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Deficiency of inducible nitric oxide synthase exacerbates hepatic fibrosis in mice fed high-fat diet☆

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Abstract

The role of inducible nitric oxide synthase (iNOS) in the progression of fibrosis during nonalcoholic steatohepatitis remains to be elucidated. This study examined the role of iNOS in the progression of fibrosis during steatohepatitis by comparing iNOS knockout ($iNOS^{-/-}$) and wild-type ($iNOS^{+/+}$) mice that were fed a high-fat diet. Severe fatty metamorphosis developed in the liver of $iNOS^{+/+}$ and $iNOS^{-/-}$ mice. Fibrotic changes were marked in $iNOS^{-/-}$ mice. Gelatin zymography showed that pro MMP-2 and pro MMP-9 protein expressions were more highly induced in $iNOS^{+/+}$ mice than in $iNOS^{-/-}$ mice. Active forms of MMP-2 and MMP-9 were clearly present only in the liver tissue of $iNOS^{+/+}$ mice. In situ zymography showed strong gelatinolytic activities in the liver tissue of $iNOS^{+/+}$ mice, but only spotty activity in $iNOS^{-/-}$mice. iNOS may attenuate the progression of liver fibrosis in steatohepatitis, in part by inducing MMP-2 and MMP-9 expression and augmenting their activity.

Keywords: Inducible nitric oxide; Knockout mice; Steatohepatitis; Zymography; Nitrotyrosine

Inducible nitric oxide synthase (iNOS) has been reported to play a pivotal role in the development of various types of liver disease [1–7]. Strong expression of iNOS was found in cirrhotic areas of diseased liver [8]. iNOS mRNA and protein activity have also been found to be induced in rats on a high-fat diet [9,10]. It has recently been reported that the levels of both iNOS and an NO (nitric oxide) derivative, nitrotyrosine, were significantly higher in severe forms of nonalcoholic steatohepatitis with liver fibrosis than in the mildest forms, suggesting NO to be important in the process of fibrogenesis in nonalcoholic steatohepatitis in obesity [11]. However, the precise role of iNOS in the process of liver fibrosis in steatohepatitis is still to be elucidated.

Liver fibrosis is characterized by increased deposition of extracellular matrix (ECM) components, including type I, III, and IV collagens, due to ECM overproduction which overwhelms its degradation. Among the ECM-degrading enzymes, matrix metalloproteinases (MMPs) are thought to be central to fibrolysis and tissue remodeling, since these enzymes are able to degrade native fibrillar collagens of types I, III, and IV. Recently,

☆ Hepatic fibrosis in $iNOS^{-/-}$ mice.

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Casini et al. [12] reported that mRNA expression of procollagen I is modulated by NO in hepatic stellate cells, which are the major source of collagen in the liver. In addition, activation and expression of MMPs have been reported to be regulated by NO in various cell types [13–16].

Thus, we hypothesized that NO might participate in the process of liver fibrosis in steatohepatitis by directly modulating the expression of collagens and MMPs. In this study, we fed a high-fat diet to iNOS-knockout ($iNOS^{-/-}$) and wild-type ($iNOS^{+/+}$) mice, and we observed increased susceptibility of $iNOS^{-/-}$ mice to the development of liver fibrosis. Markedly attenuated MMP activity and overexpression of procollagen I were demonstrated in the hepatic tissues of $iNOS^{-/-}$ mice.

Materials and methods

Mice and experimental protocol. We used wild-type ($iNOS^{+/+}$) C57 Bl/6J mice (Charles River, Krea, Japan) and inducible NO synthase knockout ($iNOS^{-/-}$) mice derived from C57 Bl/6J × 129SvEv (Merck, NJ, USA) with the identical genetic background [17]. The animals were maintained in a pathogen-free barrier facility with a 12-h light/dark cycle and had free access to food and water. From 8 weeks of age, mice of each strain were fed a high-fat diet that contained 15% fat, 1% cholesterol, and 0.5% cholic acid for 12 weeks [18]. Control mice of each strain were fed normal chow for the same period of time. The study was approved by the Animal Care Committee of Tokai University.

Tissue preparation and histological analysis. Mice from all experimental groups were fasted for 12 h and sacrificed with a pentobarbital overdose at 20 weeks. The blood was collected and the concentrations of total cholesterol, HDL cholesterol, triglycerides, and ALT were measured. The liver was excised and embedded in OCT compound (Tissue-Tek) and paraffin for histological analysis. The OCT-embedded samples were serially sectioned at 4 μm. For the evaluation of fatty deposition, the liver tissues were stained with Oil red O, and counterstained with hematoxylin and eosin. The presence of collagen in the lesions was examined in Azan-stained and Masson's trichrome-stained sections. For quantification, 50 fields were microscopically examined at 40× magnification using a grid of 0.0625 mm^2 with 100 points. Values were expressed as the number of stained points/100 points. Other liver tissues were snap-frozen in liquid nitrogen and stored at liquid nitrogen or −70 °C until use for molecular and biochemical determinations.

