The role of iNOS in cholesterol-induced liver fibrosis

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Accumulation of cholesterol in the liver is associated with the development of non-alcoholic steatohepatitis-related fibrosis. However, underlying mechanisms are not well understood. The present study investigated the role of inducible nitric oxide synthase (iNOS) in cholesterol-induced liver fibrosis by feeding wild-type (WT) and iNOS-deficient mice with control or high-cholesterol diet (HCD) for 6 weeks. WT mice fed with HCD developed greater liver fibrosis, compared with iNOS-deficient mice, as evident by Sirius red staining and higher expression levels of profibrotic genes. Enhanced liver fibrosis in the presence of iNOS was associated with hypoxia-inducible factor-1α stabilization, matrix metalloproteinase-9 expression, and enhanced hepatic DNA damage. The profibrotic role of iNOS was also demonstrated *in vivo* using a selective inhibitor of iNOS as well as *in vitro* in a rat liver stellate cell line (HSC-T6). In conclusion, these findings suggest that iNOS is an important mediator in HCD-induced liver fibrosis.
Laboratory Investigation (2015) **95,** 914–924; doi:10.1038/labinvest.2015.67; published online 22 June 2015

Non-alcoholic fatty liver disease (NAFLD) is currently one of the most common liver diseases worldwide, affecting both adults and children. The disease encompasses a wide spectrum of liver damage ranging from simple hepatic steatosis and non-alcoholic steatohepatitis (NASH) to liver cirrhosis and increased risk for developing hepatocellular carcinoma. Pathological characterization of NASH includes the presence of steatosis and inflammation and recently it has also been recognized as a major cause of liver fibrosis. Liver fibrosis represents the liver's wound healing response to virtually all forms of chronic liver injury. This dynamic process involves the accumulation of the extracellular matrix (ECM) following injury. Under acute or limited insults, parenchymal cells regenerate and replace the necrotic or apoptotic cells. However, if the liver injury is sustained, regeneration fails and liver parenchymal cells are substituted by scar tissue.[1,2]

Fibrosis associated with NASH has been suggested to be the physiological consequence of chronic hepatic injury, necrosis, and inflammation as well as unbalanced intrahepatic lipid metabolism (steatosis), insulin resistance, oxidative stress, and lipid peroxidation.[3–5]

Different lipid molecules may mediate the pathogenesis and the progression of NASH. To date, the identity of a dominant lipid molecule/s, which causes liver lipotoxicity, is still unknown. Recently, attention has been given to the role of hepatic cholesterol content in NASH pathology.[6–10] Evidence collected from both human and animal models of NASH point to cholesterol as a potential mediator of lipotoxicity in NASH, including the development of hepatic fibrosis. In the first National Health and Nutrition Examination Survey in the United States, dietary cholesterol consumption was positively associated with the risk of cirrhosis or liver cancer. Consistent with these findings, cholesterol-lowering agents, such as statins and ezetimibe, were shown to improve liver fibrosis among patients with hypercholesterolemia and mice with NASH.[8–11] Enhanced liver fibrosis is also evident in experimental studies conducted in rodents and rabbits following the consumption of a high-cholesterol diet (HCD) containing cholic acid or a high-fat/HCD diet or methionine–choline-deficient diet supplemented with cholesterol.[8,9]

Initiation of chronic liver diseases commonly involves an inflammatory phase, which progresses to fibrosis after continuous oxidative stress. Under these conditions inducible nitric oxide synthase (iNOS) is upregulated, leading to the production of large amounts of nitric oxide (NO).[12] The role of iNOS in fibrosis formation is not clearly understood. Data from animal models of fibrosis or NASH-related liver fibrosis have shown conflicting results of both beneficial and deleterious effects of iNOS.[12–19] The exact reason/s for these paradoxical effects is difficult to explain. Therefore, the

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present study was undertaken to elucidate the role of iNOS in the pathophysiology of liver fibrosis due to consumption of a HCD.

MATERIALS AND METHODS
Animal Experiments
Adult male wild-type (WT) C57BL/6J mice and C57BL/6J iNOS-deficient (iNOS−/−) mice were purchased from Harlan Laboratories, (Jerusalem, Israel) and Jackson Laboratories (Bar Harbor, ME), respectively. Mice were maintained in a temperature- and light-controlled facility, and permitted *ad libitum* consumption of water and pellet chow. Experiment 1 (genetic inhibition of iNOS): WT and iNOS-deficient mice were fed with standard diet (*n* = 3–4 per group) or with HCD (1% cholesterol+0.5% cholic acid, *n* = 6–7 per group). Experiment 2 (pharmacological inhibition of iNOS): WT mice were fed a standard diet or HCD with or without the addition of iNOS-specific inhibitor S,S′-1,3-phenylene-bis (1,2-ethanediyl)-bis-isothiourea dihydrobromide (PBIT, 100 p.p.m. in diet, *n* = 7–9 per group). In both experiments, after 6 weeks of diet consumption, animals were killed to obtain plasma and liver tissue. All animals received humane care, and all study protocols were approved by Institutional Animal Care and Use Committee.

Blood Parameters and Biochemical Analyses
Analysis of plasma alanine aminotransferase (ALT/SGPT) and aspartate aminotransferase (AST/SGOT) levels were performed by American Laboratories (Hertzlia, Israel). Total lipid was extracted from livers using the Folch method.[20] Cholesterol levels were determined colorimetric following saponification. In brief, about 4 ml of absolute ethanol and 1 ml 60% KOH were added to samples and mixed thoroughly. The capped tubes were placed in 65 °C water bath for 1 h. After cooling to room temperature, 3 ml of distilled water were added and the non-saponifiables were extracted twice with petrol ether. Following petrol ether evaporation, cholesterol was quantified using a color reagent of glacial acetic acid–$FeSO_4$–H_2SO_4. The absorbance of the cooled, color mixture was read at 490 nm against the reagent blank.

Liver Histology and Immunohistochemistry
Livers were fixed in 4% formaldehyde (Bio-Lab, Jerusalem, Israel), and microtome (Leica Microsystems) sections (5 μm) were prepared. Hematoxylin and eosin staining was used for tissue section visualization and Sirius red was used to stain collagen in tissue sections. Immunohistochemistry was done with anti-proliferating cell nuclear antigen (PCNA) or alpha smooth muscle actin (αSMA) antibodies.

Quantitative Real-Time PCR
Total RNA was isolated using the Tri-Reagent (Sigma-Aldrich, Israel) method, according to the manufacturer's protocol. Complementary DNA was prepared using a high-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using the 7300 real-time PCR System (Applied Biosystems, Warrington, UK) using specific primers (Supplementary Information). Fold change in gene expression was determined by normalizing to 18S mRNA.

