

# 2-(Allylthio)pyrazine, a Cancer Chemopreventive Agent, Inhibits Liver Fibrosis Induced by Dimethylnitrosamine in Rats: Role of Inhibition of Transforming Growth Factor- $\beta$ 1 Expression

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**Abstract:** Exposure to nitrosamines may be the occupational risk factor for liver cirrhosis. 2-(Allylthio)pyrazine, a chemopreventive agent, inhibits CYP2E1 and induces phase II enzymes. We examined the effects of 2-(allylthio)pyrazine on hepatic fibrosis, a prepathologic state of cirrhosis, and on the expression of transforming growth factor- $\beta$ 1 induced by dimethylnitrosamine. Treatment of rats with dimethylnitrosamine for 4 weeks increased plasma alanine/aspartate aminotransferase and  $\gamma$ -glutamyl transpeptidase activities, and bilirubin content, whereas the total plasma protein and albumin levels were decreased. 2-(Allylthio)pyrazine inhibited dimethylnitrosamine-induced increases in the enzyme activities and bilirubin, and restored the plasma protein and albumin contents. Masson's trichrome staining showed that dimethylnitrosamine induced liver fibrosis, the extent of which was reduced by 2-(allylthio)pyrazine treatments. Reverse transcription-polymerase chain reaction analysis revealed that 2-(allylthio)pyrazine inhibited production of transforming growth factor- $\beta$ 1 mRNA by dimethylnitrosamine. These results demonstrated that 2-(allylthio)pyrazine might inhibit dimethylnitrosamine-induced liver fibrosis due to suppression of CYP2E1 expression and transforming growth factor- $\beta$ 1 production.

2-(Allylthio)pyrazine has been studied as a cancer chemopreventive agent active against a variety of chemical carcinogens including vinylcarbamate, azoxymethane, benzopyrene and aflatoxin B1 (Kim & Kim 1999). Formation of aflatoxin B1-induced preneoplastic foci is significantly inhibited by 2-(allylthio)pyrazine in the rat liver (Ha *et al.* 1999). 2-(Allylthio)pyrazine also effectively suppresses the liver injury induced by toxicants including acetaminophen, isoniazid and carbon tetrachloride (Kim *et al.* 1997a). Chemopreventive and hepatoprotective effects of 2-(allylthio)pyrazine may result from the inhibition of cytochrome P450 2E1 (an alcohol-inducible form of P450, CYP2E1) expression and the metabolic activity, and from the induction of phase II detoxifying enzymes such as glutathione S-transferases (Kim & Kim 1999; Kim *et al.* 1999).

Epidemiological studies have shown that rubber workers died excessively from liver cirrhosis, which might be associated with the occupational risk factors such as exposure to N-nitroso compounds (Straif *et al.* 1999). Case reports and animal data raised the possibility that exposure to nitrosamines may be a risk factor for incidence of liver cirrhosis and the resultant mortality (Chao *et al.* 1995; Park & Mirer 1996; Straif *et al.* 1999). Nitrosamine, in particular dimethylnitrosamine, is bioactivated by the catalysis of

CYP2E1 (Peng & Yang 1982; Koop 1992). Hence, CYP2E1 may be an important target for the prevention of liver cirrhosis inducible by N-nitroso compounds.

Cirrhosis plays a role in the carcinogenesis of several types of cancer. In particular, the risk of hepatocellular carcinoma was substantially increased in the patients with liver cirrhosis (Sørensen *et al.* 1998). Hepatic fibrosis is a prepathologic state of cirrhosis that occurs as a consequence of severe liver damage in diverse chronic liver diseases. The prevention of hepatic fibrosis may serve as an important potential target for chemoprevention (Sakaida *et al.* 1994). Fibrosis arises from overproduction of extracellular matrix (e.g. type I, III and IV collagens) as a result of activation of hepatic nonparenchymal cells including Kupffer cells and stellate cells (Pinzani *et al.* 1998). Activation of hepatic stellate cells in the fibrotic liver causes morphological changes in myofibroblast-like cells, which is accompanied by synthesis of large quantities of extracellular matrix (Savolainen *et al.* 1988; Freidman 1993).

Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is involved in the regulation of cell growth and differentiation. TGF- $\beta$ 1 appeared to play a role in fibrogenesis after liver injuries (Cheever *et al.* 1998; Kimura *et al.* 1999), although the complex pathogenesis of hepatic fibrosis is poorly understood yet. TGF- $\beta$ 1, as a key fibrogenic mediator, can directly enhance deposition of extracellular matrix and inhibit collagenase activity in the liver (Freidman 1993). Treatment of rats with the adenoviral vector expressing the mutant TGF-

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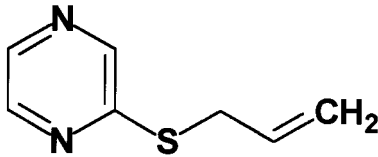


Fig. 1. Chemical structure of 2-(allylthio)pyrazine.

$\beta$ 1 receptor completely inhibited the process of hepatic fibrosis induced by DMN (Qi *et al.* 1999). Conversely, the transgenic mice overexpressing TGF- $\beta$ 1 developed severe hepatic fibrosis (Clouthier *et al.* 1997). Hence, inhibition of TGF- $\beta$ 1 production may serve as the potential therapeutic approach against liver fibrogenesis. Considering the chemoprevention and efficient liver protection by 2-(allylthio)pyrazine as an inhibitor of CYP2E1 against chemical carcinogens and toxicants, we determined the antifibrotic effect of 2-(allylthio)pyrazine in rats treated with dimethylnitrosamine activated primarily by CYP2E1. The role of TGF- $\beta$ 1 expression was further investigated as part of mechanistic studies.

### Materials and methods

**Materials.** 2-(Allylthio)pyrazine was obtained from Yuhan Research Center (Gunpo, Korea). Chemical structure of the agent is shown in fig. 1. The purity of the compound was greater than 99%, as assayed by high-performance liquid chromatography. Reverse transcriptase and *Taq.* polymerase were obtained from Takara Co. (Tokyo, Japan). Dimethylnitrosamine and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

**Animals.** Animal studies were conducted in accordance with the institutional guidelines for care and use of laboratory animals. Male Sprague-Dawley rats at 6 weeks of age (140–160 g) were supplied from Korea Food and Drug Administration (Seoul, Korea) and maintained under 12 hr light and dark cycles in an air-conditioned room with commercially-available rat chow (Purina, Korea) and water available *ad libitum*. Dimethylnitrosamine (10  $\mu$ l/kg) was intraperitoneally injected, as dissolved in sterile saline (10  $\mu$ l/kg body weight) 3 times a week for 4 weeks, as described previously (Ala-Kokko *et al.* 1987; Tsukamoto *et al.* 1990). Data were obtained from two groups of dimethylnitrosamine-treated animals (N=16 animals). Control animals received vehicle. 2-(Allylthio)pyrazine was orally administered 24 hr after each treatment with dimethylnitrosamine (3 times per week for 4 weeks). Animals were sacrificed on day 28 under light anaesthesia with diethyl ether. Blood was collected from *vena cava*. The left lateral lobe was sub-

Table 2.

Total plasma protein and albumin contents.

Treatment	Total plasma proteins (g/dl)	Plasma albumin (g/l)
Untreated	7.4 $\pm$ 0.2	4.1 $\pm$ 0.2
2-AP	7.3 $\pm$ 0.5	3.9 $\pm$ 0.2
DMN	5.7 $\pm$ 0.1*	3.0 $\pm$ 0.1*
DMN + 2-AP	6.9 $\pm$ 0.3 <sup>†</sup>	3.8 $\pm$ 0.1 <sup>†</sup>

Animals were treated as described in table 1. The values are mean $\pm$ S.E. (N=8–16). One way analysis of variance was used for comparisons of multiple group means, followed by Newman-Keuls test. Comparison with untreated control (\* P<0.05) or with DMN alone (<sup>†</sup> P<0.01).

jected to histopathological examinations. For determination of TGF- $\beta$ 1 mRNA level, animals were gavaged with 2-(allylthio)pyrazine at the dose of 100 mg/kg 18 hr after a dimethylnitrosamine injection (10  $\mu$ l/kg) and killed at 24 hr after 2-(allylthio)pyrazine treatment.

**Blood chemistry.** Alanine aminotransferase (ALT), aspartate aminotransferase (AST),  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GT), bilirubin, total protein and albumin in the plasma were analyzed using an automatic blood chemistry analyzer, Selectra II (Merck, Darmstadt, Germany).