Reverse-transcription-polymerase chain reaction and quantitative real-time RT-PCR. To determine iNOS mRNA levels, the frozen liver was homogenized in ISOGEN (Nippon Gene, Tokyo, Japan). Isolation of total RNA and reverse-transcription-polymerase chain reaction (RT-PCR) were performed; the primer for amplification of iNOS was as previously described [19]. GAPDH mRNA was used as an internal control.

To measure mRNA levels of α-smooth muscle actin (α-SMA) and procollagen I (αI), real-time quantitative RT-PCR using an ABI PRISM 7700 Sequence Detection system and TaqMan EZ RT-PCR Core Reagents (Applied Biosystems, Japan) was performed according to the manufacturer's protocol (TaqMan EZ RT-PCR kit protocol). To correct for RNA quality differences, GAPDH mRNA expression was measured as an internal control (TaqMan Rodent GAPDH Control Reagents-VIC labeled, Applied Biosystems, Japan) and relative gene expression was calculated as the ratio of the signal for the target gene (FAM) to that of GAPDH (VIC). The threshold cycle and the standard curve method were used for calculating the relative amount of the target RNA. The conditions for the TaqMan PCR were as follows: 50 °C for 2 min, 60 °C for 30 min, 95 °C for 5 min, and 40 cycles of 94 °C for 20 s, and annealing/extension at 60 °C for 1 min. The following primers were used for the quantification of smooth muscle α-actin (SMA) (GenBank NM_007392) and collagen pro-α-1 type I (proCOLI) (GenBank U080209): (SMA689F: 5′-GAGCGT GAGATTGTCCGTCCGTGA), (SMA839R: 5′-AGCGTTCGTTT CCAATGGTG), and (SMA780P: 5′-FAM-CCCTGGAGAAGAGC TACGAACTGCCTGA-TAMRA-3′); and (proCOLI3896F: 5′-AGA ACTGGTACATCAGCCCGA), (proCOLI4046R: 5′-GTGGACATT AGGCGCAGGAA), and (proCOLI3955P: 5′-FAM-ATGACCGAT GGATTCCCGTTCGAGTAC-TAMRA-3′), respectively. Specificity of the desired PCR products was confirmed by agarose gel electrophoresis and ethidium bromide staining.

Immunohistochemistry. Serial cryostat sections were used for staining nitrotyrosine. The primary antibody was a polyclonal rabbit anti-nitrotyrosine antibody (No.06-248, Upstate Biotechnology, USA, diluted 1:2000) as previously described [20]. As a negative control, normal mouse or rabbit IgG was used instead of the primary antibody.

Gelatin zymography. Frozen liver tissue was homogenized in protein extraction buffer (50 mM Tris/HCl, pH 7.6, 150 mM NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, and 0.01% (v/v) Brij-35). The supernatant of a centrifuged liver sample (20 μg of protein extract per line) was mixed 1:3 with sample buffer and separated by SDS–polyacrylamide gel electrophoresis in 7.5% polyacrylamide gel which had been copolymerized with 1 mg/ml gelatin as described elsewhere [21]. Gelatinase activity was quantified by an image analysis system (CS Analyser ver.3) based on densitometry of digested bands and compared with that of mouse MMPs.

In situ zymography. The procedures for in situ zymography were essentially based on the methods developed by Ikeda et al. [22] and the manufacturer's instructions (Wako Pure Chemical Industries, Osaka, Japan). Fresh tissue was embedded in OCT compound and sectioned at 4 μm. These thin sections were placed on a polyethylene terephthalate base film coated with cross-linked gelatin (7 μm thickness; MMP in situ Zymo-Film, Wako Pure Chemical Industries, Osaka, Japan) as described previously [22]. The film with sections was incubated in a moist chamber at 37 °C for 6 h. After the incubation it was stained for 4 min with Biebrich Scarlet (Wako Pure Chemical Industries, Osaka, Japan). The film was then rinsed for 10 min with distilled water. The degraded gelatin is not stained with Biebrich Scarlet, and areas of gelatinolytic activity are identifiable as white faded areas on the red background. In order to distinguish MMP activity from nonspecific proteinase activities in the liver tissue, serial tissue sections were placed on MMP-PT in situ Zymo-Film which is composed of a 7 μm-thick layer of special gelatin containing an MMP inhibitor, 1,10-phenanthroline. The faded area in this procedure represents nonspecific proteinase activities and the areas which are faded without the MMP inhibitor, but remain red (unfaded) with the MMP inhibitor, represent specific MMP activity. For quantification, numbers of points in the faded area were counted using the same method as described in the histology section.

Statistical evaluation. Values are expressed as means ± SEM. Significant differences were analyzed by using Student's t test for parametric data and Mann–Whitney U test for nonparametric data.

Results

iNOS deficiency did not alter plasma levels of lipids and ALT in animals on a high-fat diet (Table 1)

Plasma total cholesterol and ALT levels were markedly elevated and triglyceride levels were decreased with the high-fat diet in both $iNOS^{+/+}$ and $iNOS^{-/-}$ mice.

