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Role of nitric oxide in the regulation of fibrogenic factors in experimental liver fibrosis in mice

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Summary. Previously, we have shown that an increased expression level of iNOS but a reduction in the expression of eNOS is associated with increased oxidative stress markers in CCl_4-induced experimental liver fibrosis. The present study aimed to investigate the effect of L-arginine and 5-methylisothiourea hemisulfate (SMT) in the expression of profibrogenic factors in chronic liver injury. ICR mice were treated with CCl_4 with or without treatment of L-arginine, an NO donor, or SMT, an iNOS inhibitor. The expression of matrix metalloptroteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs), α-smooth muscle actin (α-SMA), tumor necrosis factor-α (TNF-α) and cyclooxygenase-2 (COX-2) were investigated by RT-PCR. The activity of the MMP-2 and MMP-9 were measured by zymography. Our results showed that CCl_4-treated mice showed significant up-regulation of expression of pro-fibrogenic factors, TNF-α and COX-2. Treatment with L-arginine or SMT showed a significant reduction in CCl_4-induced expression of these pro-fibrogenic factors, TNF-α and COX-2. In conclusion, both SMT and L-arginine effectively attenuated the progression of CCl_4-induced liver fibrosis. SMT suppresses iNOS mediated NO production. However, L-arginine augments NO production. The similar effect of the two drugs on liver fibrosis indicates that there may be two distinct pathways of NOS mediated fibrogenesis in chronic liver injury by iNOS and eNOS. Our results suggest that eNOS-mediated liver fibrogenesis may play a more important role than that of iNOS in chronic liver injury. Taken together, these results support the contention that NO plays an active role in the progression of liver fibrosis and hepatocellular damage.

Key words: Liver fibrosis, SMT, L-arginine, Nitric oxide, Nitric oxide synthases

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Introduction

Liver fibrosis, a common occurrence in chronic liver diseases, is characterized by the accumulation of extracellular matrix (ECM) in sinusoids (Bataller and Brenner, 2005; Friedman, 2008). The degradation and formation of matrix in the liver is characterized as a remodeling process due to tissue damage from different causes (Bissell, 1998; Li and Friedman, 1999). Tissue damage in the liver causes inflammatory responses and triggers the remodeling process in association with the formation of extracellular matrix (Bedossa and Paradis, 2003; Ramadori and Saile, 2004; Lotersztajn et al., 2005). This remodeling process includes the activation of the quiescent hepatic stellate cells into fibroblast-like cells, which then produces matrix degradation proteins, collectively known as the family of matrix metalloproteinases (MMPs) (Arthur et al., 1989; Benyon and Arthur, 2001). The activity of the MMPs is regulated by their inhibitors, tissue inhibitor of metalloproteinases (TIMPs), which prevents damage of the non-injured cells (Iredale, 1997). However, when the injury persists, the overproduction of TIMPs eliminates the matrix degradation process and results in fibrosis due to net ECM accumulation in the liver (McCrudden and Iredale, 2000).

Accumulating evidence shows that nitric oxide (NO) plays an important role in the progression of liver

diseases (Hon et al., 2002). Nitric oxide is a very small molecule and is also a very active free radical. Nitric oxide is involved in numerous physiological processes, including the regulation of vascular tone, host immune defense system, neurotransmission, etc. (Moncada et al., 1991). Nitric oxide is produced together with L-citrulline through the enzymatic action of nitric oxide synthases (NOS) on the substrate L-arginine (Moncada and Higgs, 1993). There are three isoforms of NOS identified in the body, in which the constitutively expressed endothelial NOS (eNOS) and the inducible NOS (iNOS) are the most abundant forms found in liver (Clemens, 1999). Inducible NOS is readily induced in both acute and chronic liver injury.

The present study aimed to investigate the effect of L-arginine and 5-methylisothiourea hemisulfate (SMT) in the expression of profibrogenic factors in experimental liver fibrosis. Liver fibrosis was induced by chronic carbon tetrachloride (CCl_4) intoxication in ICR mice. In order to modulate the NO concentration in chronic liver injury, a substrate for NOS, L-arginine or an iNOS inhibitor, SMT was administered together with CCl_4.

Materials and methods

Animal model and treatments

Male ICR mice weighing approximately 30g were obtained from the Laboratory Animal Unit, The University of Hong Kong and used in the experiments. The mice were given free access to water and animal chow in compliance with the requirements of The University of Hong Kong and the National Institute of Health guidelines. The Laboratory Animal Unit of The University of Hong Kong is fully accredited by the Association for Assessment and Accreditation for Laboratory Animal Care (AAALAC International).

ICR mice were divided into eight groups (8 animals/group) with various treatments and CCl_4. Group (1) Control (injection of vehicles only); (2) Carbon tetrachloride (CCl_4, 50 µl/kg); (3) D-arginine (200 mg/kg, Sigma, St. Louis, MO, USA); (4) D-arginine + CCl_4; (5) 5-methylisothiourea hemisulfate (SMT, 10 mg/kg, Sigma, St. Louis, MO, USA); (6) L-arginine (200 mg/kg, Sigma, St. Louis, MO, USA); (7) SMT + CCl_4; (8) L-arginine + CCl_4. All the drugs used were dissolved in normal saline while the hepatotoxin was dissolved in olive oil. The administration of CCl_4 was performed through intraperitoneal injection twice a week and the drugs were injected subcutaneously daily for a course of 8 weeks. The optimum dose of CCl_4 was previously determined at a level at which no mortality was observed (Chen et al., 2004). The animals were sacrificed at the end of the treatment and the liver tissues and blood samples were collected and stored at -80°C.

Tissue processing

Briefly, liver tissues taken from the animals were cut into small slices and fixed in 4% phosphate buffered formalin for 72 hours. Liver tissues were routinely processed for histology and embedded in paraffin wax. Tissue blocks were sectioned with a thickness of 5 µm.

