Biochimica et Biophysica Acta 1832 (2013) 660–673

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

Protective effects of lipocalin-2 (*LCN2*) in acute liver injury suggest a novel function in liver homeostasis☆,☆☆

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ARTICLE INFO

Article history:
Received 16 November 2012
Received in revised form 10 January 2013
Accepted 16 January 2013
Available online 31 January 2013

Keywords:
Lipocalin
LCN2
NGAL
BDL
CCl_4
Acute phase response

ABSTRACT

Lipocalin-2 is expressed under pernicious conditions such as intoxication, infection, inflammation and other forms of cellular stress. Experimental liver injury induces rapid and sustained LCN2 production by injured hepatocytes. However, the precise biological function of LCN2 in liver is still unknown. In this study, $LCN2^{-/-}$ mice were exposed to short term application of CCl_4, lipopolysaccharide and Concanavalin A, or subjected to bile duct ligation. Subsequent injuries were assessed by liver function analysis, qRT-PCR for chemokine and cytokine expression, liver tissue Western blot, histology and TUNEL assay. Serum LCN2 levels from patients suffering from liver disease were assessed and evaluated. Acute CCl_4 intoxication showed increased liver damage in $LCN2^{-/-}$ mice indicated by higher levels of aminotransferases, and increased expression of inflammatory cytokines and chemokines such as IL-1β, IL-6, TNF-α and MCP-1/CCL2, resulting in sustained activation of STAT1, STAT3 and JNK pathways. Hepatocytes of $LCN2^{-/-}$ mice showed lipid droplet accumulation and increased apoptosis. Hepatocyte apoptosis was confirmed in the Concanavalin A and lipopolysaccharide models. In chronic models (4 weeks bile duct ligation or 8 weeks CCl_4 application), $LCN2^{-/-}$ mice showed slightly increased fibrosis compared to controls. Interestingly, serum LCN2 levels in diseased human livers were significantly higher compared to controls, but no differences were observed between cirrhotic and non-cirrhotic patients. Upregulation of LCN2 is a reliable indicator of liver damage and has significant hepato-protective effect in acute liver injury. LCN2 levels provide no correlation to the degree of liver fibrosis but show significant positive correlation to inflammation instead.

1. Introduction

Lipocalins are a distinct family of over thirty small soluble secreted proteins involved in the transport of small hydrophobic proteins [1]. Although most of these proteins share three conserved motifs, they have a large degree of diversity with limited regions of sequence homology. However, they contain a single characteristic eight-stranded, continuously hydrogen-bonded anti-parallel β-barrel [1]. Lipocalin-2 (LCN2) also known as 24p3 protein was first identified in urine taken from mice with SV40-infected kidneys [2]. Neutrophil gelatinase-associated lipocalin (NGAL), the human homologue of LCN2, was subsequently purified from neutrophils and shown to be associated with gelatinase that does not directly affect its enzymatic activity [3]. Rodent forms of this lipocalin are not associated with gelatinase, and most NGAL is exocytosed from neutrophils in a form that is not complexed with gelatinase [3]. Several functions of LCN2 have been identified, but the precise cellular and extracellular roles are not yet defined. Functions related to cancer have been suggested [4–6], but overall, the role of LCN2 in cell signaling, proliferation, and apoptosis is still unclear. Some data suggest a role in inflammation [7], while other studies point at an important LCN2 role in iron metabolism [8]. A number of inducers of this gene have been found, including serum, lipopolysaccharide (LPS), various growth factors, retinoic acid, glucocorticoids, and phorbol esters [4,9]. Also MK-886, nordihydroguaiaretic acid (NDGA), and several compounds acting as cyclooxygenase-2 inhibitors that induce apoptosis stimulate LCN2 expression [10,11]. LCN2 may serve as an acute kidney injury biomarker [12] and exhibits important beneficial functions in renal damage in experimental ischemia–reperfusion injury [13,14]. It induces iron-dependent responses, possibly *via* renal epithelial

Abbreviations: HRS, hepatorenal syndrome; NGAL/LCN2, lipocalin 2 protein; LPS, lipopolysaccharide; ConA, Concanavalin A; BDL, bile duct ligation; MCP-1/CCL2, monocyte chemoattractant protein-1/C–C chemokine ligand-2

☆ Conflict of interest: None of the authors have something to declare.