Table 1
Plasma lipids and ALT levels, and body weight in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice

	Normal diet		High-fat diet	
	iNOS$^{+/+}$	iNOS$^{-/-}$	iNOS$^{+/+}$	iNOS$^{-/-}$
Total cholesterol (mg/dl)	72.9 ± 23.2	90.4 ± 5.3	192.0 ± 14.6*	186.5 ± 27.8*
HDL cholesterol (mg/dl)	54.4 ± 18.4	68.8 ± 5.3	70.5 ± 4.8*	75.2 ± 8.0*
Triglyceride (mg/dl)	16.4 ± 3.5	15.6 ± 3.3	4.7 ± 1.5*	5.2 ± 1.9*
ALT (U)	23.4 ± 11.5	20.3 ± 4.0	180.3 ± 63.0	135.8 ± 27.4
Body weight (g)	25.0 ± 0.7	23.2 ± 0.5	24.9 ± 1.2	24.1 ± 0.8

Both iNOS knockout (iNOS−/−) and wild-type (iNOS+/+) mice were fed a high-fat diet or normal diet, starting from 8 weeks of age for 12 weeks. Sera were obtained 20 weeks ($n = 5$–7). ALT, alanine amino t.
* $p < 0.01$ normal diet vs high-fat diet.

Neither of these serum parameter levels was significantly different between the two strains. Body weights remained in the same ranges in all four groups.

iNOS mRNA expression and nitrotyrosine protein level

As shown in Fig. 1, iNOS mRNA was not observed in the liver of iNOS$^{-/-}$ mice, as expected. It was also not observed in the liver of iNOS$^{+/+}$ mice fed normal chow. However, iNOS mRNA expression was clearly observed in the liver of iNOS$^{+/+}$ mice fed a high-fat diet (Fig. 1). To demonstrate localization of NO-induced nitration reaction in the liver, nitrotyrosine staining was performed in iNOS$^{+/+}$ and iNOS$^{-/-}$mice (Fig. 2). Nitrotyrosine staining was observed only in the liver of iNOS$^{+/+}$ mice fed a high-fat diet, but not in that of iNOS$^{-/-}$ mice fed a high-fat diet. Nitrotyrosine staining was localized diffusely around the hepatocytes in the hepatic lobules. Notably, some relatively strong nitrotyrosine staining was seen in the periportal zone of the liver acinus, and in some spindle-shaped cells resembling Kupffer cells or stellate cells in the sinusoidal area. Nitrotyrosine staining was not observed in sections of liver from either strain fed normal chow (data not shown).

Association of iNOS deficiency with severe hepatic fibrosis in mice fed high-fat diet

The livers of mice fed normal chow showed slight fatty deposits in HE and Oil red O stainings (Fig. 3). In the livers of mice fed the high-fat diet, severe fatty metamorphosis was seen. Fine granular droplets of lipid were observed in the cytoplasm of hepatocytes throughout the hepatic lobules. The severity of fatty metamorphosis appeared to be similar in both strains with a high-fat diet.

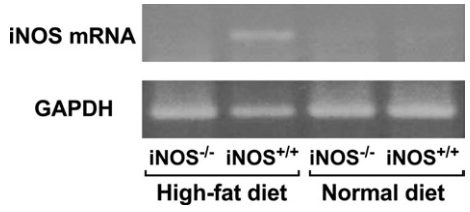

Fig. 1. iNOS mRNA expression in the liver (RT-PCR analysis). GAPDH is an internal control.

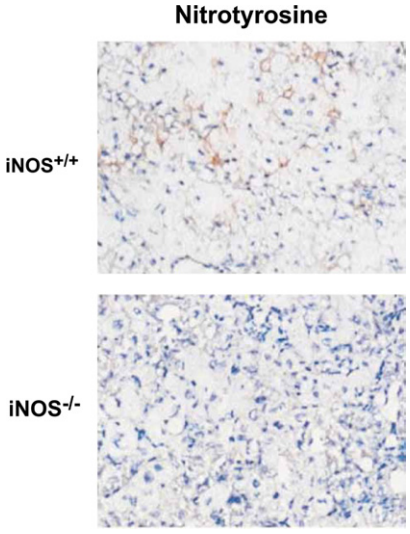

Fig. 2. Nitrotyrosine staining of the liver of animals in the high-fat diet groups. Immunohistochemical study was performed using a polyclonal rabbit anti-nitrotyrosine antibody as a primary antibody. The presence of nitrotyrosine is shown by the brown color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

In contrast, Azan staining revealed strong fibrotic changes throughout the hepatic tissues, especially around the degenerated hepatocytes and the central vein area in the liver of iNOS$^{-/-}$ mice fed a high-fat diet (Fig. 4A). However, only minor fibrotic change, such as pericellular fibrosis, was present in the liver of iNOS$^{+/+}$ mice. Calculated data revealed that the collagen fraction was significantly smaller in iNOS$^{+/+}$ mice than in iNOS$^{-/-}$ mice (Fig. 4B). Masson's trichrome staining also showed a similar staining pattern to that seen in Azan staining (data not shown).

