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Oral *N*-acetylcysteine rescues lethality of hepatocyte-specific *Gclc* knockout mice providing a model for hepatic cirrhosis

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Abstract

Background/Aims—Certain liver diseases have been associated with depletion of glutathione (GSH), the major antioxidant in liver. A recent report about *Gclc^{h/h}* mice having a hepatocyte-specific ablation of *Gclc* (the gene encoding the catalytic subunit of the rate-limiting enzyme in GSH synthesis) has shown an essential role of GSH in hepatic function. *Gclc^{h/h}* mice develop severe steatosis and die of liver failure within one month, due to ~95% depletion of hepatic GSH; mitochondria are the major affected organelles, displaying abnormal ultrastructure and impaired function.

Methods—*Gclc^{h/h}* mice were fed with *L*-*N*-acetylcysteine (NAC; 10 g/L) in drinking water starting at postnatal day 18.

Results—*Gclc^{h/h}* mice were rescued by NAC supplementation to adulthood. NAC replenished the mitochondrial GSH pool and attenuated mitochondrial damage, accompanied with diminished hepatic steatosis; however, abnormal liver biochemical tests, hepatocytes death and hepatic oxidative stress persisted in the rescued mice. At age 50 days, the liver from rescued *Gclc^{h/h}* mice started to display characteristics of fibrosis, and at age 120 days, macronodular cirrhosis was observed. Immunohistostaining for liver-specific markers and the expression profile of hepatic cytokines indicated that the repopulation of hepatocytes in the cirrhotic nodules involves the expansion of oval cells.

Conclusions—Replenishment of mitochondrial GSH and restoration of mitochondrial function by NAC prevent mortality caused by loss of hepatocyte GSH *de novo* synthesis, allowing the progression of steatosis to a chronic stage. Thus, with NAC supplementation, *Gclc^{h/h}* mice provide a model for the development of liver fibrosis and cirrhosis.

Keywords

glutathione; conditional knockout; mitochondria; steatohepatitis; liver fibrosis and cirrhosis

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1. Introduction

Because of the hepatocytes' unique role in drug, xenobiotic and endogenous compound metabolism, these cells are a target of reactive oxygenated metabolites (ROMs). To prevent cellular damage, hepatocytes are equipped with robust antioxidant defense systems. Hepatocytes maintain the highest level of and synthetic capacity for glutathione (GSH), a tripeptide thiol-based antioxidant known to be important in the elimination of endogenous and xenobiotic ROMs (1). Numerous studies suggest that overproduction of ROMs and/or depletion of hepatic GSH is a common thread, allowing association of various forms of liver disease regardless of etiology (2;3).

The function of hepatic GSH has been studied using a hepatocyte-specific knockout mouse model of glutamate-cysteine ligase catalytic subunit (*Gclc*) (4), the gene encoding the essential enzyme in GSH biosynthesis. The *Gclc^{h/h}* hepatocyte-specific knockout mice reveal steatosis and necroinflammation in the liver, first documented at postnatal day 21 (PND21), and succumb to hepatic failure by PND30. Loss of hepatocyte *Gclc* results in a dramatic decrease in hepatic GSH, which precedes the depletion of mitochondrial GSH. Hepatic failure in *Gclc^{h/h}* mice parallels loss of mitochondrial function, accumulation of mitochondria with an abnormal ultrastructure and a dramatic decline in cellular ATP, suggesting mitochondrial failure as the underlying cause of hepatic failure.

Hepatic mitochondrial dysfunction has been suggested to participate in steatohepatitis occurring in liver injuries of various etiologies (5). In patients with steatohepatitis, liver fibrosis and cirrhosis commonly occur as the disease progresses (6). In *Gclc^{h/h}* mice, hepatocyte GSH depletion is perhaps too severe to observe the cirrhotic phenotype seen in steatohepatitis. In this current study, we test this hypothesis by providing *Gclc^{h/h}* mice with the antioxidant *L*-N-acetylcysteine (NAC), starting at PND18. NAC lessened the mitochondrial damage associated with GSH depletion, allowing mice to evade hepatic failure; however, the rescued mice developed macronodular hepatic cirrhosis by PND120.

2. Materials and methods

2.1. Mice and treatment

The *Gclc^{h/h}* knockout mouse line was generated as reported earlier (4). All studies were conducted on littermates and approved by the Institutional Animal Care and Use Committee (IACUC). NAC (Sigma, 10g/L) was dissolved in regular tap water and the solution was adjusted to pH 7.0 using NaOH. Freshly made NAC-containing water was supplied to mice every 2 days.

