ORIGINAL RESEARCH COMMUNICATION

Activation of a Novel c-Myc-miR27-Prohibitin 1 Circuitry in Cholestatic Liver Injury Inhibits Glutathione Synthesis in Mice

Heping Yang,^{1,2,*} Tony W.H. Li,^{1,2,*} Yu Zhou,² Hui Peng,^{1,2,*} Ting Liu,³ Ebrahim Zandi,^{2,4} María Luz Martínez-Chantar,⁵ José M. Mato,⁵ and Shelly C. Lu^{1,2,*}

Abstract

Aims: We showed that chronic cholestatic liver injury induced the expression of c-Myc but suppressed that of glutamate-cysteine ligase (GCL, composed of catalytic and modifier subunits GCLC and GCLM, respectively). This was associated with reduced nuclear antioxidant response element (ARE) binding by nuclear factor-erythroid 2 related factor 2 (Nrf2). Here, we examined whether c-Myc is involved in this process. **Results:** Similar to bile duct ligation (BDL), lithocholic acid (LCA) treatment *in vivo* induced c-Myc but suppressed GCL subunits expression at day 14. Nrf2 expression and Nrf2 ARE binding fell markedly. However, Nrf2 heterodimerization with MafG was enhanced by LCA, which prompted us to examine whether LCA treatment *in vivo* altered proteins that bind to ARE using biotinylated ARE in pull-down assay followed by proteomics. LCA treatment enhanced c-Myc but lowered prohibitin 1 (PHB1) binding to ARE. This was a result of c-Myc-mediated induction of microRNA 27a/b (miR27a/b), which target both PHB1 and Nrf2 to reduce their expression. Knockdown of c-Myc or miR27a/b attenuated LCA-mediated suppression of Nrf2, PHB1, and GCL subunit expression, whereas overexpression of PHB1 protected against the fall in Nrf2 and GCL subunits. Both c-Myc and PHB1 directly interact with Nrf2 but c-Myc lowers Nrf2 binding to ARE while PHB1 enhances it. **Innovation:** This is the first work that shows how activation of this circuit in cholestatic liver injury inhibits GCL expression. **Conclusions:** LCA feeding and BDL activate c-Myc-miR27a/b-PHB1 circuit, with the consequence of inhibiting Nrf2 expression and ARE binding, resulting in decreased reduced glutathione synthesis and antioxidant capacity. *Antioxid. Redox Signal.* 22, 259–274.

Introduction

CHRONIC CHOLESTATIC LIVER INJURY remains a major cause of chronic liver disease with limited treatment options (14). Ursodeoxycholic acid (UDCA), the only Food and Drug Administration-approved medication for treating primary biliary cirrhosis (PBC), is ineffective in other chronic cholestatic liver disorders and elicits a complete response in only 25–30% of PBC patients (24). This makes a better understanding of the pathogenesis of injury and im-

provement in therapy imperative. The underlying pathophysiology of chronic cholestatic liver injury is thought to be related largely to retention of toxic bile acids that induce oxidative stress, cell death, and fibrosis, leading to cirrhosis (26). Using bile duct ligation (BDL) in mice as a model for chronic cholestasis, we described marked reduction in the expression of the rate-limiting enzyme for reduced glutathione (GSH) synthesis, glutamate-cysteine ligase (GCL, composed of catalytic and modifier subunits, GCLC and GCLM, respectively), and hepatic GSH levels at 2 weeks

¹Division of Gastroenterology and Liver Diseases and ²USC Research Center for Liver Diseases, Keck School of Medicine, University of Southern California, Los Angeles, California.

³Department of Gastroenterology, Xiangya Hospital, Central South University, Changsha, China.

⁴Norris Comprehensive Cancer Center, Department of Molecular Microbiology and Immunology, Keck School of Medicine, University of Southern California, Los Angeles, California.

⁵CIC bioGUNE, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (Ciberehd), Bizkaia, Spain.

*Current affiliation: Division of Gastroenterology, Department of Medicine, Cedars-Sinai Medical Center, Los Angeles, California.

Innovation

Expression of GSH synthetic enzymes and GSH levels are markedly reduced in chronic cholestasis, and this can further exacerbate liver injury. Here, we elucidated a key mechanism responsible, which is activation of a novel c-Myc-microRNA 27a/b (miR27a/b)-prohibitin 1 (PHB1) circuit, where c-Myc induces the expression of miR27a/b, which, in turn, inhibit the expression of both nuclear factor-erythroid 2 related factor 2 (Nrf2) and PHB1. In addition, both c-Myc and PHB1 directly interact with Nrf2 to influence its antioxidant response element binding, with c-Myc acting as a corepressor while PHB1 acts as a coactivator. These findings provide new therapeutic targets for drug design in cholestatic liver injury.

after BDL (33). Since GSH is a key intracellular antioxidant that protects against fibrogenesis, these changes likely facilitate worsening of liver injury and fibrosis (18, 23).

The molecular mechanism for the fall in GCL subunit expression was thought to be due to displacement of Nrf2 by c-Maf and MafG from binding to the antioxidant response element (ARE), a positive regulator of these genes (31). However, another potential mechanism not yet investigated is the role of c-Myc, which is also induced after BDL (32). Although c-Myc has been shown to activate both GCL subunits at the transcriptional level (3), recent work from Levy and Forman showed it interacts with Nrf2 and negatively regulates ARE-dependent genes by destabilizing Nrf2 (16). Our current work was aimed at examining whether c-Myc induction in cholestasis plays a role in the downregulation of GCL subunit expression and in the course of our investigation, we have uncovered a novel circuit that involves c-Myc, microRNA 27 (miR27), and prohibitin 1 (PHB1) that is activated by BDL and toxic bile acids such as lithocholic acid (LCA) to inhibit GSH synthesis and impair antioxidant defense. Chronic BDL or LCA treatment leads to induction of c-Myc, which set in motion a series of events that culminate in reduced Nrf2 expression, ARE binding and *trans*-activation. Interrupting this circuit protects against reduced expression of GCL subunits and presents a potential therapeutic strategy in the treatment of cholestatic liver injury.

