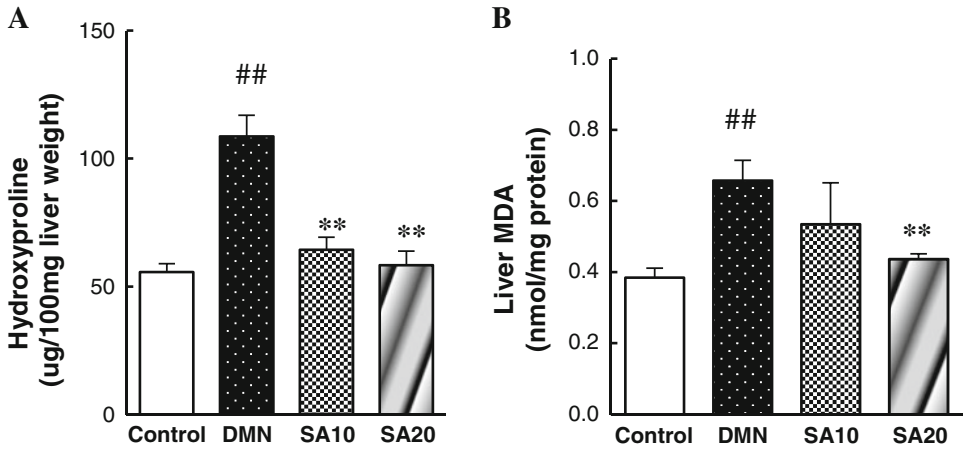

Fig. 5 Effects of sinapic acid on the hydroxyproline (**a**) and the MDA content (**b**) in the liver of rats treated with DMN. Values are mean ± SE of n = 6 rats/group. $^{**}p < 0.01$ compared with the DMN group, and $^{\#\#}p < 0.01$ compared with the control group

Lipid peroxidation

The lipid peroxidation was measured by MDA determination in liver homogenates (Fig. 5b). Compared with the control group (0.38 ± 0.03 nmole/mg protein), the MDA content in the DMN group (0.66 ± 0.06 nmole/mg protein) was significantly higher. The liver MDA content was significantly reduced in rats treated with sinapic acid (0.53 ± 0.12 nmole/mg protein and 0.44 ± 0.02 nmole/mg protein in the SA10 and SA20 groups, respectively). These results indicate that sinapic acid inhibits lipid peroxidation on DMN-induced liver damage.

The analyses of mRNA expressions of TGF-β1, COL1 and α-SMA

We next quantified the mRNA expression level of TGF-β1, COL1 and α-SMA by RT-PCR (Fig. 6a), normalized the data to the corresponding GAPDH values and expressed the results relative to the control group (Fig. 6b). DMN treatment increased the expression of TGF-β1 and COL1, but sinapic acid administration abrogated this effect. In addition, the expression of α-SMA in the SA10 and SA20 groups treated with sinapic acid was also decreased compared that in the DMN group, consistent with the results of α-SMA immunohistochemistry.

Western blotting analyses of NF-κB p65 and α-SMA

The expression of nuclear NF-κB p65 and cytosolic α-SMA was measured to evaluate the effect of sinapic acid on DMN-induced liver fibrosis (Fig. 7a). Values were expressed as the relative levels of NF-κB p65 and α-SMA over the control group on the base of average ratios of p65/β-actin and α-SMA/β-actin (Fig. 7b), respectively. The expression of NF-κB p65 was markedly increased in the DMN group, whereas its expression was slightly reduced in sinapic acid-treated groups. Similar to NF-κB p65, the expression of cytoplasmic α-SMA in the sinapic acid-treated groups also significantly decreased, consistent with