2.2. Measurement of hepatic ATP and GSH levels

Fresh liver pieces were processed for ATP measurement using the ATP luminescence kit (Sigma) according to manufacturer's protocol. Whole liver homogenates and liver cytosolic fractions were prepared from frozen liver pieces and GSH levels were determined spectrophotofluorometrically using *o*-phthalaldehyde (OPA) as described (7). Although cysteine, including NAC, does not react appreciably with OPA, in mice supplemented with NAC, the authenticity of GSH-OPA conjugates was verified chromatographically (8). In addition, chromatographic separation of the γ -glutamylcysteine (γ -GC) peak from the GSH peak revealed that γ -GC was always at low concentrations (<15%), compared to GSH.

2.3. Mitochondrial isolation and in vitro mitochondrial analysis

Liver mitochondrial suspension were prepared from freshly excised liver as previously described (9). Aliquots were subject to measurements of oxygen consumption (9), membrane

potential using 5,5',6,6',-tetrachloro-1,1,3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Sigma) (7) and GSH levels using OPA (10) as described. Mitochondrial GSH as percent of total hepatic GSH was calculated as: Percent (%) = $(\text{GSH}_{\text{mit}} \times V_{\text{mit}} \times 100) / (\text{GSH}_{\text{liv}} \times W_{\text{liv}})$, where GSH_{mit} is the concentration of mitochondrial GSH ($\mu\text{mol}/\mu\text{l}$), V_{mit} is the total volume of mitochondrial suspension (μl), GSH_{liv} is the concentration of hepatic GSH ($\mu\text{mol}/\text{g}$ liver), and W_{liv} is the wet weight of liver (g) used for the mitochondrial isolation.

2.4. Histopathological examination and histochemical staining of the liver

Liver paraffin sections (5- μm) and thin sections (1- μm) were prepared for light microscopy and electron microscopy, respectively, as described (4). HE and Masson's trichrome staining on paraffin sections were performed by the Department of Pathology at the University of Cincinnati using standard procedures. Liver histopathology was examined and scored by a blinded pathologist (DJO) using the Brunt scoring system (11). TUNEL stain was performed on paraffin sections using In Situ Cell Death Detection kit (Roche; Indianapolis, IN) according to manufacturer's protocol. Thin sections were stained with uranyl acetate and lead citrate. Images were obtained using a Nikon Eclips TE-300 microscope.

2.5. Immunohistochemistry (IHC) and immunofluorescence (IHF)

Deparaffinized and rehydrated liver sections (5- μm) were prepared in the same manner as for light microscopy. IHC was conducted using TSA™ Biotin System Kit according to the manufacturer's protocol (NEN Life Science Products, Boston, MA). IHF was performed in a similar manner except that sections were incubated with fluorescence-labeled secondary antibodies and DAPI to stain the nuclei. Images were obtained using a Nikon Eclips TE-300 microscope. Specific primary antibodies were used at dilutions of 1:100 for anti-rat α -fetoprotein (AFP; Santa Cruz Biotechnology, Santa Cruz, CA), 1:10 for rat monoclonal A6 antibody (generous gift from Dr. Valentina Factor, National Institutes of Health), 1:50 for anti-CK19 (ICN/Cappel, Aurora, OH), 1:400 for anti-mouse α -smooth muscle actin (α -SMA; Sigma, St Louis, MO), and at 25 $\mu\text{g}/\text{ml}$ for anti-bovine $\text{TGF}\beta_{1,2}$ (R&D Systems, Minneapolis, MN). Corresponding secondary antibodies were purchased from SouthernBiotech (Birmingham, AL) and used at dilutions of 1:200–500.

2.6. Plasma liver biochemical tests and lipid peroxidation assay

Mouse blood was collected by cardiac puncture using heparin-coated syringes. Plasma was collected and immediately assayed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), and bilirubin levels in the Clinical Laboratory following standard procedures. A whole liver homogenate was prepared from frozen liver pieces to measure hepatic lipid peroxidation using the malondialdehyde (MDA) assay kit according to the manufacturer's protocol (Oxford Biomedical Research, MI).

2.7. Reverse transcription and real-time quantitative PCR (Q-PCR)

Mouse livers were harvested, frozen in liquid nitrogen, and stored at -80°C until use. Total RNA was isolated using Tri-Reagent (Molecular Research Center, Inc.). Synthesis of first strand cDNA and Q-PCR reactions using gene-specific primer sets were performed as described (4). Primers used for Q-PCR are summarized in Table 1. For comparison, the mRNA levels in untreated *Gcle^{fl}* mice at PND28 were set as control (=1) and relative mRNA levels were reported as fold of control, after normalization with housekeeping gene β -2 microglobulin (B2M).

