Recql5 protects against lipopolysaccharide/D-galactosamine-induced liver injury in mice

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Data sharing statement: No additional data are available.

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Received: April 9, 2015 Peer-review started: April 9, 2015 First decision: May 18, 2015 Revised: May 26, 2015 Accepted: July 3, 2015 Article in press: July 3, 2015 Published online: September 28, 2015

Abstract

AIM: To investigate the effects of Recql5 deficiency on liver injury induced by lipopolysaccharide/D-galactosamine (LPS/D-Gal).

METHODS: Liver injury was induced in wild type (WT) or Recql5-deficient mice using LPS/D-Gal, and assessed by histological, serum transaminases, and mortality analyses. Hepatocellular apoptosis was quantified by transferase dUTP nick end labeling assay and Western
INTRODUCTION

The RecQ family is a highly conserved group of DNA helicases that play a critical role in DNA replication, recombination, transcription, and repair[21]. Mammals express five RecQ homologues: RECQL1, BLM, WRN, REQL4, and REQL5[22], which share a conserved helicase domain, but differ in their C- and/or N-terminal domains[3]. Mutations in BLM, WRN, and REQL4 are linked to the human genetic disorders Bloom’ syndrome (BS), Werner’s syndrome (WS), and Rothmund-Thomson’s syndrome (RTS), respectively. These disorders are characterized by increased genomic instability and cancer susceptibility[22]. REQL5 has not been directly linked to human genetic disease, but has been implicated in DNA double strand break (DSB) repair and DNA transcription[4-10].

Several lines of evidence suggest that RECQ helicases also play a role in hepatic cell proliferation and metabolism. For example, RECQL1 expression is significantly correlated with histological grade and MIB-1 indices of hepatocellular carcinoma (HCC) development. Silencing RECQL1 expression suppresses HCC cell proliferation both in vitro and in vivo[11], BLM-deficient cells from patients with BS show slower growth and increased irradiation-mediated apoptosis. Deletion of BLM in mice leads to a reduced number of fetal liver cells and increased cell death[12]. In addition, a recent report has shown that Wrn-mutant mice exhibit accelerated typical age-related liver changes, including pseudocapillarization that directly affects hepatocyte apoptosis and oxidative stress and modulation of CYP450 expression.

RESULTS: Following LPS/D-Gal exposure, Recql5-deficient mice exhibited enhanced liver injury, as evidenced by more severe hepatic hemorrhage, higher serum aspartate transaminase and alanine transaminase levels, and lower survival rate. As compared to WT mice, Recql5-deficient mice showed an increased number of apoptotic hepatocytes and higher cleaved caspase-3 levels. Recql5-deficient mice exhibited increased DNA damage, as evidenced by increased γ-H2A.X levels. Inflammatory cytokine levels, neutrophil infiltration, and ERK phosphorylation were also significantly increased in the knockout mice. Additionally, Recql5-deficient mice exhibited increased malondialdehyde production and elevated inducible nitric oxide synthase, superoxide dismutase, glutathione peroxidase, catalase, and glutathione reductase activity, indicative of enhanced oxidative stress. Moreover, CYP450 expression was significantly downregulated in Recql5-deficient mice after LPS/D-Gal treatment.

CONCLUSION: Recql5 protects the liver against LPS/D-Gal-induced injury through suppression of hepatocyte apoptosis and oxidative stress and modulation of CYP450 expression.

Key words: Recql5; Liver injury; Apoptosis; Oxidative stress; CYP450

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Core tip: Wild type and Recql5-deficient mice were intraperitoneally injected with lipopolysaccharide and D-galactosamine (LPS/D-Gal). The aim of the study was to explore the effects of Recql5 deficiency on LPS/D-Gal-induced liver injury. Our findings reveal that Recql5 protects against liver injury via inhibition of hepatocyte apoptosis and oxidative stress and regulation of hepatic CYP450 expression levels.