☆☆ Financial support: This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB/TRR57, P13) and the IZKF Aachen of the RWTH Aachen University.

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http://dx.doi.org/10.1016/j.bbadis.2013.01.014

delivery of catechol–iron complexes [15]. Hence, LCN2 may comprise an endogenous nephron-protective mechanism limiting repeated bouts of tubular insult [16]. In contrast, LCN2 is on the other hand reported to be essential for chronic kidney disease progression in mice and humans [17].

In murine liver, LCN2 is markedly induced during experimental sepsis and supposed to participate in antimicrobial host defenses by binding and scavenging bacterial iron-containing siderophores [18–21]. We recently identified that LCN2 is induced during experimental liver injury [22]. Moreover, immunohistochemistry and cell-based experiments revealed that injured hepatocytes are the main source of hepatic LCN2 [22]. Previous findings further suggest that LCN2 is induced in heart, kidney and liver after X-ray exposure most likely by the activity of reactive oxygen species [23].

To further elucidate the causes of LCN2 induction and its functioning, we here performed a set of experiments in different models of experimental liver injury. LCN2$^{-/-}$ mice were subjected to acute and chronic liver injury by application of CCl_4, Concanavalin A (ConA), LPS and ligation of the common bile duct (BDL). Acute single dose CCl_4 intoxication showed more liver damage in LCN2$^{-/-}$ mice indicated by significant higher levels of aminotransferases and increased expression of inflammatory cytokines and chemokines including IL-1β, TNF-α and the monocyte chemoattractant protein-1/C–C chemokine ligand-2 (MCP-1/CCL2). Additionally, hepatocytes of LCN2$^{-/-}$ mice showed enhanced hepatic lipid droplet accumulation and apoptosis. Increased apoptosis in LCN2 deficient mice was also found after application of ConA or LPS and in livers of animals that were subjected to BDL for five days. During chronic insult, LCN2$^{-/-}$ mice showed more severe fibrosis compared to wild type controls. In humans, patients with chronic liver disease displayed overall higher serum LCN2 levels that were associated with impaired renal function.

2. Material and methods

2.1. Primary liver cell isolation and culturing

Hepatocytes were isolated using the collagenase method of Seglen as described previously [24] and cultured on collagen-coated dishes in Hepatozyme-SFM medium (Gibco, Invitrogen, St. Louis, MO).

2.2. RNA isolation, RT-PCR, and qRT-PCR

Total RNA from hepatocytes and liver tissue were isolated through QIAzol Lysis Reagent containing a monophasic solution of phenol and guanidine thiocyanate, followed by chloroform and isopropanol precipitation, DNAse digestion and RNeasy clean up with Mini Kits (Qiagen, Hilden, Germany). Amplification primers were selected from sequences deposited in the GenBank database (Table 1) using the Primer Express software (Applied Biosystems Invitrogen, Darmstadt, Germany).

Table 1
Primers used in this study.

Gene[1]	Acc. no.	Forward primer	Reverse primer
Col αI	NM_007742	catgttcagctttgtggacct	gcagctgacttcagggatgt
α-SMA	NM_009606	aatgagcgtttccgttgc	atccccgcagactccatac
IL-1α	NM_010554	ttggttaaatgacctgcaaca	gagcgctcacgaacagttg
IL-1β	NM_008361	gagctgaaagctctccacctc	ctttcctttgaggcccaaggc
IL-6	NM_031168	gctaccaaactggatataatcagga	ccaggtagctatggtactccagaa
TNF-α	NM_013693	accacgctcttctgtctactga	tccacttggtggtttgctacg
CCL2	NM_011333	gtgttggctcagccagatgc	gacacctgctgctggtgatcc
CCR2	NM_009915	tcgctgtaggaatgagaagaagagg	caaggattcctggaaggtggtcaa
IFN-γ	NM_008337	ggaggaactggcaaaaggatgg	tgttgctgatggcctgattgtc
IL-2	NM_008366	gctgttgatggacctacagga	ttcaattctgtggcctgctt
IL-4	NM_021283	cgtcctcacagcaacgaagaagcac	aagagtctctgcagctccatga
IL-10	NM_010548	ggctgaggcgctgtcatcg	tcattcatggccttgtagacacc
β-Actin	NM_007393	ctctagacttcgagcaggagatgg	atgccacaggattccatacccaaga
rS6	BC092050	cccatgaagcaaggtgttct	acaatgcatccacgaacaga

