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Liver fibrosis and hepatocyte apoptosis are attenuated by GKT137831 a novel NOX4/NOX1 inhibitor in vivo

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Abstract

Reactive oxygen species (ROS) play a key role in chronic liver injury and fibrosis. Homologues of NADPH oxidases (NOXs) are major sources of ROS, but the exact role of the individual homologues in liver disease is unknown. Our goal was to determine the role of NOX4 in liver fibrosis induced by bile duct ligation (BDL) with the aid of the pharmacological inhibitor GKT137831, and genetic deletion of NOX4 in mice. GKT137831 was either applied for the full term of BDL (preventive arm), or starting at 10 days post-operatively (therapeutic arm). Primary hepatic stellate cells (HSC) from control mice with and without BDL were analyzed and the effect of NOX4 inhibition on HSC activation was also studied. FasL or TNF α /actinomycin D induced apoptosis was studied in wild type and NOX4^{-/-} hepatocytes. Results: NOX4 was upregulated by a TGF- β /Smad3-dependent mechanism in HSC. Downregulation of NOX4 decreased ROS production and the activation of NOX4^{-/-} HSC was attenuated. NOX4^{-/-} hepatocytes were more resistant to FasL or TNF α /actinomycin D induced apoptosis. Similarly, after pharmacological NOX4 inhibition, ROS production, the expression of fibrogenic markers and hepatocyte apoptosis were reduced. NOX4 was expressed in human livers with stage 2–3 autoimmune hepatitis. Fibrosis was attenuated by the genetic deletion of NOX4. BDL mice gavaged with GKT137831 both in the preventive or the therapeutic arm displayed less ROS production; significantly attenuated fibrosis and decreased hepatocyte apoptosis. In conclusion, NOX4 plays a key role in liver fibrosis. GKT137831 is a potent inhibitor of fibrosis and hepatocyte apoptosis; therefore it is a promising therapeutic agent for future translational studies.

Keywords

liver fibrosis; nicotinamide adenine dinucleotide phosphate reduced oxidase 4; hepatocyte apoptosis; stellate cell activation

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Introduction

Liver fibrosis leading to cirrhosis is one of the major health burdens worldwide with currently limited therapeutic options available[1]. Chronic liver injury of various etiologies results in hepatocyte apoptosis, and subsequent transdifferentiation of hepatic stellate cells into myofibroblasts with an upregulation of profibrogenic cytokines such as TGF- β [1], and an increased production of ECM compounds. Chronic oxidative stress is an important factor in initiating the fibrogenic process in the liver[2]. We and others have previously shown that the phagocytic NADPH oxidase NOX2 is expressed in HSC and its activation leads to the induction of early fibrogenic cascades[3–4]. Angiotensin II-mediated induction of NOX1 was also described as profibrogenic [4], and NOX1 was shown to promote HSC proliferation and aggravate fibrosis[5]. NOX4, a non phagocytic NOX homologue is expressed in the liver[6–7], and is different from the other NOX isoforms as it does not require the recruitment of cytosolic structural subunits to form the active enzyme, and is constitutively able to generate ROS, mainly hydrogen peroxide. NOX4 was shown to be critical in lung and kidney fibrosis by mediating activation of myofibroblasts[8–9]. The role of NOX4 in liver injury and fibrosis however; has not been elucidated yet. In the liver, NOX4 is primarily expressed in hepatocytes, stellate cells, and endothelial cells[10]. NOX4 has been found to be upregulated in hepatitis C, and to contribute to the formation of ROS, most likely *via* TGF- β induction[6]. On the other hand, NOX4 is also known to mediate TGF- β induced hepatocyte apoptosis[11]. These observations prompted us to test the hypothesis that NOX4 is an important pro-apoptotic and fibrogenic factor in the liver. Recently, small molecule NOX4/NOX1 dual inhibitors have been developed showing good oral bioavailability and tolerability when administered orally in an animal model of pulmonary fibrosis[12]. GKT137831, a pyrazolopyridine dione core inhibitor of the enzymatic activity is a candidate drug currently being developed as a new therapy for diabetic nephropathy. This compound is currently undergoing phase I clinical testing, and was used in this study to determine the role of NOX-mediated liver injury and fibrosis.