Liao WQ, Qi YL, Wang L, Dong XM, Xu T, Ding CD, Liu R, Liang WC, Lu LT, Li H, Li WF, Luo GB, Lu XC. Recql5 protects against lipopolysaccharide/D-galactosamine-induced liver injury in mice. World J Gastroenterol 2015; 21(36): 10375-10384 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i36/10375.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i36.10375

MATERIALS AND METHODS

Animals

Male, 6-8-wk-old Recql5-deficient and wild type...
(WT) C57BL/6 mice were used in this study. The Recql5-deficient mice used in this study have been characterized previously\(^{[4-7]}\). Mice were fed a commercial diet and maintained in a controlled environment at 20-25 ℃ and 50% ± 5% relative humidity with a 12:12 h dark-light cycle. All animal studies were approved by the Wenzhou Medical University Institutional Animal Care and Use Committee.

Reagents and antibodies
LPS (E. coli, 0111:B4) and D-Gal were purchased from Sigma (St. Louis, MO, United States). Caspase-3 (rabbit polyclonal, 1:1000), ERK (rabbit polyclonal, 1:2000), phospho-ERK (rabbit polyclonal, 1:2000), JNK (rabbit polyclonal, 1:1000), phospho-JNK (mouse monoclonal, 1:2000), phospho-p65 (mouse monoclonal, 1:1000), β-actin (rabbit polyclonal, 1:2000), and GAPDH (rabbit polyclonal, 1:3000) antibodies were obtained from Cell Signaling Technology (Waltham, MA, United States). Peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, United States).

Liver injury induction
Liver injury was induced in 6-8-wk-old male mice via an intraperitoneal injection of LPS/D-Gal. For mortality assay, mice were intraperitoneally injected with 30 μg/kg LPS and 20 mg/kg D-Gal, and mortality was recorded for 72 h. To induce acute liver injury, mice were intraperitoneally injected with 10 μg/kg LPS and 300 mg/kg D-Gal. Mice were scarified at 1 and 6 h after LPS/D-Gal administration. Blood and liver samples were collected for further experiments.

Serum analysis
The serum activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was measured with a commercial assay kit (Nanjing Jiancheng Biological Technology, Inc., Nanjing, China). The activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was measured with a commercial assay kit (Nanjing Jiancheng Biological Technology, Inc.).

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The serum activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was measured with a commercial assay kit (Nanjing Jiancheng Biological Technology, Inc., Nanjing, China). The enzyme activity was expressed as international units per liter (U/L). Serum tumor necrosis factor (TNF)-α and interleukin (IL)-6 levels were measured using commercial assay kits (Nanjing Jiancheng Biological Technology).

Histopathological analysis
Formalin-fixed specimens were embedded in paraffin and stained with hematoxylin-eosin for conventional morphological evaluation under a light microscope.

Myeloperoxidase activity analysis
Myeloperoxidase (MPO) activity was determined using an MPO detection kit according to the manufacturer’s instructions (Nanjing Jiancheng Biological Technology, Inc.).

Oxidation stress analysis
Lipid peroxidation was determined by measuring malondialdehyde (MDA) levels using an assay kit (Beyotime Institute of Biotechnology, Inc., Shanghai, China). The activity of inducible nitric oxide synthase (iNOS), superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), and glutathione reductase (GR) was tested using commercial assay kits (Nanjing Jiancheng Biological Technology, Inc.).

Terminal deoxynucleotidyl transferase dUTP nick end labeling assay
Hepatocellular apoptosis was evaluated by transferase dUTP nick end labeling (TUNEL) assay using the DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI, United States). The terminal transferase reactions produced a dark-brown precipitate. For each mouse liver section, the number of TUNEL-positive cells in five randomly selected fields was counted.