Protein Extraction and Western Blot Analyses
Whole-cell lysates were prepared in RIPA buffer and nuclear extracts were prepared as previously described.[21] Aliquots of protein were then subjected to western blot analysis. Ponceau S (Sigma) staining was used to verify equal loading and transfer. Primary antibodies used were: iNOS (BD Biosciences), hypoxia-inducible factor 1(HIF-1) α (Novus Biologicals), αSMA, matrix metalloproteinase-9 (MMP-9; Abcam), and histone 2AX (H2AX) phospho-specific (Ser139; Cell Signaling Technology). Secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA).

Gelatin Zymography
Gelatinolytic activity of liver homogenates was examined by gelatin zymography as described previously.[22]

Cell Culture and Treatments
HSC-T6 cells, an immortalized cell line of rat hepatic stellate cells (HSCs; generously provided by Dr S.L. Friedman), were maintained at 37 °C in 5% CO_2 in Dulbecco's modified Eagles medium supplemented with 10% heat inactivated FBS, 1 × glutamine, penicillin (100 U/ml), and streptomycin (100 mg/ml). Approximately 24 h after seeding, HSC-T6 cells were washed twice with PBS, and the medium was replaced by serum-free medium. HSCs were then incubated with or without water-soluble cholesterol (50 μM) for 24 h. N-nitro-L-arginine methyl ester hydrochloride (L-NAME; 0-2 mM) was used to inhibit iNOS. Alternatively, cells were incubated with the NO donor, DETA-NONOate (1 mM) for 0–12 h.

Statistical Analysis
Statistical analysis was performed using the multiple groups. Student's *t*-test. Data are expressed as mean ± s.e.m., and *P* < 0.05 was considered statistically significant.

RESULTS
Effects of HCD on Liver Steatosis in WT and iNOS-Deficient Mice
Following consumption of the HCD, a marked activation of iNOS was observed in WT mice, whereas no protein expression was observed in iNOS-deficient mice (Figure 1a). However, as shown in Figure 1b, HCD induced similar levels of liver steatosis in both the groups of mice. HCD feeding also led to increased liver weights in the WT and iNOS-deficient mice, whereas total body weights were decreased, leading to higher liver to body weight ratio. It should be noted that liver weight and liver to body weight ratio were slightly, but significantly, lower in HCD-fed iNOS-deficient mice (Figure 1c). Conversely, total lipids and cholesterol contents
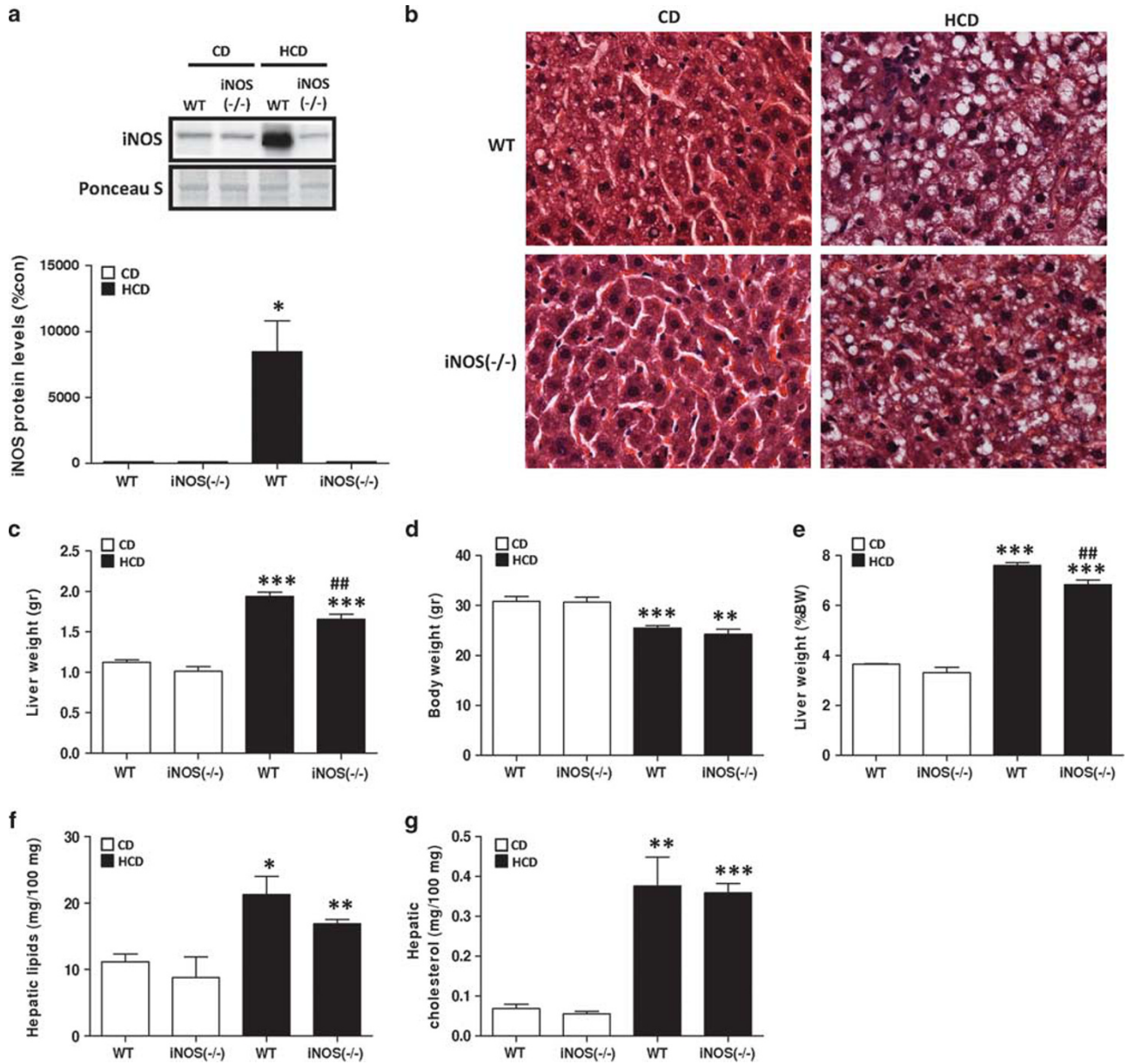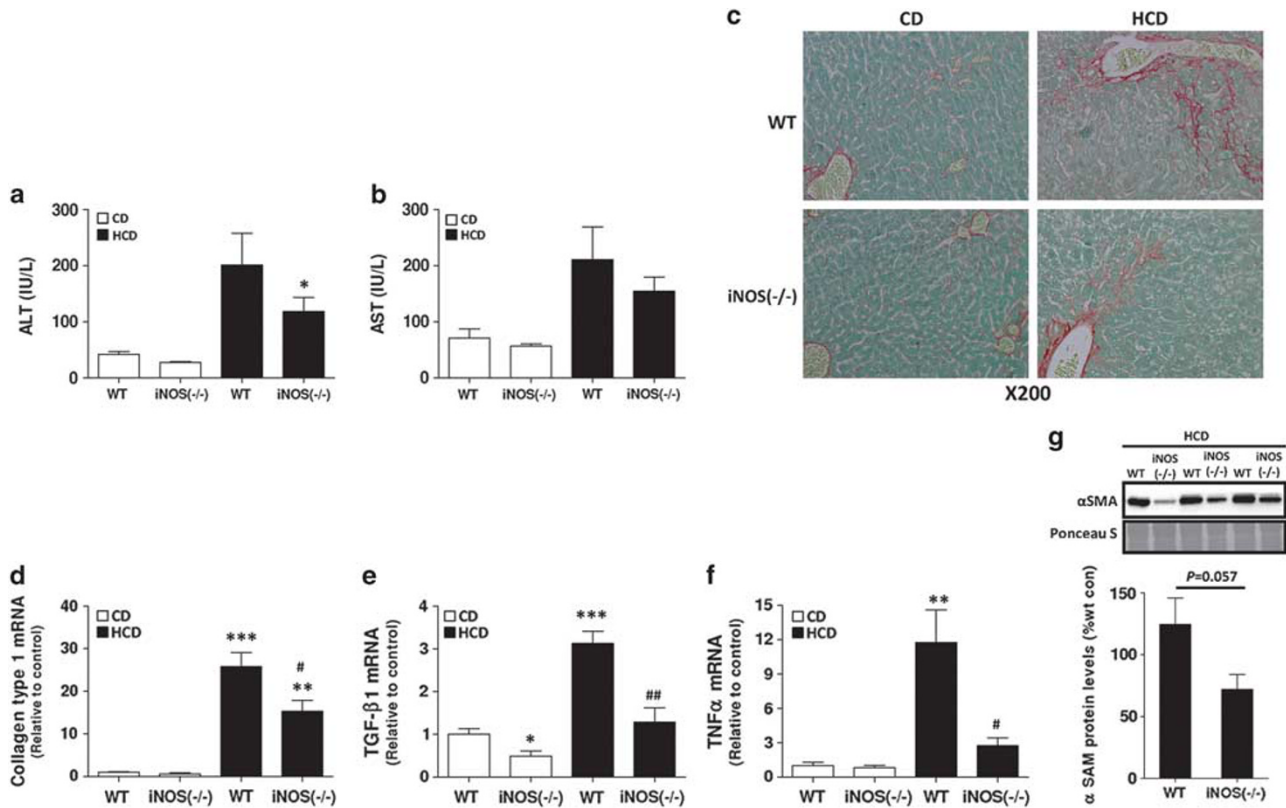
Figure 1 Effects of high-cholesterol diet (HCD) on liver steatosis in wild-type (WT) and iNOS-deficient mice (iNOS(−/−)). WT and iNOS-deficient mice were fed with control diet (CD) (n=3–4, white bars) or HCD (n=6–7, black bars) for 6 weeks. (a) iNOS protein levels. (b) Hematoxylin and eosin (H&E)-stained sections of representative liver samples. (c) Liver weight (g); (d) body weight (BW) (g); (e) liver weight (% BW); and hepatic (f) lipids and (g) cholesterol levels. Results are means±s.e.m. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ vs WT fed with CD; ##$P \leq 0.01$ vs WT fed with HCD. NS, not specific.

