Published in final edited form as:

Gastroenterology. 2010 October ; 139(4): 1375–1384.e4. doi:10.1053/j.gastro.2010.05.074.

NOX2 plays a key role in stellate cell activation and liver fibrogenesis in vivo

Joy X. Jiang¹, Senthil Venugopal², Nobuko Serizawa¹, Xiangling Chen¹, Fiona Scott¹, Yong Li¹, Roger Adamson³, Sridevi Devaraj⁴, Vijay Shah⁵, M. Eric Gershwin⁶, Scott L Friedman⁷, and Natalie J Török¹

¹Department of Internal Medicine, Division of Gastroenterology and Hepatology, UC Davis Medical Center, Sacramento, CA

²Division of Transplant Medicine, UC Davis Medical Center, Sacramento, CA

³Dept. of Human Physiology, UC Davis Medical Center, Sacramento, CA

⁴Laboratory for Atherosclerosis and Metabolic Research, UC Davis Medical Center, Sacramento, CA

⁵Department of Gastroenterology and Hepatology, Mayo College of Medicine, UC Davis Medical Center, Sacramento, CA

⁶Division of Rheumatology, UC Davis Medical Center, Sacramento, CA

⁷Division of Liver Diseases, Mount Sinai School of Medicine, New York, NY

Abstract

BACKGROUND & AIMS—Hepatocyte apoptosis and activation of hepatic stellate cells (HSC) are critical events in fibrogenesis. We previously demonstrated that phagocytosis of apoptotic hepatocytes by HSC is profibrogenic. Based on this, as well as the observation that NADPH oxidase induction is central to fibrogenesis, our aim was to study the phagocytic NADPH oxidase, NOX2.

METHODS—An *in vivo* phagocytosis model was developed by injecting wild type (wt) or NOX2^{-/-} mice with lentiviral-GFP containing a hepatocyte-specific promoter, and ad-TRAIL. Fibrosis was evaluated in bile duct ligated (BDL) wt and NOX2^{-/-} mice with or without gadolinium treatment. NOX2 expression was studied in human liver samples and in HSC isolated from fibrotic livers. The fibrogenic activity of NOX2 was assessed by collagen reporter assays.

Correspondence: Natalie Török, M.D., UC Davis Medical Center, Patient Support Services Building, 4150 V Street, Suite 3500, Sacramento, CA 95817. Natalie.Torok@ucdmc.ucdavis.edu; fax: 916-734-7908.

Author's involvement in the manuscript:

Joy X. Jiang: performing experiments and writing paper

Senthil Venugopal: performing experiments

Nobuko Serizawa: performing experiments

Xiangling Chen: performing experiments

Fiona Scott: performing experiments

Yong Li: performing experiments

Roger Adamson: technical support

Sridevi Devaraj: technical support

Vijay Shah: collaboration, technical support

M. Eric Gershwin: critical revision of the manuscript for intellectual content

Scott L Friedman: critical revision of the manuscript for intellectual content

Natalie J Török: concept and design, analysis of the data, funding

Disclosures: Nothing to disclose

RESULTS—In the phagocytosis model engulfment of GFP-labeled apoptotic bodies was seen, and the expression of α -SMA and collagen I increased significantly in the wt, but not in the NOX2^{-/-} mice. Inhibiting apoptosis decreased the profibrogenic response. NOX2^{-/-} animals exhibited significantly less fibrosis following BDL. Inactivating macrophages in wt BDL mice did not lower collagen production to the level observed in NOX2^{-/-} mice suggesting that NOX2-expressing HSC are important in fibrogenesis. NOX2 was upregulated in HSC from fibrotic livers, and phagocytosis-induced NOX2 expression and activity were demonstrated. Based on reporter assays, NOX2-mediated ROS directly induced collagen promoter activity in HSC.

CONCLUSIONS—Apoptosis and phagocytosis of hepatocytes directly induce HSC activation and initiation of fibrosis. NOX2, the phagocytic NADPH oxidase plays a key role in this process and in liver fibrogenesis *in vivo*.

Keywords

liver fibrosis; apoptosis; NADPH oxidase

Liver fibrosis is the end result of chronic liver injury of various etiologies¹. Apoptosis of hepatocytes is a common feature of this process independent of the type of liver injury². The apoptotic cells are usually efficiently eliminated by the professional phagocytes by efferocytosis however, when the phagocytic system is overwhelmed, non professional phagocytes start to increasingly play a role in phagocytosis³. We have recently identified hepatic stellate cells (HSC) performing phagocytic function during chronic liver injury⁴. HSC are central to liver fibrosis; upon activation they transdifferentiate into myofibroblasts, produce extracellular matrix (ECM) components and profibrogenic cytokines. Phagocytosis of hepatocyte-derived apoptotic bodies (AB) by HSC is profibrogenic as it induces collagen α 1 (I) and TGF- β 1 upregulation, and NADPH oxidase (NOX)-dependent superoxide production⁴. In addition, phagocytosis and NADPH oxidase-mediated signaling events induce myofibroblast survival⁵. Thus, phagocytosis and resulting HSC activation could be amongst the first, initiating events in liver fibrogenesis linking hepatocyte apoptosis to HSC activation and production of ECM. HSC express the p22phox, p47phox, p67phox and gp91phox (NOX2), subunits of the NADPH oxidase enzyme complex, as well as NOX1⁶. During activation of the phagocytic NOX, the cytosolic subunits (p47phox, p67phox) migrate to the membrane, where they associate with the subunits gp91phox and p22phox to assemble the active oxidase. Gp91phox is the rate-limiting catalytic component, and its upregulated transcription was shown to increase respiratory burst capacity during inflammatory responses⁷. Rac GTPases are also important regulatory elements of NOXs⁸. Upon activation, Rac-GTP translocates to the membrane where it interacts with p67phox. Rac1 is ubiquitously expressed while Rac2 is only expressed in myeloid cells⁹. Rac1 was shown to activate NOX2 in heterologous systems resulting in reactive oxidative species (ROS production)⁸. Expression of the constitutive active Rac1 induced accelerated liver fibrosis in mice¹⁰, underlying the importance of Rac1 in fibrogenesis. On the other hand, Rac1 activation is also linked to the induction of phagocytosis¹¹. Several profibrogenic stimuli can activate NOXs in HSC, such as PDGF¹², angiotensin II¹³, and leptin¹⁴. However, in HSC the mechanism of activation, function and downstream signaling events elicited by the different NOXs have not yet been clearly elucidated.