Quantitative real-time PCR
Total RNA was isolated from liver tissue using TRIZOL reagent (Invitrogen) and was treated with DNase to remove contaminating DNA before cDNA synthesis. RNA (2 μg) was reverse-transcribed to cDNA with murine leukemia virus (MLV)-reverse transcriptase (Invitrogen). Each cDNA sample was analyzed in triplicate on an ABI 7300 Real-Time Detection system (Applied Biosystems, Foster City, CA, United States) using SYBR Green (Tiangen, Beijing, China). The primer sequences are shown in Table 1. The primer concentration used in the PCR assay was 0.5 μmol/L. Cycle conditions were as follows: 95 ℃ for 2 min followed by 40 cycles of 95 ℃ for 15 s, 60 ℃ for 30 s, and 68 ℃ for 30 s. Relative mRNA quantification was calculated using the comparative threshold cycle (ΔΔCt) method. ∆∆Ct was converted to a fold change of expression using the formula 2^-ΔΔCt.

Table 1  Primers used in this study

| Gene       | Forward primer (5’→3’)                      | Reverse primer (5’→3’)                      | Length of product |
|------------|--------------------------------------------|--------------------------------------------|------------------|
| TNF-α      | GAAGGATTTGGTCGTATTGGGCA                    | AGGGGTCTGAGCCATAGAAGCT                   | 203 bp           |
| IL-6       | CCGAAGAGCAGAAAGGTCGTTCC                    | AAGTGACATCTGGTGTCTCATAC                  | 141 bp           |
| Cyp2A4     | AGGAGCTTCCAGCATCATCCTGTTTC                 | GAGGCTTCCAGCATCATCCTGTTTC                | 123 bp           |
| Cyp2A5     | GGAGGCTTCCAGCATCATCCTGTTTC                 | GCTTCCACGATCTGGAGAAGC                    | 124 bp           |
| Cyp2B9     | GCTGGAAGACGAACGGTGCTTT                    | AGGGTCTGGGCCATAGAAGCT                   | 147 bp           |
| Cyp2B10    | TGAAGGATTTGGTCGTATGGGCA                    | CCACACAAATGGGAGCAGAT                    | 68 bp            |
| GAPDH      | CAGGATTTGGTCGTATGGGCA                      | TGGCCTTGGGAGATGGGAGC                    | 216 bp           |
first examined the effect of Recql5 deficiency on mouse mortality after LPS shock. Following injection of 20 μg/kg LPS and 400 mg/kg D-Gal, the mortality was significantly increased in Recql5-deficient mice as compared to WT mice (Figure 1A). Lethal shock in D-Gal/LPS-treated mice is characterized by acute liver injury [20]. To further elucidate the direct effects of Recql5 on liver injury, we used a low-dose LPS/D-Gal model (10 μg/kg LPS and 300 mg/kg D-Gal). Liver morphology analysis [21] showed that the liver of Recql5-deficient mice was swollen and exhibited more bleeding on the surface, as compared to the liver of WT mice, indicating that there was more severe liver hemorrhage in knockout mice after treatment (Figure 1B). These data were further confirmed by HE staining (Figure 1C). Moreover, the serum ALT and AST levels, two well-established biochemical markers of hepatocellular damage, were significantly increased in Recql5-deficient mice 6 h after injection (Figure 1D and E). Together, these results indicate that Recql5 has a protective role in liver injury induced by LPS/D-Gal.

Increased hepatocellular apoptosis and DNA damage in Recql5-deficient mice

Hepatocyte apoptosis is considered a main cause of liver injury in the LPS/D-Gal model [22]. Thus we evaluated whether Recql5 deficiency affected hepatocyte apoptosis. The TUNEL assay showed that the number of apoptotic hepatocytes was significantly increased in Recql5-deficient mice compared to WT mice (Figure 1F). Western blotting

Liver tissue was lysed in lysis buffer [50 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40 and 0.1% (w/v) SDS] supplemented with a protease/phosphatase inhibitor cocktail (Cell Signaling Technology). After sonication, the lysate was centrifuged at 12000 rpm for 15 min at 4 ℃, and the supernatant was collected. Proteins were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, United States). After blocking with 5% (w/v) milk in TTBS (TBS plus 0.1% Tween-20), the membranes were incubated with primary antibodies followed by horseradish peroxidase (HRP)-conjugated secondary antibodies. Protein bands were visualized with the Immun-Star HRP chemiluminescence kit (Bio-Rad). For densitometric analysis, Image J software was used.