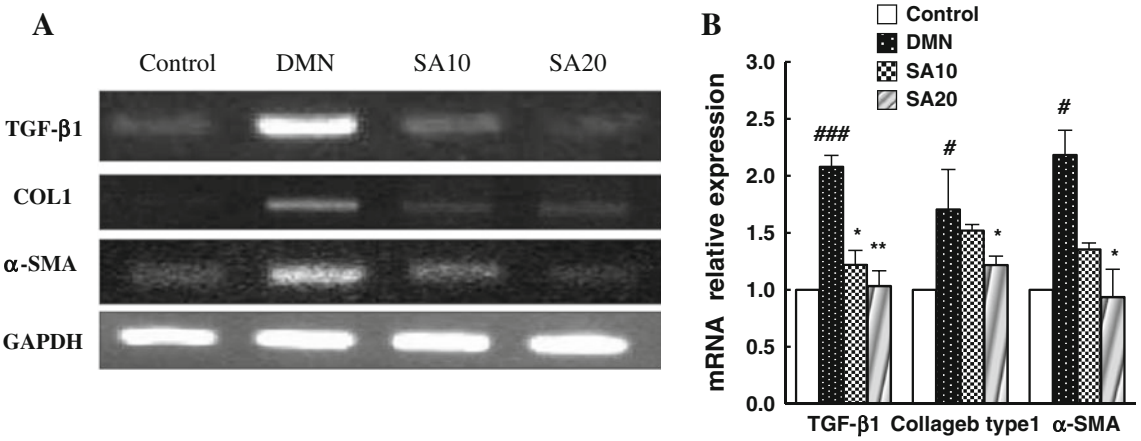

Fig. 6 **a** RT-PCR analysis of TGF-β1, COL1 and α-SMA mRNA expressed in the livers of control and DMN-treated rats with or without sinapic acid treatment. **b** Quantification of TGF-β1, COL1 and α-SMA mRNA after normalization against GAPDH levels. Data are the mean ± SE from 3 replicate experiments. $^{**}p < 0.01$, and $^{*}p < 0.05$ compared with the DMN-treated group. $^{\#\#\#}p < 0.001$ and $^{\#}p < 0.05$ compared with the control group

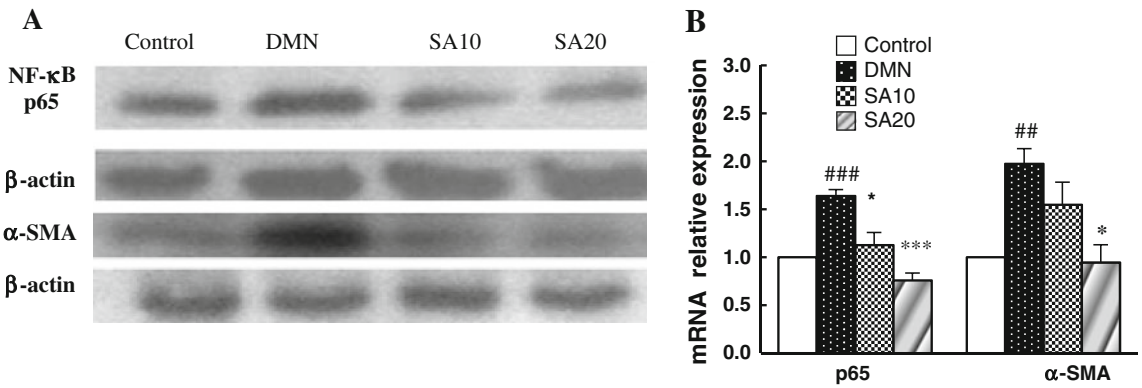

Fig. 7 **a** Western blot of NF-κB p65 and α-SMA in the livers of control and DMN-treated rats with or without sinapic acid treatment. **b** Quantification of NF-κB and α-SMA expression after normalization against β-actin levels. Data are the mean ± SD from 3 replicate experiments. $^{***}p < 0.001$ and $^{*}p < 0.05$ compared with the DMN-treated group. $^{###}p < 0.001$, and $^{##}p < 0.01$ compared with the control group

results of immunohistochemical staining and RT-PCR analysis of α-SMA.

Discussion

Various types of chronic liver injury including viral, metabolic and toxic stress, can cause liver fibrosis. Regardless of the source of injury, liver fibrosis is characterized by extensive deposition of ECM and accompanied by hepatocelluar necrosis and inflammation. In the initial stage of inflammation, injured hepatocytes, Kupffer cells, platelets, and leukocytes are recruited and involved in the response. As a result, reactive oxygen species (ROS) and inflammatory mediators, including platelet-derived growth factor (PDGF), TGF-β, tumor necrosis factor-alpha (TNF-α) and connective tissue growth factor are released. These factors likely act as paracrine mediators and activate quiescent HSCs localized in the perisinusoidal space, resulting in abnormal deposition of ECM (Wu and Zern 2000).

Kupffer cells (KCs), the resident macrophages of the liver, carry out an important function in modulating inflammation and are involved in the initiation of liver fibrosis. In response to liver damage, KCs produce a variety of proinflammatory cytokines such as TNF-α and monocyte chemoattractant proteins that stimulate the activation of HSCs. This leads to the production of ECM protein, in particular collagen I, and subsequently contributes to hepatic injury. Secretion of proinflammatory cytokines and the modulation of HSC activity by KCs has been shown in vitro but not in vivo (Nieto 2006). It has previously been reported that suppression of macrophage infiltration inhibits the activation of HSCs and liver fibrogenesis in rats (Imamura et al. 2006).

