Lipocalin 2 Upregulation Protects Hepatocytes from IL1-β-Induced Stress

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Abstract

Background: Lipocalin 2 (LCN2), a protein primarily produced by hepatocytes, is highly upregulated under various conditions that induce cellular stress, such as intoxication, infection or inflammation. However, the precise biological functions and underlying mechanisms of LCN2 in hepatocytes remains unknown. Methods: Hepatocyte stress was successfully induced by treating Huh7 cells with interleukin-1β (IL-1β). Interleukin-6 (IL-6), Tumor Necrosis Factor-α (TNF-α) and LCN2 levels were measured in IL-1β treated Huh7 cells and supernatant. Additionally, microarray analysis was conducted to identify genes differentially expressed in LCN2-silenced and control Huh7 cells. Results: TNF-α, IL-6 and LCN2 were significantly elevated in Huh7 cells after IL-1β treatment. In LCN2-silenced Huh7 cells, expression of IL-6 and TNF-α was significantly increased when compared with the expression levels of control Huh7 cells. Furthermore, differentially expressed genes were observed between the LCN2-silenced and control cells. Microarray analysis indicated that LCN2 acted by influencing genes involved in protein metabolism, stress response, cell cycle and proliferation. Conclusions: Our results suggest that LCN2 upregulation protects hepatocytes from IL-1β-induced stress. Additionally, our microarray analysis of LCN2-silenced and control cells provides a better understanding of the mechanisms that may be influenced by LCN2 induction.

Introduction

The liver is an important immunological organ that responds quickly to bacterial infection, toxins and other sources of damaging cellular stress. Hepatic immune cells,
including Kupffer, dendritic, NK and NK T cells, constitute the bodies first line of defense against infection [1]. However, many of the cytokines produced by hepatic immune cells can cause hepatocyte injury, and IL-1β, IL-6 and TNF-α are especially dangerous [2, 3]. Furthermore, IL-1β has been reported to induce high levels of TNF-α [4, 5] and IL-6 [6] in hepatocytes and osteoblasts, respectively. Because 80% of the liver volume consists of hepatocytes [7], the prevention of hepatocyte injury is an important consideration when designing therapeutic strategies [2]. Hepatocytes have been demonstrated to synthesize numerous protective proteins in response to deleterious cytokine exposure [7], and one potentially protective protein whose induction has been reported in hepatocytes after cytokine exposure is Lipocalin 2 (LCN2).

LCN2, also known as neutrophil gelatinase-associated lipocalin (NGAL), is a 25-kDa secreted glycoprotein [8]. The production of LCN2 in hepatocytes has primarily been seen in response to bacterial infection or partial hepatectomy [9]. LCN2 is highly upregulated in response to intoxication, infection, inflammation and other forms of cellular stress [10-15]. Growing evidence suggests that LCN2 plays a protective role in hepatic injury [16]. Studies have reported that activation of the transcription factors STAT3 and NF-kB are responsible for LCN2 synthesis [9, 11, 17]; however, the mechanisms underlying LCN2’s induction and cellular protective role are still not fully understood. Among IL-1β, IL-6 and TNF-α, IL-1β is the strongest LCN2 inducer [10, 18]. In the present study, we assessed the protective effects of LCN2 on the liver, with special emphasis on the underlying molecular mechanisms, by establishing an hepatocyte IL-1β induced stress condition.

Materials and Methods

Cell culture

Huh7 cell were grown in DMEM medium (Gibco, Life Technologies, Grand Island, NY, USA) with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT, USA), 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified environment with 5% CO2 at 37°C.

Small Interfering RNA (siRNA) Gene Silencing

Huh7 cells were divided into the following groups: a blank group, an IL-1β treatment (10 ng/ml) group, an LCN2-silenced (siRNA) group and a negative control group. LCN2-siRNA (100 nM) and negative control siRNA were synthesized by RiboBio (RiboBio Co. Ltd., Guangzhou, China). siRNA constructs were transfected into Huh7 cells using Lipofectamine® RNAiMAX Reagent (Invitrogen, Life Technologies) according to the manufacturer’s protocol. After 24 h, IL-1β (10 ng/ml) was added to the media, and the cells were incubated for another 24 h. After incubation with IL-1β, the cell culture media was collected and cells were lysed for further analysis. siRNA sequences were as follows:

Sense: 5’ – GAAUGCAAUUCUCAGAGAAdTdT – 3’
Antisense: 3’ - dTdTCUUACGUUAAGAGUCUCUU – 5’