Statistical analysis

Statistical comparisons were performed using Student’s t-test or analysis of variance (ANOVA) where appropriate. Data are expressed as the mean ± SD. Kaplan-Meier survival analysis was performed using the log-rank test. P-values less than 0.05 were considered significant.

RESULTS

Enhanced liver injury in Recql5-deficient mice

To investigate the role of Recql5 in liver injury, we

Figure 1 Enhanced lipopolysaccharide/D-Gal-induced liver injury in Recql5-deficient mice. A: Survival curves after lipopolysaccharide (LPS)/D-Gal injection. Wild type (WT) and Recql5-deficient (Recql5− /−) mice were treated with 20 μg/kg LPS and 400 mg/kg D-Gal (WT, n = 14; KO, n = 16). Survival curves were created using the Kaplan-Meier method and compared by log-rank (Mantel-Cox) test; B: Images of the whole livers demonstrate the different degree of hemorrhage. WT and Recql5-deficient mice were treated with 300 mg/kg D-Gal and 10 μg/kg LPS (n = 4-7 per group). Livers were removed 6 h after injection; C: HE staining of liver sections 6 h after injection; D: Serum alanine transaminase (ALT) activity 6 h after injection; E: Serum aspartate transaminase (AST) activity 6 h after treatment. bP < 0.01, WT vs Recql5− /−.
increased in Recql5-deficient mice, as compared to WT mice after LPS/D-Gal administration (Figure 2A). In agreement with this observation, Western blot analysis showed that the cleaved caspase-3 levels were significantly elevated in Recql5-deficient mice (Figure 2B). Given the important role of Recql5 in genomic stability, we assumed that LPS/D-Gal treatment would result in elevated DNA damage in Recql5-deficient mice, which, in turn, would trigger apoptosis. Indeed, Western blot showed that the level of γ-H2A.X, a biomarker of DNA damage, was significantly increased in Recql5-deficient mice (Figure 2C). Together, these results suggest that Recql5 deficiency results in increased LPS/D-Gal-induced DNA damage and hepatocyte apoptosis, thereby inducing liver injury.

**Increased inflammatory response in Recql5-deficient mice**

The release of pro-inflammatory cytokines is involved in liver injury stimulated by LPS/D-Gal. Among these, cytokines TNF-α and IL-6 are key mediators of hepatocyte apoptosis. To examine whether Recql5 deficiency could alter TNF-α and IL-6 expression, we measured their hepatic mRNA levels. As compared with WT mice, the mRNA levels of TNF-α and IL-6 were significantly elevated in Recql5-deficient mice at 1 and 6 h, respectively. Similar results were found for serum TNF-α and IL-6 levels (Figure 3C and D). Consistent with these data, the neutrophil infiltration, which can be triggered by TNF-α signaling, was significantly increased in knockout mice, as evaluated by MPO activity (Figure 3E). LPS/D-Gal-induced secretion of inflammatory cytokines is primarily dependent on the activation of the mitogen activated protein kinase (MAPK) and nuclear factor (NF)-κB pathways. We then tested whether deletion of Recql5 affected these pathways. Our results showed that the phosphorylation of extracellular signal-related kinase (ERK) was significantly increased in Recql5-deficient mice, whereas there was no significant difference in c-Jun N-terminal kinase (JNK) and p65 phosphorylation between Recql5-deficient mice and control mice (Figure 3F), suggesting that Recql5 deficiency results in ERK activation.