2.8. Statistical analyses

Statistics were performed using SigmaStat Statistical Analysis software (SPSS Inc., Chicago, IL). Group means were compared by 1-way ANOVA, followed by the student *t*-test for pairwise

comparison-of-means. Normality test (Kolmogorov-Smirnov test with Lilliefors' correction) was performed with residuals of the dependent variable from 1-way ANOVA and the data did not deviate from the normality assumption. Results are reported as the means $\pm$ S.E.M. of 7–10 mice for growth curve, survival rate and Brunt score, or of 4–6 mice for other measurements. The *P*-values of <0.05 were considered as statistically significant.

3. Results

3.1. Rescue of *Gclc^{h/h}* mice with NAC-supplemented drinking water

We previously reported the generation of a conditional null *Gclc* allele, flanked by *loxP* sites (“floxed”) and termed this allele *Gclc(f)* (4). Cre-mediated recombination of *Gclc(f)*, using a hepatocyte-specific *ALB-Cre* transgene was complete and detected only in hepatocytes by PND14. The resultant hepatocyte-specific *Gclc* null allele was termed *Gclc(h)*. Progressive depletion of hepatic GSH, up to 95%, caused growth retardation and early death in *Gclc^{h/h}* mice.

We therefore attempted to rescue these mutant mice by administering GSH ethylester (GSH-EE) or NAC at PND18. Delivery of the GSH-EE by intraperitoneal injection (5 mmol/kg) was not successful in rescue, possibly due to the difficulty of using this methodology chronically in pre-weanling mice. When supplied in drinking water, GSH-EE (10 g/L) did not increase hepatic GSH levels or rescue the mice (data not shown), suggesting that this route of delivery for GSH-EE may be inappropriate. In contrast, we were successful in averting death in ~80% *Gclc^{h/h}* mice by supplying NAC (10 g/L) in the drinking water (Fig. 1A). Rescued *Gclc^{h/h}* mice showed a much slower weight gain, similar to untreated *Gclc^{h/h}* mice at early times, but began to gain weight significantly around PND50 and attained a body weight comparable to that of control by the age of 3 months (Fig. 1B). In this study, mice were fed with standard rodent diet containing 0.4% methionine and 0.34% cystine (Harlan Teklad).

3.2. NAC supplementation preferentially replenishes mitochondrial GSH pool

Oral NAC is absorbed in the small intestine and rapidly deacetylated by the small intestine and liver providing cysteine for GSH synthesis (12). We compared the GSH levels between treated and untreated animals (Fig. 2A). Hepatic cytosolic and mitochondrial GSH in untreated *Gclc^{ff}* mice representing control levels were 44 ± 4 and 7.9 ± 0.9 nmol/mg protein, respectively. NAC supplementation increased cytosolic GSH in *Gclc^{ff}* mice by 25% at PND120, whereas it doubled (untreated = 6% and treated = 13% of control) the cytosolic GSH in *Gclc^{h/h}* mice. Compared to control levels, this increase in cytosolic GSH is modest. On the other hand, the mitochondrial GSH from NAC-treated *Gclc^{h/h}* mice increased to >40% of control levels. We calculated the amount of mitochondrial GSH as the percent of total hepatic GSH. In untreated *Gclc^{h/h}* mice, mitochondrial GSH accounted for ~30% at PND28 (comparing ~10% in untreated control mice); NAC supplementation increased mitochondrial GSH in *Gclc^{h/h}* mice to 56%, ~52%, and ~58% of total hepatic GSH at PND28, 50 and 120, respectively.

Mitochondrial GSH derives from transport of cytoplasmic GSH by the function of transporters present in the mitochondrial inner membrane (13). The dicarboxylate carrier (DIC, *Slc25a10*) and the oxoglutarate carrier (OGC, *Slc25a11*) have been identified to partially account for GSH transport across the mitochondrial membrane in the liver (13). We therefore assessed the mRNA levels of these transporters (Fig. 2B). Upregulation of *Slc25a10* by 1.9- and 2.5-fold was observed in untreated and treated *Gclc^{h/h}* mice, respectively. NAC also caused mild but significant increase (1.3-fold) of *SLC25A10* mRNA in *Gclc^{ff}* mice. However, upregulation of *SLC25A11* mRNA (~1.5-fold) was only observed in NAC-treated *Gclc^{h/h}* mice. Taken together, our data suggested that induction of these transporters may contribute

to the preferential accumulation of mitochondrial GSH in *Gclc^{h/h}* mice following NAC supplementation.