**Histopathology.** Hepatic morphology was assessed by light microscopy. Left lateral lobe of the liver was sliced (3 slices per rat) and tissue slices were fixed in 10%-buffered neutral formalin for 6 hr. Fixed liver tissue slices were processed and embedded in a paraplast automatic tissue processor, Citadel 2000 (Shandon Scientific, Cheshire, U.K.). Sections of 4  $\mu$ m in thickness were subjected to haematoxylin and eosin and Masson's trichrome staining prior to examinations. A certified pathologist scored samples in a blinded fashion. Extents of fibrosis were graded as 0=no increase; 1=slight increase; 2=moderate increase; 3=distinct increase; 4=severe increase. Extents of periportal bridging, intralobular degeneration, portal inflammation and fibrosis were also graded according to the Knodell's scoring method (Moragas *et al.* 1998).

**Reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA (0.5  $\mu$ g) obtained from the liver was reverse-transcribed using an oligo(dT) adaptor as a primer to produce cDNAs. PCR was performed using the selective primers for TGF- $\beta$ 1 (sense primer: 5'-CTT CAG CTC CAC AGA GAA GAA CTG C-3', antisense primer: 5'-CAC GAT CAT GTT GGA CAA CTG CTC C-3') (298 bp) and glyceraldehyde-3-phosphate dehydrogenase genes (sense: 5'-TCG TGG AGT CTA CTG GCG T-3', antisense: 5'-GCC TGC TTC ACC ACC TTC T-3') (510 bp). PCRs were carried out for 34, 36 and 38 cycles using the following conditions: denaturation at 94

Table 1.

Plasma aminotransferase and  $\gamma$ -glutamyl transpeptidase activities, and total bilirubin content.

Treatment	ALT (units/l)	AST (units/l)	$\gamma$ -GT (units/l)	Bilirubin (mg/dl)
Untreated	49 $\pm$ 2	113 $\pm$ 6	0.2 $\pm$ 0.1	0.15 $\pm$ 0.01
2-AP	52 $\pm$ 5	147 $\pm$ 19	0.1 $\pm$ 0.1	0.17 $\pm$ 0.04
DMN	190 $\pm$ 12**	412 $\pm$ 39**	12.1 $\pm$ 4.1**	0.93 $\pm$ 0.15**
DMN+2-AP	133 $\pm$ 11 <sup>†</sup>	231 $\pm$ 16 <sup>‡</sup>	4.7 $\pm$ 1.1 <sup>‡</sup>	0.20 $\pm$ 0.02 <sup>‡</sup>

Dimethylnitrosamine (DMN) (10  $\mu$ l/kg) was intraperitoneally injected, as dissolved in sterile saline (10  $\mu$ l/kg body weight) 3 times a week for 4 weeks. 2-(Allylthio)pyrazine (2-AP) (50 mg/kg) was orally administered 24 hr after each DMN treatment (3 times a week for 4 weeks). The values are mean $\pm$ S.E. (N=8–16). One way analysis of variance was used for comparisons of multiple group means, followed by Newman-Keuls test. Comparison with untreated control (\*\* P<0.01) or with DMN alone (<sup>†</sup> P<0.05; <sup>‡</sup> P<0.01).

° for 0.5 min., annealing at 49° for 0.5 min., and elongation at 72° for 1 min. Band intensities of the amplified DNAs were compared after visualization on a UV transilluminator.

**Enzyme-linked immunosorbent assay (ELISA).** Blood was collected from retroorbita of rats 1 hr after an lipopolysaccharide (LPS) injection and the plasma was used for the assay of TNF- $\alpha$ . 2-(Allylthio)pyrazine (50–100 mg/kg) was orally administered 2 hr prior to an LPS injection (1  $\mu$ g/kg, intravenously). The TNF- $\alpha$  level was measured by ELISA using rabbit anti-rat TNF- $\alpha$  antibody and biotinylated secondary antibody (Endogen, Woburn, MA, USA).

**Data analysis.** One-way analysis of variance procedures were used

to assess significant differences among treatment groups. For each significant effect of treatment, the Newman-Keuls test was used for comparison of multiple group means. The criterion for statistical significance was set at  $P < 0.05$  or  $P < 0.01$ .

## Results

### *Hepatoprotective effects of 2-(allylthio)pyrazine against dimethylnitrosamine.*

Dimethylnitrosamine is toxic to hepatocytes and induces coagulative necrosis and eventually fibrosis in the liver.