Results

Changes in the expression of GCL subunits, Nrf2, and c-Myc after LCA, UDCA, and S-adenosylmethionine treatment

We previously showed that LCA treatment (100 μ M) in Huh-7 cells resulted in a fall in *GCLC* and *GCLM* mRNA levels at 20 h that could be prevented by UDCA or S-adenosylmethionine (S-AdoMet) (31). In the same model, c-Myc protein levels increased by 8 h and remained elevated for approximately 48 h (32). We first examined whether these changes also occur after *in vivo* LCA treatment. Figure 1A shows that *Gclc*, *Gclm*, and *Nrf2* mRNA levels increased transiently from day 1 to day 3 but fell to below baseline by day 7 and remained at 50% below basal levels from day 14 to day 28. In contrast, *c-Myc* and *MafG* mRNA levels progressively increased and remained elevated till day 28. Liver injury occurred rapidly as illustrated by serum alanine transaminase (ALT) levels (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/ars).

Similar to BDL (33), S-AdoMet and UDCA treatment individually protected against LCA-induced biochemical and histologic liver injury but protection was more effective when combined (Supplementary Fig. S1). S-AdoMet and UDCA treatment for 14 days prevented LCA-induced fall in *Gclc*, *Gclm*, and *Nrf2* expression as well as the increase in *c-Myc* and *MafG* (Fig. 1B). Similar protection by S-AdoMet and UDCA was observed in Huh-7 cells treated with LCA for 20 h (Fig. 1C). Protection by S-AdoMet and UDCA on the fall in *Nrf2* and the increase in *c-Myc* protein expression was also confirmed on immunohistochemistry after 14 days of LCA treatment (Supplementary Fig. S2).

Effects of LCA treatment on protein binding to ARE of GCLC and protein-protein interactions

LCA treatment *in vivo* for 14 days or in Huh-7 cells for 20 h resulted in decreased Nrf2 binding to ARE, whereas c-Myc and MafG binding to ARE increased (Fig. 2A). These changes were blunted with either UDCA or S-AdoMet treatment and were largely prevented when the two agents were used in combination (Fig. 2A). Using sequential chromatin immunoprecipitation (Seq-ChIP), LCA treatment increased MafG and c-Myc co-occupancy to the same ARE region despite reduced Nrf2 binding and UDCA and S-AdoMet treatments were similarly protective (Fig. 2B).

After LCA treatment *in vivo*, there is increased interaction between Nrf2 and c-Myc as well as between Nrf2 and MafG (Fig. 3A). This is surprising, as increased heterodimerization of Nrf2 and MafG typically induces ARE-dependent gene expression (18). This prompted us to examine whether LCA treatment led to a change in the composition of proteins that bind to ARE to result in a reduced Nrf2 binding and ARE-dependent gene expression. Using biotinylated ARE fragment in pull-down followed by proteomics, Figure 3B shows that LCA treatment *in vivo* for 14 days resulted in a change in the type and amount of proteins that bind to ARE. Candidate proteins with high scores are shown in the table in Figure 3B (full list in Supplementary Table S1). Several candidate proteins, including Nrf2, showed a fall in binding to ARE, and several showed an increase in binding that included MafG, c-Maf, and c-Myc. Interestingly, a protein candidate that exhibited decreased binding after LCA treatment is prohibitin 1 (Phb1 for mouse, PHB1 for human), which, to our knowledge, has not been shown to bind to ARE or interact with Nrf2. Western blotting of the proteins pulled down with biotinylated ARE confirmed that LCA treatment reduced Nrf2 and Phb1, while increasing c-Myc, c-Maf, and MafG binding to ARE (Fig. 3B, bottom). Reduced Phb1 protein expression and protection by S-AdoMet and UDCA was confirmed on immunohistochemistry (Supplementary Fig. S2).

miR27 links c-Myc to PHB1 changes in LCA treatment

PHB1 is transcriptionally activated by c-Myc (6) but LCA and BDL treatment suppresses PHB1 protein levels despite increased c-Myc expression (Fig. 4A). Recently, PHB1 has been shown to be negatively regulated by miR27 (9), which is, in turn, positively regulated by c-Myc in breast cancer cells (17). To see whether miR27 may be the link between c-Myc and Phb1 in LCA-treated livers, we examined changes in miR27a and miR27b expression after LCA treatment. LCA

FIG. 1. Effect of LCA treatment on mRNA levels of *GCL* subunits, *c-Myc*, *Nrf2*, and *MafG* in mice livers from day 1 to day 28 (A), protection by UDCA and SAME on these changes at day 14 (B), and in Huh-7 cells after 20 h (C). RNA was extracted from mice livers and Huh-7 cells after various treatments, and mRNA levels were measured using real-time PCR as described in “Materials and Methods.” Results are expressed as % of respective controls in mean $\pm$ SEM from six mice per condition (A, B) or three individual experiments (C) done in duplicate, * $p < 0.05$ versus control (Con), † $p < 0.05$ versus LCA, § $p < 0.05$ versus SAME, # $p < 0.05$ versus UDCA, ‡ $p < 0.05$ versus LCA + SAME, ** $p < 0.05$ versus LCA + UDCA. LCA, lithocholic acid; GCL, glutamate-cysteine ligase; Nrf2, nuclear factor-erythroid 2 related factor 2; UDCA, ursodeoxycholic acid; SAME, S-adenosylmethionine; PCR, polymerase chain reaction; SEM, standard error of the mean.

treatment increased miR27a/b mRNA levels by day 1 and similar to *c-Myc*, they remain elevated till day 28 (Fig. 4B). *Phb1* mRNA levels fell below baseline by day 3, were 50% lower at day 7, and persisted to day 28 (Fig. 4B). A similar change in *Phb1* and miR27a/b expression also occurred after BDL (Fig. 4C).

We next used Huh-7 cells to establish a causal relationship and define the sequence of events after LCA treatment.

