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Proteomic profiling in Lipocalin 2 deficient mice under normal and inflammatory conditions

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ABSTRACT

Lipocalin 2 (LCN2) belongs to the superfamily of lipocalins which represent a group of small secreted proteins classified as extracellular transport proteins expressed in many tissues. LCN2 is strongly increased in experimental models of acute and chronic liver injuries. To investigate the function of LCN2 in normal liver homeostasis and under conditions of inflammatory liver injury, we comparatively analyzed hepatic extracts taken from *Lcn2*-deficient and wild type mice under basal conditions and after stimulation with lipopolysaccharides. Liver was chemically and mechanically lysed and extracts were subjected to 2-D-DIGE after minimal labeling (G200 and G300 dyes) using an appropriate internal standard (G100). Afterwards MALDI TOF MS and MS/MS were used to identify differentially expressed proteins. Proteins that were identified to be differentially expressed include for example the chloride intracellular channel protein 4 (CLIC4), aminoacylase 1 and transketolase. The altered expression of respective genes was confirmed by Western blot analysis and further validated by quantitative real time PCR. Altogether, the complex expression alterations in mice lacking LCN2 under normal conditions and after exposure to inflammatory stimuli reveal that LCN2 has essential function in liver homeostasis and in the onset of inflammatory responses in which LCN2 expression dramatically increases.

1. Introduction

Liver damage is generally associated with an inflammatory response, oxidative stress and decline of hepatocytes [1]. The inflammatory process is significantly triggered through various cytokines that are released by the different hepatic cell subpopulations and are embedded in a tight network of signaling cascades that are mainly triggered by the Janus kinase signal transducers and activators of transcription [2].

We have recently shown that the expression of Lipocalin 2 (LCN2) is strongly increased in experimental models of acute and chronic liver injuries due to its activation by pro-inflammatory

Abbreviations: CLIC4, chloride intracellular channel protein 4; IEF, isoelectric focusing; LCN2, Lipocalin 2; LPS, lipopolysaccharide; PMF, peptide mass fingerprint; qRT-PCR, quantitative real time polymerase chain reaction.

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cytokines including interleukin-1β via nuclear factor-κB activation [3]. LCN2 belongs to the superfamily of lipocalins that are classified as extracellular transport proteins with a barrel-shaped tertiary structure and expressed in many tissues [4]. This group comprises several small proteins that contain hydrophobic pockets allowing them to bind with high affinity to lipophilic molecules such as retinoids, fatty acids, cholesterol, bacteria-derived lipopolysaccharides (LPS), and prostaglandins.

LCN2 is a 25-kDa protein that is also known as neutrophil gelatinase-associated lipocalin (NGAL), 24p3 protein, α1-microglobulin-related protein, or uterocalin [5–7]. It preferably binds lipophilic substances and plays a role in iron metabolism, hematopoiesis, and inflammation, and has recently been implicated in epithelial-to-mesenchymal transition and restoration of tissue homeostasis following LPS-induced injury [8–10]. Consistently, mice targeted disrupted for the *Lcn2* gene showed an increased susceptibility to bacterial infections [8]. Several reports have demonstrated that LCN2 binds bacterial siderophores, thus reducing the availability of free iron for the bacteria [5,8,11–13]. It was further demonstrated that oxidative stress induces *Lcn2* expression [14]. Serum LCN2 is elevated in the setting of many inflammatory diseases and correlates well with established markers of inflammations and disease activity.

Through proteomics and microarray screening, LCN2 was detected as an adipokine that potentially connects obesity and its related adipose inflammation [15]. Additionally, individual lipocalins have been further associated with the activity of the c-Myc oncogene [16,17] suggesting that lipocalins are potentially relevant for tumor formation. In line, the *Lcn2* gene was identified as an attractive candidate for the diagnosis of hepatocellular carcinoma [18,19]. However, the regulatory network via which LCN2 influences all these biological processes remains still unknown.