First-strand cDNA was synthesized from 1 μg RNA in 20 μl volume using SuperScriptTM II RNAse H reverse transcriptase and random hexamer primers (Invitrogen). First-strand cDNA derived from 25 ng RNA was subjected to real-time quantitative PCR, using qPCR Core Kits (Eurogentec, Cologne, Germany). PCR conditions were 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All Taqman primers used in this study are given in Table 1. RNA normalization was obtained through Taqman Ribosomal RNA Control Reagents (Applied Biosystems) designed for 18S ribosomal RNA (rRNA) detection.

2.3. Patient samples

We included patients with chronic liver diseases of any etiology, who were treated at our hospital as in- or outpatients [25]. Informed consent was obtained prior to recruitment. A total of n = 192 serum specimens was analyzed encompassing patients without fibrosis, patients with histologically proven fibrosis and individuals that were diagnosed for cirrhosis based on the conjunction of imaging studies, liver histology, laboratory parameters and the presence of typical cirrhosis-related sequela [25]. Cirrhotic patients were further stratified according to the Child–Turcotte–Pugh-Score [26]. Healthy blood donors (n = 91) from the local blood bank served as controls.

2.4. Animal experiments and specimen collection

All animal protocols were in full compliance with the guidelines for animal care approved by the German Animal Care Committee.

a) acute injury models: To investigate the effect of LCN2 in acute liver injury in mice, we used 6–8 week-old C57BL/6 wild type and LCN2$^{-/-}$ mice subjected to either (i) a single intraperitoneal injection of 0.8 ml/kg body weight CCl_4 (in mineral oil) for 48 h or (ii) a single intravenous injection of 20 mg/kg body weight ConA (Sigma, Taufkirchen, Germany) for 8 or 24 h or the respective tracer controls, as previously described [27], (iii) single dose i.p. of LPS (2.5 mg/kg) for 2 and 6 h respectively and (iv) 5 day-BDL [28,29].
b) chronic injury models: For chronic liver injury, we used long term application of CCl_4 for 8 weeks or BDL for 4 weeks. Mice were sacrificed, serum samples analyzed by standard techniques and liver specimens snap frozen in liquid nitrogen for protein and RNA isolation. Frozen tissue section were preserved with Tissue-Tek (Sakura Finetek, The Netherlands) in ice-cold 2-methylbutane (Roth, Karlsruhe, Germany) and kept at −80 °C, or fixed in 4% buffered paraformaldehyde for histological examination.

2.5. Immunohistochemistry

Paraffin-embedded liver tissue sections were treated as described [30]. Non-specific staining was blocked with 50% FCS and 0.3% Triton X-100 in PBS for 30 min at 37 °C followed by incubation with peroxidase, avidin and biotin. Blots were incubated with primary antibodies at 4 °C overnight followed by incubation with biotinylated secondary antibodies (BA-9200, Vector Laboratories, Eching, Germany), avidin-conjugated peroxidase (Vectastain ABC-Elite reagent, Vector Laboratories) and developed using the 3,3′-diaminobenzidine substrate (DAKO, Hamburg, Germany).

2.6. SDS-PAGE and Western blot analysis

Cell and tissue lysates were prepared using RIPA buffer containing 20 mM Tris–HCl (pH 7.2), 150 mM NaCl, 2% (w/v) NP-40, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate and the Complete™-mixture of proteinase inhibitors (Roche Diagnostics, Mannheim, Germany). Equal amounts of cellular or liver protein extracts were diluted with Nu-PAGE ™ LDS electrophoresis sample buffer and DTT as reducing agent, then heated at 95 °C for 10 min. and separated in 4–12% Bis–Tris gradient