In this study, we showed that NOX4 is a key element in HSC activation, and liver fibrosis *in vivo*. GKT137831 applied both in the preventive or therapeutic way inhibited hepatocyte apoptosis, improved serum ALT, and attenuated liver fibrosis.

Materials and Methods

Human liver tissue

Liver biopsy samples from patients with autoimmune hepatitis (Stage 2–3) were obtained from the UCD Cancer Center shared tissue repository funded by the NCI (N=6).

Animals

Sprague Dawley rats and C57/B6 mice and NOX4^{-/-} mice with the same background generated by the coauthors[13] were used in this study. HSC and hepatocytes were isolated from rats or mice as described by Geerts et al. [14], by sequential *in situ* perfusion with collagenase and pronase. BDL was performed on mice as described[3]. Mice were then fed by gavage with either GKT137831 (dissolved in 1.2w% Methylcellulose + 0.1w% Polysorbate 80 in water, 60 mg/kg) or solvent once a day. The treatment started either on day 1 (preventive protocol, 3 weeks of treatment) or day 10 (therapeutic protocol, 1.5week of treatment) after the surgery. Sham-operated animals were used as control. Three weeks later, the mice were sacrificed, and the liver specimens and sera were collected. The animals were housed in facilities approved by the National Institute of Health. All procedures were reviewed and approved by the Animal Welfare Committee of the University of California Davis.

siRNA transfection

Primary rat HSC were cultured as above for a day then the medium was changed to DMEM, 0.5% FBS and transfection with the siRNA to NOX4 (Santa Cruz Biotechnology), or scrambled siRNA was performed using the RiboJuice transfection reagent (EMD Chemicals Inc., Darmstadt, Germany) according to the instruction.

Adenovirus preparation

The adenoviral dominant negative (DN) Smad 3, and wild type Smad 3 were gifts by Dr. Rebecca Wells (University of Pennsylvania). HEK293 cells were incubated for 24–48 hrs at 37°C. The multiplicity of infection (MOI) was 5–10 pfu/cell. The cells were collected when 80% showed cytopathic effects. After lysis by consecutive freeze-thaw cycles and centrifugation, the supernatant was collected, and further purified using the ViraBindtm Adenovirus Purification Kit (Cell Biolabs). The adenovirus titer was obtained using the QuickTitertm Adenovirus Titer Immunoassay Kit (Cell Biolabs).

Immunohistochemistry

Tissue samples were sectioned, deparaffinized and processed for staining. The tissue was incubated with the primary antibodies targeting NOX4 (1:200, Novus, St. Charles MO) and α SMA (1:200, Epitomics Inc., Burlingame, CA), or albumin (Novus, Littleton, CO). After probing with the appropriate AlexaFluor conjugated secondary antibodies (Invitrogen), the fluorescent signals were detected and analyzed by confocal microscopy. Hematoxylin–eosin (H&E) staining and picosirius red staining were performed by the Department of Pathology, UC Davis Medical Group following a standard protocol. The images were assessed by the NIH ImageJ software.

Apoptosis Studies

Primary wild type or NOX4^{-/-} mouse hepatocytes were incubated with FasL (5ng/ml, 16 hours) or TNF α /actinomycin D (28 ng/ml and 0.2 μ g/ml), in the presence or absence of glutathione monoethyl ester (10 mmol/l, Calbiochem). Actinomycin D is commonly used as inhibitor of DNA transcription, to allow the TNF- α -induced apoptotic response[15]. To assess the effect of the inhibitor on apoptosis, GKT137831 (20 μ M for 5 hours) then by Fas ligand (FasL 5ng/ml, 16 hours) were used. The cells were then stained with an antibody against active caspase-3 (Cell Technology, Inc., Mountain View, CA). The positive cells were counted from 5 random views using a fluorescence microscope and divided by the total cell number to obtain the apoptotic rate. Immunohistochemistry on the liver tissue from BDL mice treated with the solvent, GKT137831 for 1.5 weeks (treatment arm), and 3 weeks (preventive arm) using the above antibody was done, co-stained with DAPI to label nuclei, and positive cells were counted as above.