did not significantly differ between WT and iNOS-deficient mice following HCD administration (Figure 1f and g).

Effects of HCD on Liver Damage and Fibrosis in WT and iNOS-Deficient Mice

Liver damage, as evaluated by plasma liver enzyme levels, increased in both the HCD-fed groups (Figure 2a and b). Liver samples were then stained with Sirius red to evaluate the development of tissue fibrosis. In control groups of WT and iNOS-deficient mice, staining was only observed in areas surrounding the blood vessels. In HCD-fed WT mice, extensive

intralobular liver fibrosis was observed. iNOS-deficient mice fed with the same diet demonstrated much less fibrosis (Figure 2c). Consistent with these findings, the expression levels of collagen type-1 and the profibrotic cytokine tumor necrosis factor α (TNFα) and transforming growth factor β1(TGF-β1) were also induced to a greater extent in HCD-fed WT mice compared with HCD-fed iNOS-deficient mice (Figure 2d–f). Overall, αSMA protein levels tended to be lower in HCD-fed iNOS-deficient mice compared with WT mice fed with the same diet, as was demonstrated by western blot (Figure 2g). However, significant variability in protein levels in this group

Figure 2 Effects of high-cholesterol diet (HCD) on liver damage and fibrosis in wild-type (WT) and iNOS-deficient mice (iNOS(−/−)). WT and iNOS-deficient mice fed with control diet (CD) (n=3–4, white bars) or HCD (n=6–7, black bars) for 6 weeks. Plasma levels of (**a**) ALT and (**b**) AST. (**c**) Sirius red stained sections of representative liver samples. Hepatic mRNA expression levels of (**d**) collagen type 1 (**e**), transforming growth factor-β1 (TGF-β1), (**f**) tumor necrosis factor-α (TNF-α), and (**g**) α-smooth muscle actin (α-SMA) protein levels. Results are means±s.e.m. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ vs WT fed with CD; #$P \leq 0.05$, ##$P \leq 0.01$ vs WT fed with HCD.

was observed. Variance was also high in immunohistochemistry analysis (Supplementary Figure S1).

Effects of HCD on HIF-1 Activation in WT and iNOS-Deficient Mice

We have recently demonstrated the importance of iNOS in HIF-1α stabilization and accumulation.[23,24] Consistent with previous findings, in the current study HIF-1α was significantly increased in HCD-fed WT mice and was higher than in iNOS-deficient mice (Figure 3a). The elevated HIF-1 induction in HCD-fed WT mice was concomitant with a greater increase in the expression of profibrotic genes, ie, platelet-derived growth factor (PDGF)-A, PDGF-B, fibroblast growth factor-2 (FGF-2), and a trend toward higher plasminogen activator inhibitor-1 (PAI-1) expression, all of which were previously shown to be regulated in HIF-1α-dependent manner (Figure 3b–e). These results demonstrate the importance of the HIF/iNOS axis in HCD-induced liver fibrosis.

Effects of HCD on MMP-2 and MMP-9 Activation in WT and iNOS-Deficient Mice

Compelling evidence has documented the association of MMPs with liver fibrosis.[25,26] MMP-2 and MMP-9 expression

was significantly enhanced by HCD in WT mice, whereas only MMP-2 expression was significantly increased in iNOS-deficient mice. Yet, as shown in Figure 4a and b, both MMPs gene expression was significantly lower in the iNOS-deficient group compared with WT mice. MMP-9, but not MMP-2, activity was much lower in HCD-fed iNOS-deficient mice, compared with WT mice that were fed with the same diet. Moreover, the activity of high-molecular weight gelatinase, which was previously identified as an MMP-9 dimer, was also increased to a greater extent in HCD-fed WT mice (Figure 4c-e). Along with these changes in MMP-9 expression and activity, MMP-9 protein levels also tended to be lower in iNOS-deficient mice (Supplementary Figure S2).