In this study we have shown that apoptosis of hepatocytes is directly linked to stellate cell activation *in vivo*, via the activation of NOX2 (gp91phox) in HSC. This represents an essential early event in liver fibrogenesis with a direct effect on ROS-mediated collagen upregulation in HSC. NOX2 thus is a central enzyme in the induction of early fibrogenic changes.

Materials and Methods

Liver tissue samples

The liver biopsy samples were obtained from the UC Davis Cancer Center Biorepository funded by the NCI.

Animal studies

In vivo phagocytosis model: wt (C56BL6) or NOX2^{-/-} mice (same background, Jackson Lab, Bar Harbor, ME) were injected with lentiviral (LV)-GFP with the hepatocyte-specific α 1 antitrypsin (α 1-AT) promoter (3×10^7 pfu/g) (gift from Dr. Zern, UC Davis) into the portal vein, then 7 days later Ad-TRAIL (2×10^7 pfu/g) (gift from Dr. Gores, Mayo Clinic), into the tail vein of the same mice. A separate group of mice were treated by i.p. injection of the pancaspase inhibitor Q-VD-OPH (10 mg/kg, MP Biomedicals, Solon, OH; twice on the day of Adeno-TRAIL injection and the day after), and controls were injected with the vehicle, DMSO. As for control injections, mice were injected either with α 1-AT-LV-GFP (3×10^7 pfu/g), or Ad-TRAIL (2×10^7 pfu/g), or Ad-GFP (2×10^7 pfu/g, Vector Biolabs, Philadelphia PA), only. The animals were sacrificed 3 days after the Ad-TRAIL injection.

BDL was performed on wt and NOX2^{-/-} mice, as described⁴. Sham operation was conducted in parallel. Gadolinium chloride (GdCl₃, 10 mg/kg in saline, Sigma-Aldrich) was injected i.p. every other day throughout the experiment. The mice were sacrificed 3 or 6 weeks after surgery. To study NOX2 expression HSC were isolated from BDL or sham-operated Sprague Dawley rats (Charles River Laboratories Inc., Wilmington, MA) as described below.

Statistical analysis

All data represented at least three experiments and expressed as the mean $\pm$ SED. Differences between groups were compared using one-way Analysis of Variance (ANOVA) associated with the Dunnett's test. Statistical significance was assumed when $p < 0.05$.

For details regarding histology, immunohistochemistry, TUNEL assay, hydroxy-proline assay, serum biochemical measurements, preparation of apoptotic bodies and phagocytosis experiments, virus preparation, siRNA experiments, real time PCR, lucigenin assay, reporter assays assessing collagen promoter activity, and Rac1 pull down assay, western blotting please see Supplementary Materials and Methods.

Results

NOX2 is expressed in hepatic stellate cells during liver fibrogenesis

We performed immunohistochemistry and confocal microscopy to study NOX2 expression in liver specimens of patients with HCV or PBC. NOX2 was expressed in α -SMA positive HSC (Figure 1A) in both HCV and PBC. To recapitulate this in an animal model of liver fibrosis, BDL was performed in rats, and HSC were isolated (the purity of the HSC was confirmed by α -SMA staining). NOX2 mRNA (12.7 ± 1.2 fold, $*p < 0.05$) and protein expression (4.22 ± 0.004 fold, $p < 0.05$, densitometry, not shown) were significantly increased in primary HSC from BDL rats while low levels of expression were seen in HSC from sham-operated animals (Figure 1B). As the signals inducing the phagocytic NOX2 expression in activated HSC are not known, we postulated that engulfment of apoptotic bodies (AB) could be a trigger. To test this, primary HSC were exposed to AB and NOX2 expression was tested by real-time PCR. We found that NOX2 expression was significantly induced (4.25 ± 0.98 fold, $**p \leq 0.0001$) in HSC following phagocytosis (Figure 1C). Taken together with our earlier data, upregulation of NOX2 following phagocytosis may translate into increased collagen I production by HSC.

Superoxide production and collagen upregulation following phagocytosis of apoptotic bodies are NOX2-dependent

Primary rat HSC were transfected with a NOX2 siRNA or scrambled siRNA, then exposed to AB. Superoxide production was assessed by the lucigenin assay (Figure 2A). In the scrambled siRNA-transfected cells phagocytosis induced superoxide production (1.75 ± 0.24 fold, $*p < 0.05$), and this was inhibited in the NOX2 siRNA-transfected cells. Next, we assessed if collagen upregulation was NOX2-mediated following phagocytosis by real-time PCR using scrambled or NOX2 siRNA-transfected HSC. Collagen IA1 expression was upregulated in the scrambled siRNA-transfected HSC following AB exposure ($**p \leq 0.01$), and this decreased significantly in the NOX2 siRNA-transfected primary HSC (by $88\% \pm 4$, $##p \leq 0.0001$), suggesting that this is a central enzyme in phagocytosis-induced fibrogenic responses (Figure 2B).