RNA isolation and real-time PCR

Total RNA was isolated from cells using Trizol (Invitrogen) according to the manufacturer’s instructions [19, 20]. RNA was quantified using a GeneQuant pro RNA/DNA Calculator spectrophotometer (Amersham Biosciences, Freiburg, Germany), and then first-strand cdNA was synthesized from 1 µg RNA in a 20 µl reaction volume using a PrimeScriptTM RT reagent Kit with gDNA Eraser (RR047A; Takara, Shiga, Japan) according to the manufacturer’s instructions. β-actin was used as a control to normalize total mRNA input and confirm cdNA synthesis efficiency. LCN2, IL-6, TNF-α, HSPA1A, SOCS5, CITED2 and β-actin mRNA expression was quantified via quantitative PCR using Power SYBR Green PCR Master MIX (RR820A; Applied Biosystems, Life Technologies) in an ABI Prism 7900 (Applied Biosystems) according to the manufacturer’s instructions. The primers were purchased from Sangon Biotech (Shanghai, China), and their sequences were as follows:

LCN2:
5’ - AAAGACCCGCAAAGATGTATG - 3’ (sense)
5’ - AACCTGGAACAAAAGTCCTGAT - 3’ (anti-sense)
IL-6:
5’ – ACCCCTGACCCAACCACAAAT - 3’ (sense)
5’ – AGCTGGGAGAATAGATGAGT - 3’ (anti-sense)

TNF-α:
5’ – CTGTAGCCATGTTGACAAAC - 3’ (sense)
5’ – GCTGTGATCTCTCAGCTCCAC - 3’ (anti-sense)

HSPA1A:
5’ –AGCTGGAGCAGTGTTAAC - 3’ (sense)
5’ – CAGCAATCTTGGAAGGCC - 3’ (anti-sense)

SOCS5:
5’ –AGAGCGCGCACCCAAG - 3’ (sense)
5’ – AGAGGAGGAGGTAGGCTC - 3’ (anti-sense)

CITED2:
5’ –GGCTGATTTAATGCCTGAAGACT - 3’ (sense)
5’ – TATGTGCTCGCCCATTAGGG - 3’ (sense)

β-actin:
5’ – GTGGCCGAGGACTTTGATTG - 3’ (sense)
5’ – AGTGGGGTGGCTTTTAGGATG - 3’ (anti-sense)

Western blot
Cell lysates (40 µg) were subjected to 10% SDS-PAGE and transferred onto PVDF membranes. Membranes were incubated in blocking solution containing 5% non-fat dry milk in TBST buffer for 1 h at room temperature, followed by overnight incubation at 4°C with the following primary antibodies: anti-NGAL (1:1,000, Abcam, USA) and anti-β-actin (1:3,000, MAI BIO Technology Co, Ltd., China). Antibodies were diluted in TBST with 5% milk powder. After incubation, the membranes were washed three times in TBST for 10 min, followed by incubation in goat anti-rabbit or goat anti-mouse secondary antibody (1:3,000) for 1 h at room temperature. After incubation with the secondary antibody, the washing step was repeated. Signals were detected by chemiluminescence (ECL) and exposure to X-ray film [21, 22].

ELISA
Huh7 cells were cultured in 24-well plates. After 24 hr of pre- and post-transfection IL-1β exposure, the cells were lysed (as described above), the supernatants were collected and the levels of IL-6 (Dakewe Biotech Company Limited, Beijing, China), TNF-α (R&D Systems, Inc., Minneapolis, MN, USA) and LCN2 protein (Ray Biotech, Inc. Norcross, GA, USA) were detected using commercial ELISA kits according to the manufacturer’s instructions. Absorbance was read at 450 nm with a microtiter plate reader (Bio-Rad, Hercules, CA, USA) [21]. Standard curves prepared with various concentrations of purified recombinant IL-6, TNF-α and LCN2 were used to calculate the levels of IL-6, TNF-α and LCN2 protein.

Microarray and gene ontology (GO) analysis
Total RNA was isolated from LCN2-silenced and negative control Huh7 cells. Differential expression was detected using an Agilent gene chip (Agilent Technologies Inc. Santa Clara, CA, USA) and subsequent data analysis was done using GO-analysis. Microarray analysis of 14,900 transcripts was performed. GO-analysis classified significantly different gene expression from three different angles, biological processes, cellular components and molecular functions [23]. Within the each category, a p value < 0.05 was used as the threshold of significance.

