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Liver receptor homolog-1 regulates mouse superoxide dismutase 2

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ABSTRACT

Liver receptor homolog-1 (LRH-1) is a nuclear receptor that plays an important role in the regulation of bile acid biosynthesis, cholesterol reverse transport, steroidogenesis, and exocrine pancreatic enzyme production. In the current study, previously published data from a genome wide analysis of LRH-1 binding in the liver were re-analyzed to identify new LRH-1 targets and propose new roles for LRH-1 in the liver. Superoxide dismutase 2 (*Sod2*) was identified, which contains putative LRH-1 binding sites in the proximal promoter. When hepatocytes were treated with the LRH-1 agonist RJW101, *Sod2* expression was dramatically increased and reactive oxygen species (ROS) production, which was induced by a high concentration of palmitate, was significantly reduced. A LRH-1 binding site was mapped to −288/−283 in the *Sod2* promoter, which increased *Sod2* promoter activity in response to LRH-1 and its agonist. LRH-1 binding to this site was confirmed using a chromatin immunoprecipitation assay. These results suggest that *Sod2* is a target gene of LRH-1, and that LRH-1 agonists can mediate a reduction in ROS production and oxidative stress driven by an excess of fatty acids, as exhibited in nonalcoholic fatty liver disease.

1. Introduction

Liver receptor homolog-1 (LRH-1), also known as nuclear receptor 5A2 (NR5A2), is a member of the NR5A subfamily. LRH-1 is expressed mainly in the liver, exocrine pancreas, intestine, and ovaries [1], and plays a critical role in endoderm development, differentiation, and metabolism [2]. The role of LRH-1 in the regulation of hepatic bile acid synthesis [3–6], reverse cholesterol transport [7–9], and hepatic acute phase response [10] have been extensively studied using *Lrh-1*$^{-/-}$ mice. LRH-1 also regulates glucose homeostasis, via regulation of the glucokinase gene [11], steroid synthesis, ovulation, follicle maturation, and normal gestation in the ovaries [2,12–14]. In the pancreas, LRH-1 controls the production and secretion of digestive enzymes [15,16].

LRH-1 was classified as an orphan nuclear receptor and could be activated in the absence of a ligand [17]. However, a synthetic small molecule agonist specific to LRH-1, named RJW101, was identified that further enhances the transcriptional activity of LRH-1 [18]. Based on structural studies of LRH-1, phospholipids were suggested as potential LRH-1 ligands, and dilauroylphosphatidylcholine (DLPC) was identified as a potent ligand of human and mouse LRH-1 *in vitro* [19–21]. When hepatocytes were treated with DLPC, the expression of known LRH-1 target genes, such as those involved in bile acid biosynthesis, was increased [21]. DLPC treatment of mice revealed additional roles for LRH-1. In animal models of insulin resistance, such as leptin receptor deficient *db/db* mice and animals with diet-induced obesity, DLPC treatment caused anti-diabetic effects. It improved glucose homeostasis, lowered hepatic steatosis, and increased bile acid levels, which were not seen when *Lrh-1* was deleted in the liver. The reduced hepatic TG levels were due mainly to reduced SREBP-1c expression, thereby causing a decrease in expression of its target genes, which are main players in *de novo* lipogenesis [21]. Recent studies using *Lrh-1* null mice and agonists revealed that LRH-1 was able to resolve ER stress, suggesting that the LRH-1 pathway could be a new target for the treatment of diseases associated with chronic ER stress [22].

LRH-1 binds to the consensus sequence 5′PyCAAGGPyCPu3′ as a monomer [23]. Genome-wide analysis of LRH-1 binding to chromatin in the liver revealed over 10,600 LRH-1 binding sites in the

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genome, over 20% of which were located in the proximal promoter region. Many of these genes were involved in lipid metabolism [24]. To identify new LRH-1 target genes and novel functions of LRH-1 in the liver, previously published data from hepatic ChIP-seq of LRH-1 were searched [24], and superoxide dismutase 2 (*Sod2*) was identified as a potential candidate.

SOD2, also known as manganese-dependent superoxide dismutase (MnSOD), is a mitochondrial enzyme that converts superoxide (O_2^-) to hydrogen peroxide and molecular oxygen. Superoxide can generate other ROS such as reactive nitrogen species (RNS), peroxynitrite ($ONOO^-$) and peroxynitrite degradation products (OH, NO_2, CO_3^-), lipid peroxyl (RO_2), and alkoxyl (RO) radicals. Therefore, SOD2 is considered to be a ROS detoxifying enzyme and an antioxidant in cells [25,26].

In the present study, *Sod2* was identified as a direct target of LRH-1. LRH-1 agonists induced *Sod2* expression and reduced palmitate-induced ROS production in hepatocytes.