The collagen promoter is induced by a NOX2-dependent mechanism following phagocytosis

Collagen consists of two $\alpha 1$ chains and one $\alpha 2$ chain. Both COL1A1 and COL1A2 genes are highly sensitive to ROS¹⁵. The promoters contain a H₂O₂-responsive area therefore we studied whether the NOX2-mediated superoxide and peroxide production could directly result in collagen promoter activity. Primary wild type or NOX2^{-/-} HSC were transfected by the constructs containing the truncated promoter Col1A2 P1-Luc (-378/+58), or with a construct where the peroxide-sensitive area was intact [Col1A2 P1-Luc (-2900/+58)], or empty vector. The cells were then exposed to AB in the presence or absence of the reducing agent GSH or catalase. Engulfment of AB by HSC resulted in a significant induction of the COL1A2 promoter activity in the Col1A2 P1-Luc (-2900/+58)-transfected cells (35.7 ± 6.2 fold, $**p \leq 0.01$), compared to control cells. This was abrogated in the Col1A2 P1-Luc (-378/+58)-transfected cells (1.95 ± 0.21 fold, $**p \leq 0.01$), or decreased after exposure to catalase (5.66 ± 0.4 fold, $**p \leq 0.01$), indicating that the promoter activity resulted from peroxide produced following phagocytosis (Figure 2C). In NOX2^{-/-} cells the luciferase activity was significantly blunted following phagocytosis (1.71 ± 0.21 fold, $*p < 0.05$). NOX2 hence is a candidate enzyme regulating superoxide and peroxide-mediated induction of collagen expression during fibrogenesis.

Intact NOX2 is required for Rac1 recruitment and phagocytosis of apoptotic bodies by HSC

In our previous studies we found that phagocytosis of AB induced Rac1 activation, an important regulatory element of NOX2 in non-hemopoietic cells; and constitutive active Rac1 also augmented the phagocytic activity of HSC¹⁶. Rac recruitment to the enzyme complex is known to be significantly decreased in CGD neutrophils (with the mutation of the gp91phox)¹⁷. Thus it is plausible that intact NOX2 is required for Rac1 recruitment and activation in HSC for phagocytosis to occur. To test this, first, the phagocytic rate in wt and NOX2^{-/-} HSC was studied, and we found that NOX2^{-/-} HSC engulfed significantly less AB ($\#p < 0.001$, Figure 3A). To assess whether this decline in the phagocytic activity was actually due to decreased GTP-ase activity and/or decreased recruitment of Rac1 to the enzyme complex in the membrane, we performed Rac1 pull-down assays in wt and NOX2^{-/-} HSC following exposure to AB and tested the membrane fractions for GTP-Rac1. In the NOX2^{-/-} HSC the amount of active Rac1 in the membrane fraction was decreased (Figure 3B). While the antibody against active Rac1 labeled the phagosomal membrane in wt cells, the phagosomal labeling was not seen in NOX2^{-/-} cells. Tethering of TAMRA-labeled AB in NOX2^{-/-} cells did occur, but the engulfment did not take place or was often incomplete. Intact NOX2 is thus required for Rac1 recruitment, activation and for phagocytosis. As phagocytosis could be an important early event in hepatocyte apoptosis-induced fibrogenic activity, we next will test the validity of these *in vitro* data by a novel model of *in vivo* phagocytosis.

Hepatocyte apoptosis and phagocytosis *in vivo* directly induce hepatic stellate cell activation

In this study we induced selective apoptosis of GFP positive hepatocytes to track the fate of their AB. This model was based on the notion that TRAIL is not apoptotic on normal hepatocytes, but it induces cell death of virus-infected hepatocytes¹⁸⁻¹⁹. To track apoptotic hepatocytes we used a lentiviral approach where GFP only expressed by hepatocytes because of the expression of the hepatocyte-specific promoter $\alpha 1$ -AT. The animals were first injected with the $\alpha 1$ -AT-LV-GFP *via* the portal vein then 7 days later Ad-TRAIL was injected into the tail vein of the same mice. TRAIL-mediated apoptosis of GFP positive, virus-infected hepatocytes was then detected. As control, mice were injected only with $\alpha 1$ -AT-LV-GFP, or only with Ad-TRAIL, or Ad-GFP. A separate group of mice were injected with the pancaspase inhibitor Q-VD-OPH prior to the viral injections. The liver tissues from only Ad-TRAIL, or LV-injected mice showed no significant injury or infiltration by inflammatory cells, and the ALT values remained normal (Figure 4b, c). In the LV plus Ad-TRAIL-injected animals the liver showed mild to moderate hepatocyte injury with associated regenerative activity, increased ALT values, and no significant infiltration by inflammatory cells (Figure 4d). In the pancaspase inhibitor-treated LV plus Ad-TRAIL-injected group, the liver histology was normal (Figure 4e), and the ALT decreased significantly ($p \leq 0.05$). To validate our model, and demonstrate apoptosis of hepatocytes, TUNEL assays were done on all samples. In the LV plus Ad-TRAIL infected livers hepatocyte apoptosis was increased (Figure 4d') compared to only Ad-TRAIL (Figure 4b'), or only LV (Figure 4c') injected animals. In the Q-VD-OPH treated group the number of apoptotic cells has decreased (Figure 4e'). Studying liver histology in each experimental condition, we have not seen infiltration by inflammatory cells. To confirm this, immunohistochemistry for CD11b (Mac-1) was done and we found similar numbers of CD11b positive cells in each experiment (Supplementary Figure 1A). In addition, real-time PCR showed comparable levels of TNF- α expression in each condition.