Lucigenin Assay

Fresh liver tissues were homogenized on ice in homogenization buffer (250 mM sucrose, 0.5 mM EDTA, 50 mM HEPES with protease inhibitor, pH 7.4). The scrambled or NOX4 siRNA-treated cells were collected, viability assessed by propidium iodide (Suppl. Fig. 1). The cells were then lysed, and clarified by centrifugation at 1500g for 10 min at 4°C. Twenty μ l of supernatant was added to 0.68 ml of lucigenin working solution (50 mM KH₂PO₄, 150 mM sucrose, 1 mM EGTA, 5 μ M lucigenin, 100 IM NADPH, pH 7.0). The lucigenin intensity was read by a luminometer (Monolight Luminometer 3010, BD, Franklin Lakes, NJ) every 20 s, up to 10 counts. The data were adjusted to the protein amount in each sample.

Hydroxy proline Assay

The liver tissue was denatured in 6N HCl at 100°C for 16 hours, then washed and resuspended in H₂O. The tissue suspension was then mixed with chloramines-T (50mM) and incubated at room temperature for 20 min. after adding perchloric acid (3.15M, Sigma-Aldrich), and 20% p-dimethylaminobenzaldehyde (Sigma-Aldrich), the absorbance at 557 nm was measured. The hydroxy-proline amount was calculated based on a standard curve generated from a series of hydroxy-proline solution with known concentrations. The data were expressed as µg of hydroxy-proline per gram of wet liver.

Western blot analysis

Fifty µg of protein were denatured in the sample buffer and separated on SDS-PAGE gel. The proteins were transferred onto nitrocellulose membrane. After blocking, the blot was probed with the anti-NOX4 antibody (1:1000, Pierce, Rockford, IL.) followed by secondary IgG-HRP (1:2000, Santa Cruz Inc.). The immunocomplexes were visualized using the ECL method (Pierce Biotechnology, Rockford, IL). GAPDH was used as internal control.

Quantitative RT-PCR

Total RNA from hepatic stellate cells (control or treated with TGF-β, 4 ng/ml) or liver tissues were extracted by using an RNA purification kit (QIAGEN, Valencia, CA). Reverse transcription was performed by using Superscript III kit based on the random hexamer method (Superscript III first strand synthesis supermix for qRT PCR, Bio-rad, Hercules, CA). The primers for real-time PCR reactions are listed in Table 1.

Statistics

All data represented at least three experiments and expressed as the mean ± SED. Differences between groups were compared using the analysis of variance (ANOVA) with the Dunnett's test. Statistical significance was assumed when p<0.05.

Results

NOX4 expression is induced *in vitro* during stellate cell activation by a TGF-β and Smad 3-dependent mechanism, and *in vivo* during BDL

Primary hepatic stellate cells are known to spontaneously undergo transdifferentiation when plated on plastic [16],[17]. To study whether NOX4 was induced during culture-activation, primary HSC were cultured for 8 days and the expression of NOX4 tested by real time PCR. NOX4 was significantly upregulated (p<0.01) in cells that transdifferentiated to myofibroblasts compared to day 1 quiescent cells (Figure 1A). As NOX4 is a transcriptionally inducible NOX, next we tested if TGF-β plays a role in its induction (Figure 1B). TGF-β induced a significant upregulation of NOX4 (p<0.05) whereas this was blocked by Ad-DNSmad 3, (p<0.01) suggesting that the induction of NOX4 during HSC activation was TGF-β and Smad3 dependent. NOX4 expression was also assessed in HSC isolated from BDL mice at different time points post operatively, and there was a gradual and significant induction (#p<0.05, *p<0.01) of NOX4 both at the transcript (Figure 1C) and protein (Figure 1D) levels during fibrogenesis in HSC. In contrast in the control, sham operated mice no induction was seen. To determine whether NOX4 is induced in patients with liver disease we studied patients with autoimmune hepatitis, a disease which is characterized by hepatocyte cell death and ensuing fibrosis. Immunohistochemistry was performed on control livers and liver biopsy samples from patients with stage 2–3 fibrosis. In control livers NOX4 immunoreactivity was low in hepatocytes (Supplementary Fig. 1). In autoimmune hepatitis NOX4 was expressed by myofibroblasts (Figure 1E, α-SMA