2. Materials and methods

2.1. Cell culture

AML12 immortalized mouse hepatocytes (ATCC CRL-2254) were cultured in DMEM/F-12 media (Gibco, NY, USA) supplemented with 10% fetal bovine serum, 1 × Insulin-Transferrin-Selenium-X Supplement (Gibco), dexamethasone (40 ng/mL; Sigma-Aldrich), and penicillin-streptomycin (100 U/mL). Human embryonic kidney (HEK)-293T cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum and penicillin-streptomycin (100 U/mL). Cells were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.2. Real-time PCR

Total RNA was isolated from cells using Trizol (Life Technologies, Carlsbad, CA, USA), as previously described [27]. Complementary DNA (cDNA) was synthesized using a cDNA superscript kit (Bio-Rad, Hercules, CA, USA) and used for quantitative real-time PCR (qRT-PCR) using a CFX96 real-time PCR detection system (Bio-Rad). Messenger RNA (mRNA) expression levels of genes were normalized to ribosomal protein L32 gene expression. Primer sequences used for qRT-PCR were as follows: *Lrh-1*, 5′-GCCCTCATTCGAGCCAATGG-3′ (forward), 5′-CTGGGTACTCAGACTTGATGGC-3′ (reverse); *Sod2*, 5′-GCACATTAACGCGCAGATCA-3′ (forward), 5′-AGCCTCCAGCAACTCTCCTT-3′ (reverse); and mouse *L32*, 5′-ACATTTGCCCTGAATGTGGT-3′ (forward), 5′-ATCCTCTTGCCCTGATCCTT-3′ (reverse).

2.3. Analysis of genome-wide ChIP-Seq data for LRH-1

Mouse genome-wide ChIP-Seq data for LRH-1 from a published study [24] were analyzed to identify new genes with LRH-1 binding regions, using the University of California at Santa Cruz (UCSC) genome browser. BED files were uploaded as custom tracks to visualize within the UCSC Genome Browser (http://genome.ucsc.edu/index.html).

2.4. Preparation of BSA-bound palmitate

Sodium palmitate (P9767, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 50% ethanol to make a 0.15 M solution and heated at 65 °C for 15 min. The solution was diluted with 10% fatty acid-free BSA solution to a final concentration of 7.5 mM palmitate, which was filtered and stored at −20 °C.

2.5. Measurement of reactive oxygen species (ROS) production using flow cytometry

AML12 cells (5×10^5 cells/well) were cultured in the absence or presence of 10 μM RJW101 in serum-free media for 5 h, then 0.5 mM palmitate was added and incubated for 1 h. Ten μM carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFHDA; Molecular Probes, Eugene, OR, USA) was added to the cells and incubated at 37 °C for 20 min. Cells were harvested by treating with 0.05% trypsin then washed twice with cold PBS. Cells conjugated with carboxy-H_2DCFHDA-FITC (excitation, 494 nm; emission, 524 nm) were detected using the FL1 setting of FACS Calibur (BD Biosciences, San Jose, CA, USA). RJW101 was a kind gift from Dr. Richard J. Whitby (University of Southampton, UK).

2.6. Construction of plasmids and transient transfection

The promoter region of *Sod2* containing the region −1132 to +207 bases from the transcription initiation site (+1) was synthesized by PCR and inserted into the pGL3-basic vector (Promega, Madison, WI, USA). These plasmids were designated pmSod2 (−1132/+207). Putative LRH-1 binding sequences in the promoter region were deleted by site-directed mutagenesis using a QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA). Primers were as follows: −288Δ forward: 5-ACGTGATTTAATGTCAGGTTATTAACTGGGTCAGAGGGGC-3′ and reverse: 5′-GCCCCTCTGACCCAGTTAATAACCTGACATTAAATCACGT-3′; −788 Δ forward: 5′-TGTTCACAGCAGTAAAACCCCGGTCTGACGCCTGTGGACA-3′ and reverse: 5′-TGTCCACAGGCGTCAGACCGGGGTTTTACTGCTGTGAACA-3′; −1065 Δ forward: 5′-CATCTGGAGGTGTGGCCTTGTGTGTCACTGTGGGTGTGGG-3′ and reverse: 5′-CCCACACCCACAGTGACACACAAGGCCACACCTCCAGATG-3′. HEK-293T cells were plated onto 12-well plates at a density of 5×10^4 cells/well in 1 mL media on day 0. On day 1, cells were transfected with pmSod2 (−1132/+207) (0.2 μg) together with murine LRH-1 (0 or 0.1 μg) and pCMV-β-galactosidase (0.1 μg) expression plasmids, as previously described [28]. On day 2, 10 μM RJW101 or DLPC was added to the cells and incubated for 24 h. On day 3, the cells were lysed and luciferase assays were performed as previously described [28].

2.7. Chromatin immunoprecipitation (ChIP) assay

Chromatin was prepared from AML12 cells for a ChIP assay as previously described [29]. Briefly, AML12 cells were treated with 10 μM DLPC for 24 h. Cells were fixed with 4% paraformaldehyde for 15 min, then glycine was added to a final concentration of 0.125 M and incubated for 10 min before harvesting. Cells were subjected to ChIP using an anti-LRH-1 antibody. Ct values of ChIP and input samples were used and presented as fold changes. Values were normalized to L32 expression levels. Primers used for ChIP PCR were as follows: *Sod2*, forward 5′- GCAACAAAGATGAACACACG-3′ and reverse 5′-CTGCTGGTCAGAATTATGGA-3′; *L32*, forward 5′-ACATTTGCCCTGAATGTGGT-3′ and reverse 5′-ATCCTCTTGCCCTGATCCTT-3′.

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism 3−5.0 software. Data were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) was used to determine statistical differences among the experimental groups. A p value less than 0.05 was considered statistically significant.

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