Statistical analysis
Data were analyzed using SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical analysis was performed using independent t-tests to evaluate differences between groups. Each in vitro experiment was repeated at least three times, and data were expressed as means ± SEM. p < 0.05 was considered statistically significant.
Results

Expression of LCN2, IL-6 and TNF-α were increased in IL-1β-induced hepatocytes

When compared with the control group, the mRNA expression levels of LCN2, IL-6 and TNF-α were significantly increased in Huh7 cells after IL-1β exposure (p < 0.01; Fig. 1). Furthermore, LCN2, IL-6 and TNF-α protein levels were also markedly higher after IL-1β exposure than in the control group (p < 0.01).

LCN2 silencing increased IL-6 and TNF-α mRNA and protein levels

After LCN2-siRNA treatment, LCN2 mRNA and protein levels were significantly lower than in control cells (p < 0.01; Fig. 2). In contrast, IL-6 and TNF-α mRNA expression levels in the LCN2-silenced group were significantly elevated (p < 0.01; Fig. 3A). The IL-6 and TNF-α protein levels in the supernatant of the LCN2-silenced group were also significantly higher when compared with the negative controls (p < 0.05).

GO-analysis results

When compared with negative controls, 180 genes were upregulated and 268 genes were downregulated in the LCN2-silenced group. Biological processes related to genes with differential expression were analyzed (Fig. 4A and Table 1). Genes related to the stress response, protein metabolism, cell cycle, proliferation, developmental processes and RNA metabolism were significantly differentially expressed (Table 2).

All five differentially expressed stress response genes were upregulated. Notably, among these genes, heat shock 70 kDa protein 1a (HSPA1A) and crystallin alpha B (CRYAB) were elevated 3.6- and 6.2-fold, respectively. Nine differentially regulated genes were involved in protein metabolism: Asb11, Dnajb5, Stk40, Plk2, Limk2, Bhs10, Abi1, Socs5, Cct8 and Pik3ca. Of these, the first five were upregulated and the last four were downregulated. Among the differentially regulated cell cycle and proliferation genes, four were upregulated and three
were downregulated. Notably, CITED2 exhibited a 7.1-fold decline. Additionally, three RNA metabolism genes were upregulated and four were downregulated, and five developmental genes were upregulated and six were downregulated.

Differentially expressed genes were localized in the membranes (32%), nucleus (19%), mitochondrion (4%), ER/Golgi (4%) and cytoskeleton (3%; Fig. 4B). Functionally, 13% of the differentially expressed genes were related to nucleic acid binding activity and 11% to signal transduction activity. A small percentage of these genes were also related to transcription...
regulatory, kinase, enzyme regulator, transporter and cytoskeletal activity. However, no molecular functions could be assigned to 56% of the differentially expressed genes (Fig. 4C).

In addition, the significance of the variations found for genes, such as \( HSPA1A, SOCS5 \) and \( CITED2 \), was verified by qRT-PCR (Fig. 5).

**Table 1.** Summary of differentially expressed genes in thirteen GO terms

| GO Term                             | Count | p-value | Up-count | Down-count |
|-------------------------------------|-------|---------|----------|------------|
| cell cycle and proliferation        | 31    | 0.001025| 14       | 17         |
| Stress response                     | 24    | 0.026066| 15       | 9          |
| transport                           | 33    | 0.545953| 11       | 22         |
| developmental processes             | 47    | 0.033359| 22       | 25         |
| RNA metabolism                      | 51    | 0.010529| 18       | 33         |
| DNA metabolism                      | 5     | 0.675099| 5        | 0          |
| Protein metabolism                  | 57    | 0.00065 | 25       | 32         |
| Other metabolic processes           | 42    | 0.322742| 14       | 28         |
| Cell organization and biogenesis    | 34    | 0.073693| 18       | 16         |
| cell-cell signaling                 | 6     | 0.319699| 3        | 3          |
| Signal transduction                 | 49    | 0.677046| 21       | 28         |
| Cell adhesion                       | 13    | 0.085557| 6        | 7          |
| death                               | 14    | 0.25941 | 5        | 9          |

**Discussion**

Hepatocyte cellular stress was successfully induced via IL-1β treatment of Huh7 cells to investigate the precise biological functions and underlying mechanisms of LCN2. We demonstrated that TNF-α, IL-6 and LCN2 levels were significantly elevated in Huh7 cells after IL-1β treatment. Additionally, after siRNA knockdown of \( LCN2 \), IL-6 and TNF-α levels...
were significantly increased. Our results are supported by the results of a previous study by Erawan Borkham-Kamphorst et al. that reported that liver damage and the levels of pro-inflammatory cytokines in LCN2-/ mice were significantly increased after the mice were subjected to LPS, CCl4 and ConA treatment [16].