During liver injury, damaged hepatocytes, activated KCs, activated HSCs, and neutrophils secrete ROS that cause oxidative stress (Bataller and Brenner 2005; Svegliati-Baroni et al. 2001). Oxidative stress is one of the major causes of liver damage due to stimulation of lipid peroxidation, and is reported to be associated with HSC activation (Friedman 2008). There is sufficient evidence suggesting that lipid peroxidation can occur during both acute and chronic liver injury (Chen et al. 2001). This may compromise the integrity of membranes and cause reactive intermediates to bind covalently to important biological molecules such as glutathione (Muriel 1997). Recent studies have shown that antioxidants such as resveratrol, quercetin, N-acetylcysteine can inhibit HSC activation (Kawada et al. 1998). Silymarin is a natural flavonoid component that exhibits antifibrotic activity in an experimental liver injury model (Boigk et al. 1997). Our previous studies have shown that that resveratrol and proanthocyanidins exert antifibrotic activities in DMN-induced liver fibrosis (Lee et al. 2010; Shin et al. 2010).

In the present study, we investigated the hepatoprotective effect of sinapic acid on DMN-induced chronic liver injury on the base of various parameters including AST and ALT. MDA and thiobarbituric reactivity are indices of lipid peroxidation, which is also associated with liver injury (Janero 1990). We found that sinapic acid exerts protective effects on DMN-induced hepatic fibrosis in rats. The results showed that the levels of MDA, AST and ALT, markedly increased by DMN, were significantly attenuated upon treatment with sinapic acid. Histopathological examination also showed less necrotic areas of liver tissue following treatment with sinapic acid. These observations indicate sinapic acid has potent scavenger abilities and acts to protect hepatocytes from oxidative stress-induced iver injury by reducing lipid peroxidation.

TGF-β1 is a key mediator of the transformation of HSCs to myofibroblast-like cells that express the characteristic markers α-SMA and COL1. A role for TGF-β1 in

perpetuating rather than initiating HSC activation has been established by examining the behavior of stellate cells in TGF-β1 knockout mice undergoing acute liver injury (Friedman 2000). These animals have markedly reduced collagen accumulation in response to liver injury as expected but still have increased α-SMA, indicative of stellate cell activation (Hellerbrand et al. 1999).

We focused on the antifibrotic effects of sinapic acid by measuring hydroxyproline, SR staining for collagen content, and immunohistochemical detection of α-SMA. Additionally, we performed molecular profiling to provide an indication of the mechanism of action of sinapic acid with regard to its antifibrotic activities. To this end, we monitored fibrosis-related molecules including TGF-β1, COL1 and α-SMA. In addition, we performed western blot analyses of NF-κB P65 and α-SMA to assess the effect of sinapic acid on HSC activation.

In relation to the change of ECM components, sinapic acid reduced the collagen, hepatic hydroxyproline contents, and tissue distribution of α-SMA induced by DMN. At the molecular level, sinapic acid reduced the expression of COL1, one of the main components of accumulated ECM, and decreased the expression of TGF-β1, a major factor that stimulates HSCs to produce ECM. Consistent with a role for sinapic acid in the inhibition of HSC activation, western blot results showed that sinapic acid suppressed DMN-induced α-SMA production. This was also confirmed by RT-PCR and immunohistochemistry results. NF-κB activation has been reported to regulate the development of liver fibrosis. Studies have documented the hepatoprotective effects of natural antioxidants, such as curcumin (Reyes-Gordillo et al. 2007), caffeic acid (Natarajan et al. 1996) and gomicine (Ryutaro et al. 2012), 1-alpha-tocopherol and butylated hydroxytoluene (Okazaki et al. 2000). These compounds likely act via suppression of NF-κB p65, reduction of proinflammatory cytokine production and prevention of oxidative stress. Reyes-Gordillo reported that acute hepatotoxicity induced by CCl_4 leads to increased NF-κB activation and production of downstream cytokines including TNF-α, IL-1β and IL-6 (Reyes-Gordillo et al. 2007). In the DMN-induced hepatic fibrosis in vivo model, sinapic acid significantly suppressed the expression of nuclear NF-κB p65. In addition to the clear reduction of NF-κB activation, TGF-β1 expression and the expression of various fibrotic factors, such as α-SMA and COL1, decreased. Since the DPPH assay indicated that sinapic acid has ROS scavenging activity, we infer that inhibition of NF-κB activation by sinapic acid may be related to ROS scavenging activity. This, in turn would suppress NF-κB p65, leading to HSC inactivation and subsequent reduction of fibrogenesis. In our results, we observed that the inactivation of NF-κB p65 by sinapic acid was associated with TGF-β down-regulation, although we do not currently understand the underlying mechanism for this. However, the suppression of NF-κB activation may represent a target for the prevention and treatment of liver fibrosis (Muriel 2009).

In summary, we have demonstrated that sinapic acid exhibited hepatoprotective and antifibrotic effects against DMN-induced fibrosis using an in vivo model. This is most likely explained by the antioxidant activity of sinapic acid and its capacity to suppress NF-κB p65 as well as TGF-β1. Natural antioxidants have attracted increasing attention for use as potential therapeutic agents. Our data suggest that sinapic acid now joins this class, and may be useful in preventing the development of hepatic fibrosis.

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