To analyze phagocytosis, immunohistochemistry and confocal microscopy were performed to visualize GFP-labeled hepatocytes and activated HSC (α -SMA, red). There was an increase in the number of activated HSC in the liver of mice injected with $\alpha 1$ -AT-LV-GFP plus Ad-TRAIL (Figure 5d, e), while in the only LV (Figure 5c), or only Ad-TRAIL (Figure 5b) injected mice no increase was detected similar to control (Figure 5a). At higher magnification in the α -SMA-positive HSC, GFP-labeled AB could be seen (arrow), indicating phagocytosis of hepatocyte-derived AB (Figure 5f). Apoptosis and phagocytosis mediated fibrogenic changes were also confirmed in a different model where wt and galectin 3^{-/-} mice were treated as above, and a decrease in procollagen $\alpha 1(I)$ and TGF β expression were seen in the galectin 3^{-/-} mice (manuscript in preparation). Galectin 3 is necessary for phagocytosis by facilitating of the tethering of AB²⁰.

Apoptosis of hepatocytes thus directly induced HSC activation *in vivo* as no fibrogenic agents or methods were used in this model. This directly supports the hypothesis that apoptosis of hepatocytes is profibrogenic.

Stellate cell activation by phagocytosis of AB is decreased in NOX2^{-/-} mice

Based on our *in vitro* data, in NOX2^{-/-} HSC, the upregulation of collagen was blunted. To assess this in our *in vivo* model both wt and NOX2^{-/-} mice were injected with Ad-TRAIL, or LV or LV plus Ad-TRAIL, with or without the pancaspase inhibitor, as above. Immunohistochemistry showed less α -SMA positive HSC in NOX2^{-/-} mice after injection of LV plus Ad-TRAIL, suggesting that in these animals HSC activation was blunted (images for wt and NOX2^{-/-} injected with LV plus Ad-TRAIL are shown, with or without the pancaspase inhibitor, Figure 6A). To confirm these data, real-time PCR was performed on the livers of virus-injected wt and NOX2^{-/-} mice using α -SMA, collagen IA1 and TGF- $\beta 1$ specific primers.

The expression of α -SMA (31.3 ± 6.16 -fold, $*p < 0.05$), collagen IA1 (6.03 ± 0.7 -fold, $**p \leq 0.01$), and TGF- β 1 (17.03 ± 2.6 -fold, $*p < 0.05$) have significantly increased in LV plus Ad-TRAIL injected wt animals (Figure 6B), while no increase was detected in NOX2^{-/-} animals. In addition, treatment with the caspase inhibitor decreased the upregulation of the fibrogenic markers significantly ($*p < 0.05$). Taken together, these data indicate that NOX2 is essential in the early upregulation of pro-fibrogenic genes following the apoptosis of hepatocytes.

Liver fibrosis is decreased in NOX2^{-/-} mice

To further study the *in vivo* relevance of the above findings, BDL was performed in wt and NOX2^{-/-} mice. We chose BDL as fibrosis-inducing method, as both carbon tetrachloride (CCl₄) and thioacetamide (TAA)-induced liver injury cause significant oxidative stress and hepatocyte necrosis which may confound the data. The animals were sacrificed after 3 weeks and in the case of some NOX2^{-/-} mice after 6 weeks following surgery, and the liver specimens were processed for picrosirius staining to assess fibrosis stage (wt animals did not survive 6 weeks after BDL). The effects of BDL were similar in the wt and NOX2^{-/-} livers, showing the same degree of inflammation (grade 2) and bile duct proliferation (data not shown), and apoptosis (TUNEL assay, data not shown). We found that in NOX2^{-/-} animals the fibrosis stage was significantly lower compared to that of wt animals following BDL (Figure 7A b, c, d). The serum was tested for ALT and bilirubin values and we found that both ALT and bilirubin were increased in BDL animals ($*p < 0.05$); (in both wt and NOX2^{-/-}, with the ALT somewhat lower in NOX2^{-/-} mice than in wt albeit with no statistical significance). To elucidate the relative contribution of HSC and Kupffer cells to liver fibrosis in respect to their NOX2 expression, mice undergone BDL were injected with GdCl₃, throughout the experiment, to inhibit macrophages. Fibrosis stage was lower in GdCl₃-injected wt animals after BDL compared to PBS-injected controls (Figure 7B a, compared to figure 7A, b), consistent with previous data²¹. To assess collagen expression, and liver collagen content, real time PCR and OH proline assays were performed in all experimental conditions. Collagen expression ($55\% \pm 6.8$, $**p \leq 0.01$) and OH-proline incorporation ($63\% \pm 9.5$, $*p < 0.05$) have significantly decreased in NOX2^{-/-} mice compared to wt animals following BDL. In the GdCl₃-injected BDL wt mice the expression of collagen IA1 and incorporation of OH-proline have decreased to a certain extent ($24\% \pm 2.3$, $*p < 0.05$, and $42\% \pm 5.6$, $*p < 0.05$), compared to PBS-injected BDL animals. However, this decline in fibrogenic activity following macrophage inhibition in wt animals (white bars) did not reach the low level observed in NOX2^{-/-} animals (black bars), suggesting that NOX2 expressing HSC may play a major role in liver fibrosis.