## Effects of Pharmacological Inhibition of iNOS in HCD-Induced Liver Fibrosis

The role of iNOS in HCD-induced liver fibrosis, and specifically in MMP-9 activation under this feeding regime, was further tested using a highly selective and potent iNOS-specific inhibitor (PBIT),[27] which temporarily blocked iNOS activity during HCD consumption. Both control and PBIT-treated mice exhibited higher liver weight following the consumption of HCD (Figure 5a-c). Similarly, liver damage,
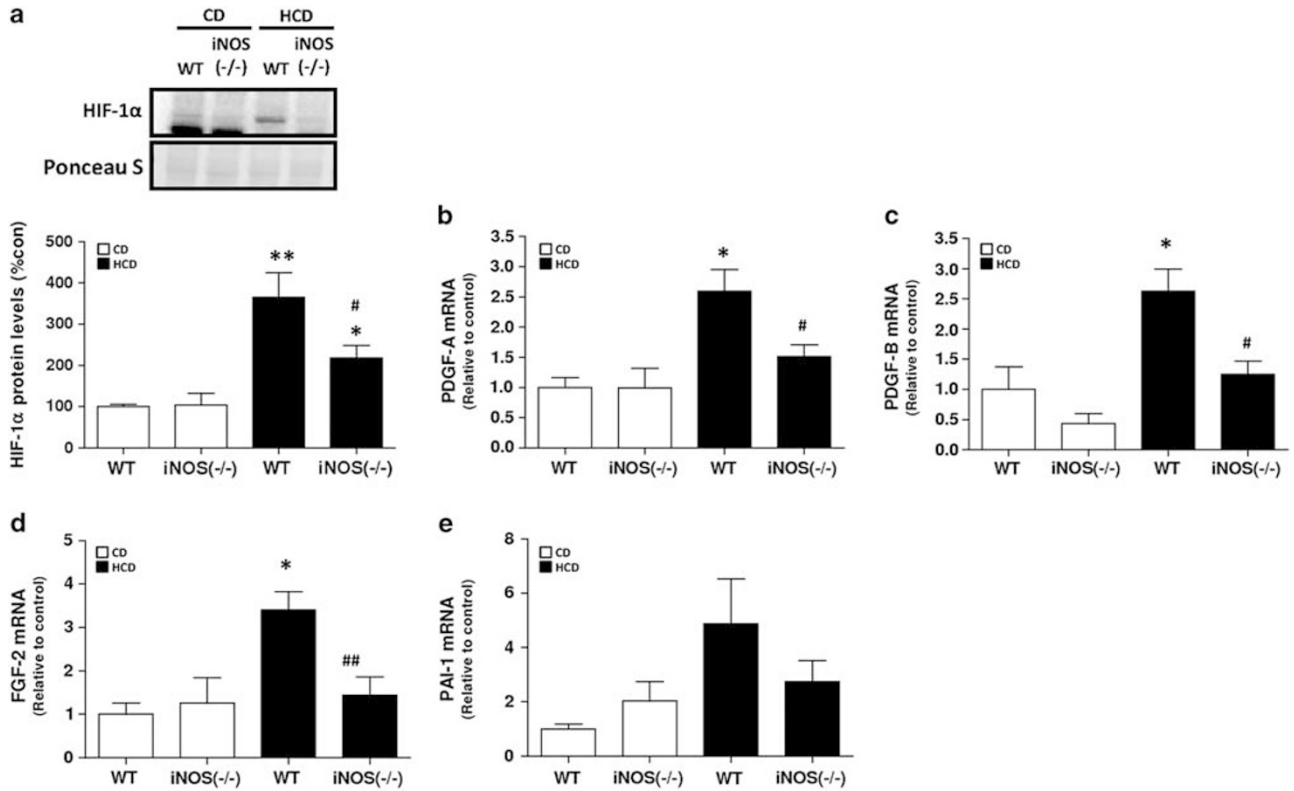
Figure 3 Effects of high-cholesterol diet (HCD) on liver hypoxia inducible factor-1 (HIF-1) activation in wild-type (WT) and iNOS deficient mice (iNOS (−/−)). WT and iNOS deficient mice fed with control diet (CD) ($n=3–4$, white bars) or HCD ($n=6–7$, black bars) for 6 weeks. (**a**) HIF-1α protein levels in the nucleus and hepatic mRNA expression levels of HIF-1 target genes (**b**) platelet-derived growth factor (PDGF)-A (**c**) PDGF-B (**d**) fibroblast growth factor-2 (FGF-2) (**e**) plasminogen activator inhibitor-1 (PAI-1). Results are means±s.e.m. *$p≤0.05$, **$p≤0.01$ vs. WT fed with CD; #$p≤0.05$, ##$p≤0.01$ vs. WT fed with HCD. NS, not specific.
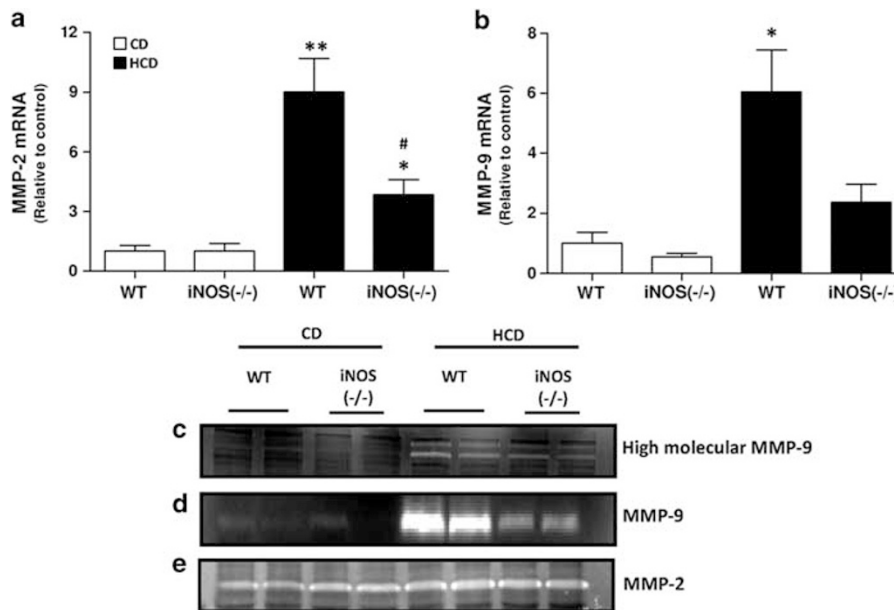
Figure 4 Effects of high-cholesterol diet (HCD) on liver matrix metalloproteinase (MMP)-2 and -9 induction in wild-type (WT) and iNOS-deficient mice (iNOS(−/−)). WT and iNOS-deficient mice fed with control diet (CD) (white bars) or HCD (black bars) for 6 weeks. Hepatic mRNA expression levels of (**a**) MMP-2 and (**b**) MMP-9. Activity of (**c**) high molecular MMP-9, (**d**) MMP-9, and (**e**) MMP-2 assessed by gelatin zymography. Results are means±s.e.m. *$P≤0.05$, **$P≤0.01$ vs WT fed with CD; #$P≤0.05$ vs WT fed with HCD.