**Elevated oxidative stress in Recql5-deficient mice**

Oxidative stress is a known contributor to LPS/D-Gal-induced liver injury. To investigate the effects of Recql5 deficiency on oxidative stress, we measured several parameters involved in this process, including MDA production and iNOS, SOD, GPX, CAT and GR activity. Our data showed that the levels of MDA, an end product of lipid peroxidation, were significantly increased in Recql5-deficient mice, as compared to WT mice (Figure 4A). Furthermore, there was a significant increase in iNOS activity in Recql5-deficient mice, indicative of enhanced NO production (Figure 4B). Additionally, SOD, GPX, CAT, and GR activity in Recql5-deficient mice was significantly reduced (Figure 4C-F). These results suggest that Recql5 deficiency leads to increased LPS/D-Gal-mediated oxidative stress.

**Decreased CYP450 expression in Recql5-deficient mice**

CYP450s are important for the metabolism of a variety of drugs and xenobiotics. Decreased expression of CYP450s in Recql5-deficient mice could affect the metabolism and clearance of these substances, potentially contributing to the increased liver injury observed. We then tested whether deletion of Recql5 affected the expression of CYP450s in the liver. Western blot analysis showed that the levels of CYP450s were significantly reduced in Recql5-deficient mice, as compared to WT mice (Figure 4D-F). This indicates that Recql5 deficiency may impair the metabolic capacity of the liver, thereby exacerbating liver injury.

**Conclusions**

Our study provides evidence that Recql5 deficiency leads to increased liver injury after LPS/D-Gal treatment. The mechanisms underlying this effect include increased DNA damage, altered cytokine expression, enhanced oxidative stress, and decreased CYP450 expression. These findings highlight the importance of Recql5 in maintaining genomic stability and regulating inflammatory and oxidative responses in the liver. Further investigation into the specific roles of Recql5 in these processes could provide insights into potential therapeutic strategies for liver injury.
of endogenous and exogenous substrates\(^{25,26}\). It has been reported that reduced CYP450 gene expression is associated with enhanced liver injury\(^{27}\). Therefore, we examined whether Recql5 deficiency could alter CYP450 expression. The mRNA levels of four CYP450 members were detected, including CYP2A4, CYP2A5, CYP2B9 and CYP2B10, which are regulated by LPS as reported previously\(^{28-30}\). Our results showed that, following LPS/D-Gal exposure, mRNA levels of CYP2A4, CYP2A5, CYP2B9, and CYP2B10 were significantly reduced in Recql5-deficient mice, as compared to WT mice (Figure 5). These data indicate that Recql5 deficiency results in the downregulation of CYP450 expression, which may impair LPS and/or D-Gal disposition.

**DISCUSSION**

In the present study, we demonstrated that Recql5 has a protective role against LPS/D-Gal-induced liver injury, as Recql5-deficient mice exhibited increased hepatic hemorrhage, elevated serum aminotransferase levels, and decreased survival rate. LPS/D-Gal-induced liver injury is a well-established experimental animal model of acute hepatic failure. The outcomes of this model are associated with increased hepatocyte apoptosis, inflammation, and oxidation\(^{18,31,32}\). First, we speculated that Recql5 deficiency might increase hepatocyte apoptosis, which could lead to enhanced liver damage. Indeed, TUNEL assays and Western blot confirmed an increase in hepatocyte apoptosis...
in Recql5-deficient mice. Moreover, consistent with the role of Recql5 in genomic stability, we found that Recql5-deficient liver exhibited increased DNA damage, which has been recognized as an inducer of apoptosis[33]. Our data suggest that Recql5 is a regulator of cell apoptosis. In agreement with these findings, a previous study suggested that BLM also regulates cell apoptosis, and that BLM deficiency results in increased cell death and apoptosis, which is associated with p53 dysfunction[12].