Sirius Red staining and quantification of collagen

The collagens are the basic substances of connective tissue. They can be visualized by Sirius Red staining. Briefly, the fixed and sectioned tissues were dewaxed and rehydrated followed by staining with 0.1% picro-Sirius Red (Polysciences Inc., Washington, DC, USA) in saturated aqueous picric acid for 1 hour. The tissues were then differentiated in 0.01% hydrochloric acid for 30 minutes. The stained slides were immediately dehydrated and mounted with Permount medium (Fisher Scientific, NJ, USA).

Reverse transcription polymerase chain reaction (RT-PCR) analysis

The mRNA expression of various factors involved in liver fibrosis was analyzed by semi-quantitative RT-PCR. Total RNA was extracted from liver tissue using the NucleoSpin Nucleic Acid Purification Kits (CLONTECH Laboratories, Inc., Palo Alto, CA, USA). Liver tissue of 10 mg was homogenized in lysis buffer and total RNA was extracted according to the procedures stated in the manufacturer's manual. The preparartion of the first-strand cDNA was performed by following the instructions in SuperScript™ First-Strand Synthesis System. The cDNA obtained was used for PCR and the target genes were amplified with the primers and the corresponding thermal cycle listed in Table 1. The optical density of mRNA and GADPH was expressed as a ratio represented by arbitrary units in the Figures.

Protein extraction and western blotting

The cytosolic and nuclear proteins were extracted from the liver tissue isolated from the mice. Briefly, 100 mg of liver tissue was homogenized in 500 µl lysis buffer (250 mM sucrose, 15 mM NaCl, 5 mM EDTA, 1 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 1 mMdithiothreitol, 15 mM Tris-HCl pH 7.9, 60 mM KCl, 0.1% Leupeptin, 0.1% Aprotinin, 0.5% Phenylmethyl-sulfonylfluoride (PMSF)). The final lysate was centrifuged at 5,000 rpm at 4°C for 10 min. The supernatant fraction was brought to centrifuge at 13,000 rpm at 4°C for 20 min to obtain the cytosolic protein in the supernatant. The pellet fraction from the lysate was resuspended in 500 µl Buffer A (10 mM Hepes pH 7.9, 0.75 mM $MgCl_2$, 0.5 mM EDTA, 0.5 M KCl, 0.1% Leupeptin, 0.1% Aprotinin, 0.5% PMSF) and centrifuged at 6,000 rpm at 4°C for 10 min. 400 µl of buffer B (0.5 mM Hepes pH 7.9, 0.75 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM KCl, 12.5% Glycerol, 0.1% NP-40, 0.1% Leupeptin, 0.1% Aprotinin, 0.5% PMSF) was added to resuspend the pellet and rocked for 30 min at

4°C. After centrifugation at 13,000 rpm at 4°C for 30 min, the supernatant was collected and followed by overnight dialysis against Buffer C (10 mM Tris-HCl pH 7.9, 5 mM $MgCl_2$, 10 mM EDTA, 50 mM Dithiothreitol) at 4°C. The extracted cytosolic and nuclear proteins were stored at -80°C in aliquots. Protein concentration was measured by Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

The cytosolic or nuclear protein was diluted and mixed with 2x sample buffer (0.1 M Tris-HCl pH 6.8, 20% Glycerol, 4% Sodium Dodecyl Sulfate, 0.2% Bromophenol Blue, 5.25% ß-Mercaptoethanol). The mixture was denatured at 95°C for 5 min and followed by electrophoresis in a 7% or 10% polyacrylamide gel as appropriate. The protein was then transferred to a Immun-Blot™ PVDF Membrane (Bio-Rad Laboratories, Inc, CA, USA) in a TE series transfer electrophoresis unit (Hoefer Pharmacia Biotech Inc., CA, USA). The membrane was then incubated in blocking buffer (5% nonfat milk powder in TBST) for 3 hours followed by incubation with primary antibody in TBST (100 mM Tris-HCl pH 7.5, 0.9% NaCl, 0.1% Tween 20) overnight at 4°C with gentle agitation: α-SMA (50 µg cytosolic protein; 1:2000 dilution; Santa Cruz Biotecnhology, CA, USA); COX-2 (50 µg cytosolic protein; 1:500 dilution; Cayman Chemical, MI, USA); C/EBP-α (30 µg nuclear protein; 1:1000 dilution; Santa Cruz Biotechnology, CA, USA). The membrane was washed with TBST and incubated with anti-mouse (α-SMA) or anti-rabbit (COX-2 and C/EBP-α) secondary antibody (1:2000 dilution in TBST) for 2 hours at room temperature. After washing off the unbound antibody with TBST, the expression of the antibody linked protein was determined by ECL™ Western Blotting Detection Reagents (Amersham Pharmacia Biotech Inc., NJ, USA). The optical density of protein products was expressed as arbitrary units in the Figures.

Zymography to show the activity level of the matrix degrading enzymes, MMP-2 and MMP-9

Protein samples were collected differently from those used in western blotting. Briefly, the liver tissues were homogenized in 1 ml extraction buffer (1% Triton X-100, 500 mM Tris/HCl pH 7.6, 200 mM NaCl, 10 mM $CaCl_2$, 0.1% Leupeptin, 0.1% Aprotinin, 0.5% PMSF). The lysate was then centrifuged at 12,000 rpm for 30 min at 4°C. The concentration of the resulting proteins was measured. A protein sample of 20 µg was diluted with distilled water to a final volume of 10 µl and then mixed with 2 volumes of sample buffer (62.5 mM Tris-HCl pH 6.8, 25% glycerol, 4% SDS, 0.01% Bromophenol Blue). The mixture was then loaded to a pre-casted 10% SDS-polyacrylamide gel containing 1 mg/ml gelatin. Electrophoresis was carried out at 50 mA. The gel was then washed with wash buffer (50 mM Tris-HCl pH 7.6, 5 mM $CaCl_2$, 2.5% Triton X-100) for 15 min and followed by an overnight washing with gentle agitation. The wash buffer was removed by washing with distilled water three times for 15 min each. The gel was then allowed to incubate in developing buffer (1% Triton X-100, 50 mM Tris-HCl pH 7.6, 5 mM $CaCl_2$, 0.02% NaN_3, pH 7.6) at 37°C for 12 hours. The gel was stained with Coomassive Blue staining solution (0.5% Coomassive Brillant Blue, 30% methanol, 10% acetic acid) for 4 hours followed by destaining in Destain I (30% methanol, 10% acetic acid) and Destain II (30% methanol, 5% glycerol). The gel was then brought to air dry. The optical density of zymography bands was expressed as arbitrary units in the Figures.