3.3. Progression from steatosis to fibrosis and cirrhosis in rescued *Gclc^{h/h}* mice

NAC-rescued *Gclc^{h/h}* mice appeared healthy, until studies were concluded at PND120. To our surprise, livers from these mice at PND120 were nodular in appearance (Fig. 3A, *right*). At this age, the liver weight as percent of body weight for *Gclc^{h/h}* mice almost doubled, when compared with that for *Gclc^{ff}* mice (Table 2). In addition, plasma ALT, AST, and bilirubin levels remained elevated in rescued *Gclc^{h/h}* mice, indicating the persistence of hepatic functional abnormalities (Table 2).

Examination of liver sections from these mice revealed multiple age-dependent pathological changes (Fig. 3B, *top row*). At PND30, hepatic steatosis of both macrovesicular and microvesicular forms were focally present (*right upper inset*) and localized inflammatory infiltrate surrounding necrotic foci were noted (*right lower inset*). At PND50, steatosis enhanced and pericellular inflammation was more noticeable. At PND120, steatotic hepatocytes and lobular inflammation diminished; however, regular hepatic acinar architecture was lost and replaced with nodules (*left inset, **). At all time points, hepatocellular ballooning degeneration and hepatocyte apoptosis detected by TUNEL staining (Fig. 3B, *second row*) were observed. We performed Mason's trichrome staining for collagen accumulation and IHC detection of α -smooth muscle actin (α -SMA) for activated hepatic stellate cells (HSCs), the key player in the pathogenesis of liver fibrosis. As shown in Fig. 3B (*third row*), periportal fibrosis was minimally present at PND30, whereas both perisinusoidal and periportal fibrosis were noted at PND50, and further progressed at PND120 when extensive portal fibrosis was observed (*left inset, arrows showing fibrous septa*). Consistent with the timing and intensity of hepatic fibrosis, immunopositivity of α -SMA was identified (Fig. 3B, *bottom row*). The overall liver histopathology for each age group of NAC-rescued *Gclc^{h/h}* mice (N = 7~8) was scored by the Brunt scoring system (Fig. 3C). This result showed the presence of steatosis, inflammation and hepatocyte injury in these mice at all ages, all of which were most severe at PND50; in contrast, hepatic fibrosis progressively developed with age and advanced to cirrhosis by PND120.

3.4. Restoration of mitochondrial function by NAC

In our previous study with *Gclc^{h/h}* mice, hepatic failure paralleled declines in mitochondrial structure and function (4). Moreover, several studies have suggested a pivotal role of mitochondrial dysfunction in determining the severity of steatohepatitis (5). In *Gclc^{h/h}* mice, NAC supplementation replenished mitochondrial GSH to >40% of control levels; however, steatosis still developed. We therefore examined the mitochondrial morphology and function (Fig. 4). In untreated *Gclc^{h/h}* mice at PND28, ~20% of hepatocyte mitochondria appeared swollen, with tubular cristae uncharacteristic of hepatic mitochondria (4). In NAC-treated *Gclc^{h/h}* mice from PND28 through PND50 (Fig. 4A), the mitochondria appeared much healthier than that of untreated *Gclc^{h/h}* mice showing lamellar cristae and proportional size, although in our judgment mitochondria from NAC-treated mice still appeared more damaged than mitochondria from untreated or treated *Gclc^{ff}* mice. At PND120, mitochondria appeared normal (Fig. 4A). Overall, all parameters related to mitochondrial *in vitro* function in NAC-treated *Gclc^{h/h}* mice were improved, when compared to those in untreated *Gclc^{h/h}* mice (Fig. 4B), which is consistent with the improved mitochondrial ultrastructure. Nevertheless, hepatic mitochondria from NAC-rescued *Gclc^{h/h}* mice remained significantly uncoupled (respiratory control ratio less than 3.0) by PND50; similarly mitochondrial membranes appeared somewhat depolarized relative to that from *Gclc^{ff}* mice. At PND120, mitochondrial coupling and membrane potential were within the normal ranges. Mitochondrial energetics, reflected by hepatic ATP levels, were drastically improved by NAC (untreated = 1.3 versus NAC-treated

= 4.5 $\mu\text{mol/g}$ at PND28), although they remained significantly lower than controls. Thus, we show above that NAC treatment specifically restores mitochondrial GSH; consequently, mitochondrial ultrastructure and function are largely restored. It is worth noting that NAC was not effective at restoring ER structure in *Gclc^{h/h}* mice by PND50, which still appeared vesicular rather than lamellar; however, by PND120 the ER appeared normal (Fig. 4A).