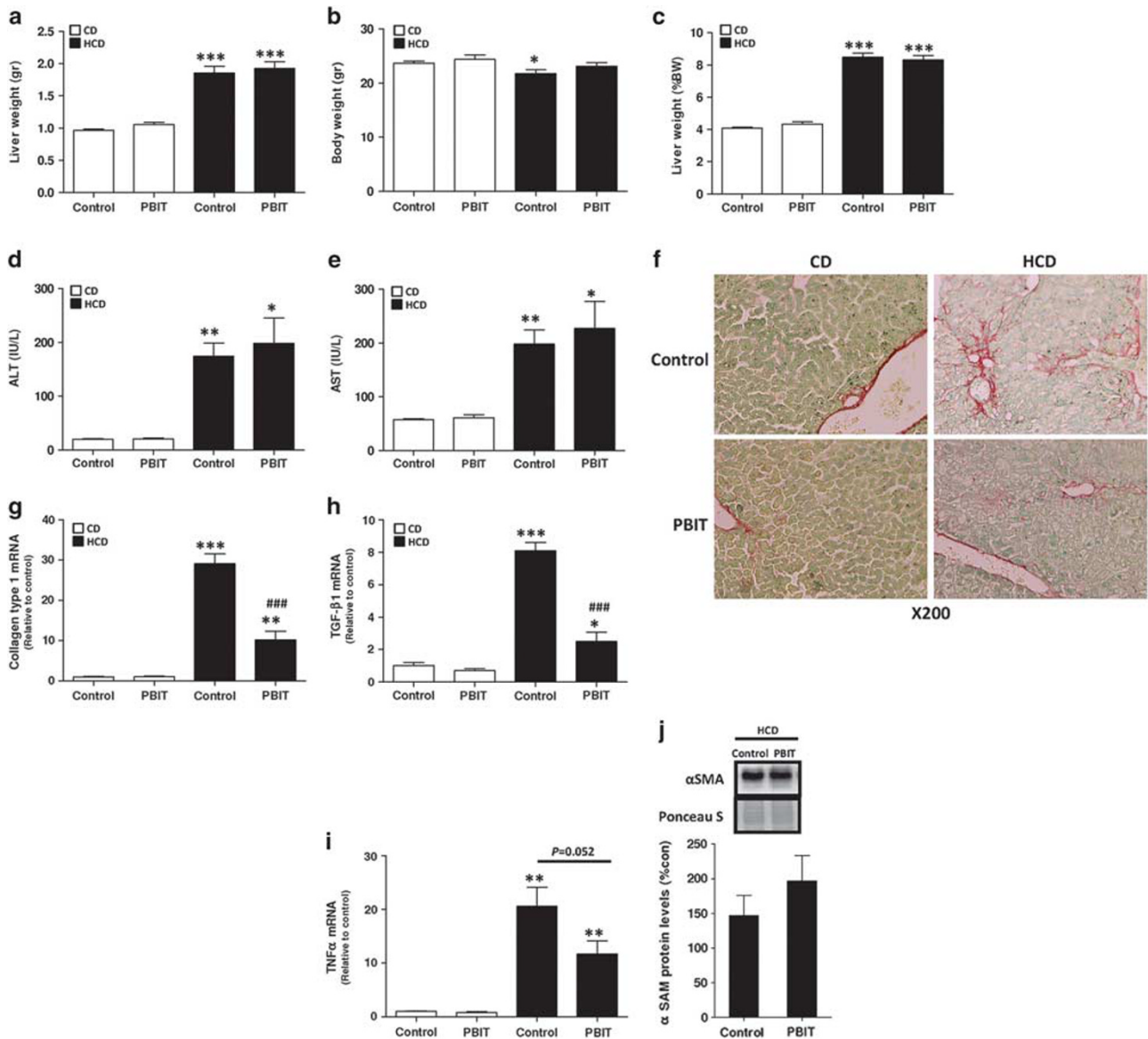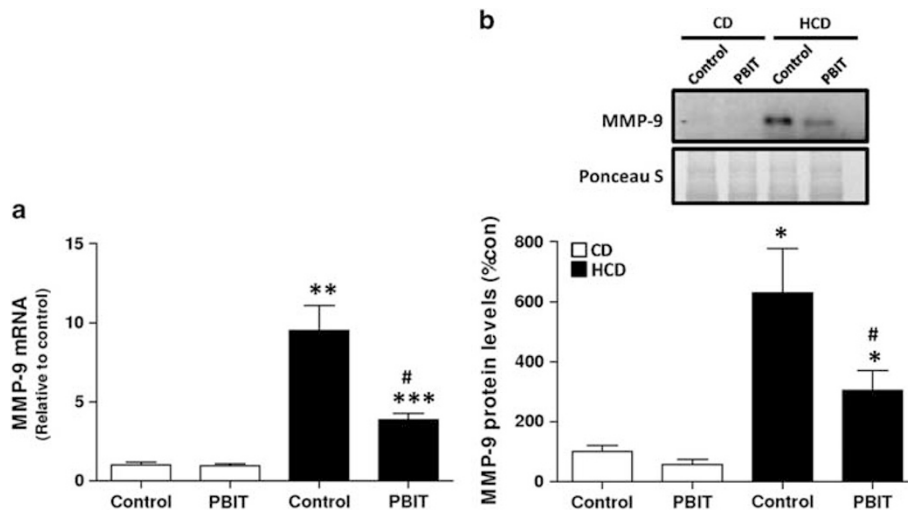
Figure 5 Effects iNOS inhibition by PBIT during high-cholesterol diet (HCD) treatment on liver steatosis, damage, and fibrosis. Control and PBIT-treated mice fed with control diet (CD, white bars) or HCD (black bars) for 6 weeks. (**a**) Liver weight (g), (**b**) body weight (BW) (g), (**c**) liver weight (% BW), and plasma levels of (**d**) ALT and (**e**) AST. (**f**) Sirius red-stained sections of representative liver samples. Hepatic mRNA expression levels of (**g**) collagen type 1, (**h**) transforming growth factor-β1 (TGF-β1), (**i**) tumor necrosis factor-α (TNF-α), and α-smooth muscle actin (α-SMA) protein levels. Results are means ±s.e.m. *$P\leq0.05$, **$P\leq0.01$, ***$P\leq0.001$ *vs* control mice fed with CD; $^{###}P\leq0.001$ *vs* control mice fed with HCD.

based on measurement of plasma liver enzyme levels, was also enhanced in both the groups to a comparable extent (Figure 5d and e). Consistent with genetic elimination of iNOS expression, pharmacological inhibition of iNOS reduced HCD-induced liver fibrosis, as illustrated by Sirus Red staining and the expression of collagen type-1 and profibrotic cytokines (Figure 5f–i). However, similar αSMA levels were observed in control and iNOS-inhibited mice fed with HCD (Figure 5j). Regarding MMP-9, there was a lower induction of gene expression and protein levels in the livers of HCD-fed mice treated with iNOS-specific inhibitor (Figure 6a and b). Overall, these findings provide further evidence to

support the important role of iNOS in liver fibrosis caused by consumption of a HCD.