Statistical analysis

All data were expressed as mean ± standard error of mean (SEM). The comparison between different groups

Table 1. Primers used in RT-PCR.

Target Gene		Sequence	Annealing Temp (°C)	Number of cycle
COX-2	forward	5'-GGAGAGACTATCAAGATAGTGATC-3'	60	37
	reverse	5'-ATGGTCAGTAGACTTTTACAGCTC-3'		
TNF-α	forward	5'-ATGAGCACAGAAAGCATGATC-3'	50	34
	reverse	5'-TACAGGCTTGTCACTCGAATT-3'		
TIMP-1	forward	5'-GCATCTGGCATCCTCTTGTT-3'	60	33
	reverse	5'-AAGAAGCTGCAGGCATTGAT-3'		
TIMP-2	forward	5'-ATTTATCTACACGGCCCC-3'	55	31
	reverse	5'-CAAGAACCATCACTTCTCTTG-3'		
MMP-2	forward	5'-GCTGATACTGACACTGGTACTG-3'	55	30
	reverse	5'-CAATCTTTTCTGGGAGCTC-3'		
MMP-9	forward	5'-CGTCGTGATCCCCACTTACT-3'	55	40
	reverse	5'-AGAGTACTGCTTGCCCAGGA-3'		
α-SMA	forward	5'-CTGGAGAAGAGCTACGAACTGC-3'	53	30
	reverse	5'-CTGATCCACATCTGCTGGAAGG-3'		
GAPDH	forward	5'-CCTTCATTGACCTCAACTACATGGT-3'	55	25
	reverse	5'-TCATTGTCATACCAGGAAATGAGCT-3'		

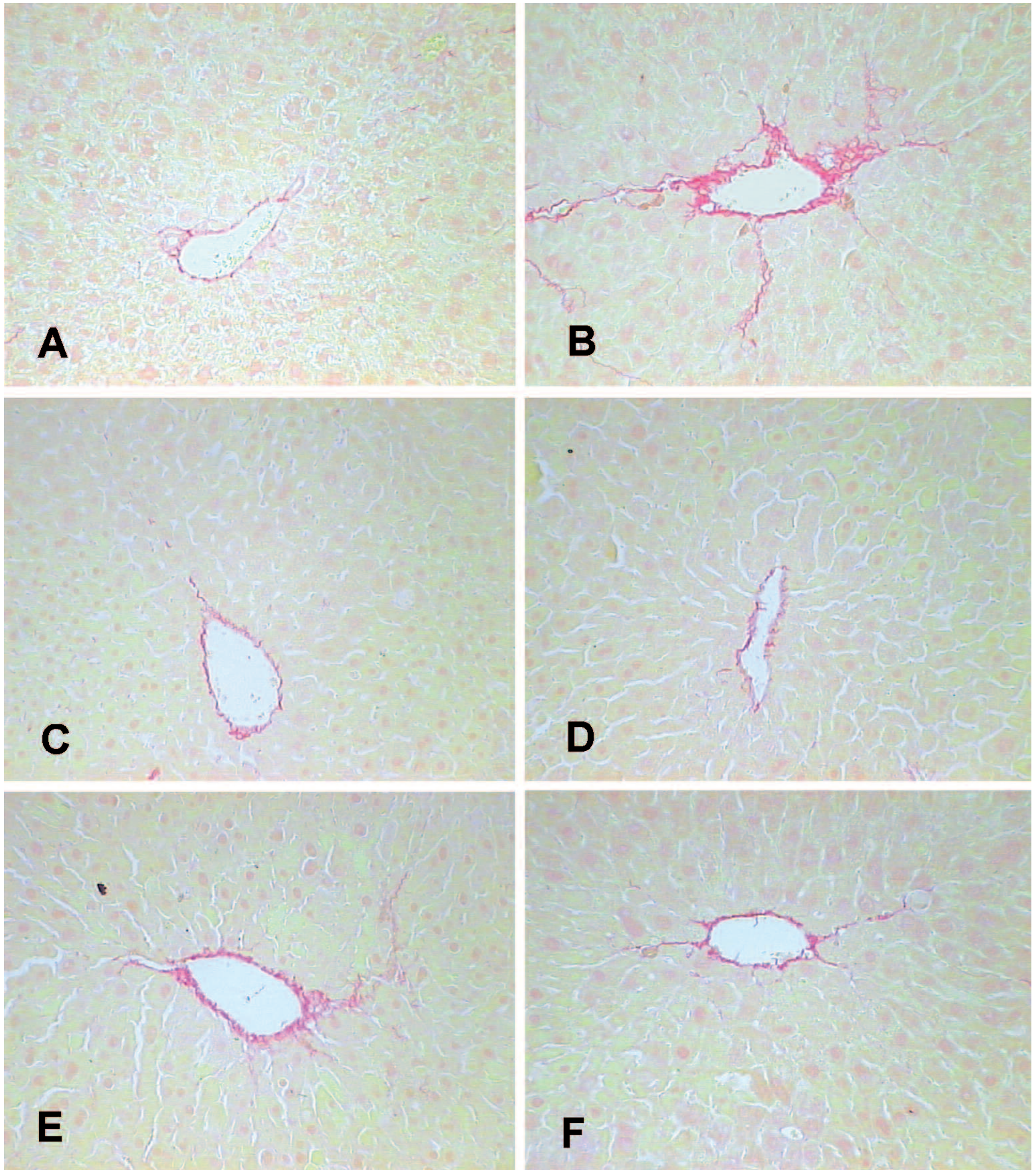

Fig. 1. Sirius Red staining of the liver sections from each treatment group. The collagen stained in red. **A.** Vehicle control shows there is a thin layer of collagen along the central vein under normal conditions. **B.** CCl_4 shows an extensive accumulation of collagen in the thickened layer of extracellular matrix along the central vein and in the perihepatic area. SMT **(C)** and L-arginine **(D)** show that the treatment with drugs alone did not alter the amount of collagen in the liver. SMT + CCl_4 **(E)** and L-arginine + CCl_4 **(F)** show that pretreatment with drugs prior to CCl_4 markedly inhibit CCl_4-induced extracellular matrix deposition in the liver. x 200

was detected by Kruskal-Wallis followed by post hoc Dunns test using GraphPad Prism (GraphPad Software, Inc., San Diego CA, USA). A p-value <0.05 was regarded as statistically significant.