2-D-DIGE is a state of the art proteomics technique. We already analyzed different materials obtained from experimental hepatic disease models and human subjects suffering from various liver insults to gain insight into the processes of hepatic inflammation and fibrogenesis [20,21].

In the present study, we performed comparative proteomic analysis of livers taken from mice with targeted disruptions of both *Lcn2* alleles and wild type controls that were left either untreated or challenged with bacterial LPS mimicking the characteristics of chronic bacterial infection and inflammatory insult. Using DIGE and MALDI mass spectrometry in addition to peptide mass fingerprinting (PMF) and MS/MS, we identified several proteins that were differentially either up- or downregulated in *Lcn2* deficient mice. The majority of the proteins identified have functions in detoxification, amino acid metabolism, cell adhesion, and glucose metabolism. Under unchallenged conditions, we found that the expression of aminoacylase 1 (ACY1) was overrepresented in livers of *Lcn2* deficient mice, while the expression of transketolase (TKT) was significantly reduced in respective mice. After stimulation with LPS, we found that calcium intracellular channel 4 (CLIC4) previously associated with tumorigenesis, angiogenesis, myofibroblast differentiation and peroxisome proliferator activated receptor-β (PPARβ) signaling was significantly upregulated in *Lcn2* deficient mice compared to wild type mice. Respective findings were confirmed by Western blot analysis and quantitative real time PCR. Therefore, we suggest that the LCN2 protein be involved in the control of liver homeostasis and further be a crucial mediator of hepatic inflammation and immune function evolving its biological activities with or via other protein constituents including CLIC4.

2. Material and methods

2.1. Animals

In the first set of experiments, a liver specimen was sampled from five *Lcn2*$^{-/-}$ mice [8] and respective C57BL6/J wild type mice. In the second experimental set up, the same amount and type of mice were treated with LPS (i.p., 2.5 μg/g body weight) 4 h before they were sacrificed. The age and gender of all animals used in this study are listed in Supplementary Table 1. Animal studies were conducted according to the National Animal Welfare Regulations and all protocols were approved by the local Animal Care and Use Committee of Aachen University.

2.2. Sample preparation

The liver tissue was snap frozen and stored at −80 °C for RNA and protein isolation. Frozen liver tissue was pulverized with mortar and pestle in liquid nitrogen and a tip of a spatula of the resulting powder was suspended in 0.5 ml lysis buffer that was prepared according to the labeling protocol for the Refraction 2D kit (NH DyeAGNOSTICS, Halle, Germany). The extracts were sonicated on ice and centrifuged (4 °C, 15 min, 13,000 rpm). The protein content of the supernatants was determined by Bradford assay (BioRad, Hercules, CA, USA) and lysates were stored at −80 °C until final use.

2.3. Protein labeling with G-Dyes

50 μg of total protein from *Lcn2*$^{-/-}$ and wild type control samples were labeled with 200 pmol of the fluorophores G-Dye300 and G-Dye200 (NH DyeAGNOSTICS) respectively, using the Refraction-2D™ labeling kit (NH DyeAGONSTICS) according to the manufacturer's instructions. These dyes have absorbance and emission properties that allow accurate analysis of 2D-electrophoresis gel images. As an internal standard, equal amounts of protein from each sample were pooled and labeled with G-Dye100. Samples of LPS treated mice were handled the same way.

2.4. 2D DIGE

Prior to isoelectric focusing (IEF), the G-Dye100, G-Dye200 and G-Dye300 labeled samples for each gel were mixed and rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 1% DTT, 1% IPG buffer pH 3–11, 0.002% bromophenol blue) was added to a total volume of 450 μl. Gel rehydration was performed using 24 cm pH 3–11 Immobiline DryStrips NL (GE Healthcare, Chalfont St. Giles, UK) in an Immobiline DryStrip Reswelling Tray (GE Healthcare). IEF was carried out by the IPGphor system III (GE Healthcare) with a total of 53 kVh of isoelectric focusing at 20 °C. Following IEF, the IPG strips were