Figure 5A shows the effect of *c-Myc* knockdown at baseline and in combination with LCA treatment for 20 h. Knocking down *c-Myc* raised the mRNA levels of *PHB1*, *Nrf2*, both *GCL* subunits by 40–60%, while lowering miR27a and miR27b by 60%. Knockdown of *c-Myc* attenuated LCA-mediated induction of miR27a/b and suppression of *PHB1*, *Nrf2*, *GCLC*, and *GCLM*. Knockdown of miR27a or miR27b had no influence on basal *c-Myc* expression but resulted in

FIG. 2. Effects of LCA, SAME, and UDCA treatments on ARE nuclear binding activity and co-occupancy by Nrf2, MafG, and c-Myc. (A) ChIP assays were performed using Huh-7 cells (left panel) treated with 100 μ M of LCA (L), SAME (S) or UDCA (U), alone or together or vehicle control for 20 h, or hepatocytes isolated from livers of mice after 14 days of LCA feeding (right panel) as described in “Materials and Methods.” PCR products from amplification of the ARE sites after immunoprecipitation with antisera against Nrf2, MafG, and c-Myc demonstrate that LCA treatment led to decreased Nrf2 binding to the ARE site, while MafG and c-Myc binding increased. SAME and UDCA cotreatment protected against these changes with the combined treatment being most effective. Input genomic DNA (gDNA input) was used as a positive control, and IgG was used as a negative control. Panel (B) shows co-occupancy of ARE by Nrf2 and MafG or Nrf2 and c-Myc. Seq-ChIP was done on the same samples as described in “Materials and Methods.” In both Huh-7 cells and mouse hepatocytes isolated after 14 days of LCA treatment, despite decreased Nrf2 binding, co-occupancy by c-Myc and MafG to the same region was higher. SAME and UDCA cotreatment protected against these changes. Representative results from at least three experiments are shown. Numbers below the pictures are % of control values after normalizing to input DNA, * p < 0.05 versus control (C), [†] p < 0.05 versus LCA (L), [§] p < 0.05 versus SAME (S), [#] p < 0.05 versus UDCA (U), [‡] p < 0.05 versus LCA + SAME (L + S), and ** p < 0.05 versus LCA + UDCA (L + U). ARE, antioxidant response element; IgG, immunoglobulin G; Seq-ChIP, sequential chromatin immunoprecipitation.

induction of *PHB1*, *Nrf2*, *GCLC*, and *GCLM*, and additive induction was observed with combined knockdown (Fig. 5B). Individual knockdown of either miR27a or miR27b tended to attenuate LCA-mediated suppression of *PHB1*, *Nrf2*, *GCLC*, and *GCLM*, whereas combined knockdown

completely prevented LCA's inhibitory effect on their expression despite marked c-Myc induction (Fig. 5C).

We next examined the role of PHB1. Overexpression of PHB1 resulted in higher Nrf2, GCLC, and GCLM protein levels (Fig. 6A). The PHB1 overexpression vector maintained

FIG. 3. Effects of LCA *in vivo* treatment on interactions between Nrf2 and c-Myc or MafG, and on proteins that bind to biotinylated ARE. Panel (A) shows co-immunoprecipitation of liver extracts from LCA (L), SAME (S), and UDCA (U) treatments for 14 days using anti-Nrf2 antibody followed by Western blotting for c-Myc, MafG, or Nrf2. Total protein levels of Nrf2, c-Myc, and MafG are shown next. Graph on the right represents % of control Nrf2 binding densitometric values (normalized to beta-actin). Representative blots from at least three mice per condition are shown, * $p < 0.05$ versus control (Con), † $p < 0.05$ versus LCA (L), § $p < 0.05$ versus SAME (S), # $p < 0.05$ versus UDCA (U), ‡ $p < 0.05$ versus LCA + SAME (L + S), LCA + UDCA (L + U), and SAME + UDCA (S + U). Panel (B) summarizes key candidate proteins that were identified by mass spectrometry analysis to exhibit a change in amount bound to ARE after LCA treatment. Some of these were confirmed by Western analysis of the eluted fractions shown below the table.

higher PHB1 expression despite LCA treatment (20% reduction but still nearly 60% above baseline, Fig. 6B), and this prevented LCA's ability to lower Nrf2, GCLC, and GCLM mRNA levels below baseline. Overexpressing PHB1 had no effect on the mRNA levels of c-Myc and MafG at baseline or

after LCA treatment (Fig. 6B). Since overexpression may not reflect physiological effect, we also examined knockdown of PHB1 in Huh-7 cells. After 24 h of knockdown, mRNA levels of Nrf2, GCLC, and GCLM fell (Fig. 6C) and this translated to reduced Nrf2 binding to the GCLC ARE region (Fig. 6D).

FIG. 4. Phb1 and miR27a/b expression in hepatocytes in cholestatic liver injury. Panel (A) shows Western blot analysis of Phb1 in hepatocytes after LCA treatment (left) and BDL (right) in mice from day 0 (Con) to day 28 (D28). Representative blots from six mice per condition are shown. Numbers below the blot represent % of day 0, * $p < 0.05$ versus day 0. Panel (B) shows mRNA levels of miR-27a, miR-27b, and Phb1 in hepatocytes isolated from livers of LCA- or BDL-treated mice at the same time points as (A). Results are expressed as mean $\pm$ SEM in % over Con from at least six mice per condition, * $p < 0.05$ versus Day 0 (Con). Phb1, prohibitin 1; miR, microRNA; BDL, bile duct ligation.

miR27a/b regulate Nrf2 expression directly at the mRNA level

Since miR27a/b siRNA treatment raised Nrf2 mRNA level and there are consensus binding sites in the Nrf2 untranslated region (UTR), we suspected that these miRNAs can directly regulate Nrf2. Using wild-type and mutated Nrf2 3'UTR constructs, Figure 7 shows that knockdown of either miR27a or miR27b raised Nrf2 3'UTR reporter activity by about 20% and combined knockdown had an additive effect, all of which were eliminated when the binding site was mutated.

c-Myc and PHB1 interact with Nrf2 and influence Nrf2 binding to ARE

Since both c-Myc and PHB1 can interact with Nrf2, we next addressed whether they can directly interact with Nrf2 and how this affects Nrf2-ARE binding. Figure 8A and B show an interaction between these recombinant proteins *in vitro*. All three proteins can interact with each other directly. To see whether c-Myc and PHB1 compete for Nrf2 binding, cells were treated with c-Myc siRNA and co-immunoprecipitation (co-IP) shows that an interaction between Nrf2 and PHB1 increased, in parallel to their expression level (Fig. 8C). Conversely, when cells were treated with over-expression vector of PHB1, there is a preferential increase in interaction between PHB1 and Nrf2 as an interaction between

PHB1 and c-Myc was unchanged (Fig. 8D). These changes translated to Nrf2 ARE binding as demonstrated by ChIP and Seq-ChIP. Thus, either c-Myc knockdown or PHB1 over-expression increased Nrf2 and PHB1 co-occupancy of ARE (Fig. 8E).