Results

Administration of L-arginine showed a greater suppression than SMT in the accumulation of collagen under chronic liver injury

The results of Sirius Red staining are shown in Fig. 1, which includes normal, fibrotic liver tissues and other treatment groups. Qualitatively, only a thin lining of connective tissue along the central vein was stained with Sirius Red in normal tissues. However, numerous collagen staining were observed in the CCl_4 treated mice, and were concentrated around the central vein and in the perihepatic area. The amount of Sirius Red staining was markedly reduced in the SMT + CCl_4 and L-arginine + CCl_4 groups. Our previous quantitative data (Leung et al., 2008) support these findings.

Increased expression of α-SMA in chronic CCl_4-induced liver injury

Treatment with CCl_4 only or D-arginine with CCl_4 showed a significant increase in the expression of mRNA and protein levels of α-SMA when compared with control groups ($p<0.01$) (Fig. 2). A significant reduction was observed in the groups treated with L-arginine or SMT + CCl_4 when compared with CCl_4 or D-arginine + CCl_4 group ($p<0.01$). The expression level of α-SMA in the vehicle control group showed no difference to the groups treated with L-arginine, SMT or D-arginine only, or the groups pretreated with L-arginine or SMT followed by CCl_4.

L-arginine and SMT reduced the expression of TNF-α and COX-2 in CCl_4-induced liver injury

The mRNA expression levels of the pro-inflammatory cytokine TNF-α significantly increased after CCl_4 administration ($p<0.01$) (Fig. 3A). The treatment of L-arginine or SMT + CCl_4 effectively suppressed CCl_4-induced TNF-α expression to a level similar to control ($p <0.01$). The treatment with the drug only (D-arginine, SMT or L-arginine) showed no difference relative to the vehicle control group. Carbon tetrachloride treatment also showed a significant increase in both mRNA and protein levels of COX-2 ($p <0.01$) (Fig. 3B,C). As for treatment with either L-arginine or SMT followed by CCl_4, both drugs reduced the COX-2 mRNA level to about half of the value when compared with the CCl_4 group ($p<0.01$). However, the level of COX-2 mRNA expression in the groups of L-arginine + CCl_4 and SMT + CCl_4 was still higher than that of other control groups administered with drugs only (Fig. 3B). Treatment with L-arginine and SMT followed by CCl_4 also showed a significant reduction in CCl_4-induced COX-2 protein expression when compared with the CCl_4 group ($p<0.01$). The COX-2 protein levels were found to be similar among the control, L-arginine + CCl_4 and SMT + CCl_4, groups. The induced expression of COX-2 in chronic liver injury was confirmed by the down-regulation of C/EBP-α after CCl_4 injection (Fig.

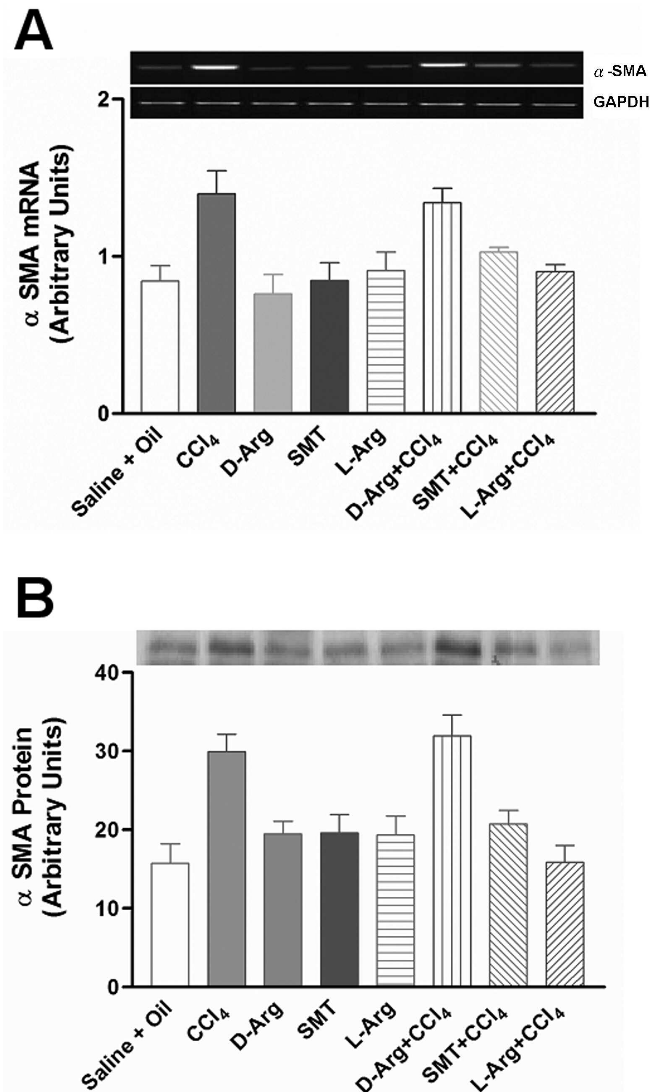

Fig. 2. Alpha smooth muscle actin in different groups of rats. The mRNA **(A)** and protein **(B)** expression levels of α-smooth muscle actin (α-SMA). A high level of α-SMA mRNA and protein levels are seen in CCl_4-induced liver fibrosis and in the group treated with D-arginine + CCl_4 compared with the controls ($p<0.01$). In treatment with L-arginine or SMT followed by CCl_4 groups shows a significant reduction in both mRNA and protein levels of α-SMA ($p<0.01$) comparable to the control groups. The optical density of mRNA and GADPH was expressed as a ratio represented by arbitrary units in the Figures. The optical density of protein products was also expressed as arbitrary units in the Figures. Animals per group (n=8)

3D). C/EBP-α is known to be an important regulator of COX-2 expression. The CCl_4 and D-arginine + CCl_4 groups showed the lowest expression of C/EBP-α protein when compared with other groups (p <0.01). In contrast, the expression of C/EBP-α protein in the control and treated groups were significantly up-regulated.