Quantitative real-time RT-PCR for procollagen I and αSMA mRNA expression was performed to further evaluate hepatic fibrosis (Fig. 5). High-fat diet-induced procollagen I mRNA expression in both iNOS$^{+/+}$mice and iNOS$^{-/-}$mice. α-smooth muscle actin mRNA

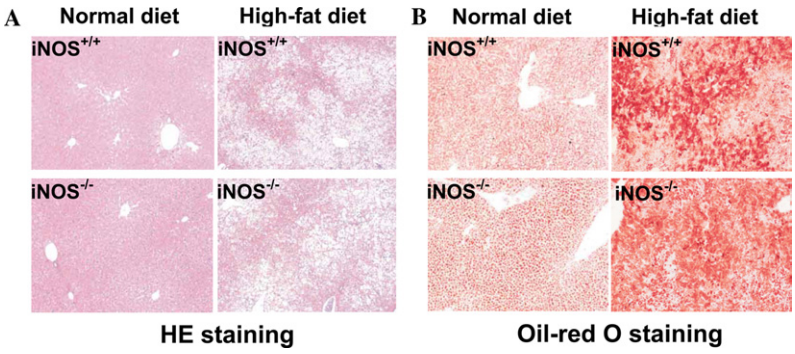

Fig. 3. HE (A) and Oil red O (B) stainings of the liver.

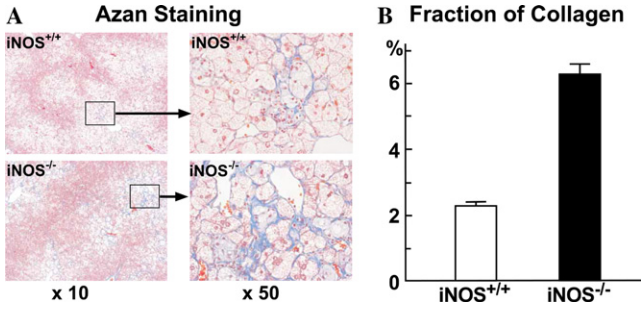

Fig. 4. (A) Azan staining of the liver with high-fat diet groups. (B) The collagen fraction determined by the point counting method was significantly smaller in $iNOS^{+/+}$ than in $iNOS^{-/-}$ mice ($2.3 \pm 01\%$ vs $6.3 \pm 03\%$, mean $\pm$ SEM, $n = 6$).

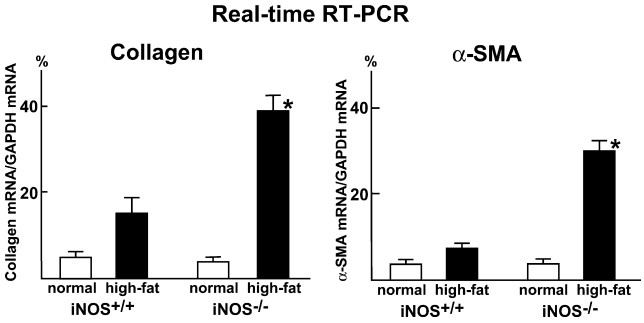

Fig. 5. Quantitative real-time RT-PCR for procollagen I mRNA and αSMA mRNA. Real-time RT-PCR was used to quantify expression levels of procollagen I and αSMA mRNA. The increases of expression were normalized to GAPDH. The mRNA levels are shown as a fold over that of $iNOS^{+/+}$ mice fed a normal diet. Data were obtained from 4 independent livers for each group and are expressed as means $\pm$ SEM. $*p < 0.01$, $iNOS^{+/+}$ vs $iNOS^{-/-}$.

expression was significantly induced only in the $iNOS^{-/-}$ mice with the high-fat diet.

iNOS deficiency suppressed expression of both MMP-2 and MMP-9

As shown in Fig. 6A, gelatin zymography of liver tissues showed that expression of proMMP-2 and proMMP-9 is induced by a high-fat diet in $iNOS^{+/+}$ and $iNOS^{-/-}$ mice. However, their expression was weak in $iNOS^{-/-}$ mice as compared to that in $iNOS^{+/+}$ mice. Importantly, active forms of MMP-9 and MMP-2 were clearly observed in the liver tissue of $iNOS^{+/+}$ mice, but were only barely apparent in that of $iNOS^{-/-}$ mice.

Similar results were obtained by in situ zymography of the liver tissues. As shown in Fig. 7, strong gelatinolytic activity was seen only in $iNOS^{+/+}$ mice. The activity was stronger around the centrilobular area in hepatic parenchyma than around the portal area. Minor spotty activity was found in the liver of $iNOS^{-/-}$ mice. In the presence of MMP inhibitor, the gelatinolytic activity was almost completely abolished, indicating that the activity is specifically due to MMPs, and not nonspecific proteinases. The fraction with gelatinolytic activity in $iNOS^{+/+}$ mice was significantly higher than that in $iNOS^{-/-}$ mice.