Role of MafG and c-Maf in the c-Myc-miR27-PHB1 circuit

We previously reported that MafG or c-Maf knockdown protected against LCA-mediated suppression of Nrf2 ARE binding and GCLC and GCLM expression (31). We examined whether they have an impact on the c-Myc-miR27-PHB1 circuit. Single MafG or c-Maf knockdown had no influence on the mRNA levels of c-Myc, PHB1, or miR27a/b, but combined knockdown raised PHB1 mRNA levels by 47% (Supplementary Fig. S3).

Discussion

GSH is the most abundant nonprotein thiol present in all mammalian cells that defend against oxidative stress (18). Liver has the highest concentration of GSH in all organs and determines systemic GSH homeostasis (18). Reduced hepatic GSH level facilitates fibrogenesis and inflammatory response to endotoxin (18, 23, 28). GSH is synthesized in the cytosol *via* a two-step enzymatic process that involves the

FIG. 5. Effect of knocking down c-Myc, miR27a or miR27b on expression of Nrf2 and GCL subunits at baseline and following LCA. mRNA levels of *GCLC*, *GCLM*, *c-MYC*, *NRF2*, miR27a, miR27b, and *PHB1* were assessed after (A) c-MYC knockdown with or without LCA treatment, (B, C) miR27a or miR27b individual (27a si or 27b si) or combined knockdown (27a/b si), with or without LCA treatment in Huh-7 cells. Knockdown was for 24 h, followed by LCA treatment for 20 h. Results are from three to five independent experiments, done in duplicate (qPCR results) are expressed as mean $\pm$ SEM in % of scrambled control (SC). For panel A, * p < 0.05 versus SC, † p < 0.05 versus c-MYC siRNA, ‡ p < 0.05 versus LCA + SC, for panels B and C, * p < 0.05 versus SC + dimethylsulfoxide (DMSO) or SC + SC + DMSO, † p < 0.05 versus 27a si, ‡ p < 0.05 versus 27b si, $^{\#}$ p < 0.05 versus LCA + SC, § p < 0.05 versus 27a/b si + DMSO. GCLC, GCL-catalytic subunit; GCLM, GCL-modifier subunit.

rate-limiting enzyme GCL and the second-step GSH synthetase (18). The genes encoding GCL subunits are coordinately regulated in many instances and are positively regulated by Nrf2 *trans*-activation of critical ARE present in their promoters (18). Nrf2 is a member of the cap “n” collar-

basic leucine zipper proteins that is kept in the cytosol by Keap1 under nonstressful conditions and undergoes proteasomal degradation (10). Signals imparted by oxidative and electrophilic molecules release Nrf2 from Keap1, enabling Nrf2 to escape proteasomal degradation and translocate to the

FIG. 6. Effect of PHB1 on expression of Nrf2 and GCL subunits at baseline and after LCA, and on Nrf2 ARE binding. (A) Western analyses were done to determine the effect of PHB1 overexpression (OE) for 24 h in Huh-7 cells. (B) PHB1 overexpression, in the presence or absence of LCA, was done to look at the effect on mRNA expression. After 24 h of treatment with PHB1 OE or empty vector (EV), cells were then treated with LCA for another 20 h. Results are from three independent experiments, done in duplicate (qPCR results), and are expressed as mean $\pm$ SEM in % of EV, * p < 0.05 versus EV + DMSO, † p < 0.05 versus EV + LCA, ‡ p < 0.05 versus PHB1 OE. Huh-7 cells were treated with PHB1 siRNA or scrambled control (SC) for 24 h and mRNA levels of Nrf2, GCLC, and GCLM (C) or Nrf2 binding to GCLC ARE4 region (D) were determined as described in “Materials and Methods.” Results are from three to four independent experiments, expressed as mean $\pm$ SEM in % of SC, * p < 0.05 versus SC.

nucleus to induce genes involved in defense and survival (8). Nrf2 forms heterodimers with small Maf (MafG, MafK, and MafF) and Jun (c-Jun, Jun-D, and Jun-B) proteins to bind ARE (8). Small Mafs can form homodimers to repress ARE-mediated gene expression (4). C-Maf, a large Maf protein, can also heterodimerize with small Mafs (but not Nrf2) to repress ARE-mediated gene expression (5). In our previous studies, we found increased nuclear levels of MafG and c-Maf and a switch from Nrf2 to MafG/c-Maf binding to ARE during prolonged cholestasis after BDL (31, 33). These findings supported a role for these changes in causing decreased expression of GSH synthetic enzymes. However, BDL also induces the expression of c-Myc (32), which has been shown to destabilize Nrf2 protein without affecting Nrf2 mRNA level in human bronchial epithelial cells (16). In contrast, c-Myc was shown to transcriptionally activate GCLC and GCLM promoters in human melanoma cells (3). These conflicting reports prompted us to examine the role of c-Myc induction in the downregulation of GCL expression during cholestatic liver injury.

In this study, we used two *in vivo* models, BDL and LCA *in vivo* treatment, as well as *in vitro* LCA treatment in Huh-7 cells. The *in vitro* model recapitulates BDL-induced changes in c-Myc, GCL subunits, Nrf2 ARE binding, and effects of UDCA and SAME treatments (31–33) and allows us to dissect the molecular mechanisms responsible more easily. The *in vivo* models serve to confirm changes that are due to higher levels of toxic bile acids rather than due to increased ductal pressure from ligating the bile duct.