L-arginine showed a greater suppression of the expression of remodelling factors in chronic liver injury than that of SMT

In the group showing chronic liver injury with CCl_4 or D-arginine + CCl_4 treatment, there was a marked increase in mRNA expression of the matrix

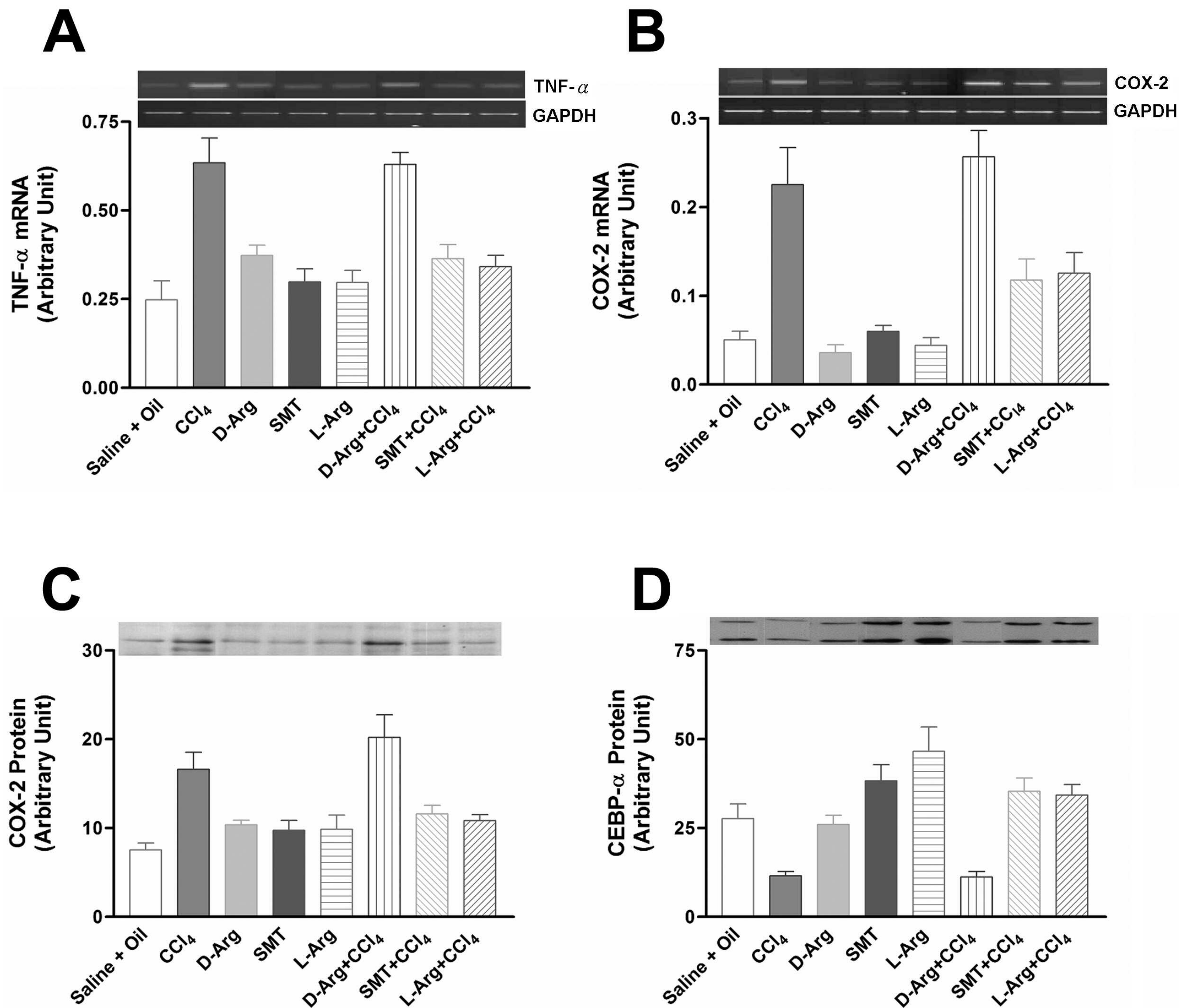

Fig. 3. Liver TNF alpha and Cox-2 expression. **A.** The mRNA expression level of TNF-α. The expression level of TNF-α is higher in CCl_4 and D-arginine + CCl_4 groups when compared with the controls (p<0.01). In treatment with SMT or L-arginine + CCl_4, the expression of TNF-α is significantly reduced when compared with CCl_4 or D-arginine + CCl_4 groups (p<0.01). **B.** The mRNA expression level of COX-2 and the protein level of COX-2 **(C)** and C/EBP-α **(D)**. The expression of COX-2 is higher in CCl_4 and D-arginine + CCl_4 groups when compared with the controls (p<0.01). In the treatment with SMT or L-arginine + CCl_4, the expression of COX-2 is significantly reduced when compared with CCl_4 or D-arginine + CCl_4 groups (p<0.01). C/EBP-· shows a reduced expression in the groups treated with CCl_4 and D-arginine + CCl_4 when compared with the controls (p<0.01). Treatment with L-arginine or SMT abrogates the effect of CCl_4-induced altered expression of C/EBP-α protein level (p<0.01). The optical density of mRNA and GADPH was expressed as a ratio represented by arbitrary units in the Figures. The optical density of protein products was also expressed as arbitrary units in the Figures. Animals per group (n=8)