Discussion

In the present study, we have shown that iNOS induction attenuates the progression of liver dysfunction from fatty liver to fibrosis in mice fed a high-fat diet. Previous reports [9,10] have shown that a high-fat diet results in a fatty liver and induces iNOS mRNA expression in liver tissues. However, it is still not clear how iNOS contributes to the development of fatty liver, or to its progression to liver fibrosis; some workers found that iNOS induction-mediated liver injury [1,2], but others found that iNOS induction prevented the injury [5,7]. To elucidate the role of iNOS in the progression of liver damage induced by a high-fat diet, we compared the pathological findings in $iNOS^{+/+}$ and $iNOS^{-/-}$ mice fed a high-fat diet. The livers of both strains on the high-fat diet presented severe fatty metamorphosis, while strong fibrotic changes were present in the liver of $iNOS^{-/-}$ mice. As the histopathological characteristics of $iNOS^{-/-}$ mice are similar to those of nonalcoholic steatohepatitis (NASH) [23,24], $iNOS^{-/-}$ mice may be a good model of NASH. Regarding the role of iNOS, prominent fibrotic changes were seen in the

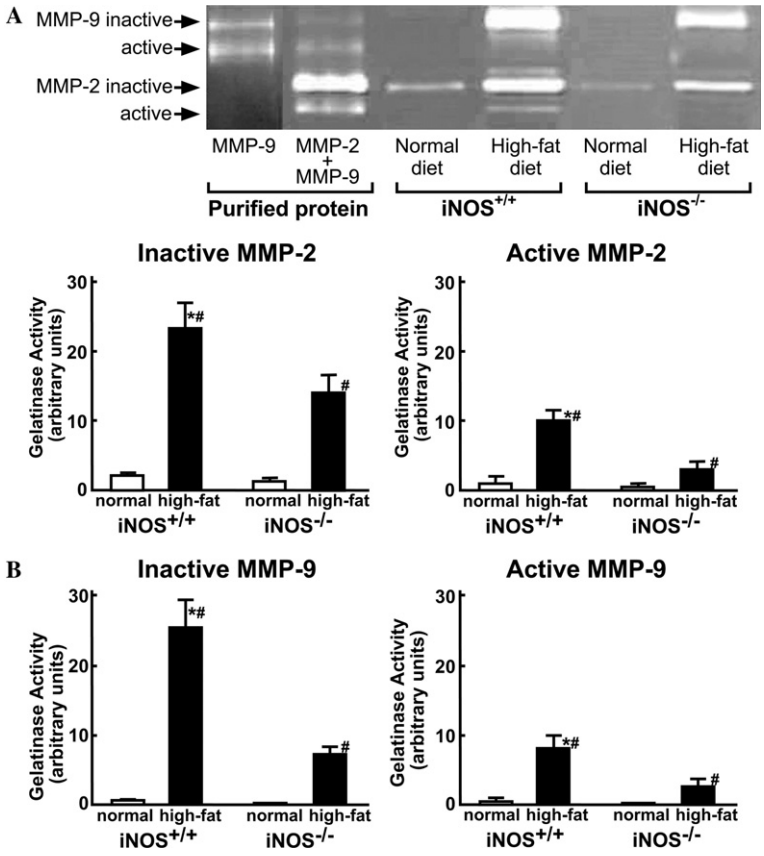

Fig. 6. MMP-2 and MMP-9 protein expression in the liver. (A) Gelatin zymography was performed to determine expression of MMP proteins in terms of gelatinase activity in liver homogenates. Purified inactive and active MMP-2 and MMP-9 proteins, and protein extracts of homogenized liver (20 μg in each line) from the four groups were loaded separately. Purified human MMP-2 and MMP-9 proteins were loaded in separate lanes as positive controls. (B) Gelatinase activity was quantified by densitometry. Data were obtained from 4 independent livers for each group and are expressed as means ± SEM. $^{\#}p < 0.01$, high-fat diet vs normal diet. $^{*}p < 0.01$, iNOS$^{+/+}$ vs iNOS$^{-/-}$.

hepatic tissues of iNOS$^{-/-}$ mice compared with those of iNOS$^{+/+}$ mice, but there were little differences of fatty deposition and inflammatory cell infiltration, or of plasma ALT level, between the two strains, iNOS expression appears to contribute to the progression to liver fibrosis without influencing fatty deposition. This supports the idea that iNOS acts as a protector against a presently unknown second "hit" [25] in the progression of NASH.

Our finding of the presence of minor fibrotic changes even in iNOS$^{+/+}$ mice fed a high-fat diet may seem to be surprising, since the majority of previous studies in which a high-fat diet was given to mice or rats revealed fatty liver without fibrotic changes [9,10]. However, we found one report of steatohepatitis with mild fibrosis in wild-type mice fed a high-fat diet for 6 months [26]. Therefore, long-term feeding with a high-fat diet can induce mild hepatic fibrosis.