LCA treatment *in vivo* led to similar changes in expression of c-Myc, GCL subunits, and MafG as BDL (31, 33). SAME and UDCA treatment also protected against the fall in GCL subunits and induction in MafG as in BDL (33). Both LCA and BDL treatment resulted in marked reduction in Nrf2 binding to ARE, while MafG binding to ARE increased. Our previous works did not examine changes in MafG or Nrf2 at the mRNA level, although nuclear Nrf2 protein level was reduced by 50% in LCA-treated Huh-7 cells (31, 33). This work showed that *in vivo* LCA treatment lowered hepatic Nrf2 mRNA level by 50% at day 14 that persisted to day 28

FIG. 7. Nrf2 is regulated by miR27a and miR27b. Panel (A) shows Nrf2 3'UTR binding site for miR27a/b and site-directed mutagenesis. Panel (B) shows the effect of knocking down miR27a, miR27b, alone or together on Nrf2 3'UTR reporter activity in wild-type (W) or mutant (M) Nrf2 3'UTR constructs. Huh-7 cells were treated with siRNA targeting miR27a (27a si), miR27b (27b si), both (27a/b si), or scrambled siRNA control (SC) for 24 h, followed by transfection with Nrf2 3'UTR reporter constructs as described in "Materials and Methods." Results are expressed as % of W+SC from three experiments done in duplicate, * $p < 0.05$ versus WT+SC, † $p < 0.05$ versus WT+27a si, and ‡ $p < 0.05$ versus WT+27b si.

(Fig. 1), which suggests that a key mechanism for reduced Nrf2 expression and Nrf2 ARE binding after LCA treatment lies at the pretranslational level. Consistent with a report from Levy and Forman (16), we found that c-Myc can physically interact with Nrf2 and co-occupy ARE with Nrf2. Using recombinant proteins, we found that c-Myc can directly interact with Nrf2. Also consistent with findings from Levy and Forman is that knockdown of c-Myc raised *GCLC* and *GCLM* mRNA levels and protected against LCA-induced GCL subunit suppression. However, while the mechanism reported by Levy and Forman was due to increased Nrf2 protein stability with c-Myc knockdown (16), we found increased Nrf2 mRNA level in our model. The difference may be at least in part due to different cell types.

We had previously hypothesized that decreased Nrf2 ARE binding during BDL and after LCA treatment of Huh-7 cells was likely due to induction in MafG and c-Maf, which could have formed MafG/MafG homodimer and MafG/c-Maf heterodimer to compete with Nrf2 for ARE binding (31). While this mechanism may still be important, the observation that LCA treatment enhanced an interaction between Nrf2 and MafG (Fig. 3A) seems to be at odds with this hypothesis as enhanced heterodimerization between

Nrf2 and MafG generally leads to increased ARE *trans*-activating activity (8). This prompted us to investigate further whether LCA treatment altered the type and abundance of proteins that interact with ARE that could have interfered with Nrf2 ARE *trans*-activating activity. Using biotinylated ARE fragment in pull-down assay followed by proteomics, we identified changes in the type and abundance of several proteins that bind to ARE after LCA treatment. Some are expected, such as reduced Nrf2 and increased c-Myc, c-Maf, and MafG. However, Phb1 is an unexpected interacting protein with ARE that was found to be reduced after LCA treatment (Fig. 3B). PHB1 belongs to an evolutionary conserved and ubiquitously expressed family of proteins with a variety of suggested activities in different cellular compartments (19, 21). PHB1 is best known as a mitochondrial chaperone protein that is essential for mitochondrial function and biogenesis (6). PHB1 is also found in the nucleus, where it has been shown to interact with Rb and p53 among other proteins to bring about a change in transcriptional activities of E2F (29) and p53 (7). These nuclear events have been associated with inhibition of cell cycle progression (29) and induction of apoptosis (7). Interestingly, reports show that PHB1 expression is reduced

during cholestatic liver injury plays a significant role in reducing Nrf2 and GCL subunits expression. The finding that acute knockdown of PHB1 lowered the mRNA levels of Nrf2 and GCL subunits and Nrf2 ARE binding (Fig. 6C, D) further supports this notion. The next question is how c-Myc induction is linked to Phb1 downregulation.

PHB1 expression is higher in many cancers (21), which may be related to the fact that its promoter region contains many c-Myc binding sites and c-Myc is often induced in cancer (6). Thus, finding lower PHB1 mRNA levels during cholestasis when c-Myc expression is induced was a surprise. Recent publications showing PHB1 is targeted by miR27a and miR27b (9) and c-Myc positively regulates miR27a (17) prompted us to examine whether the expression of miR27a and miR27b changed during cholestasis. In both BDL and LCA treatment, miR27a/b expression increased by day 1 in conjunction with c-Myc. Knockdown of c-Myc lowered the expression of both miR27a/b and also attenuated LCA-mediated induction of miR27a/b (Fig. 5A). These results clearly show c-Myc is upstream of miR27a/b, which lie upstream of PHB1, as knocking down miR27a or miR27b raised PHB1 mRNA level. However, how c-Myc knockdown or miR27a/b knockdown raised Nrf2 mRNA level was unclear. In examining Nrf2 3'UTR region, we identified potential miR27 binding site and, indeed, knockdown of miR27a or miR27b individually raised Nrf2 3'UTR reporter activity with combined knockdown exerting an additive effect and this was completely eliminated when the binding site was mutated (Fig. 7). Recently, miR27a was shown to regulate Nrf2 in human neuronal SH-SY5Y cells (20). We confirmed this is also true in liver and hepatocytes and showed that miR27b also regulates Nrf2 at the same binding site. To the best of our knowledge, this is the first demonstration of hepatic Nrf2 expression being regulated by miR27a/b and it shows that c-Myc induction can lower *Nrf2* mRNA level via this mechanism.