metalloproteinases. Three-fold and one-fold increases were observed in the mRNA expression of MMP-2 and MMP-9, respectively, after CCl_4 treatment when compared with the controls (p<0.01) (Fig. 4A,B). The treatment with L-arginine + CCl_4 showed a stronger suppression than SMT + CCl_4 in the expression of MMP-2 mRNA (p <0.01). The MMP-2 mRNA level in the SMT + CCl_4 group was approximately 2-fold higher than in the L-arginine + CCl_4 group while a slight difference was observed between the two groups in the expression of MMP-9 mRNA. No observable change in the expression level was detected in the treatment with drugs (D-arginine, L-arginine or SMT) only. CCl_4 also showed a significant increase in the expression of TIMP-1 and TIMP-2, 3-fold and almost 2-fold, respectively, when compared with the controls (p <0.001) (Figs. 4C,D). Treatment with both L-arginine and SMT followed by CCl_4 effectively down-regulated the expression of TIMP-1 and TIMP-2 induced by CCl_4 (p<0.001). All the groups treated with the drugs alone

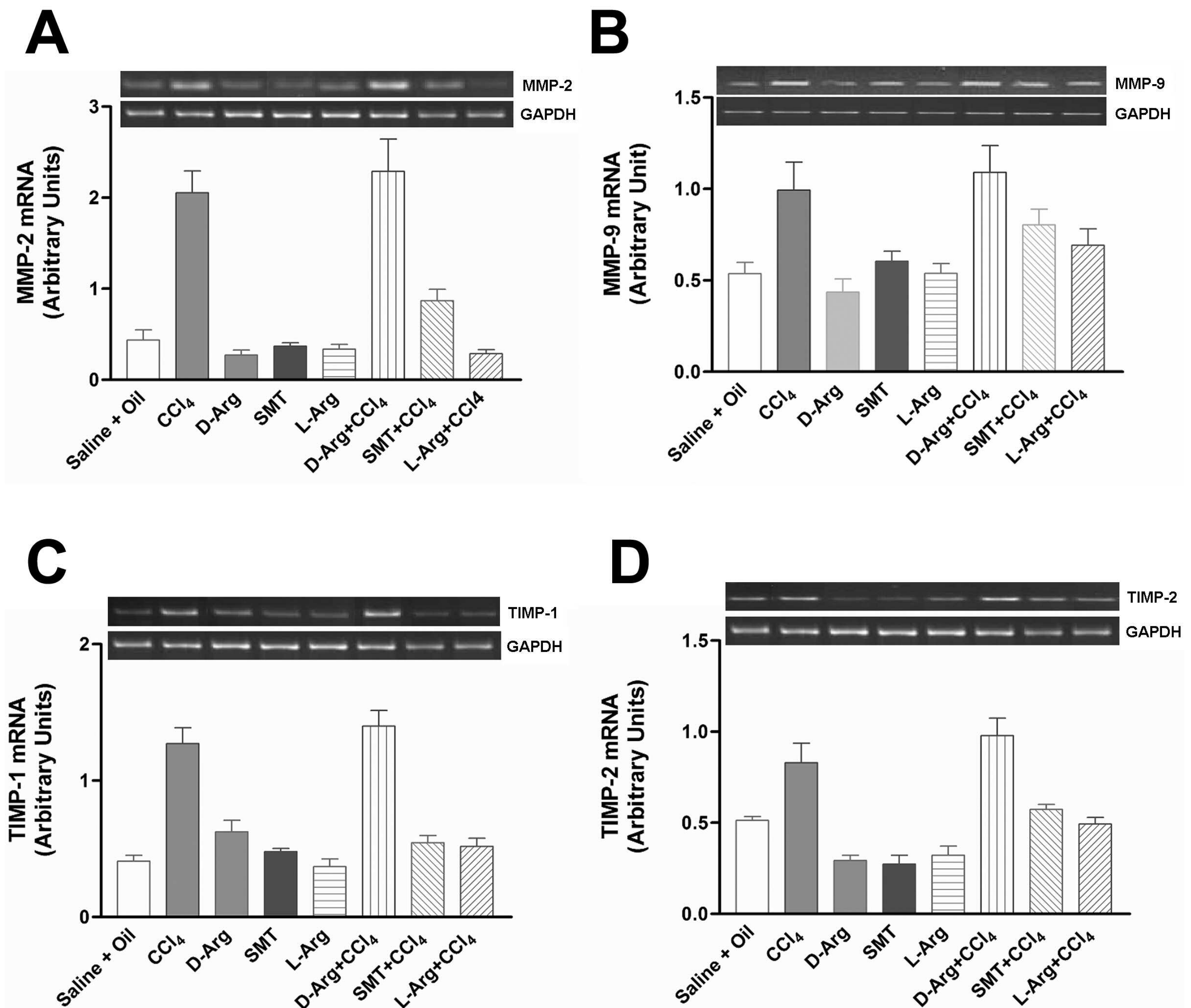

Fig. 4. The expression of fibrogenic factors in the liver. The mRNA level of MMP-2 **(A)** and MMP-9 **(B)**. The groups treated with CCl_4 and D-arginine + CCl_4 show a significant increase in the expression of MMP-2 and MMP-9 when compared with the controls (p<0.01). The treatment with L-arginine + CCl_4 shows a greater inhibitory effect on CCl_4-induced expression of MMPs than that of SMT + CCl_4 (p<0.01). The mRNA level of TIMP-1 **(C)** and TIMP-2 **(D)**. Both TIMP-1 and TIMP-2 mRNA levels are markedly increased in the groups treated with CCl_4 and D-arginine + CCl_4 when compared with the controls (p<0.001). Treatment with L-arginine or SMT followed by CCl_4 effectively reduces the expression of TIMP-1 and TIMP-2 when compared with CCl_4 or D-arginine + CCl_4 groups (p<0.001). The optical density of mRNA and GADPH was expressed as a ratio represented by arbitrary units in the Figures. Animals per group (n=8)

showed basal and low expression of both TIMP-1 and TIMP-2. The effectiveness of L-arginine and SMT in suppressing CCl_4-induced expression of TIMP-1 and TIMP-2 was of the same degree.