The Role of iNOS in Cholesterol-Induced MMP-9 Expression in HSC-T6 Cells

Recent evidence indicates that during fibrogenesis HSCs are an important source of MMP-9.[13,28] Our work also elucidated the role of cholesterol and NO in MMP-9 induction in an *in vitro* model of HSC-T6 cells. As shown in Figure 7a, MMP-9 expression was significantly increased in HSC-T6 cells incubated with cholesterol. In addition, increased iNOS mRNA and protein levels were observed (Figure 7b and c).

Figure 6 Effects of iNOS inhibition by PBIT during high-cholesterol diet (HCD) treatment on liver matrix metalloproteinase-9 (MMP-9) induction. Control and PBIT-treated mice fed with control diet (CD, white bars) or HCD (black bars) for 6 weeks. (**a**) Liver mRNA expression and (**b**) protein levels of MMP-9. Results are means±s.e.m. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ *vs* control mice fed with CD; #$P \leq 0.05$ *vs* control mice fed with HCD.

The involvement of iNOS in cholesterol-induced MMP-9 expression was verified using an iNOS inhibitor. Inhibition of iNOS using L-NAME successfully ameliorated cholesterol-induced MMP-9 expression (Figure 7d). The ability of NO to affect MMP-9 expression was further verified using an NO donor. HSC-T6 cells were treated with an NO donor, which significantly enhanced MMP-9 expression (Figure 7e). These results demonstrate the importance of iNOS-derived NO in MMP-9 induction in HSC-T6 cells.

Effects of HCD on DNA Damage in WT and iNOS-Deficient Mice

Liver fibrosis has been previously found to be associated with DNA damage.[29] Recently, both iNOS and MMP activation have separately and together been connected to DNA damage.[9,30–34] Therefore, it was important to evaluate the effect of HCD on DNA damage. Phosphorylation status of histone 2AX (H2AX) at Ser139 is a sensitive reliable marker of early DNA damage response.[35] As shown in Figure 8a, phosphorylation of H2AX was significantly higher in HCD-fed WT mice than in HCD-fed iNOS-deficient mice and tend to be lower in PBIT-treated mice fed with HCD compared with mice fed with HCD (Supplementary Figure S3). PCNA is an auxiliary factor required for DNA repair. Similar to H2AX phosphorylation status, PCNA levels were also enhanced to a greater extent in HCD-fed WT mice (Figure 8b). These findings indicate augmented DNA damage and attempts to repair damaged tissue in the presence of iNOS during HCD.

DISCUSSION

The role of iNOS in liver fibrosis is highly controversial. The current study found a profibrotic effect of iNOS following feeding with a HCD. In iNOS-deficient mice and in mice with pharmacological inhibition of iNOS, lower levels of liver fibrosis were observed following the consumption of HCD for 6 weeks. Although the exact mechanism by which iNOS promotes fibrosis progression is still not entirely understood, our results suggest that iNOS has a role in this process through the induction of HIF-1 and MMP-9 and by promoting DNA damage.

Interesting findings were reflected by αSMA levels in iNOS-deficient and iNOS-inhibited mice. Despite a decrease in liver fibrosis, αSMA levels were not reduced in comparison with control animals. This implies that iNOS could have a role in the late phase of HSC activation, during perpetuation, rather than in the activation step. Although αSMA is a sensitive marker of activated stellate cells, its expression is commonly upregulated even before ECM accumulates.[36] Indeed, experimental studies have shown a temporal sequence of events with HSC activation preceding liver fibrogenesis.[37] HSC activation has been demonstrated in patients with NAFLD with variable rates of fibrosis, including greater HSC activation scores relative to the stage of fibrosis.[36–39] Consistent with this finding, liver biopsies taken from alcoholic patients contained significantly greater numbers of activated HSC than control biopsies, although there was no correlation between numbers of activated HSC and number of Kupffer cells or the extent of fibrosis.[40] HSC activation and fibrosis in many cases of NASH may be explained by the fact that HSC activation is a dynamic process relative to fibrosis.[39]