In addition to regulating Nrf2 at the mRNA level, c-Myc and PHB1 regulate Nrf2 ARE *trans*-activating activity. Both c-Myc and PHB1 physically interact with Nrf2 and knockdown of c-Myc resulted in increased interaction between Nrf2 and PHB1, as well as their co-occupancy of the ARE as shown on ChIP and Seq-ChIP (Fig. 8). This occurred because the expression of both Nrf2 and PHB1 increased, as miR27a/b expression fell. Overexpression of PHB1 did not alter c-Myc expression, but increased Nrf2 expression and interaction between PHB1 and Nrf2 (Fig. 8D). Despite higher Nrf2 expression, Nrf2 interaction with c-Myc was not changed, suggesting a preferential interaction between Nrf2 and PHB1. This also translated to increased Nrf2 and PHB1 co-occupancy of ARE (Fig. 8E). Since Nrf2 is itself positively regulated by ARE at the promoter level (15), the finding of higher *Nrf2* mRNA levels with PHB1 overexpression supports the notion that PHB1 serves as a coactivator for Nrf2 to activate ARE.

Although this work illustrates the novel role of PHB1 in the regulation of Nrf2-ARE binding and ARE-dependent gene expression, there are many other aspects of PHB1 functionality that also likely impact cholestatic liver injury. As a key mitochondrial chaperone protein, a fall in PHB1 expression would lead to impaired mitochondrial function and increased susceptibility to cell death, including apoptosis and necrosis, which could trigger an inflammatory response

(6, 27). These, along with decreased expression of ARE-dependent genes, could work in concert to perpetuate the liver injury.

A recent report emphasized the antioxidant action of bilirubin during cholestatic liver injury (11). In both BDL and LCA treatment, serum bilirubin levels are markedly elevated from day 7 on. We previously showed that hepatic GSH levels fell by 50% and more than 75% by days 14 and 28 after BDL, respectively (33). Although high bilirubin levels may help defend against oxidative stress, mice treated with UDCA or SAME for 28 days had no significant impact on serum bilirubin levels but prevented the fall in hepatic GSH levels and attenuated the increase in necrosis, apoptosis, and fibrosis induced by BDL (33). These results strongly support that the fall in GSH plays a critical role in liver injury induced by chronic cholestasis.

An area of controversy is whether Nrf2 plays a defensive role against cholestatic liver injury. There are conflicting data on the effect of cholestatic liver injury in Nrf2 knockout (KO) mice. While some reported no worsening in liver injury after BDL in Nrf2 KO mice (30), others reported Nrf2 KO mice were more susceptible to LCA treatment-induced liver injury (25) and increased Nrf2 functionality was protective against cholestatic liver injury, such as in Keap1 KO mice subjected to BDL (22). Thus, the results from the Nrf2 KO mice are mixed. In addition, there can be compensatory mechanisms that develop in the KO mice to further confuse this issue. Since ARE-dependent genes include those involved in detoxification, antioxidant defense, and efflux transporters (8, 30), Nrf2-ARE activation is most likely a major defense mechanism against cholestatic liver injury.

Our previous work showed that induction of MafG and c-Maf played a key role in the downregulation of GCL subunit expression during cholestatic liver injury (31). This was supported by the finding that knocking down MafG or c-Maf was protective (31). Since the expression of both Maf proteins is tremendously induced during cholestasis, their up-regulation could have formed complexes such as MafG/MafG homodimer or c-Maf/MafG heterodimer that would *trans*-repress ARE (4, 5). However, this work uncovered another mechanism, namely regulation of PHB1 expression. This was observed only with combined knockdown of MafG and c-Maf (individual knockdown raised *PHB1* mRNA levels by 16% and 22%, respectively, while combined knockdown raised it by 47%). How Maf proteins regulate the expression of PHB1 is unclear and will be a subject of future study.

SAME and UDCA, which exerted added benefits when combined in preventing BDL-induced liver injury, also had the same protective effect in LCA-induced liver injury. When used alone, each attenuated changes in c-Myc and MafG, and when combined, completely prevented their changes. Since treatment of chronic cholestatic liver disease remains largely ineffective, our results showing combining SAME with UDCA can target multiple upstream players that are responsible for downregulation of ARE-dependent genes support studies to examine this treatment strategy.

In conclusion, we have identified a novel circuit that is activated during chronic cholestatic liver injury that links c-Myc induction to downregulation of Nrf2 and PHB1

expression. Both Nrf2 and PHB1 are direct targets of miR27a and miR27b, which are induced by c-Myc. In addition, c-Myc acts as a corepressor while PHB1 acts as a coactivator of Nrf2 for ARE. Figure 9 summarizes our proposed molecular mechanism(s) of downregulation of GSH synthetic enzymes during chronic cholestasis based on existing results.

Materials and Methods

Materials

UDCA and LCA were obtained from Sigma-Aldrich. SAME in the form of disulfate p-toluenesulfonate dried powder was generously provided by Gnosis SRL. All other reagents were of analytical grade and obtained from commercial sources.

Cell culture and mouse hepatocytes

Huh-7 cells and isolated mouse hepatocytes were obtained from the Cell Culture Core of the USC Research Center for Liver Diseases. Cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 1% penicillin-streptomycin. Mouse hepatocytes were isolated from LCA-treated mice at day 14, centrifuged, and purified through Percoll as described (13).

LCA, UDCA, SAME, and siRNA or overexpression treatments in Huh-7 cells

Huh-7 cells were treated with LCA (100 μ M) for 20 h and processed for gene and protein expression analysis as

FIG. 9. Mechanisms leading to downregulation of GCLC and GCLM during chronic cholestatic liver injury. Chronic cholestasis induces expression of MafG and c-Maf, which can homodimerize (MafG:MafG) and heterodimerize (c-Maf:MafG) to repress ARE-dependent gene expression, as blocking their induction prevented BDL-mediated suppression of GCLC and GCLM expression (31, 33). This study showed that another mechanism is also important, where increased c-Myc expression induces miR27a/b, which suppress expression of Nrf2 and PHB1 at the post-transcriptional level. In addition, c-Myc and PHB1 can directly interact with Nrf2 but c-Myc acts as a corepressor while PHB1 acts as a coactivator for ARE-dependent gene expression.

described next. In other experiments, Huh-7 cells were treated with LCA, UDCA, SAME (all 100 μ M), UDCA plus SAME, or a combination of LCA with UDCA and/or SAME for 20 h.