The activity of MMPs was attenuated by L-arginine and SMT in chronic liver injury

As shown in Fig. 5, the activity of MMP-2 and MMP-9 were significantly higher in CCl_4-induced liver injury when compared with the controls ($p<0.05$). Furthermore, the increase in the activity of MMP-2 was greater than that of MMP-9. The high level of MMP activity in chronic liver injury was significantly suppressed by the administration of L-arginine or SMT, comparable to the control levels ($p<0.05$). All the groups treated with drugs alone showed no effect in modulating the activity of MMP-2 and MMP-9.

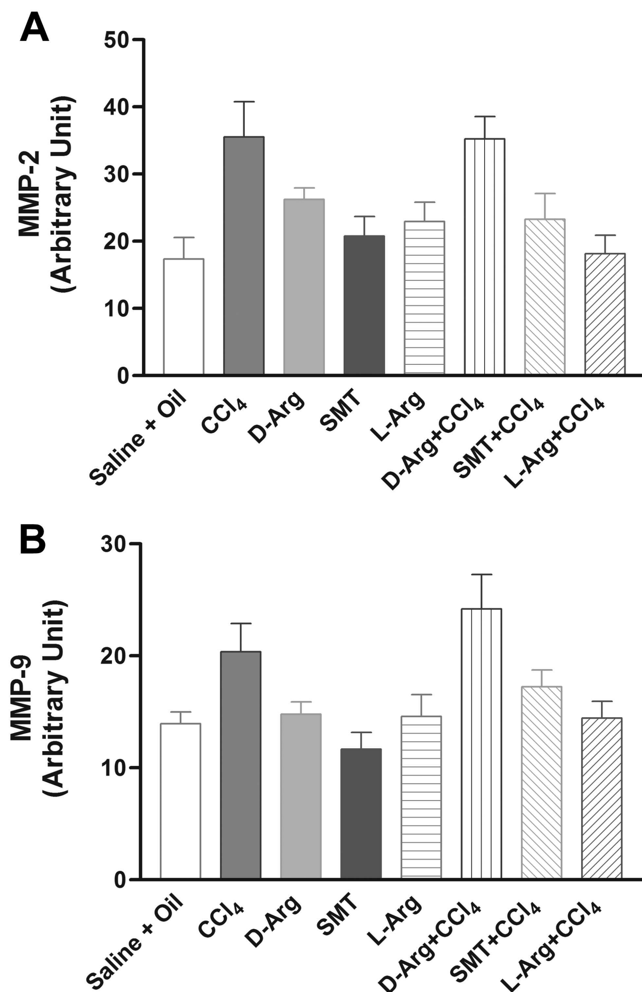

Fig. 5. The enzymatic activity of MMP-2 **(A)** and MMP-9 **(B)** indicated by zymography assay. Chronic liver injury induced by CCl_4 and D-arginine + CCl_4 shows a significant increase in the activity of the two MMPs when compared with the controls ($p<0.05$). In treatment with L-arginine or SMT + CCl_4 this induced activity of MMP-2 and MMP-9 is attenuated when compared with the CCl_4 or D-arginine + CCl_4 groups ($p<0.05$). The optical density of zymography band was expressed as arbitrary units in the Figures. Animals per group (n=8).

Discussion

Liver fibrosis is a common feature in chronic liver diseases. It is a result of net accumulation of extracellular matrix in the liver. Liver fibrosis involves the activation of hepatic stellate cells and the release of a number of cytokines (TNF-α, TGF-ß) and pro-fibrogenic factors (matrix metalloproteinases and tissue inhibitors for metalloproteinases). In chronic CCl_4 injury, there is a continuous production of free radicals either from the conversion of CCl_4 to CCl_3• and CCl_3OO• or from the inflammatory cells (Losser and Payen, 1996; Weber et al., 2003). Injury to the liver triggers a regenerative process better known as wound healing process. In this restructuring process, the damaged tissue is degraded and replaced by newly synthesized extracellular matrix.

The present study showed that treatment of the CCl_4 mice with either L-arginine, an NO donor, or SMT, a selective iNOS inhibitor, effectively down-regulated the formation of extracellular matrix. However, L-arginine had a greater effect than SMT in attenuating the expression of various pro-fibrogenic factors and the formation of extracellular matrix. The exact mechanism of how L-arginine inhibits the accumulation of collagen is unclear but it is hypothesized that the protective effect of L-arginine may be related to NO production.

Reduced liver injury by L-arginine and SMT is related to a decreased inflammatory response

It is generally believed that inflammation promotes liver fibrosis. We therefore investigated the expression level of two pro-inflammatory mediators, TNF-α and COX-2, which have been shown to be important in liver injury. The expression of these pro-inflammatory mediators was found to be significantly increased in CCl_4-treated mice. Increased expression of COX-2 was observed in association with reduced C/EBP-α in the CCl_4 treated group. Callejas et al. (2000) reported that the expression of COX-2 is inhibited by high levels of C/EBP-α. COX-2 is involved in metabolizing the formation of prostanoids from arachidonic acid that is released from plasma membranes. The expression of COX-2 and the COX-prostanoid pathway are believed to play a pivotal role in enhancing the inflammatory process (Chan and Rodger, 1997; Hu, 2003).

In the present study, treatment of mice with L-arginine or SMT followed by CCl_4 showed a similar decrease in the expression levels of both TNF-α and COX-2. This observation indicated that both drugs had similar efficacy in reducing the inflammatory response of the liver tissue due to chronic CCl_4 insult. The

C/EBP-α protein expression of mice treated with L-arginine or SMT followed by CCl_4 were elevated when compared to the CCl_4 group. Thus, we suggest that the decrease in COX-2 expression in the L-arginine or SMT + CCl_4 groups were suppressed by the higher level of C/EBP-α protein expression.