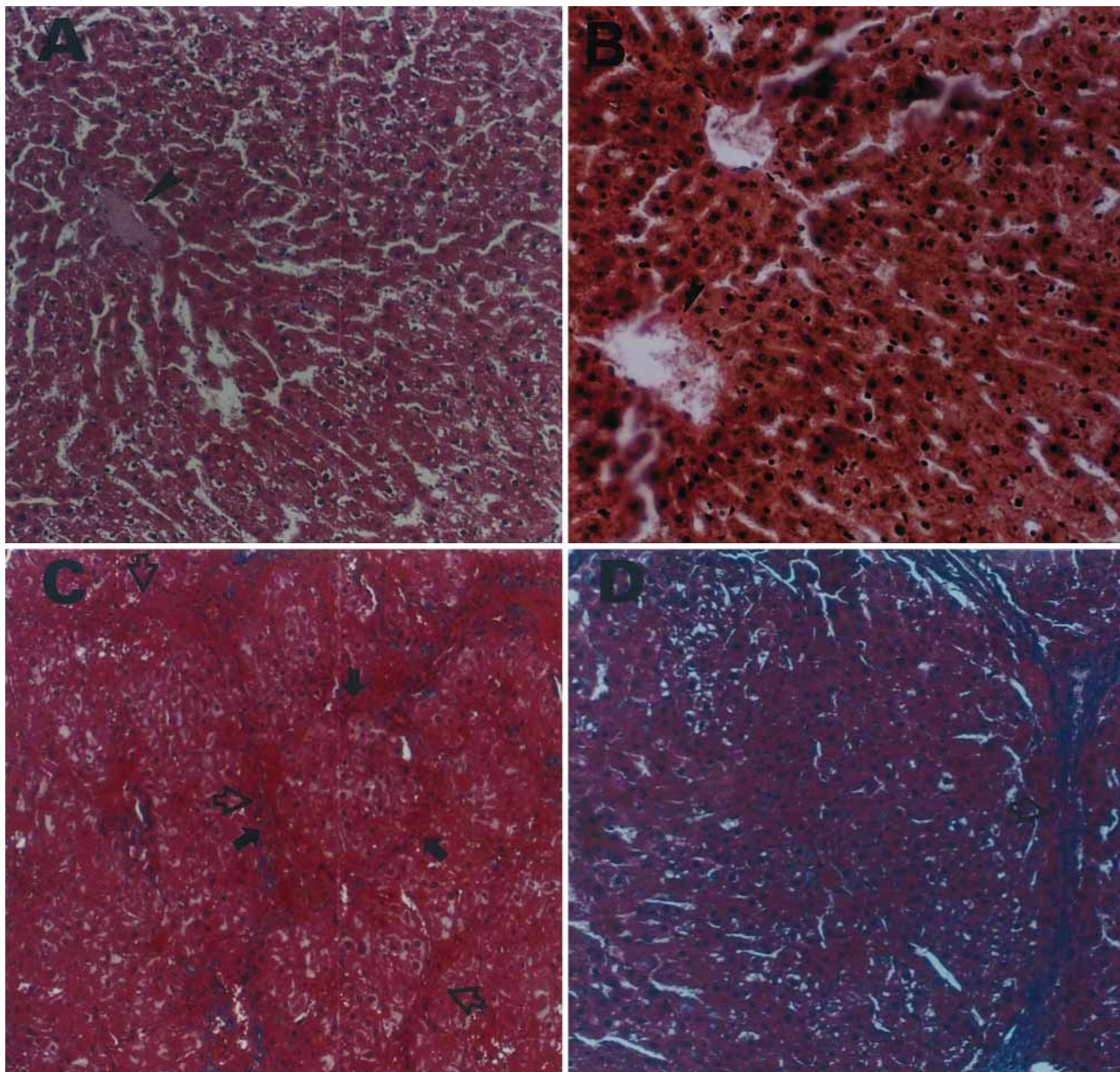


Fig. 2. Masson's trichrome stainings of liver sections. Shown above are from rats treated with vehicle (A, magnification  $\times 100$ ), 2-(allylthio)pyrazine (2-AP) (50 mg/kg, 3 times a week for 4 weeks) (B, magnification  $\times 200$ ), dimethylnitrosamine (DMN) (10  $\mu$ l/kg, 3 times a week for 4 weeks) (C, magnification  $\times 100$ ), or 2-AP+DMN (D, magnification  $\times 100$ ). A. Low power view of normal control liver section showing no pathological changes; B. Low power view of liver section showing well preserved central vein; C. Low power view of liver section showing multinodular appearance surrounded by thin fibrous band; D. Low power view of liver section showing infrequent nodular formation separated by thin fibrous band.  $\blacktriangleright$ , central vein;  $\diamond$ , fibrosis region;  $\blacklozenge$ , cirrhotic nodule.