positive), and hepatocytes (albumin positive, Supplementary Figure 1), assessed by confocal microscopy

NOX4 plays a role in ROS production and HSC activation *in vitro* and *in vivo*

To study the role of NOX4 in ROS production of primary, culture-activated HSC, the cells were transfected with scrambled or NOX4 siRNA and the released ROS were measured by lucigenin chemiluminescence. We found that ROS release was significantly inhibited by the NOX4 siRNA (Figure 2A, * $p < 0.05$). Activated HSC (myofibroblasts) express procollagen $\alpha 1(I)$, and α SMA, the hallmarks of transdifferentiation[1]. We found that in wild type cells procollagen $\alpha 1(I)$ (9.1fold $\pm$ 0.83, * $p < 0.001$), and α SMA were significantly induced (3.4fold $\pm$ 0.33, * $p < 0.001$) whereas no induction was seen in the NOX4^{-/-} HSC (Figure 2B, ** $p = 0.003$, # $p < 0.05$). BDL was performed on wt and NOX4^{-/-} mice to assess fibrosis. Both procollagen $\alpha 1(I)$ and α SMA were downregulated in the NOX4^{-/-} BDL livers compared to the wt livers (0.37fold $\pm$ 0.02, * $p < 0.05$, and 0.38 $\pm$ 0.03, * $p < 0.05$, N=5, Figure 2C), and the α SMA immunoreactivity decreased in NOX4^{-/-} BDL mice (Figure 2D).

GKT137831 inhibits ROS production and fibrogenic activation of HSC

GKT137831, a member of the pyrazolopyridine dione family is an efficient inhibitor of both Nox4 and Nox1 isoforms with K_i in the range of 100–150nM in cell free assays of ROS production using membranes prepared from cells heterologously over-expressing specific NOX enzyme isoforms. GKT137831 shows only weak inhibitory activity on the NOX2 isoform in cell free assay and does not significantly inhibit neutrophil oxidative burst at concentrations up to 100uM, and did not inhibit innate microbial bacterial killing *in vitro* or *in vivo* (when used at a concentration of up to 20uM or administered at 100mg/kg orally, respectively). Furthermore, GKT137831 has neither scavenging nor antioxidant activity when tested at 10 μ M, and does not inhibit H₂O₂ production in the xanthine oxidase assay using the same read out and conditions as in the NOX assays[12]. It has an excellent specificity for NOX4 and NOX1 enzymes as shown in an extensive *in vitro* off-target pharmacological profile on 170 different proteins including ROS producing and redox-sensitive enzymes [12]. To study the effects of GKT137831, primary HSC were treated with GKT137831, and the ROS release was measured, and found to be significantly decreased. (Figure 3A, ** $p = 0.01$). GKT137831 also significantly blunted HSC activation as assessed by real time PCR of procollagen $\alpha 1(I)$, α SMA and TGF- β (Figure 3B, ** $p = 0.01$, *** $p < 0.01$, # $p < 0.001$).