To investigate the mechanisms underlying LCN2’s hepatocyte protective role, microarray analysis of the differences between the LCN2-silenced and negative control groups was conducted. The resultant data indicated that the protective role of LCN2 was tightly related to genes involved in the stress response, protein metabolism, cell cycle and proliferation.

Heat shock proteins (HSPs) are molecular chaperones produced by cells in response to various stressful conditions. They are named according to their molecular weight, and HSP family members include HSP100, HSP90, HSP70, HSP60 and small HSP (sHSP) [24]. When activated by stressful stimuli, HSPs protect against inappropriate protein interactions and misfolding [25]. In the current study, heat shock 70kDa protein 1a (HSPA1A) was significantly elevated in the LCN2-silenced group. HSPA1A is a member of the HSP70 family, and protects proteins from aggregation and works together with other HSPs to mediate the folding of newly translated proteins in the cytosol and organelles [26]. In our study, the increased HSP expression in the LCN2-silenced cells indicated that the cells were under increased stress when LCN2 was knocked down. Supporting this conclusion, others have reported that LCN2 plays a protective role.

| Table 2. Differentially expressed genes involved in protein and RNA metabolism, developmental processes, stress response, cell cycle and proliferation |
|---|---|---|---|
| **Stress response** | Up | Fold change | Down | Fold change |
| Ndhp1 | 2.006045 | Elf2a | 2.284116 |
| Vgf | 4.842413 | Fbox31 | 2.199312 |
| Mtr1 | 2.264259 | Jasp2 | 2.222018 |
| P parad | 2.658682 | Rux1 | 2.390595 |
| Mapk9 | 2.976285 | Cat | 2.154408 |
| Cry2 | 2.996746 | Npm1 | 2.225445 |
| Foxo3 | 2.325413 | Tnf | 2.293686 |
| Hspal1 | 3.603816 | Prg | 2.965046 |
| Cyrb | 2.020697 | Mdk | 2.347856 |
| F9 | 13.292180 | | |
| Akrin | 2.531604 | Gita | 2.163245 |
| Herpud | 2.319134 | | |

| **Protein metabolism** | Up | Fold change | Down | Fold change |
|---|---|---|---|---|
| Asb11 | 15.370274 | Ab1 | 3.394974 |
| Hpn | 2.242927 | Elf2a | 2.410745 |
| Psmh9 | 2.697259 | March1 | 2.739084 |
| Dnajk1 | 4.802749 | Cpd | 2.407891 |
| Ube2e | 2.564038 | Pmch | 2.374533 |
| Cskl1d | 2.111092 | Pesa27 | 2.084690 |
| Trbl1 | 2.213055 | Hsc | 2.538136 |
| Fbox33 | 2.682089 | Utp2 | 2.214078 |
| Elf5 | 2.444075 | Pigs | 2.063632 |
| Ppp1rb | 2.190372 | Pik3r1 | 2.718698 |
| Pcm1 | 0.970623 | Socs5 | 6.253598 |
| Red | 2.290869 | Tolr1 | 2.089313 |
| Mrp16 | 2.424925 | Cellt | 3.060617 |
| Sdk40 | 3.235775 | Pik3r1 | 2.351721 |
| Sox7 | 2.103468 | Rgl7 | 2.660059 |
| Pdcd4 | 2.407791 | Cat2 | 2.759068 |
| Eps6ka1 | 2.322223 | Acaca | 2.173593 |
| Uspl2 | 2.529919 | Prpf4b | 2.404165 |
| Limk2 | 4.503347 | Ppnt2 | 2.290155 |
| Bbs10 | 14.320525 | Eps6ka3 | 2.077773 |
| Elf3h | 2.034143 | Pik3ca | 6.901063 |
| Wbs1 | 2.598028 | | |

| **Cell cycle and proliferation** | Up | Fold change | Down | Fold change |
|---|---|---|---|---|
| Mync | 3.996117 | Adrn | 11.463231 |
| Aktr2 | 3.345361 | Slk | 2.235528 |
| Trd1 | 2.945981 | Det | 2.246368 |
| H2afx | 2.603217 | Calc3 | 2.635705 |
| Rarg | 2.575744 | Kfl | 2.249511 |
| Fox1c | 2.332431 | Rasb | 2.164372 |
| Cebpe | 2.787830 | Fse | 2.531148 |
| Mafg | 2.027328 | Pmfg | 2.196084 |
| Espl1 | 2.645516 | Vegla | 4.090032 |
| Tnnp1 | 3.217375 | Gnb1 | 2.322321 |
| Pkcb | 3.399897 | Cled2 | 2.125572 |
| Calm1 | 2.247160 | Smo | 2.556038 |
| Csk1 | 3.459275 | | |

