Free Radical Biology and Medicine ▮ (▮▮▮▮) ▮▮▮–▮▮▮

Contents lists available at SciVerse ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contributions

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

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ARTICLE INFO

Article history:
Received 20 January 2012
Received in revised form
23 April 2012
Accepted 3 May 2012

Keywords:
Liver fibrosis
NADPH oxidase 4
Hepatocyte apoptosis
Stellate cell activation

ABSTRACT

Reactive oxygen species (ROS) play a key role in chronic liver injury and fibrosis. Homologs of NADPH oxidases (NOXs) are major sources of ROS, but the exact role of the individual homologs 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 started at 10 day postoperatively (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 TNFα/actinomycin D-induced apoptosis was studied in wild-type and NOX4$^{-/-}$ hepatocytes. 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 TNFα/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 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.

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 nonphagocytic NOX homolog 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

Abbreviations: HSC, hepatic stellate cell; NADPH oxidase (NOX), nicotinamide adenine dinucleotide phosphate reduced oxidas; BDL, bile duct ligation; TGF-β1, transforming growth factor-β; TNF-α, tumor necrosis factor α; αSMA, α-smooth muscle actin; ALT, alanine aminotransferase

☆ This study was supported by NIH Grant DK083283 (N.J.T.). The authors declare no conflict of interest.

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http://dx.doi.org/10.1016/j.freeradbiomed.2012.05.007

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 proapoptotic 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 in both a preventive and a 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 1.2 wt% methylcellulose+0.1 wt% 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, 1.5 week 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 and 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 instructions.

Adenovirus preparation

The adenoviral dominant negative (DN) Smad 3 and wild-type Smad 3 were gifts from Dr. Rebecca Wells (University of Pennsylvania). HEK293 cells were incubated for 24–48 h 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 ViraBind adenovirus purification kit (Cell Biolabs). The adenovirus titer was obtained using the QuickTiter 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 αSMA (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 picrosirius 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 (5 ng/ml, 16 h) or TNFα/actinomycin D (28 ng/ml and 0.2 μg/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 μM for 5 h) and then by Fas ligand (FasL 5 ng/ml, 16 h) 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 and costained 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

Table 1
Primer sequences used in the experiments.

Mouse Nox4	Forward: 5′–TTGCCTGGAAGAACCCAAGT-3′
	Reverse: 5′–TCCGCACAATAAAGGCACAA-3′
Mouse Collagen IA1	Forward: 5′–AGAGGCGAAGGCAACAGTCG-3′
	Reverse: 5′–GCAGGGCCAATGTCTAGTCC-3′
Mouse/Rat αSMA	Forward: 5′–TCAGCGCCTCCAGTTCCT-3′
	Reverse: 5′–AAAAAAAACCACGAGTAACAAATCAA-3′
Mouse TGF-β	Forward: 5′–CATGGAGCTGGTGAAACGG-3′
	Reverse: 5′–GCCTTAGTTTGGACAGGATCTGG-3′
Mouse β-Actin	Forward: 5′–ACGGCCAGGTCATCACTATTG-3′
	Reverse: 5′–ATACCCAAGAAGGAAGGCTGGA-3′
Rat Nox4	Forward: 5′–TTACTACTGCCTCCATVAAGC-3′
	Reverse: 5′–GGAATGATTGGATGTCTCTGC-3′
Rat Collagen IA1	Forward: 5′–TGATCTGTATCTGCCACAATG-3′
	Reverse: 5′–ACTTCTGCGTCTGGTGATAC-3′
Rat TGF-β	Forward: 5′–CATGGAGCTGGTGAAACGG-3′
	Reverse: 5′–GCCTTAGTTTGGACAGGATCTGG-3′
Rat Arbp	Forward: 5′–AAGGAGGACCTCACCGAGAT-3′
	Reverse: 5′–CCCTCTAGGAAGCGAGTGTG-3′