Double-stranded c-Myc and scrambled siRNA were purchased from Qiagen. Hsa-miR-27a, hsa-miR-27b inhibitors, and PHB1 siRNA were purchased from Life Technologies. Huh-7 cells were transfected with c-Myc, PHB1, miR27a, miR27b, or scrambled siRNA (10 nM per 1×10^5 cells) using Lipofectamine™ RNAiMAX Transfection Reagent (Life Technologies) in six-well plates at 30% confluency for 24 h and processed for gene and protein expression analysis. Overexpression with PHB1 using overexpression vector (PHB1-pcDNA3.1) was done as described (12). In other experiments, Huh-7 cells transfected with siRNA or PHB1 overexpression vector mentioned earlier for 24 h were subsequently treated with LCA (100 μ M) for another 20 h and processed for gene expression, ChIP, and co-IP as described next.

BDL, LCA, UDCA, and SAME treatments in mice

Male C57BL/6 mice were fed chow *ad libitum*, and housed at constant temperature (22°C) with alternating 12 h of light and darkness. The mouse procedure protocols, use and the care of the animals were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Southern California. BDL and sham surgery were performed in 3-month-old mice as described (33). In other experiments, 2-month-old mice were given 200 mg/kg of LCA by oral gavage daily as described (1). BDL- and LCA-treated mice and their respective controls were divided into groups that received vehicle, UDCA [100 mg/(kg·day⁻¹) by oral gavage], SAME [100 mg/(kg·day⁻¹) by oral gavage], or both UDCA and SAME for 14 days. Mice were sacrificed on indicated days postsurgery or treatment, and livers and serum were harvested for studies described next.

Histology and immunohistochemistry

Formalin-fixed liver tissues embedded in paraffin were cut and stained with hematoxylin and eosin for routine histology and evaluation of necrosis. The percentage of necrosis was estimated by counting the number of microscopic fields with necrosis compared with the entire section in 15 different sections at 100 $\times$ magnification. Immunohistochemical staining of Nrf2, c-Myc, and Phb1 (Abcam) was performed with Dako kit according to the manufacturer's method. For quantifying immunohistochemical staining, a total of five fields at 100 $\times$ magnification were randomly selected (minimum of 1000 cells total) and positive nuclei or cells were counted and expressed as a percentage of the total using the MetaMorph imaging software (Woburn, MA). Control with normal mouse immunoglobulin G showed no staining.

RNA isolation and gene expression analysis

Total RNA was isolated by the TRIzol reagent (Life Technologies) from liver tissues and Huh-7 cells. Gene expression was assessed using real-time polymerase chain reaction (PCR). Total RNA was subjected to reverse transcription by using M-MLV Reverse transcriptase (Life Technologies). miRNA first-strand synthesis kit, the primers and TaqMan probes for human and murine *GCLC*, *GCLM*, *Nrf2*,

c-Myc, *PHB1*, *MafG*, *miR27a*, *miR27b*, and the Universal PCR Master Mix were purchased from Life Technologies. HPRT1 was used as housekeeping gene. The thermal profile consisted of an initial denaturation at 95°C for 3 min followed by 40 cycles at 95°C for 3 s and at 60°C for 30 s. The cycle threshold (Ct value) of the target genes was normalized to that of HPRT1 to obtain the delta Ct (Δ Ct). The Δ Ct was used to find the relative expression of target genes according to the formula: relative expression = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta$ Ct of target genes in experimental condition – Δ Ct of target gene under control condition.

Western blot and co-IP analysis

Liver tissues from BDL- or LCA-treated mice and Huh-7 cells after various treatments were subjected to Western blot analysis as described (33). Equal amounts of total protein extracts (15 μ g/well) were resolved on 12.5% sodium dodecyl sulfate (SDS)–polyacrylamide gels. Membranes were probed with antibodies to GCLC, GCLM, Nrf2, MafG, c-Myc, and PHB1 (Abcam). To ensure equal loading, membranes were stripped and reprobed with anti-Actin antibodies (Sigma-Aldrich). Blots were developed by enhanced chemiluminescence (Millipore Corporation). For co-IP, total proteins were immunoprecipitated by anti-Nrf2 antibodies and then subjected to Western blotting with anti-c-Myc, anti-MafG, or anti-PHB1 antibodies.

ChIP and Seq-ChIP assay

ChIP and Seq-ChIP were done to examine changes in protein binding to the ARE region of the mouse *Gclc* promoter in an endogenous chromatin configuration using the manufacturer's suggested protocol from the Pierce agarose ChIP kit (Thermo Scientific). Briefly, DNA immunoprecipitated by Nrf2 antibody was processed for a second round of immunoprecipitation using anti-MafG, anti-c-Myc, or anti-PHB1 antibodies. The purified DNA was detected by PCR analysis. PCRs of the mouse *Gclc* promoter region across ARE4 (5'-GCAGAGTGCTGAGTCACGGTG AG-3', –4073/ –4051 relative to ATG start site) (GenBank® accession No. NM-010295) used forward primer 5'-ACTG-GAGGCCATTAGGCGCTCTC-3' (bp –4301 to –4279) and reverse primer 5'-TTGTTGTGGTAGCGCCGCCAC-3' (bp –4008 to –3987). All PCR products were run on 2% agarose gels. The PCR conditions consisted of an initial denaturation at 94°C for 3 min followed by 25 cycles at 94°C for 30 s, the annealing and extension at 67°C for 90 s using the Advantage GC 2 PCR kit (Clontech), in accordance to their suggested protocol.

ChIP and Seq-ChIP for protein binding to the human GCLC promoter ARE4 was done as described (28) using the same antibodies described earlier. PCR of the human GCLC promoter region across ARE4 (GCGCTGAGTCAC, –3614/ –3603 bp relative to ATG start site, GenBank accession No. AY780794.1) used forward primer 5'-AATCCTAGCAGC AGCAGCAGCCC-3' (bp –3716 to –3694) and reverse primer 5'-CAAGCTGCATGCTCGGAGAGC-3' (bp –3478 to –3458). All PCR products were run on 2% agarose gels.