Discussion

In this study we have shown that 1) phagocytosis of apoptotic hepatocytes is directly profibrogenic *in vivo*, 2) the profibrogenic effect is mediated by NOX2 and 3) NOX2^{-/-} animals have reduced fibrosis. Phagocytosis of apoptotic cells is essential in maintaining tissue homeostasis. According to current concepts, engulfment of AB in physiological circumstances is anti-inflammatory²². In pathological situations however, such as chronic liver disease hallmarked by ongoing apoptosis of hepatocytes, non professional phagocytes such as HSC start engulfing apoptotic cells. The notion that cells of non-myeloid lineage can phagocytose is not novel: it has been described that epithelial²³ or mesenchymal cells²⁴ can engulf AB. Here we have shown that HSC can phagocytose apoptotic hepatocytes and directly induce fibrogenic responses by an ROS-mediated collagen production. Pivotal to this process was the activation of the phagocytic NADPH oxidase, NOX2. An important correlation to the role of NOX2 is the liver disease of patients with CGD with mutation of the components of NOX2, most commonly gp91phox. In these patients the liver is affected by recurrent infections, vascular abnormalities, and eventually non-cirrhotic portal hypertension may develop²⁵. The fact that these patients do not develop liver fibrosis in the face of chronic inflammation is

intriguing and pointing to the important role of NOX2 in liver fibrogenesis. Previously, by the elegant studies of Bataller et al, it was shown that NADPH oxidase activation was indeed necessary for angiotensin II-induced liver fibrogenesis¹³. In those studies p47phox^{-/-} mice were used, a subunit known to be an organizer for both NOX1 and NOX2²⁶. Deficiency in NOX2 was shown to increase hepatocellular injury (mainly necrosis) in the CCl₄-induced model of fibrosis, upregulation of collagen expression but interestingly, decrease in fibrosis²⁷. In that model increased matrix metalloproteinase 2 (MMP2) and 9 expression in NOX2^{-/-} animals were thought to lead to a decrease in fibrosis. CCl₄ is known to induce ROS-mediated liver injury (independent of NOXs) with lipid peroxidation, and consequent necrosis. Thus, the mechanism of liver injury is distinct compared to our model, where ROS production is rather a consequence than a cause of ongoing apoptosis and resulting HSC activation.

ROS are known to play a key role in HSC activation^{15, 28}. H₂O₂ derived from hepatocytes induced collagen transcription in HSC¹⁵ and we have shown that NOX-derived ROS induce survival pathways in HSC contributing to the propagation of activated HSC⁵. Here we demonstrated that NOX2 activation and peroxide production directly resulted in the induction of the collagen α I promoter in HSC. An important corollary to NOX2 activation is Rac1, an essential subunit of the enzyme and a positive regulator of phagocytosis. Constitutive activation of Rac1 lead to accelerated liver fibrosis emphasizing the role of Rac1 in ROS-mediated liver injury¹⁰, and Rac1 was shown to play an important role in the phagocytosis of lymphocytes during fibrogenesis²⁹. In our study we found impaired translocation of Rac1 to the membrane in NOX2^{-/-} HSC, consistent with previous reports³⁰. Thus decrease in the GTP-bound Rac1 at the site of the phagosome in NOX2^{-/-} cells may translate into less effective engulfment. This, together with the decrease in collagen promoter activation in NOX2^{-/-} HSC may translate into a significant reduction in fibrogenic activity. As phagocytosis may represent an early “initiator” event in fibrogenesis, it was essential to recapitulate this *in vivo*. In our model TRAIL-mediated apoptosis of hepatocytes induced α -SMA and production of collagen IA1 and TGF- β 1 in wt mice. While phagocytosis of apoptotic cells is a direct profibrogenic stimulus, we can not exclude other mechanisms of fibrosis in this model. It is possible that increase in cell death also predisposes to damage-associated molecular patterns (DAMP)-mediated liver injury and HSC activation *via* the toll like receptors (TLR). This was demonstrated earlier by CpG DNA-mediated TLR9 induction on HSC and their subsequent activation³¹. In NOX2^{-/-} mice the fibrogenic activity was not seen, confirming the role of NOX2 in early fibrosis. As Kupffer cells express NOX2, their role in early fibrosis had to be addressed. To inhibit Kupffer cells we used GdCl₃ throughout the BDL experiment, to avoid repopulation of macrophages. We found that in GdCl₃-treated wt animals the fibrosis and collagen production was decreased after 3 weeks, but did not reach the low, almost baseline level of collagen production seen in BDL NOX2^{-/-} mice. This suggests that beside macrophages, HSC *via* their NOX2 expression and activity are important contributors to early fibrogenic events. Other cells in the liver could potentially also contribute to liver fibrosis *via* their NOX2 activity, and this could be a focus of future studies. Our main goal here was to study the early, initiating events in fibrosis. At a later, propagation stage of fibrosis however, NOX2 could be also activated by inflammatory mediators or cytokines such as angiotensin II, leptin or PDGF further accelerating the production ECM.

In summary, based on our *in vitro* and *in vivo* data, NOX2 is a central enzyme in liver fibrosis. It is especially important in the initiating phase of fibrogenesis when phagocytosis of apoptotic cells is one of the main profibrogenic events. Targeted inhibition of NOX2 activation may prove to be a powerful new strategy to inhibit multiple profibrogenic pathways and halt the progression of the disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dr. Gregory Gores (Mayo College of Medicine) for supplying the Ad-TRAIL construct and to Drs. Mark Zern (UC Davis) and Antonia Follenzi (Albert Einstein School of Medicine) for supplying the lentiviral vector.

Grant Support: This study was supported by the NIH DK069765, DK080715 DK083283 (NJT) and the ALF Post-doctoral Research Awards (JXJ).