Major components of the extracellular matrix in liver fibrosis are collagen types I, III, and IV. These collagens are mainly degraded by MMP-1, 2, and 9 [13]. Both induction of collagen expression and suppression of activated MMPs expression would favor collagen deposition and fibrosis in the liver of iNOS$^{-/-}$ mice. As stellate cells are the predominant source of collagen production [27], the prominent expression of αSMA mRNA and collagen mRNA observed in this study suggests that an increase in the number of stellate cells and enhanced activation of the cells may have been induced in the liver of knockout mice. In contrast, the production of collagen is increased concomitantly with the reduction of MMP activity through induction of iNOS in the wild-type mice. The previous in vitro studies show that NO may exert a direct antifibrogenic action by inhibiting activation of hepatic stellate cells through modulating intracellular cAMP [28] or cGMP [29] levels, and NO acts as a ROS scavenger, thus inhibiting proliferation of hepatic stellate cells [30].

The induction of active forms of MMP-2 and MMP-9 in gelatin zymography and diffuse gelatinolytic activity in the in situ zymography shown only in iNOS$^{+/+}$ mice suggests that the induction of MMPs may be a defense mechanism naturally present in wild-type mice (iNOS$^{+/+}$). Several in vitro studies have suggested that NO may enhance the expression of MMP-2 and MMP-9 in various cell types [31–33] through NO-mediated cGMP-dependent mechanisms [31]. To investigate

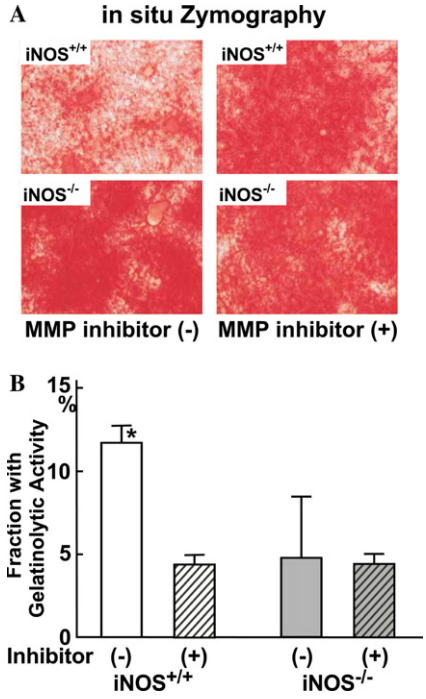

Fig. 7. In situ zymography of the liver with a high-fat diet. (A) Areas of gelatinolytic activity are identifiable as white faded areas on the red background. In order to distinguish MMP activity from nonspecific proteinase activities, serial tissue sections were placed on in situ Zymo-Film containing an MMP inhibitor, 1,10-phenanthroline. (B) Fraction showing gelatinolytic activity quantified by densitometry. Data were obtained from 4 independent mice for each group and are expressed as means ± SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

the mechanisms of upregulation of active MMPs by iNOS, we examined the production of the NO-induced oxidant, peroxynitrite, since peroxynitrite was shown to activate proMMP-1, 2, 9, and 13 [34–36]. As peroxynitrite reacts with tyrosine residues to form nitrotyrosine, which is detectable in the tissue, nitrotyrosine staining was performed. Nitrotyrosine staining was observed only in the liver of $iNOS^{+/+}$ mice fed a high-fat diet. Moreover, the nitrotyrosine staining was slightly stronger in the portal area than in the central area, which supports the distribution pattern of collagen deposit; i.e., fibrosis tends to occur more around the central vein area than the portal area in $iNOS^{-/-}$ mice fed a high-fat diet. However, the discordant distribution of nitrotyrosine and MMPs observed in wild-type mice fed a high-fat diet may be explained by the fact that peroxynitrite alone is not responsible for enhancing MMPs expression. In fact, in situ zymography showed that MMPs activities were still weakly found in $iNOS^{-/-}$ mice where nitrotyrosine staining was not seen immunohistochemically. The fact that iNOS induction and nitrotyrosine production were associated with attenuation of fibrosis is consistent with the findings of our previous study of atheroma [20]. Although a growing body of evidence indicates critical roles of NO in various processes of liver disease, it remains controversial whether NO plays an injurious or a protective role [1,2,5,7,37]. Even concerning the role of iNOS, opposite outcomes have been reported in ethanol-induced liver injury. Therefore, our finding that loss of iNOS induction favors the progression to liver fibrosis from steatohepatitis is significant. Although we focused on MMP-2 and MMP-9 expression in this study, the involvement of other factors, including other MMPs and TIMPs (tissue inhibitor of metalloproteinases), as well as differences in the rates of production of various collagens, and in the distribution of cells which produce MMPs, should be considered.

In conclusion, despite extensive evidence that NO has critical roles in various processes of liver disease, it remains controversial whether NO is injurious or protective [1,2,5,7,37]. We have shown for the first time that a high-fat diet induces fatty liver and fibrosis in iNOS-deficient mice. The mechanisms through which failure to induce iNOS leads to progression from steatohepatitis to fibrosis appear to include both a reduction of MMPs activity and an increase in collagen production. These data suggest that iNOS may act as a potent antifibrotic effector during the development of steatohepatitis, and also suggest that agents promoting NO release may have therapeutic value in steatohepatitis.