Activation of hepatic stellate cells underscored the initiation of fibrogenesis

Hepatic stellate cells are quiescent under normal conditions. These cells are activated in the presence of injury (Geerts, 2001). The activation of the hepatic stellate cells is indicated by the expression of alpha-smooth muscle actin (α-SMA) (Akpolat et al., 2005), although it can also be expressed by other cell types (Badid et al., 2000). The expression of α-SMA is known to be a reliable marker of the activation of hepatic stellate cells, preceding the deposition of fibrous tissue (Carpino et al., 2005). In this study, the expression levels of α-SMA mRNA and protein were highest in the groups administered with CCl_4 only or with the treatment with D-arginine and CCl_4. The Sirius Red staining for collagen supported this finding (Fig. 1).

The activation of the stellate cells is closely related to the progression of liver fibrogenesis (Guido et al., 1996; Kweon et al., 2001). In normal liver, the mRNA levels of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are detected in α-SMA negative hepatic stellate cells, Kupffer cells, liver myofibroblasts and hepatocytes (Knittel et al., 1999) in order to keep a steady turnover of the connective tissue through a slow process of degradation and synthesis. However, once the hepatic stellate cells are activated, they are actively involved in the progression of liver fibrogenesis by the expression of various pro-fibrogenic factors and the production of extracellular matrix (Benyon and Arthur, 2001; Schuppan et al., 2001). In the present study, the treatment with either L-arginine or SMT reduced the CCl_4-induced expression of α-SMA. This finding correlates with our Sirius Red staining results and previous observation that L-arginine and SMT effectively reduce the amount of collagen formation, suggesting the participation of activated hepatic stellate cells in the progression of liver fibrosis (Leung et al., 2008).

Expression of pro-fibrogenic factors in the progression of liver fibrosis

In the present study, we show that increased expression of various pro-fibrogenic factors, matrix metalloproteinase-2 (MMP-2) and MMP-9, and tissue inhibitors of metalloproteinases-1 (TIMP-1) and TIMP-2 in CCl_4-treated mice (CCl_4 and D-arginine + CCl_4 groups) are associated with dysregulated expression of TNF-α and TGF-ß1. Up-regulation of TGF-ß1 has been shown in our previous study (Leung et al., 2008). Knittel et al. (2000) showed that the expression of MMPs and TIMPs might be regulated by the appearance of TNF-α and TGF-ß1. In the early stage of liver injury, an increased expression of TNF-α and MMPs was detected. This was followed by the appearance of TGF-ß1 and TIMPs in the later stage, indicating different functions of these cytokines and fibrotic factors in inflammation and tissue repair reaction. The role of TGF-ß1 in fibrogenesis was shown in a transgenic mice model which chronically overproduced a mature active form of TGF-ß1 in hepatocytes (Sanderson et al., 1995). Liver cells once activated during liver injury produce a number of cytokines and inflammatory mediators such as TGF-ß1. It then activates the hepatic stellate cells and speeds up the transformation of quiescent hepatic stellate cells into activated form (Hellerbrand et al., 1999), as well as the subsequent production of extracellular matrix proteins (Grande et al., 1997). TGF-$ß_1$ also enhances fibrogenesis through inhibition of collagenase production and extracellular matrix degradation (Edwards et al., 1987). Therefore, the reduced expression of TGF-$ß_1$ shown in our previous study explains the reduction of CCl_4-induced extracellular matrix deposition in the liver in the mice pretreated with L-arginine or SMT. Futhermore, L-arginine showed a greater inhibitory effect on the suppression of TGF-ß1 expression than that of SMT. The present study showed that this resulted in the significant reduction in the extracellular matrix formation in the pretreatment with L-arginine than that of SMT.

Role of nitric oxide in liver fibrosis

We have shown that an increased expression level of iNOS but a reduction in the expression of eNOS is associated with an increase in oxidative stress markers in CCl_4-induced chronic liver injury (Leung et al., 2008). This finding suggests that the progression of liver fibrosis is closely related to the altered production of nitric oxide (NO) in different cell types in the liver. The increased activity of iNOS in the liver cells results in a high level of NO production and increased oxidative stress due to formation of peroxynitrite ($ONOO^-$) (Zhu and Fung, 2000; Leung et al., 2008). The increased level of pro-oxidants is then involved in lipid peroxidation that further induces hepatocellular damage (Weber et al., 2003). Wei et al. (2002) showed that there was a redistribution of eNOS in chronic liver injury. In normal liver, a basal level of eNOS maintains a steady production of NO to maintain the vascular tone (Mittal et al., 1994). However, eNOS protein expression is reduced in the sinusoidal endothelial cells of cirrhotic livers in bile duct ligated rats, although the total expression level of eNOS of the liver is unchanged (Wei et al., 2002). The reduced NO production in the endothelium causes vasoconstriction and increased resistance in hepatic microcirculation. Liver injury occurs as a result of persistent increase in hepatic sinusoidal resistance.

To clarify the importance of nitric oxide and nitric oxide synthases in the progression of fibrosis, we treated mice with either an iNOS inhibitor, SMT, or a substrate of NOS, L-arginine. Both agents effectively attenuated the CCl_4-induced inflammatory response as indicated by the reduction of TNF-α and COX-2 expression (Figs. 4). In fact, we found that treatment with L-arginine had a greater inhibitory effect than SMT on the expression of pro-fibrogenic factors, suggesting that a sustained and basal level of eNOS-derived NO is a critical factor in liver fibrosis. In addition, the liver injury that led to fibrogenesis could be caused by a combination of high levels of iNOS-derived NO-induced oxidative stress and the reduced eNOS-derived NO production in the endothelium. Indeed, a previous study has shown that the expression of both eNOS and iNOS was reduced in the sinusoidal endothelial cells from the rat liver after a chronic thioacetamide insult (Petermann et al., 1999). This study also further showed that a lack of the vasodilatory effect from nitric oxide leads to the development of an increase in hepatic sinusoidal resistance in chronic liver injury. Taken together, these results support the contention that NO plays an active role in the progression of liver fibrosis and hepatocellular damage. Nevertheless, further studies on the roles of eNOS and eNOS-derived NO in chronic liver injury may shed more light on the exact mechanism on how these molecules modulate the progression and treatment of liver fibrosis.

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