Abbreviations

HSC	hepatic stellate cells
TGF- β 1	transforming growth factor- β 1
AB	apoptotic bodies
ASMA	α -smooth muscle actin
NADPH oxidase	Reduced nicotinamide adenine dinucleotide phosphate oxidase
TAMRA	carboxytetramethyl rhodamine succinimidyl ester
BDL	bile duct ligation
CGD	chronic granulomatous disease

References

1. Friedman SL. Mechanisms of hepatic fibrogenesis. *Gastroenterology* 2008;134:1655–69. [PubMed: 18471545]
2. Malhi H, Gores GJ. Cellular and molecular mechanisms of liver injury. *Gastroenterology* 2008;134:1641–54. [PubMed: 18471544]
3. Birge RB, Ucker DS. Innate apoptotic immunity: the calming touch of death. *Cell Death Differ* 2008;15:1096–102. [PubMed: 18451871]
4. Zhan SS, Jiang JX, Wu J, Halsted C, Friedman SL, Zern MA, Torok NJ. Phagocytosis of apoptotic bodies by hepatic stellate cells induces NADPH oxidase and is associated with liver fibrosis in vivo. *Hepatology* 2006;43:435–43. [PubMed: 16496318]
5. Jiang JX, Mikami K, Venugopal S, Li Y, Torok NJ. Apoptotic body engulfment by hepatic stellate cells promotes their survival by the JAK/STAT and Akt/NF-kappaB-dependent pathways. *J Hepatol* 2009;51:139–48. [PubMed: 19457567]
6. Reinehr R, Becker S, Eberle A, Grether-Beck S, Haussinger D. Involvement of NADPH oxidase isoforms and Src family kinases in CD95-dependent hepatocyte apoptosis. *J Biol Chem* 2005;280:27179–94. [PubMed: 15917250]
7. Babior BM. NADPH oxidase. *Curr Opin Immunol* 2004;16:42–7. [PubMed: 14734109]
8. Hordijk PL. Regulation of NADPH oxidases: the role of Rac proteins. *Circ Res* 2006;98:453–62. [PubMed: 16514078]
9. Knaus UG, Heyworth PG, Evans T, Curnutte JT, Bokoch GM. Regulation of phagocyte oxygen radical production by the GTP-binding protein Rac 2. *Science* 1991;254:1512–5. [PubMed: 1660188]
10. Choi SS, Sicklick JK, Ma Q, Yang L, Huang J, Qi Y, Chen W, Li YX, Goldschmidt-Clermont PJ, Diehl AM. Sustained activation of Rac1 in hepatic stellate cells promotes liver injury and fibrosis in mice. *Hepatology* 2006;44:1267–77. [PubMed: 17058265]

11. Hoffmann PR, deCathelineau AM, Ogden CA, Leverrier Y, Bratton DL, Daleke DL, Ridley AJ, Fadok VA, Henson PM. Phosphatidylserine (PS) induces PS receptor-mediated macropinocytosis and promotes clearance of apoptotic cells. *J Cell Biol* 2001;155:649–59. [PubMed: 11706053]
12. Adachi T, Togashi H, Suzuki A, Kasai S, Ito J, Sugahara K, Kawata S. NAD(P)H oxidase plays a crucial role in PDGF-induced proliferation of hepatic stellate cells. *Hepatology* 2005;41:1272–81. [PubMed: 15915457]
13. Bataller R, Schwabe RF, Choi YH, Yang L, Paik YH, Lindquist J, Qian T, Schoonhoven R, Hagedorn CH, Lemasters JJ, Brenner DA. NADPH oxidase signal transduces angiotensin II in hepatic stellate cells and is critical in hepatic fibrosis. *J Clin Invest* 2003;112:1383–94. [PubMed: 14597764]
14. De Minicis S, Seki E, Oesterreicher C, Schnabl B, Schwabe RF, Brenner DA. Reduced nicotinamide adenine dinucleotide phosphate oxidase mediates fibrotic and inflammatory effects of leptin on hepatic stellate cells. *Hepatology* 2008;48:2016–26. [PubMed: 19025999]
15. Nieto N, Friedman SL, Cederbaum AI. Cytochrome P450 2E1-derived reactive oxygen species mediate paracrine stimulation of collagen I protein synthesis by hepatic stellate cells. *J Biol Chem* 2002;277:9853–64. [PubMed: 11782477]
16. Jiang JX, Mikami K, Shah VH, Torok NJ. Leptin induces phagocytosis of apoptotic bodies by hepatic stellate cells via a Rho guanosine triphosphatase-dependent mechanism. *Hepatology* 2008;48:1497–505. [PubMed: 18925608]
17. van Bruggen R, Anthony E, Fernandez-Borja M, Roos D. Continuous translocation of Rac2 and the NADPH oxidase component p67(phox) during phagocytosis. *J Biol Chem* 2004;279:9097–102. [PubMed: 14623873]
18. Mundt B, Kuhnelt F, Zender L, Paul Y, Tillmann H, Trautwein C, Manns MP, Kubicka S. Involvement of TRAIL and its receptors in viral hepatitis. *Faseb J* 2003;17:94–6. [PubMed: 12475902]
19. Volkman X, Fischer U, Bahr MJ, Ott M, Lehner F, Macfarlane M, Cohen GM, Manns MP, Schulze-Osthoff K, Bantel H. Increased hepatotoxicity of tumor necrosis factor-related apoptosis-inducing ligand in diseased human liver. *Hepatology*. 2007
20. Sano H, Hsu DK, Apgar JR, Yu L, Sharma BB, Kuwabara I, Izui S, Liu FT. Critical role of galectin-3 in phagocytosis by macrophages. *J Clin Invest* 2003;112:389–97. [PubMed: 12897206]
21. Iredale JP. Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ. *J Clin Invest* 2007;117:539–48. [PubMed: 17332881]
22. Henson PM, Bratton DL, Fadok VA. Apoptotic cell removal. *Curr Biol* 2001;11:R795–805. [PubMed: 11591341]
23. Hanayama R, Nagata S. Impaired involution of mammary glands in the absence of milk fat globule EGF factor 8. *Proc Natl Acad Sci U S A* 2005;102:16886–91. [PubMed: 16275924]
24. Wood W, Turmaine M, Weber R, Camp V, Maki RA, McKercher SR, Martin P. Mesenchymal cells engulf and clear apoptotic footplate cells in macrophageless PU.1 null mouse embryos. *Development* 2000;127:5245–52. [PubMed: 11076747]
25. Feld JJ, Hussain N, Wright EC, Kleiner DE, Hoofnagle JH, Ahlawat S, Anderson V, Hilligoss D, Gallin JJ, Liang TJ, Malech HL, Holland SM, Heller T. Hepatic Involvement and Portal Hypertension Predict Mortality in Chronic Granulomatous Disease. *Gastroenterology*. 2008
26. Takeya R, Sumimoto H. Regulation of novel superoxide-producing NAD(P)H oxidases. *Antioxid Redox Signal* 2006;8:1523–32. [PubMed: 16987008]
27. Aram G, Potter JJ, Liu X, Wang L, Torbenson MS, Mezey E. Deficiency of nicotinamide adenine dinucleotide phosphate, reduced form oxidase enhances hepatocellular injury but attenuates fibrosis after chronic carbon tetrachloride administration. *Hepatology* 2009;49:911–9. [PubMed: 19072832]
28. Kisseleva T, Brenner DA. Role of hepatic stellate cells in fibrogenesis and the reversal of fibrosis. *J Gastroenterol Hepatol* 2007;22(Suppl 1):S73–8. [PubMed: 17567473]
29. Muhanna N, Doron S, Wald O, Horani A, Eid A, Pappo O, Friedman SL, Safadi R. Activation of hepatic stellate cells after phagocytosis of lymphocytes: A novel pathway of fibrogenesis. *Hepatology* 2008;48:963–77. [PubMed: 18726940]
30. Heyworth PG, Bohl BP, Bokoch GM, Curnutte JT. Rac translocates independently of the neutrophil NADPH oxidase components p47phox and p67phox. Evidence for its interaction with flavocytochrome b558. *J Biol Chem* 1994;269:30749–52. [PubMed: 7982999]