NOX4 plays a role in death ligand induced apoptosis of hepatocytes

FasL and TNF- α are the main death-ligands inducing apoptotic cell death of hepatocytes which in turn triggers their phagocytosis and fibrogenic activity of HSC [3, 18–19]. To assess the role of NOX4 in apoptosis, primary wt or NOX4^{-/-} hepatocytes were exposed to FasL or TNF- α /Actinomycin D (ActD is commonly used to induce hepatocyte apoptosis with TNF α)[15]. Immunofluorescence staining was done to detect the active caspase 3 subunit and the rate of apoptosis was assessed. Compared to wt cells the rate of apoptosis was significantly reduced in NOX4^{-/-} hepatocytes stimulated with FasL or TNF α ./ActD (Figure 4A and B, $p < 0.05$). Hepatocytes were also treated by the NOX4/NOX1 inhibitor GKT137831, prior to FasL, and the rate of apoptosis was assessed, as above. Apoptosis by FasL was significantly reduced ($p < 0.05$) when the hepatocytes were pretreated with the inhibitor (Figure 4C).

GKT137831 reduces ROS production and apoptosis of hepatocytes *in vivo* both in the preventive and therapeutic protocols

To assess the efficacy of GKT137831 *in vivo*, the inhibitor was gavage-fed by two protocols: throughout the BDL (preventive arm) and starting from 10 days post-op (treatment arm), control animals were fed by the solvent, only. ROS production was decreased in the GKT137831-treated mice in both treatment arms (Figure 5A, $p<0.05$), and there was also a decrease in the number of apoptotic hepatocytes assessed by immunofluorescence for the active subunit of caspase 3 (Figure 5B, and Supplementary Data Figure 3). In addition, we noted a significant attenuation in the increase in serum ALT especially in mice treated with the inhibitor for 3 weeks (Figure 5C, $p<0.05$). Compared to the sham operated mice treated with the solvent, the increase in ALT was not significant in the BDL treated mice receiving therapy with GKT137831.

GKT137831 attenuates liver fibrosis *in vivo* both in the preventive and therapeutic protocols

To study liver fibrosis after BDL and the response to the inhibitor, real time PCR was carried out to assess the fibrogenic transcripts procollagen $\alpha 1$ (I), α SMA and TGF- $\beta 1$ (Figure 6A) in the liver tissue. There was a significant decrease in all markers of fibrogenesis in both treatment arms (collagen $\alpha 1$ (I) 1.5 w: $0.4\text{fold}\pm 0.03$, 3w: $0.9\text{fold}\pm 0.02$; α SMA 1.5w: $0.18\text{fold}\pm 0.01$, 3w: $0.28\text{fold}\pm 0.01$; TGF- β , 1.5w: $0.4\text{fold}\pm 0.01$, 3w: $0.5\text{fold}\pm 0.01$, $p<0.05$). Higher dose of the inhibitor (120mg/kg) was also well-tolerated but did not provide further improvement of the fibrogenic markers. The picrosirius staining showed less collagen in the GKT137831-treated livers and there was significantly less hydroxy proline in both treatment arms signifying decreased collagen deposition (Figure 6B, C, $*p<0.05$, $**p<0.01$).

Discussion

Liver fibrosis is a result of a wound healing elicited by chronic liver injury[1]. Hepatocyte apoptosis triggers stellate cell activation either directly by the phagocytosis of the apoptotic bodies[3, 20], or indirectly by the generation of damage-associated molecular patterns (DAMPs) and inducing the migration and activation of stellate cells[21–23]. Thus rational treatment approaches for liver fibrosis may include drugs that target hepatocyte apoptosis, stellate cell activation, or both. NOX4 is a nonphagocytic NADPH oxidase and its induction results in the formation of mainly hydrogen peroxide. This and other radicals e.g. peroxynitrite, were shown to be key signaling elements in fibrogenic signaling[2, 24]. We have previously shown that hydrogen peroxide derived from NOX activation directly induces the transcriptional activation of the collagen I promoter and HSC activation [3]. In addition, we found that ROS-mediated signaling also plays a role in myofibroblast survival during fibrosis[20].