Hepatotoxicity by dimethylnitrosamine depends on the formation of toxic metabolite(s) through oxidative metabolism by CYP2E1 (Peng & Yang 1982). In the present study, we determined the effects of 2-(allylthio)pyrazine on the liver fibrosis induced by dimethylnitrosamine. The body weights of rats were decreased to 55% of control rats after 4 weeks of dimethylnitrosamine treatments ( $208 \pm 8$  g versus  $114 \pm 10$  g, mean  $\pm$  SE,  $n=8-16$ ,  $P<0.01$ ). Concomitant 2-(allylthio)pyrazine treatments (50 mg/kg body weight, 3 times a week for 4 weeks) partially restored the body weight gain altered by dimethylnitrosamine ( $114 \pm 10$  g versus  $161 \pm 4$ , mean  $\pm$  S.E.,  $n=8-16$ ,  $P<0.01$ ).

Activities of the plasma ALT and AST were increased 4 times in rats after dimethylnitrosamine treatment for 4 weeks, as compared to control, resulting in 200 and 400 units per ml plasma (table 1). Concomitant treatment of rats with 2-(allylthio)pyrazine at a dose of 50 mg/kg body weight (3 times a week for 4 weeks) resulted in 35%–40% decreases in the plasma aminotransferase activities. 2-(Allylthio)pyrazine at a dose of 5 or 15 mg/kg was only marginally active (data not shown). The plasma  $\gamma$ -GT activity and total bilirubin content were assessed as representative indices for the liver function. Dimethylnitrosamine-induced increase in  $\gamma$ -GT activity was 60% suppressed by 2-(allylthio)pyrazine treatment at a dose of 50 mg/kg body weight (table 1). 2-(Allylthio)pyrazine almost completely prevented an increase in the total bilirubin content by dimethylnitrosamine (table 1). Treatments with dimethylnitrosamine caused ~20% decreases in the total plasma protein and albumin contents, which were restored by 2-(allylthio)pyrazine treatment (table 2). 2-(Allylthio)pyrazine alone caused a slight increase in ALT activity, which was not statistically significant. This was consistent with no notable changes in ALT,  $\gamma$ -GT and bilirubin levels.

#### Histopathological analysis.

The extent of liver fibrosis was histopathologically examined after 4 weeks of dimethylnitrosamine treatments. Treatment with vehicle or 2-(allylthio)pyrazine (50 mg/kg, 3 times

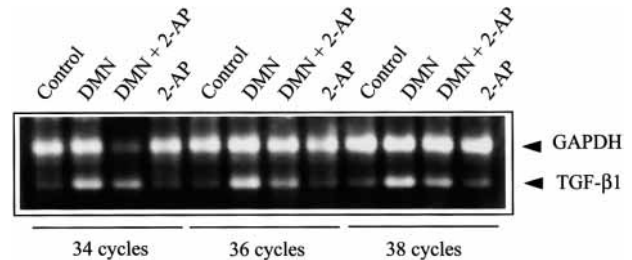


Fig. 3. Semi-quantitative duplex RT-PCR analysis of TGF- $\beta$ 1 mRNA in the livers of rats treated with DMN. Animals were gavaged with 2-(allylthio)pyrazine (2-AP) (100 mg/kg) 18 hr after a DMN injection (10  $\mu$ l/kg) and killed 24 hr after 2-AP treatment for the preparation of total RNA. TGF- $\beta$ 1 expression was assessed by RT-PCR with 34, 36 or 38 cycles as described in methods section. Four individual animals were used to confirm the data.