Acknowledgments

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References

[1] B. Chang, M. Nishikawa, E. Sato, M. Inoue, Mice lacking inducible nitric oxide synthase show strong resistance to anti-Fas antibody-induced fulminant hepatitis, Arch. Biochem. Biophys. 411 (2003) 63–72.
[2] T.M. Rahman, H.J. Hodgson, The effects of early and late administration of inhibitors of inducible nitric oxide synthase in a thioacetamide-induced model of acute hepatic failure in the rat, J. Hepatol. 38 (2003) 583–590.
[3] J. Liu, C. Li, M.P. Waalkes, J. Clark, P. Myers, J.E. Saavedra, L.K. Keefer, The nitric oxide donor, V-PYRRO/NO, protects against acetaminophen-induced hepatotoxicity in mice, Hepatology 37 (2003) 324–333.
[4] G. Sass, K. Koerber, R. Bang, H. Guehring, G. Tiegs, Inducible nitric oxide synthase is critical for immune-mediated liver injury in mice, J. Clin. Invest. 107 (2001) 439–447.

[5] I.N. Hines, H. Harada, S. Bharwani, K.P. Pavlick, J.M. Hoffman, M.B. Grisham, Enhanced post-ischemic liver injury in iNOS-deficient mice: a cautionary note, Biochem. Biophys. Res. Commun. 284 (2001) 972–976.
[6] C. Garcia-Monzon, P.L. Majano, I. Zubia, P. Sanz, A. Apolinario, R. Moreno-Otero, Intrahepatic accumulation of nitrotyrosine in chronic viral hepatitis is associated with histological severity of liver disease, J. Hepatol 32 (2000) 331–338.
[7] R.M. Rai, F.Y. Lee, A. Rosen, S.Q. Yang, H.Z. Lin, A. Koteish, et al., Impaired liver regeneration in inducible nitric oxide synthase-deficient mice, Proc. Natl. Acad. Sci. USA 95 (1998) 13829–13834.
[8] L. McNaughton, L. Puttagunta, M.A. Martinez-Cuesta, N. Kneteman, I. Mayers, R. Moqbel, et al., Distribution of nitric oxide synthase in normal and cirrhotic human liver, Proc. Natl. Acad. Sci. USA 99 (2002) 17161–17166.
[9] J.W. Kim, K.W. Kang, G.T. Oh, J. Song, N.D. Kim, Y.K. Pak, Induction of hepatic inducible nitric oxide synthase by cholesterol in vivo and in vitro, Exp. Mol. Med. 34 (2002) 137–144.
[10] G. Wan, S. Ohnomi, N. Kato, Increased hepatic activity of inducible nitric oxide synthase in rats fed on a high-fat diet, Biosci. Biotechnol. Biochem. 64 (2000) 555–561.
[11] C. Garcia-Monzon, E. Martin-Perez, O.L. Iacono, M. Fernandez-Bermejo, P.L. Majano, A. Apolinario, et al., Characterization of pathogenic and prognostic factors of nonalcoholic steatohepatitis associated with obesity, J. Hepatol. 33 (2000) 716–724.
[12] A. Casini, E. Ceni, R. Salzano, P. Biondi, M. Parola, A. Galli, et al., Neutrophil-derived superoxide anion induces lipid peroxidation and stimulates collagen synthesis in human hepatic stellate cells: role of nitric oxide, Hepatology 25 (1997) 361–367.
[13] H. Trachtman, S. Futterweit, P. Garg, K. Reddy, P.C. Singhal, Nitric oxide stimulates the activity of a 72-kDa neutral matrix metalloproteinase in cultured rat mesangial cells, Biochem. Biophys. Res. Commun. 218 (1996) 704–708.
[14] W. Eberhardt, T. Beeg, K.F. Beck, S. Walpen, S. Gauer, H. Bohles, J. Pfeilschifter, Nitric oxide modulates expression of matrix metalloproteinase-9 in rat mesangial cells, Kidney Int. 57 (2000) 59–69.
[15] C. Zaragoza, M. Balbin, C. Lopez-Otin, S. Lamas, Nitric oxide regulates matrix metalloprotease-13 expression and activity in endothelium, Kidney Int. 61 (2002) 804–808.
[16] P. Jurasz, G. Sawicki, M. Duszyk, J. Sawicka, C. Miranda, I. Mayers, M.W. Radomski, Matrix metalloproteinase 2 in tumor cell-induced platelet aggregation: regulation by nitric oxide, Cancer Res. 61 (2001) 376–382.
[17] J.D. MacMicking, C. Nathan, G. Hom, N. Chartrain, D.S. Fletcher, M. Trumbauer, et al., Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase, Cell 81 (1995) 641–650.
[18] J.S. Munday, K.G. Thompson, K.A. James, B.W. Manktelow, Dietary antioxidants do not reduce fatty streak formation in the C57BL/6 mouse atherosclerosis model, Arterioscler. Thromb. Vasc. Biol. 18 (1998) 114–119.
[19] I. Singh, M. Grams, W.H. Wang, T. Yang, P. Killen, A. Smart, et al., Coordinate regulation of renal expression of nitric oxide synthase, renin, and angiotensinogen mRNA by dietary salt, Am. J. Physiol. 270 (1996) F1027–1037.