TNF-α and IL-6 are two proximal mediators of hepatotoxicity in several models of liver damage, including LPS/D-Gal[34,35]. TNF-α-induced hepatocyte apoptosis may be an early causal event during LPS/D-Gal-induced liver injury[32]. We found that LPS/D-Gal upregulated TNF-α and IL-6 in Recql5-deficient mice. Moreover, it has been reported that LPS activates various signaling pathways, including MAPK and NF-κB, thereby inducing the production of inflammatory cytokines[18]. In line with these data, we observed an upregulation in ERK phosphorylation in knockout mice following LPS/D-Gal challenge. Together, our data suggest that, in the LPS/D-Gal model, Recql5 deficiency activates ERK signaling, resulting in inflammatory cytokine production and subsequently, hepatocyte apoptosis and damage.

Oxidative stress is associated with damage to a wide range of molecules, including lipids, proteins, and nucleic acids, and may play a crucial role in LPS/D-Gal-stimulated liver injury. For example, treatment with antioxidants significantly reduces LPS/D-Gal-induced liver injury in mice, whereas inhibition of antioxidant enzyme activity enhances liver damage[24,36]. Oxidative stress can be triggered by increased free radical production and/or decreased antioxidant activity[37]. We found that MDA and NO production, indicative of free radicals, were significantly increased in Recql5-deficient mice. In contrast, the activity of the antioxidant enzymes SOD, GPX, CAT and GR was significantly reduced in mice deficient in Recql5. These data suggest that Recql5 deficiency results in an imbalance between free radical generation and antioxidant defenses, thereby enhancing oxidative stress-induced liver injury.

CYP450 oxidases are the predominant enzymes involved in Phase I detoxification. Downregulation of CYP450 increases the risk of liver damage after hepatotoxin exposure[27,38]. We found that Recql5 deficiency resulted in reduced expression of CYP2A4, CYP2A5, CYP2B9, and CYP2B10, indicative of impaired LPS and/or D-Gal disposition, which might further aggravate liver injury. The mechanism by which Recql5 regulates CYP450 expression remains unknown. It has been shown that WRN regulates CYP2B transcription by forming complex with the Wrn binding site within the CYP2B promoter[14]. Further investigations are required to figure out whether Recql5 regulates CYP450 expression in the same manner as WRN.

In summary, the current study showed for the first time that Recql5 protects against liver injury induced
by LPS/D-Gal. The protective effect of Recql5 is attributed to the inhibition of hepatocyte apoptosis and oxidative stress, as well as the regulation of CYP450 expression. Our findings indicate a hepatoprotective role for Recql5 in liver injury.

COMMENTS

Background
RecQ helicases are regulators of genomic integrity and play important roles in hepatic cell proliferation and metabolism. Deficiency in one of the RecQ family member, Recql5, results in elevated susceptibility to various types of cancers, including liver cancer; however, the possible function of Recql5 in liver injury remains unknown.

Research frontiers
Recql5 is a member of the RecQ helicase family that plays critical roles in DNA replication, recombination, transcription, and repair. This study analyzes the possible functions of Recql5 in liver injury.

Innovations and breakthroughs
The study showed for the first time that Recql5 has a protective role against LPS/D-Gal-induced liver injury. The protection is mediated via the suppression of apoptosis, DNA damage, oxidative stress and CYP450 expression.

Applications
This study will help to improve the readers’ understanding of the mechanisms involved in acute liver injury and may represent a novel target for the acute liver failure therapy.

Terminology
Oxidative stress is a term used to describe the condition of oxidative damage resulting when the critical balance between free radical generation and antioxidant defenses is unfavorable.

Peer-review
This is an interesting study investigating the role of Recql5, a member of the RecQ helicase family, in liver injury. The authors found that Recql5 protects against LPS/D-Gal-induced liver injury. They further showed that this effect is attributed to inhibition of hepatocyte apoptosis and oxidative stress, as well as to downregulation of CYP450.

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P- Reviewer: Conti B, Pajares MA S- Editor: Ma YJ
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