a week for 4 weeks) caused no observable changes in liver morphology (fig. 2A and 2B). Rats treated with dimethylnitrosamine exhibited distinct fibrosis in the liver (fig. 2C). Masson's trichrome staining, which was used to assess extracellular matrix, revealed that rats treated with dimethylnitrosamine exhibited cirrhotic liver morphology such as multiple fibrotic nodules. 2-(Allylthio)pyrazine decreased intensities of liver fibrotic nodules inducible by dimethylnitrosamine. The extent of fibrosis was also reduced by 2-(allylthio)pyrazine (fig. 2D). However, liver fibrosis surrounding the ascini was still observed with mild portal inflammation (fig. 2D). Multiple analysis confirmed that the extent of liver fibrosis was significantly reduced by 2-(allylthio)pyrazine, as compared to that by dimethylnitrosamine alone (i.e. fibrosis score: 3.7 versus 2.9; Knodell score: 16.7 versus 9.3) (table 3).

#### Effect of 2-(allylthio)pyrazine on the production of TGF- $\beta$ 1 mRNA.

Expression of hepatic TGF- $\beta$ 1 mRNA was assessed by RT-PCR analysis. Treatment of rats with dimethylnitrosamine at the dose of 10  $\mu$ l/kg increased the level of TGF- $\beta$ 1 mRNA in 2 days. 2-(Allylthio)pyrazine treatment at the dose of 100 mg/kg 18 hr following a single dose of dimethylnitrosamine injection prevented the production of TGF- $\beta$ 1 mRNA in the liver (fig. 3). For the study of TGF- $\beta$  expression, dimethylnitrosamine was administered to rats prior to 2-(allylthio)pyrazine treatment to minimize the inhibition of CYP2E1-mediated dimethylnitrosamine activation by 2-(allylthio)pyrazine. The levels of glyceraldehyde-3-phosphate dehydrogenase mRNA, which were used for comparative purposes, were comparable among the samples. 2-(Allylthio)pyrazine at a single dose of 50 mg/kg minimally reduced TGF- $\beta$ 1 mRNA (data not shown). Data indicated that 2-(allylthio)pyrazine inhibited TGF- $\beta$ 1 expression by dimethylnitrosamine in the liver, which was in agreement with the reduction in fibrosis.

#### Effect of 2-(allylthio)pyrazine on TNF- $\alpha$ production.

The effect of 2-(allylthio)pyrazine on the plasma TNF- $\alpha$  level in rats treated with LPS was assessed by enzyme-linked

Table 3.

Extent of fibrosis after treatments with DMN in combination with 2-AP.

Treatment	Fibrosis score	Knodell score
Untreated	0	0
2-AP	0	0
DMN	$3.7 \pm 0.5$	$16.1 \pm 2.9$
DMN+2-AP	$2.9 \pm 0.9^*$	$9.3 \pm 2.4^{**}$

Animals were treated as described in table 1. The values represent the mean  $\pm$  S.D. ( $N=8-16$ ). One way analysis of variance was used for comparisons of multiple group means, followed by Newman-Keuls test (significant as compared to DMN alone, \*  $P<0.05$ , \*\*  $P<0.01$ ). Extents of fibrosis were graded as 0=no increase; 1=slight increase; 2=moderate increase; 3=distinct increase; 4=severe increase. Extents of periportal bridging (severe=10), intralobular degeneration (severe=4), portal inflammation (severe=4) and fibrosis (severe=4) were graded, and the scores were combined according to the Knodell's scoring method.

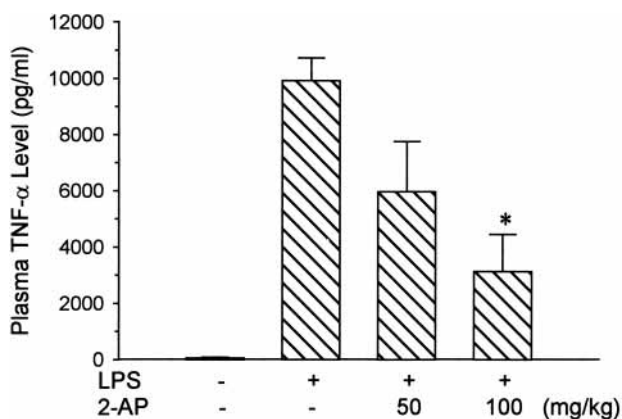


Fig. 4. Effect of 2-AP on the production of TNF- $\alpha$ . The effect of 2-AP on the plasma TNF- $\alpha$  level in rats treated with lipopolysaccharide (LPS) was assessed by enzyme-linked immunosorbent assay. One way analysis of variance was used for comparisons of multiple group means followed by Newman-Keuls test (significant as compared to LPS alone, \*  $P < 0.05$ ).