31. Watanabe A, Hashmi A, Gomes DA, Town T, Badou A, Flavell RA, Mehal WZ. Apoptotic hepatocyte DNA inhibits hepatic stellate cell chemotaxis via toll-like receptor 9. *Hepatology* 2007;46:1509–18. [PubMed: 17705260]

Figure 1. The expression of NOX2 is upregulated in HSC during liver fibrogenesis

(A) Myofibroblasts in patients with HCV (a, b, c,) and PBC (d, e, f) express NOX2 (green channel, a, d), α -SMA (red channel b, e), and co-localization images (c, f), by confocal microscopy. Bar=30 μ m. (B) HSC were isolated from BDL and sham rats and NOX2 mRNA and protein expression were assessed. Both NOX2 mRNA and protein (* p <0.05) were significantly upregulated compared to HSC from sham-operated animals (shown in fold expression). (C) HSC were isolated from rats and exposed to AB for 24 hours. NOX2 expression was determined by real-time PCR. Phagocytosis of AB induced NOX2 upregulation in HSC, shown as fold increase (** p ≤0.01). Error bars indicate 1 SED.

Figure 2. NOX2 activation in HSC results in superoxide production and upregulation of collagen I mRNA by inducing its transcriptional activation

(A) Primary rat HSC were treated with either scrambled siRNA or NOX2 siRNA and exposed to AB. After 6 hours superoxide production was assessed by the lucigenin assay. In the scrambled siRNA-transfected HSC, phagocytosis induced superoxide production, and this was inhibited in the NOX2 siRNA-transfected cells (NT: non-transfected, C: control, not exposed to AB, * $p < 0.05$, average of 4 experiments, bars indicate 1 SED, shown in fold expression).

(B) Collagen IA1 expression was assessed by real-time PCR. Collagen IA1 expression was upregulated in the scrambled siRNA-transfected cells after AB exposure (shown in fold

expression, $**p \leq 0.01$), and it decreased significantly in the NOX2 siRNA-transfected primary HSC ($##p \leq 0.0001$).

(C) Primary wild type or NOX2^{-/-} HSC were transfected by constructs containing the truncated collagen promoter Col1A2 P1-Luc (-378/+58) or with a construct where the peroxide-responsive area is intact [Col1A2 P1-Luc (-2900/+58)], or the empty vector. The cells were exposed to AB in the presence or absence of the reducing agent GSH or catalase. Phagocytosis resulted in a significant induction of the COL1A2 promoter activity in the Col1A2 P1-Luc (-2900/+58)-transfected cells. This was abrogated in the Col1A2 P1-Luc (-378/+58)-transfected cells, or decreased after exposure to catalase, or GSH. In wt cells the collagen promoter activity thus resulted from NOX2-mediated peroxide production following phagocytosis. In the NOX2^{-/-} cells the luciferase activity was significantly blunted after exposure to AB (C: control cells, AB: apoptotic bodies, cata:catalase, GSH: glutathione $*p < 0.05$, $**p \leq 0.01$).

Figure 3. The phagocytic activity is decreased in NOX2^{-/-} HSC

(A) Primary wt or NOX2^{-/-} HSC were exposed to TAMRA-labeled AB, and the rate of phagocytosis was assessed after 24 hours. The engulfment was significantly decreased in HSC isolated from NOX2^{-/-} mice compared to wt mice (#p<0.001). (B) Pull-down of active GTP-bound Rac1 from the membrane fractions of wt and NOX2^{-/-} HSC demonstrated significantly more GTP-Rac1 in the membrane fraction of wt cells following AB exposure compared to NOX2^{-/-} cells. Rac1 immunostaining was detectable at the phagosomal membrane forming around the TAMRA-labeled AB (red) in wt HSC (arrow), while in the NOX2^{-/-} HSC AB attachment is seen but impaired phagosome formation (arrowhead), bar=10μm.