There is substantial evidence that NOX4 is involved in hepatitis C mediated injury[6] furthermore it plays a role in TGF- β induced cell death of hepatocytes[11]. The profibrogenic effects of ROS are compounded by the fact that NOX4 induction in hepatocytes leads to their apoptosis further triggering the cascade of events leading to cirrhosis. Therefore NOX4 as a therapeutic target is particularly appealing as both of these key processes could be targeted. Moreover, because this NOX homologue has no known antimicrobial effects, its inhibition would not interfere with host defense. NOX4^{-/-} mice appear grossly normal, do not express a particular phenotype at baseline and they are not overtly prone to acquire infections (unpublished observations). GKT137831 is a drug-like inhibitory molecule of NOX4/NOX1 isoforms that has shown to be well tolerated in several species and currently is in phase I clinical trials, with excellent pharmacological and safety

profiles[12, 25]. In previous studies it was found to be markedly more efficient than pirfenidone in murine models of bleomycin-induced pulmonary fibrosis[25].

Here we studied NOX4 as a source of ROS during fibrogenesis and found that NOX4 is induced during fibrogenesis by TGF- β 1 and Smad3, and NOX4 mediates ROS production during HSC activation. NOX4 also plays a role in death ligand induced hepatocyte apoptosis, and as hepatocyte apoptosis and activation of HSC are necessary for the propagation of fibrosis, finding an agent which may affect both processes may have a great therapeutic utility. We tested GKT137831 and found that it inhibits culture-activation and ROS production of HSC, furthermore has an anti-apoptotic effect on hepatocytes. To recapitulate these findings *in vivo*, we chose the BDL model of fibrosis, as in this model the primary fibrogenic stimulus is not based on direct liver toxicity (in contrast to CCl₄ or thioacetamide, where ROS release independently of NOX activation may play a role). Compared to wt mice NOX4^{-/-} mice developed attenuated fibrosis. Nevertheless, the lack of NOX4 did not completely prevent fibrosis, potentially suggesting that other NOXs are also important in this process. GKT137831 effectively decreased ROS production, improved hepatocyte apoptosis and reduced ALT levels and fibrosis. Upon NOX4 inhibition, the decrease in TGF- β expression was less pronounced than that of procollagen α 1(I) and α SMA suggesting that regulation of TGF β is largely independent of NOX4; and putting NOX4 distal to TGF β in the signaling cascade. GKT137831 has been described as a NOX4/NOX1 isoform-selective inhibitor, thus the pharmacological effects we observed in this study are likely to be combined effects due to inhibition of both NOXs. NOX1 is a non-phagocytic NADPH oxidase homologue, and also plays a role in liver fibrosis, its activation, however; is mainly induced by angiotensin II[4]. In a recent study by Aoyama et al. when SOD1 mutant mice with CCl₄-induced fibrosis were treated with GKT137831, significant reduction of fibrosis was seen, similarly to our study[26]. Interestingly however, according to previous studies NOX1 and NOX4 may play different roles in hepatocyte apoptosis, as NOX1 knockdown by siRNA increased caspase-3 activity and cell death, whereas NOX4 knockdown attenuated the apoptotic process in hepatocytes[27], suggesting that the inhibitory effect of GKT137831 on apoptosis would mostly be due to NOX4 inhibition. By testing the efficacy of GKT137831 in both the preventive and therapeutic models we found significant reduction of fibrosis, albeit more pronounced when the inhibitor was used daily for 21 days.

In conclusion, we have shown that NOX4 plays an important role in liver fibrosis and genetic deletion of NOX4 or oral administration of the NOX4 inhibitor GKT137831 during liver fibrogenesis resulted in a significant attenuation of liver injury, apoptosis and fibrosis. Inhibition of NOX4 may thus become a promising new strategy for translational trials in liver fibrosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of abbreviations

HSC	hepatic stellate cell
NADPH oxidase (NOX)	nicotinamide adenine dinucleotide phosphate reduced oxidase

BDL	bile duct ligation
TGF-β1	transforming growth factor- β
TNF-α	tumor necrosis factor α
αSMA	α -smooth muscle actin
ALT	alanine aminotransferase

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Highlights

- NOX4 is a key enzyme in liver fibrosis
- NOX4^{-/-} mice have attenuated fibrosis
- The NOX4 inhibitor protects against liver injury