immunosorbent assay (fig. 4). A single dose of lipopolysaccharide treatment (1  $\mu$ g/kg, intravenously) increased TNF- $\alpha$  level from 35 to 9920  $\pm$  820 pg per ml plasma at 1 hr. 2-(Allylthio)pyrazine treatment at the dose of 100 mg/kg prevented the elevation of plasma TNF- $\alpha$  by lipopolysaccharide, resulting in 3130  $\pm$  1330 pg per ml of plasma (mean  $\pm$  S.D.,  $n = 4$ ,  $P < 0.05$ ).

### Discussion

A series of previous studies have shown that 2-(allylthio)pyrazine prevents experimental carcinogenesis and liver injuries (Kim *et al.* 1997a; Surh *et al.* 1998; Ha *et al.* 1999). Chemoprotective effect of 2-(allylthio)pyrazine may be due to the inhibition of cytochrome P450 (e.g. CYP2E1 and CYP3A) and the induction of phase II detoxifying enzymes. 2-(Allylthio)pyrazine also inhibited nitric oxide production by iNOS in lipopolysaccharide-treated animals, which raised the possibility that 2-(allylthio)pyrazine regulates inflammatory responses (Kim *et al.* 1997b).

The present study showed that 2-(allylthio)pyrazine inhibited the liver toxicity induced by multiple treatments with dimethylnitrosamine, which is primarily activated by CYP2E1 (Peng & Yang 1982). The plasma ALT, AST and  $\gamma$ -GT activities increased by dimethylnitrosamine were significantly reduced by 2-(allylthio)pyrazine treatments. The liver occupies a central role in the metabolism of bile pigments in the phase of hepatic uptake, conjugation and excretion. Excretion of bile pigments is susceptible to impairment when the liver cell is damaged. 2-(Allylthio)pyrazine inhibited the increases in the plasma bilirubin content in rats treated with dimethylnitrosamine. Furthermore, 2-(allylthio)pyrazine restored the total plasma protein and albumin levels decreased by dimethylnitrosamine treatment. These results showed that 2-(allylthio)pyrazine was capable of protecting the liver against multiple treatments with dimethylnitrosamine.

Dimethylnitrosamine is a hepatocarcinogen and hepatotoxicant commonly employed for the induction of experimental liver fibrosis. A recent study revealed that the liver cirrhosis induced by the drug in animal models appeared to parallel with the results of epidemiological studies (Straif *et al.* 1999). Dimethylnitrosamine is toxic to hepatocytes through bioactivation and induces coagulative necrosis in the centrilobular and periportal areas in the liver. CYP2E1 metabolizes nitrosamines and produces toxic metabolite(s) (Peng & Yang 1982; Yamazaki *et al.* 1992). Inflammatory cells release cytokines (e.g. TGF- $\beta$ 1 and platelet-derived growth factor) and may contribute to fibrogenesis by dimethylnitrosamine. Replacement of the necrotic areas with extracellular matrix occurs in 4 weeks (Jejävquell *et al.* 1987; Tsukamoto *et al.* 1990). Hepatic stellate cells are activated from quiescent states in response to fibrogenic stimuli, which accompanies formation of extracellular matrix. Activated stellate cells are the predominant source for the production of extracellular matrix, which characterizes hepatic fibrosis and cirrhosis (Pinzani *et al.* 1998). 2-(Allylthio)pyrazine was efficacious in reducing the extent of liver fibrosis induced by dimethylnitrosamine, as evidenced by histopathological examinations.