DNA pull-down

Biotin 3' end DNA Labeling Kit was purchased from Thermo scientific (Waltham, MA). TdT incorporated 1–3

biotinylated ribonucleotides onto the 3' end of triple ARE4 element [5'-GCAGAGTGCTGAGTCACGGTGAGGCAGAGTGCTGAGTCACGGTGAG-3' in mouse *Gclc* promoter (2)]. Dynabeads® M-280 Streptavidin was purchased from Life Technologies. Fifty microliters beads were washed with 2× binding & washing buffer (10 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), 2.0 M sodium chloride, adjust the pH to 7.0 by hydrochloric acid) twice. ARE4 element was immobilized on beads (0.39 pM DNA/μl beads) in 1× binding & washing buffer and incubated with gentle agitation for 15 min under room temperature. Beads-DNA were washed twice with 100 μl of 1× binding & washing buffer, followed by washing in incubation buffer (50 mM Tris, 1 mM EDTA, 100 mM potassium chloride, 5% Glycerol, 0.1% TritonX100, 1 mM dithiothreitol added fresh, pH 7.0). Beads-ARE4 element complex was incubated with 500 μg of liver tissue extract for 30 min at 70°C on a heater. Beads were gently shaken and suspended every 2–3 min in a glas-col rotator. The supernatant fraction (extra liver tissue extract) was removed after incubation. Beads-bound DNA-protein complex was washed thrice with 100 μl washing buffer at room temperature. DNase (Life Technologies) was added to digest the Beads-ARE4 element complex for 30 min at 37°C with gentle agitation. Fifty microliters 1× Laemmli buffer was then added and heated at 50°C for 10 min to elute binding proteins. The eluted samples were then heated at 100°C for 5 min. The eluates were then run and separated on an 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel.

Proteomics assays by mass spectrometry

Excised gel bands were cut into ~1 mm³ pieces. Gel pieces were then subjected to a modified in-gel trypsin digestion procedure. Gel pieces were washed and dehydrated with acetonitrile for 10 min followed by acetonitrile removal. Mass spectrometry (MS) assays are previously described (35).

Direct protein–protein interaction

Recombinant human Nrf2 and c-Myc proteins (Nrf2r and c-Mycr) were from Novus; recombinant human PHB1 (PHB1r) was from Mybiosource. Two micrograms PHB1r protein was immobilized to agarose beads by anti-PHB1 antibody (Abcam), followed by washing; beads were mixed with 1 μg Nrf2r and c-Mycr proteins, and rotated at 4°C for 4 h. Beads were then washed six times, boiled in SDS sample buffer and proteins were separated on SDS-PAGE. Nrf2r and c-Mycr proteins were also loaded to serve as indicators for proteins pulled down by PHB1 as described (34). Similarly, 2 μg Nrf2r was immobilized to agarose beads and mixed with 1 μg c-Mycr and PHB1r proteins for SDS-PAGE as described earlier.

Nrf2 3'UTR construct and dual luciferase assay

Nrf2 3'UTR (+1 to +478 bp) into vector pEZEX-MT01 was purchased from Genecopoeia. QuikChange™ Site-Directed Mutagenesis Kit was purchased from Agilent. A forward primer (5'-TCCTACCCTGAGTGAATGCTCATAC-3') and reverse primer (5'-GCTTTTAGTATAATAGTACAAAAAACTAGC-3') were used for mutant miR-27a/b targeted *Nrf2* 3'UTR site (Fig. 7A). Huh-7 cells (1×10⁵)

were placed in six-well plates the day before transfection, treated with miR27a/b inhibitor or scrambled control for 24 h. The wild type, mutated 3'UTR of *Nrf2* pEZEX-MT01 vector or empty vector (200 ng), and a control Renilla luciferase expression vector (2.5 ng) were subsequently cotransfected into Huh-7 cells with Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's instructions. Luciferase assays were performed 24 h later using the Dual Luciferase Reporter Assay System (Promega) as directed by the manufacturer's suggested protocol. Firefly luciferase activity was normalized to the Renilla luciferase activity.

Serum bilirubin and ALT levels

Serum bilirubin (Thermo Electron Corp.) and ALT (RAICHEM) levels were measured following the manufacturer's instructions.

Statistical analysis

Data are given as mean ± standard error of the mean. Statistical analysis was performed using analysis of variance followed by Fisher's test for multiple comparisons. For changes in mRNA and protein levels, ratios of genes or proteins to housekeeping genes or proteins densitometric values were compared. Significance was defined by $p < 0.05$.

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Author Disclosure Statement

No competing financial interests exist for any of the authors.

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Address correspondence to:
Dr. Shelly C. Lu
Division of Gastroenterology
Davis Building, 2097
Department of Medicine
Cedars-Sinai Medical Center
8700 Beverly Blvd.
Los Angeles, CA 90048

E-mail: Shelly.lu@cshs.org

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Abbreviations Used

3'UTR = 3' untranslated region
 ALT = alanine transaminase
 ARE = antioxidant response element
 BDL = bile duct ligation
 co-IP = co-immunoprecipitation
 DMSO = dimethylsulfoxide
 EDTA = ethylenediaminetetraacetic acid
 GCL = glutamate-cysteine ligase
 GCLC = GCL-catalytic subunit
 GCLM = GCL-modifier subunit
 GSH = reduced glutathione
 KO = knockout
 LCA = lithocholic acid
 miR27 = microRNA 27
 MS = mass spectrometry
 Nrf2 = nuclear factor-erythroid 2 related factor 2
 PBC = primary biliary cirrhosis
 PCR = polymerase chain reaction
 PHB1 = prohibitin 1
 PHB1r = recombinant human PHB1
 SAdMe = S-adenosylmethionine
 SC = scrambled control
 SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis
 SEM = standard error of the mean
 Seq-ChIP = sequential chromatin immunoprecipitation
 UDCA = ursodeoxycholic acid