Although discrepancies exist, results obtained from alcoholic and non-alcoholic fatty livers suggest HSC activation is associated with severity of steatosis rather than with inflammatory activity or fibrosis. The findings imply that the presence of fat alone can result in liver injury leading to HSC activation.[39] Therefore, it is logical to suggest that liver fat accumulation induced by the HCD, which was similar in all mice regardless of iNOS status, led to HSC activation. Although
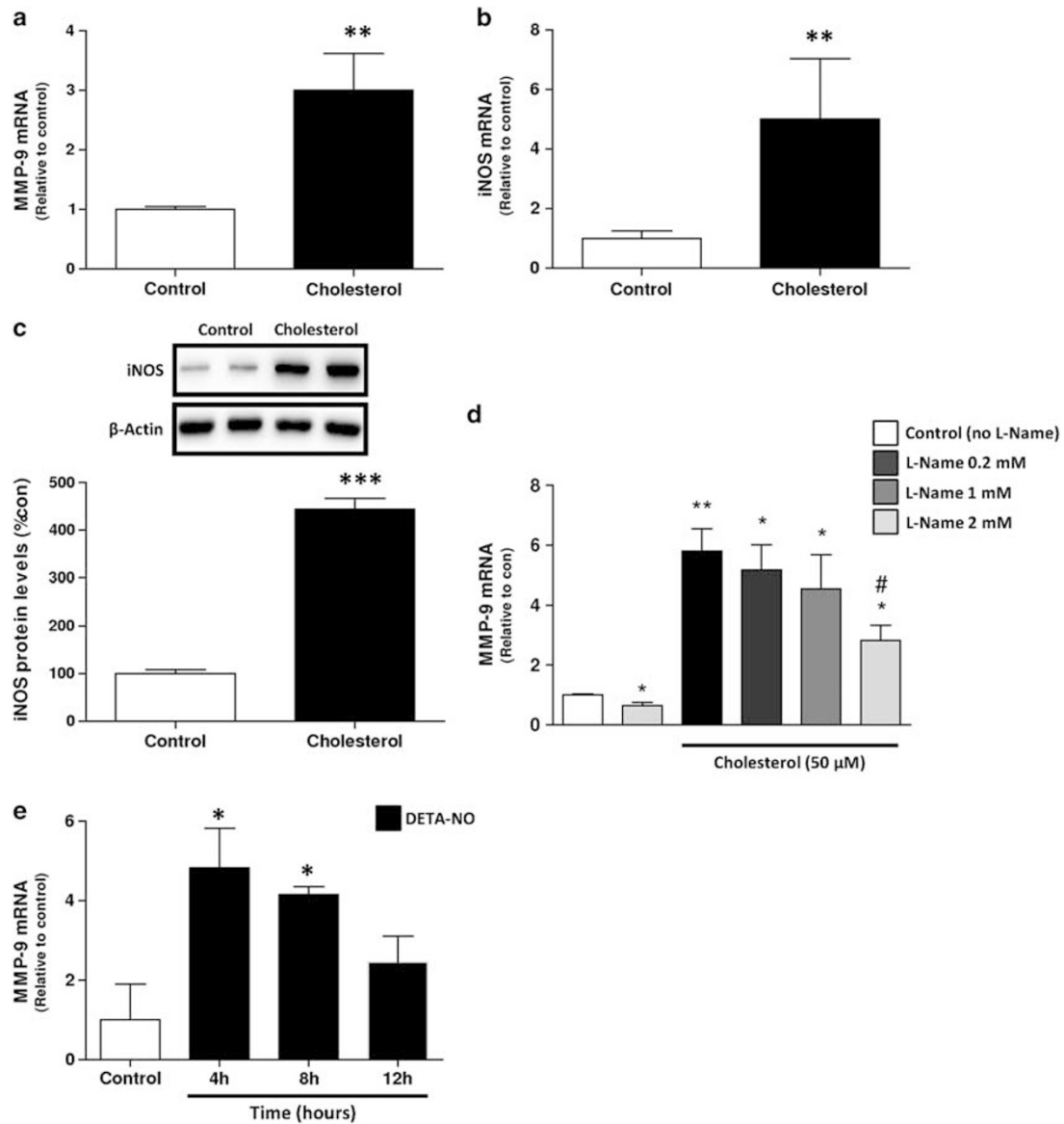
Figure 7 iNOS-mediated cholesterol induced MMP-9 expression in HSC-T6 cells. (**a**) iNOS mRNA, (**b**) protein levels, and (**c**) MMP-9 mRNA levels in HSC-T6 cells incubated with water-soluble cholesterol (50 µM) for 24 h. (**d**) MMP-9 expression levels in HSC-T6 cells incubated with or without water soluble cholesterol in the presence of various concentrations of iNOS inhibitor, L-NAME, or (**e**) with NO donor, EDTA NONOate, for 4, 8, and 12 h. Results are means±s.e.m. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ vs control group; #$P \leq 0.05$ vs cholesterol-treated cells.

αSMA staining is commonly used as a marker to evaluate the amount/activity of fibrogenic cells in the liver, recent evidence challenges this paradigm. Magness et al.[41] provided in vitro and in vivo evidence that there is heterogeneity in HSCs/myofibroblasts with regard to gene expression. The study elegantly demonstrated that αSMA and collagen α1(I) are not always coexpressed in fibrogenic cell types. This further highlights the importance of collagen expression in the liver tissue when assessing liver fibrogenesis. Moreover, it is also important to mention that while the conventional perception is that α-actin expression enhances tissue fibrosis, mice lacking αSMA protein in myofibroblasts have increased renal fibrosis in experimental glomerulonephritis.[42] This further indicates

that αSMA induction may be a counter-regulatory response to enhanced fibrogenesis.[36,42]

Stimulation of hepatic fibrosis by HCD could be mediated by the activation of HIF-1. HIF-1α was induced to a greater extent in HCD-fed WT mice compared with iNOS-deficient mice. This result is in accordance with our previous findings in which cholesterol induced the HIF-1 pathway in the liver and was iNOS dependant.[23,24] Recent data suggest an important role for HIF-1 in the pathogenesis of fibrotic diseases, including liver fibrosis. Indeed, many genes under the regulation of HIF-1 have been implicated in the pathogenesis of liver fibrosis as well as in ECM modifications.[43–49] HIF-1-deficient mice develop less liver
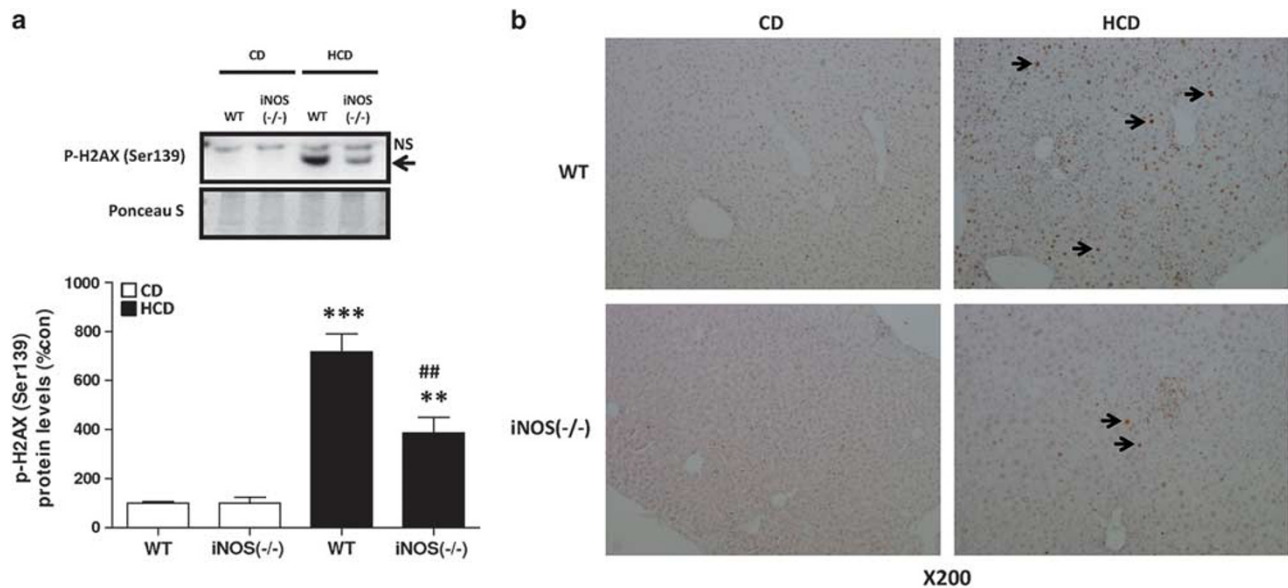
Figure 8 Effects of high-cholesterol diet (HCD) on DNA damage in wild-type (WT) and iNOS-deficient mice (iNOS(−/−)). WT and iNOS-deficient mice fed with control diet (CD) or HCD for 6 weeks. (**a**) Phosphorylation status of histone 2AX (H2AX) at Ser139 and (**b**) immunohistochemical staining of proliferating cell nuclear antigen (PCNA) in liver sections. Arrows indicate positive signals. Results are means±s.e.m. **$P \leq 0.01$, ***$P \leq 0.001$ vs WT fed with CD; ##$P \leq 0.01$ vs WT fed with HCD. NS, not specific.