| **RNA metabolism** | Up | Fold change | Down | Fold change |
|---|---|---|---|---|
| Zlfp503 | 2.478194 | Ifd2 | 2.561707 |
| Med26 | 2.488779 | Rnmt | 2.128601 |
| Mync | 3.996117 | Nfl3 | 3.947778 |
| Dnajk1 | 2.499442 | Cen1425 | 2.816671 |
| Rarg | 2.573744 | Srr1m | 2.622486 |
| Fox1c | 2.332431 | Evr1r | 2.166625 |
| Pup3e | 4.689774 | Img14c | 2.216034 |
| Akrin2 | 2.531604 | Nud1r2 | 3.336175 |
| Ctnf | 2.557107 | Irtp2p2 | 2.804514 |
| Hoxc | 2.984191 | Sp5 | 2.697480 |
| Ets2 | 2.067439 | Zfip3602 | 2.185453 |
| Zfip3602 | 2.185453 | Id2 | 2.343360 |
| Ccnl1 | 2.286740 | Dcpx | 2.058455 |
| Eppc1 | 2.290560 | Atf2 | 2.221728 |
| Zrnb2b | 2.470666 | Vps36 | 2.413733 |
| Crpr | 2.296580 | Axl1 | 2.573926 |
| Sertad2 | 3.451000 | | |
against cellular stress [12, 27]. In LCN2 deficient cells, HSPs were overexpressed to protect against protein denaturation and cellular stress. Therefore, LCN2 appears to act in collaboration with HSPs to protect hepatocytes from environmental insults.

The balance between protein synthesis and degradation is crucial for the maintenance of a healthy cellular environment. The accumulation of misfolded or redundant proteins causes toxicity to the cells [28]. Many of the differentially expressed genes identified by the current study, such as the suppressor of cytokine signaling 5 (SOCS5), are involved in protein metabolism. SOCS5 is a negative regulator of the JAK-STAT pathway [29-31], and was substantially decreased in LCN2-silenced cells. Recent data indicate that hepatocyte production of LCN2 is primarily regulated by the transcriptional factors STAT3 and NF-κB [9]. IL-1β and IL-6 are thought to enhance LCN2 levels by activating the NF-κB and JAK-STAT pathways, respectively [9, 10]. In our experiments, IL-1β treatment induced IL-6 expression, which may have contributed, in turn, to LCN2 elevation, indicating that IL-1β is a strong inducer of LCN2. It was reported that STAT1 and STAT3 phosphorylation were sustained and acute liver injury was enhanced in LCN2-/- mice exposed to LPS [16]. Additionally, bacteria-mediated serum LCN2 protein elevation was significantly reduced in IL-6RHep-/- mice. These results suggest that the LCN2 protein is closely associated with the JAK-STAT pathway [9]. In brief, our data strongly suggest that LCN2 plays a regulatory role in cytokine-induced hepatocyte stress by activating SOCS5, which, in turn, negatively regulates the JAK-STAT pathway.

LCN2 is reported to act as a growth factor in multiple cell types [32]. Our data supported this view by demonstrating that LCN2 expression affected the expression of cell cycle and proliferation related genes. Although leading to some proliferation inhibitor genes upregulation, LCN2 knockdown was more associated with the downregulation of genes whose downregulation has been closely linked with cell death. Our data also indicated that CITED2 expression, which has been reported to increase G1/S cell cycle progression and inhibit cellular quiescence [33], was significantly downregulated in LCN2-silenced cells. These findings indicate that LCN2 may improve hepatocyte survival by promoting cell cycle progression and proliferation.

Fig. 5. qRT-PCR verification of gene differential expression obtained with chip data. The differential expression of HSP1A1, SOCS5 and CITED2 was confirmed by qRT-PCR.
Conclusions

Our results suggest that LCN2 upregulation protects hepatocytes from IL-1β-induced stress. Additionally, microarray analysis indicates that LCN2 regulates protein metabolism, stress response, cell cycle and proliferation. Further studies are needed to investigate the mechanisms accounting for the protective effect of LCN2.

Abbreviations

IL-1β (interleukin-1β); IL-6 (interleukin-6); TNF-α (Tumor Necrosis Factor-α); LCN2 (Lipocalin 2); HSPs (heat shock proteins).

Disclosure Statement

The authors declare that they have no conflicts of interest.

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