Figure 1. NOX4 expression is induced in culture-activated hepatic stellate cells and in stellate cells isolated from bile duct ligated mice by a TGF-β/Smad 3 mediated mechanism

Real time PCR was performed to evaluate NOX4 expression in quiescent (day 1) and culture-activated HSC (day 8). There was a significant upregulation of NOX4 with HSC activation (Figure 1A; mean±SED, N=4, *p<0.05). HSC were infected with Ad-DNSmad3 or wt Smad3, exposed to TGF-β1, and the expression tested by real time PCR. TGF-β1 significantly induced the expression in control and wt Smad3-infected HSC whereas DN Smad3 blocked the induction (Figure 1B; mean±SED, N=3, *p<0.05, **p<0.01, NT=non transfected). Western blot analysis of HSC isolated at different time points during BDL shows a gradual induction of NOX4 both at a transcript (Figure 1C, expressed as fold over control, N=4, #p<0.05, *p<0.01), and protein level (Figure 1D, N=3, mean±SED). In patients with autoimmune hepatitis NOX4 (Figure 1E, green, a) colocalizes with αSMA expressing (red, b) myofibroblasts (overlay image, c). Liver biopsy samples of patients with stage 2–3 fibrosis were studied (N=6), representative images from a patient with stage 3 disease (Bar=30μm).

Figure 2. NOX4 plays a role in ROS production and activation of HSC

Culture-activated HSC were transfected with scrambled siRNA or NOX4 siRNA (viability of the cells did not change significantly after transfection, Suppl. Data 2A). Lucigenin assay showed that NOX4 siRNA decreased ROS production compared to the scrambled siRNA treated cells (NT, non transfected cells). (Figure 2A, mean±SED, fold over control, N=4 *p<0.05). Primary, wt or NOX4^{-/-} HSC were cultured for 8 days and the expression of profibrogenic genes procollagen α1(I), αSMA and also NOX4 were assessed. Culture activation induced a significant upregulation of these transcripts in wt cells (Figure 2B, data represent mean±SED, fold over control, N=4, *p<0.001,) while no induction was seen in the NOX4^{-/-} cells. BDL was performed on wt and NOX4^{-/-} mice and the expression of procollagen α1(I), and αSMA assessed by qPCR. Both of these transcripts were significantly downregulated in NOX4^{-/-} livers (Figure 2C, *p<0.05, **p<0.01, ***p<0.001, N=5). αSMA showed decreased immunoreactivity in NOX4^{-/-} livers (Figure 2D, αSMA: red, blue: DAPI).

Figure 3. Treatment with the NOX4/NOX1 inhibitor GKT137831 reduces ROS production and activation of hepatic stellate cells

Primary, culture activated HSC treated with GKT137831 (20 μ M) showed significantly reduced ROS production (Figure 3A, data represent mean $\pm$ SED, fold over control, N=4, **p=0.01). HSC treated with GKT137831 displayed significantly lower expression of profibrogenic genes procollagen α 1(I), α SMA and TGF- β . (Figure 3B, data represent mean $\pm$ SED, fold over control, N=5, *p=0.01, **p<0.01, ***p<0.001).

Figure 4. NOX4 plays a role in FasL and TNF- α induced hepatocyte apoptosis

Primary wild type (wt) or NOX4^{-/-} hepatocytes were treated with FasL, or TNF α /ActD to induce apoptosis, in the presence or absence of GSH ester. Immunofluorescence studies to detect active caspase-3 were done and the % of positive cells was counted in 5 different fields in 3 experiments, each. GSH ester reduced the rate of apoptosis (** $p < 0.01$). In hepatocytes from NOX4^{-/-} mice FasL induced significantly less apoptosis compared to wt mice (Figure 4A, % of caspase 3 active subunit positive cells, $N=3$, * $p < 0.05$). TNF α /ActD as expected induced significant apoptosis of wt hepatocytes and this was blunted in NOX4^{-/-} cells (Figure 4B, * $p < 0.05$). To assess the effect of GKT137831 on apoptosis, hepatocytes with or without pre-incubation of GKT137831 were treated with FasL. GKT137831 reduced significantly the FasL-induced cell apoptosis (Figure 4C, $N=4$, * $p < 0.05$).