[20] X.L. Niu, X. Yang, K. Hoshiai, K. Tanaka, S. Sawamura, Y. Koga, H. Nakazawa, Inducible nitric oxide synthase deficiency does not affect the susceptibility of mice to atherosclerosis but increases collagen content in lesions, Circulation 103 (2001) 1115–1120.
[21] M. Kuzuya, S. Kanda, T. Sasaki, N. Tayama-Mori, X. Cheng, T. Itoh, S. Itohara, A. Iguchi, Deficiency of gelatinase A suppresses smooth muscle cell invasion and development of experimental intimal hyperplasia, Circulation 108 (2003) 1375–1381.
[22] M. Ikeda, R. Maekawa, H. Tanaka, M. Matsumoto, Y. Takeda, Y. Tamura, R. Nemori, et al., Inhibition of gelatinolytic activity in tumor tissues by synthetic matrix metalloproteinase inhibitor: application of film in situ zymography, Clin. Cancer Res. 6 (2000) 3290–3296.
[23] C.A. Matteoni, Z.M. Younossi, T. Gramlich, N. Boparai, Y.C. Liu, A.J. McCullough, Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity, Gastroenterology 116 (1999) 1413–1419.
[24] E.M. Brunt, C.G. Janney, A.M. Di Bisceglie, B.A. Neuschwander-Tetri, B.R. Bacon, Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions, Am. J. Gastroenterol. 94 (1999) 2467–2474.
[25] C.P. Day, O.F. James, Steatohepatitis: a tale of two "hits"?, Gastroenterology 114 (1998) 842–845.
[26] G. Baffy, C. Zhang, J.N. Glickman, B.B. Lowell, Obesity-related fatty liver is unchanged in mice deficient for mitochondrial uncoupling protein 2, Hepatology 35 (2002) 753–761.
[27] S.L. Friedman, The cellular basis of hepatic fibrosis. Mechanisms and treatment strategies, N. Engl. J. Med. 328 (1993) 1828–1835.
[28] P. Failli, F.R. De, A. Caligiuri, A. Gentilini, R.G. Romanelli, F. Marra, G. Batignani, C.T. Guerra, G. Laffi, P. Gentilini, M. Pinzani, Nitrovasodilators inhibit platelet-derived growth factor-induced proliferation and migration of activated human hepatic stellate cells, Gastroenterology 119 (2000) 479–492.
[29] N. Kawada, T. Kuroki, M. Uoya, M. Inoue, K. Kobayashi, Smooth muscle alpha-actin expression in rat hepatic stellate cell is regulated by nitric oxide and cGMP production, Biochem. Biophys. Res. Commun. 229 (1996) 238–242.
[30] G. Svegliati-Baroni, S. Saccomanno, H. van Goor, P. Jansen, A. Benedetti, H. Moshage, Involvement of reactive oxygen species and nitric oxide radicals in activation and proliferation of rat hepatic stellate cells, Liver 21 (2001) 1–12.
[31] M. Marcet-Palacios, K. Graham, C. Cass, A.D. Befus, I. Mayers, M.W. Radomski, Nitric oxide and cyclic GMP increase the expression of matrix metalloproteinase-9 in vascular smooth muscle, J. Pharmacol. Exp. Ther. 307 (2003) 429–436.
[32] Y. Hirai, K. Migita, S. Honda, Y. Ueki, S. Yamasaki, S. Urayama, M. Kamachi, A. Kawakami, H. Ida, M. Kita, T. Fukuda, K. Shibatomi, Y. Kawabe, T. Aoyagi, K. Eguchi, Effects of nitric oxide on matrix metalloproteinase-2 production by rheumatoid synovial cells, Life Sci. 68 (2001) 913–920.
[33] V. Novaro, A. Colman-Lerner, F.V. Ortega, A. Jawerbaum, D. Paz, F. Lo Nostro, C. Pustovrh, M.F. Gimeno, E. Gonzalez, Regulation of metalloproteinases by nitric oxide in human trophoblast cells in culture, Reprod. Fertil. Dev. 13 (2001) 411–420.
[34] Y. Ishii, T. Ogura, M. Tatemichi, H. Fujisawa, F. Otsuka, H. Esumi, Induction of matrix metalloproteinase gene transcription by nitric oxide and mechanisms of MMP-1 gene induction in human melanoma cell lines, Int. J. Cancer 103 (2003) 161–168.
[35] T. Okamoto, T. Akaike, T. Nagano, S. Miyajima, M. Suga, M. Ando, et al., Activation of human neutrophil procollagenase by nitrogen dioxide and peroxynitrite: a novel mechanism for procollagenase activation involving nitric oxide, Arch. Biochem. Biophys. 342 (1997) 261–274.
[36] V.S. Mujumdar, G.M. Aru, S.C. Tyagi, Induction of oxidative stress by homocyst(e)ine impairs endothelial function, J. Cell Biochem. 82 (2001) 491–500.
[37] D.C. Rockey, V. Shah, Nitric oxide biology and the liver: report of an AASLD research workshop, Hepatology 39 (2004) 250–257.