Figure 4. *In vivo* model of hepatocyte apoptosis and phagocytosis

To follow the fate of apoptotic hepatocytes, mice were first injected with the $\alpha 1$ -AT-LV-GFP *via* the portal vein then 7 days later Ad-TRAIL was injected into the tail vein of the same mice. As control, mice were injected only with Ad-TRAIL, or Ad-GFP (images not shown) or with $\alpha 1$ -AT-LV-GFP. To inhibit apoptosis, a separate group of mice were injected with Q-VD-OPH, pancaspase inhibitor before and after the Ad-TRAIL injection. The liver from only Ad-TRAIL, or LV-injected mice showed no significant injury or infiltration by inflammatory cells, and the ALT values remained normal (Figure 4b, c). In the LV plus Ad-TRAIL-injected animals the liver showed mild to moderate hepatocyte injury, increased ALT values, (*p<0.05), and no significant infiltration by inflammatory cells (Figure 4d). In the Q-VD-OPH treated animals the liver showed normal histology (Figure 4e) and decreased ALT values (*p<0.05) compared to LV plus Ad-TRAIL injected mice. To assess apoptosis, TUNEL assays were done on all liver samples. In the LV plus Ad-TRAIL infected livers hepatocyte apoptosis was increased (Figure 4d') compared to only Ad-TRAIL (Figure 4b') or only LV (Figure 4c')-injected animals. In the Q-VD-OPH treated animals apoptosis decreased (Figure 4e'). bar=50 μ m.

Figure 5. Phagocytosis of apoptotic hepatocytes *in vivo* results in a fibrogenic response

Immunohistochemistry and confocal microscopy were performed to visualize GFP-labeled hepatocytes and their AB and activated HSC (α -SMA, red). (GFP was only expressed in hepatocytes because of the hepatocyte specific α 1-AT promoter). There was an increase in the number of activated HSC (α -SMA, red) in the liver of mice injected with α 1-AT-LV-GFP plus Ad-TRAIL (Figure 5d, e), while the only Ad-TRAIL (Figure 5b), or only LV-GFP (Figure 5c) injected mice had the same amount of activated HSC as control, uninjected mice (Figure 5a), bar=50 μ m. At higher magnification in the α -SMA-positive HSC, GFP-labeled AB could be seen, indicating phagocytosis of hepatocyte-derived AB (arrow, arrowhead depicts apoptotic hepatocytes and AB, Figure 5f), bar=20 μ m.

Figure 6. NOX2^{-/-} mice exhibit decreased profibrogenic activity

Wt and NOX2^{-/-} mice were injected with Ad-TRAIL, or LV or LV plus Ad-TRAIL. Immunohistochemistry showed much less α -SMA positive HSC in NOX2^{-/-} mice after injection of LV plus Ad-TRAIL, suggesting that in these animals less HSC activation have occurred (Figure 6b, bar=50 μ m). In wt animals treated with Q-VD-OPH less α -SMA positive HSC are seen. Real-time PCR was performed from the liver tissues of wt and NOX2^{-/-} mice in the above experiments using α -SMA, collagen IA1 and TGF- β 1-specific primers. The expression of the fibrosis-related transcripts have increased in wt Ad-TRAIL plus LV-injected mice, while no increase was detected in NOX2^{-/-} animals (*p<0.05, **p<0.01). In wt animals treated with Q-VD-OPH the increase in α -SMA expression was significantly lower than in vehicle treated wt mice (*p<0.05), and collagen IA1 and TGF- β 1 levels decreased significantly, as well (*p<0.05). Data expressed as fold over control (control mice, not injected).

Figure 7. Liver fibrosis is decreased in NOX2^{-/-} mice

(A) Wt or NOX2^{-/-} mice were bile duct ligated (BDL) then sacrificed after 3 weeks (in the case of some NOX2^{-/-} mice after 6 weeks) following surgery. Picrosirius staining was done to assess fibrosis stage. In NOX2^{-/-} animals the fibrosis stage was significantly lower compared to that of wt animals following BDL (Figure 7A b, c, d). NOX2^{-/-} mice survived longer with less fibrosis compared to wt animals (Figure 7A, b, d). ALT and bilirubin values were increased in both the wt and NOX2^{-/-} BDL animals (*p<0.05). (B) Mice undergone BDL were injected with GdCl₃, throughout the experiment, to inhibit macrophage function. The fibrosis decreased in GdCl₃-injected wt animals after BDL (Figure 7B, a) compared to PBS-injected controls (Figure 7A, b), while no significant change was seen in NOX2^{-/-} livers, bars=50μm. Real-time PCR

was performed using collagen IA1 specific primers, and OH-proline incorporation assay to assess the amount of collagen in all samples. In NOX2^{-/-} BDL mice the expression of collagen IA1 (**p≤0.01), and OH-proline incorporation significantly decreased (*p<0.05) compared to wt BDL animals. Inhibiting macrophages decreased collagen IA1 expression (*p<0.05) and OH-proline incorporation (*p<0.05) in wt BDL animals, compared to PBS-injected animals, but not to the extent seen in NOX2^{-/-} BDL mice. In GdCl₃-injected mice the ALT values were overall lower (*p<0.05) than in PBS-injected mice however, the values among GdCl₃-injected wt and NOX2^{-/-} animals were not significantly different.