fibrosis following bile duct ligation.[46] Moon et al.[46] demonstrated reduced expression of several profibrotic mediators and reduced production of collagenous matrix in HIF-1 liver-deficient mice. Consist with these previous studies, in the present study, HIF-1 activation was accompanied with an increase in PDGF and FGF-2 and a trend towards higher PAI-1 expression. Taken together, it appears that iNOS serves as an important link in cholesterol-induced liver fibrosis.

iNOS induction regulated MMP-9 activation in vivo as well as in vitro. MMPs are a family of zinc-containing enzymes involved in degradation and remodeling of the components of the ECM under both physiological and pathophysiological conditions.[50] Among this family, MMP-9 represents the largest and most complex member.[51] Uncontrolled increases of MMP-9 activity have been implicated in contributing to tissue degradation in many pathological conditions, including scarring, following myocardial injury, central nervous system injury, lung injury, kidney disease, and liver injury.[52] The role of MMP-9 in tissues and specifically in liver fibrogenesis is not entirely understood and is widely debated. Yet, compelling evidence indicates that this MMP may have an important role in this process.[26,28,53] Supporting the notion that MMP-9 may promote liver fibrosis, studies conducted in MMP-9-deficient mice found attenuated liver fibrosis in these mice following liver injury. Moreover, clinical studies discovered a link between the increased levels of MMPs, including MMP-9, and fragments of type-IV collagen in cirrhosis.[53] Furthermore, liver MMP-9 expression and activity have also been associated with the stage of fibrosis in chronic hepatitis C.[54] In a study conducted by Kamari and colleagues,[4] decreased liver fibrosis in interleukin-1 (IL-1) α-deficient

mice fed with atherogenic diet (high-fat/cholesterol and cholate diet, HFHCD) was associated with lower MMP-9 expression independent of liver steatosis. These results, together with our present findings, illustrate the positive association between MMP-9 expression and liver fibrosis in response to high-cholesterol/cholate consumption. Chen et al.[55] also demonstrated an increase in MMP-9 in WT but not in their iNOS KO mice following the consumption of HFHCD. However, in their study, an increase in liver fibrosis was observed in iNOS KO mice compared with WT mice. The reason for this discrepancy is unknown. It is possible that the addition of fat to the diet may alter the expression and activity of iNOS and/or other factors, which modifies physiological outcome. Moreover, in the work of Chen et al.,[55] the lack of protein expression, following the ablation of the iNOS gene was not demonstrated. Thus, it is uncertain whether iNOS activity and its signaling effects were utterly abolished. This assessment is critical given that NO production at different levels produces different effects.

The mechanism by which MMP-9 participates in the activation of liver fibrogenesis is only partially understood. However, several potential mechanisms have been proposed. Han et al.[28] suggested that MMPs, and their mediated degradation of ECM in the space of Disse, are essential for fibrotic activation of HSC in ECM and in liver fibrogenesis. According to this hypothesis, particular ECM components maintain the phenotype of quiescent HSCs in normal liver. Therefore, matrix breakdown by MMPs can contribute to the activation of HSC.[28]

Although it was initially believed that MMPs were solely involved in matrix turnover and degradation, recent studies

have discovered a number of novel and unexpected substrates for these MMPs.[25] These findings suggest new biological roles of MMPs including the active involvement in the inflammatory process. MMP-9 may have a role in modulation of inflammation through proteolytic activation of proinflammatory cytokines/chemokines including IL-1β, TGF-β, and membrane-bound TNFα.[56–58]

The relationship between iNOS-induced NO and MMP-9 induction is highly complex and has received much attention in the last two decades.[59] Under inflammatory conditions there is a concurrent upregulation of both iNOS and MMP-9. Still, the crosstalk between these enzymes remains to be clarified. Elevated levels of NO were shown to positively regulate MMP-9 activation on several levels: NO induces MMP-9 activation directly and indirectly, increases its release and distribution and also promotes MMP-9 expression via cGMP in both dependent and independent manners. Although many studies, including the present study, provide evidence for the activation of MMP-9 by NO, additional evidence supporting the inhibitory role of NO on MMP-9 activation also exists.[59] The conflicting results as to whether NO enhances or inhibits MMP-9 illustrates the complexity of the interactions at the molecular level.

iNOS has been associated with the pathophysiology of certain inflammatory disorders by inducing DNA damage or hindering DNA repair.[34] Hepatic fibrosis is initiated by damage to hepatocytes that results in the recruitment of inflammatory cells and the activation of kupffer cells, which subsequently lead to enhanced production of profibrotic cytokines such as TNFα and TGF-β,[60] DNA damage is a common feature in various forms of chronic liver diseases.[29,61,62] DNA damage was evident during liver fibrosis and was also shown to correlate with liver fibrosis as assessed by serum hyaluronic acid, platelet count, and histological staining score.[29,62–65] In the current study, DNA damage, as evident by higher H2AX phosphorylation on Ser139 as well as the accumulation of PCNA, was enhanced in WT mice fed with HCD compared with iNOS-deficient mice. Several lines of evidence support the role for iNOS-induced NO in the generation of oxidative and nitrosative DNA damage. NO or its oxidation products N_2O_3 and peroxynitrite were shown to induce DNA damage through direct strand breaks and base modification.[33,66] Interestingly, the involvement of MMP-9 in iNOS-induced DNA damage was also recently documented.[31] Using a model of peribiliary fibrosis, caused by chronic infection with opsthorchis viverrini, Prakobwong et al. have shown that MMP-9 expression is associated with the accumulation of peribiliary fibrosis in conjugation of iNOS and Rac1 activation. In their study, NO-induced MMP-9 activation mediated Rac1 expression, which led to reactive oxygen and nitrogen species generation and DNA damage. The authors suggested that iNOS and MMP-9 induction promoted the accumulation of fibrosis and potentiated DNA damage and tumor progression.[31]

In summary, the current study demonstrated that HCD promoted liver damage and liver fibrosis, which were potentiated by iNOS activation. iNOS activation was associated with the induction of several pathways that are involved in the development of liver fibrosis, HIF-1, and MMP-9 along with the induction of DNA damage. All of which appear to be a part of the pathologic process. Therefore, given the critical role of iNOS in the development of liver fibrosis, long-term inhibition of iNOS in cases of liver steatotis may have therapeutic benefits.

DISCLOSURE/CONFLICT OF INTEREST
The authors declare no conflict of interest.

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