The TGF- $\beta$ 1 mRNA level was assessed in dimethylnitrosamine-treated rats as part of mechanistic studies on the antifibrotic effects of 2-(allylthio)pyrazine. TGF- $\beta$ 1 mRNA expression was not detected after 4 weeks of dimethylnitrosamine treatments due to extensive cellular injuries and fibrosis. Given the lack of responsiveness of TGF- $\beta$ 1 gene expression after 4 weeks of dimethylnitrosamine insults, we determined the effect of 2-(allylthio)pyrazine against a single exposure to dimethylnitrosamine at an early time. In order to provide sufficient time for the initial bioactivation of dimethylnitrosamine, 2-(allylthio)pyrazine was administered 18 hr after a dimethylnitrosamine injection. Dimethylnitrosamine-induced TGF- $\beta$ 1 expression was inhibited by 2-(allylthio)pyrazine treatment. In the experiment, the dose of 100 mg/kg of 2-(allylthio)pyrazine was chosen to explore the maximal pharmacological effect. A relatively larger dose of 2-(allylthio)pyrazine was required to inhibit TGF- $\beta$ 1 gene expression because we administered 2-(allylthio)pyrazine following a dimethylnitrosamine injection, which initiated injurious responses in the liver. Inhibition of TGF- $\beta$ 1 gene expression may contribute to inhibiting liver fibrogenesis. This is supported in part by the study using transgenic mice, in which overexpression of TGF- $\beta$ 1 gene developed severe hepatic fibrosis (Clouthier *et al.* 1997). Expression TGF- $\beta$ 1 protein would also be controlled by translational efficiency and post-translational modification.

Enhanced collagen synthesis may result from increased responsiveness of stellate cells to the cytokines from Kupffer cells. Kupffer cells could be activated by toxic metabolite(s) from dimethylnitrosamine, which might result in acute liver toxicity. TNF- $\alpha$  is the principal mediator of inflammatory responses, and is closely associated with the acute hepatotoxicity induced by immunological and chemical toxicants including dimethylnitrosamine (Schümann *et al.* 1998;

Kunstle *et al.* 1999). Lipopolysaccharide potently stimulates the release of cytokines including TNF- $\alpha$  from activated Kupffer cells (Beutler & Kruys 1995). In the current study, the effect of 2-(allylthio)pyrazine on the plasma TNF- $\alpha$  level was assessed in rats treated with lipopolysaccharide. 2-(Allylthio)pyrazine inhibited increase in the plasma TNF- $\alpha$  level by lipopolysaccharide, which supported the possibility that 2-(allylthio)pyrazine inhibits Kupffer cell activation.

CYP2E1 metabolizes small organic molecules including dimethylnitrosamine (Koop 1992). The active metabolite(s) produced from dimethylnitrosamine by CYP2E1 led to production of ultimate carcinogens and hepatotoxicants (Jejävquel *et al.* 1987; George & Chandrakasan 1996). 2-(Allylthio)pyrazine competitively inhibits the metabolic activity of CYP2E1 with high affinity ( $K_i=12 \mu\text{M}$ ) and suppresses constitutive and inducible CYP2E1 expression (Kim *et al.* 1997a; Kim & Kim 1999), which may be responsible for the inhibition of dimethylnitrosamine-induced liver injury. Because we treated rats with dimethylnitrosamine 18 hr prior to 2-(allylthio)pyrazine treatment, dimethylnitrosamine would be sufficiently bioactivated. Hence, the antifibrotic effect by 2-(allylthio)pyrazine involving TGF- $\beta$ 1 suppression may result from the inhibition of cytokine production (i.e. TNF- $\alpha$  and TGF- $\beta$ 1) from Kupffer cells and Ito cells as well as from the inhibition of CYP2E1.

Hepatic fibrosis may result from severe and unlimiting injury accompanied by hepatic dysfunction (e.g. chronic hepatitis). Viral hepatitis results in the relatively low level of inflammation and cellular necrosis. However, the resultant damage is persistent, and may eventually cause liver cirrhosis, which may accompany hepatocellular carcinoma (George *et al.* 1999; Sørensen *et al.* 1998). 2-(Allylthio)pyrazine may exert antifibrotic effect against liver fibrogenesis induced by chronic hepatitis, which remains to be established.

In summary, 2-(allylthio)pyrazine exhibited inhibitory effects on dimethylnitrosamine-induced liver fibrosis, which might be due to suppression of CYP2E1 expression and TGF- $\beta$ 1 production. Inhibition of TNF- $\alpha$  and TGF- $\beta$ 1 by 2-(allylthio)pyrazine supports the conclusion that the antifibrotic effect might be associated with inactivation of Kupffer cells or Ito cells as well as with CYP2E1 inhibition. The inhibition of TGF- $\beta$ 1 expression by 2-(allylthio)pyrazine may also contribute to its effectiveness against liver fibrosis induced by other N-nitroso compounds.

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