Figure 5. The NOX4/NOX1 inhibitor GKT137831 reduces ROS production, hepatocyte apoptosis and serum ALT levels *in vivo*

BDL mice were subjected to gavage with GKT 60mg/kg daily for 1.5 weeks (starting on d10 after surgery) or for 3 weeks. As control, the solvent was used (for 3 weeks). The lucigenin assay showed that BDL induced ROS production in the solvent treated group (3.1-fold over sham operated mice), whereas the 3-week treatment with GKT137831 reduced it to 1.5-fold. (Mean±SED, fold over control, N=6 mice in each group, $p<0.05$, Figure 5A).

Immunofluorescence for active caspase-3 subunits (red) showed apoptosis of hepatocytes in BDL livers treated with solvent. Decreased number of apoptotic cells was seen in GKT137831 treated livers. Blue: DAPI, nuclear stain (Figure 5B, solvent-treated data shown for the 3w BDL group). The serum was tested for ALT and bilirubin values, and both ALT and bilirubin were increased in BDL animals but decreased after 3-weeks GKT137831 treatment. Compared to the sham operated mice treated with the solvent, the ALT was not significantly increased in the BDL GKT137831-treated mice (Figure 5C, Mean±SED, N=6 mice per group, $p<0.05$).

Figure 6. Liver fibrosis is reduced in mice after BDL following gavage with GKT137831
 Real time PCR data indicate that the expression of profibrogenic genes collagen $\alpha 1(I)$, α SMA and TGF β was significantly suppressed by GKT137831 (Figure 6A, solvent-treated data shown for the 3w BDL group) in the BDL liver tissues. Using the higher inhibitor dose regimen did not improve further the fibrosis markers. Picrosirius red staining (B) showed that the BDL-solvent group developed significant liver fibrosis whereas the GKT137831 treated mice had decreased fibrosis. Hydroxy proline assay (C) revealed that the collagen amount in liver was significantly decreased by GKT137831 treatment at both 1.5w and 3w treatments (Mean $\pm$ SED, N=6 mice per group, * p <0.05, ** p <0.01).

Table 1

Primer sequences used in the experiments.

Mouse Nox4	Forward: 5'-TTGCCTGGAAGAACCCAAAGT-3' Reverse: 5'-TCCGCACAAATAAAGGCACAA-3'
Mouse Collagen IA1	Forward: 5'-AGAGGCGAAGGCAACAGTCG-3' Reverse: 5'-GCAGGGCCAAATGTCTAGTCC-3'
Mouse/Rat αSMA	Forward: 5'-TCAGGCGCTCCAGTTCTCT-3' Reverse: 5'-AAAAAAAACACGAGTAACAAATCAA-3'
Mouse TGF-β	Forward: 5'-CATGGAGCTGGTGAAACGG-3' Reverse: 5'-GCCTTAGTTTGGACAGGATCTGG-3'
Mouse β-Actin	Forward: 5'-ACGGCCAGGTCATCACTATTG-3' Reverse: 5'-ATACCCAAGAAAGGAGGCTGGA-3'
Rat Nox4	Forward: 5'-TTACTACTGCCTCCATVAAGC-3' Reverse: 5'-GGAATGATTGGATGTCTCTGC-3'
Rat CoUagen IA1	Forward: 5'-TGATCTGTATCTGCCACAATG-3' Reverse: 5'-ACTTCTGCCGTCTGGTGATAC-3'
Rat TGF-β	Forward: 5'-CATGGAGCTGGTGAAACGG-3' Reverse: 5'-GCCTTAGTTTGGACAGGATCTGG-3'
Rat Arbp	Forward: 5'-AAGGAGGACCTCACCCGAGAT-3' Reverse: 5'-CCCTCTAGGAAGCGAGTGTG-3'