3.5. Persistent oxidative stress in livers of rescued *Gclc^{h/h}* mice

Ablation of the *Gclc* gene specifically in the hepatocyte, and the resultant depletion of GSH, leads to hepatic oxidative stress (4). In the current study, livers from NAC-treated *Gclc^{h/h}* mice still accumulated lipid peroxides (Fig. 5A), as measured by malondialdehyde (MDA) levels, not less than that seen in untreated *Gclc^{h/h}* mice. Furthermore, increases in mRNA levels of oxidant-stress-responsive genes, heme oxygenase-1 (*Hmox1*) and metallothionein-1 (*Mt1*), was observed (Fig. 5B); the inductions of both genes, however, decreased at PND120. Thus, NAC treatment surprisingly did not diminish hepatic oxidant stress.

3.6. Oval cell expansion in cirrhotic livers of rescued *Gclc^{h/h}* mice

Following liver damage, hepatocyte regeneration occurs to compensate for hepatocyte loss. Hepatocyte replication is a common means of hepatic regeneration following acute liver injury. On the other hand, studies on animal models with chronic liver injury of various forms suggest the involvement during this process of intra-hepatic precursor cells, called oval cells (14;15). Oval cells are bipolar progenitor cells that are capable of generating both hepatocytes and biliary cells. To test whether oval cells contribute to hepatocyte regeneration in *Gclc^{h/h}* mice, we performed co-localization studies (Fig. 6), using a panel of antibodies for liver-specific markers, including A6 (for oval cells), α -fetoprotein (AFP, for immature hepatocytes), CK19 (for biliary cells), albumin (ALB, for mature hepatocytes). All hepatic sections showed CK19 staining in the biliary epithelial architecture. In livers from NAC-treated *Gclc^{h/h}* mice, at PND30 A6-positive cells were rarely observed and noted only in bile ducts; such staining pattern of A6 has been observed in control mice in prior studies (16). AFP-positive cells were not detected at PND30. At PND50, clusters of A6-positive cells, which did not display co-staining for either CK19 or AFP indicating that these cells were not lineage committed, were detected outside the biliary epithelium. At PND120, there was a dramatic increase in A6-positive cells, among which ~80% were co-stained with AFP indicating a commitment to hepatocyte lineage, whereas only ~10% were co-stained with CK19. Above data suggested that the expansion of hepatic oval cells likely serve as a critical source of repopulated hepatocytes in the cirrhotic liver.

3.7. Expression profile of hepatic inflammatory cytokines

Increasing lines of evidence suggest that several key cytokines [such as tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), transforming growth factor- β 1 (TGF- β 1), interferon- γ (INF- γ) and lymphotoxin- β (LT- β)] act in a paracrine manner to stimulate oval cell expansion and differentiation to hepatocytes; these factors have been found upregulated during oval cell-mediated liver regeneration (17). To begin to understand whether the hepatic microenvironment in our model is in accord with other models, in which oval cells are known to play a major role in hepatocyte repopulation, we measured the liver mRNA levels of these cytokines. As shown in Fig. 7A, increased mRNA levels of TNF- α and IL-6 were observed in livers from NAC-treated *Gclc^{h/h}* mice with peak levels occurring at PND50. The TGF- β 1 messenger, on the other hand, decreased by 2-fold in untreated *Gclc^{h/h}* mice, whereas showing control levels in NAC-rescued *Gclc^{h/h}* mice. Given that TGF- β is primarily regulated at the post-translational level (18), we examined TGF- β expression in liver sections from NAC-treated *Gclc^{h/h}* mice and controls (Fig. 7B). Under the conditions employed, TGF- β immunoreactivity was not detected in livers from *Gclc^{ff}* mice. By contrast, TGF- β

immunoreactivity was robust in livers from NAC-treated *Gclc^{h/h}* mice. At PND30 its staining was associated with necrotic foci and thereafter was robust and diffuse. These results are consistent with this cytokine's role in hepatic fibrosis. Elevated mRNA expression of both IFN- γ and LT- β , two cytokines that have been implicated to play specific roles in oval cell-mediated liver regeneration (19), were observed at PND120 when oval cells expanded in the cirrhotic liver of NAC-rescued *Gclc^{h/h}* mice.

4. Discussion

Gclc^{h/h} mice succumb to hepatic failure around PND30 due to hepatic GSH depletion up to 95% (4). In the present study, we show that *Gclc^{h/h}* mice survive to adulthood, when orally supplied with NAC starting at PND18. Comparison of untreated versus NAC-treated hepatocytes from *Gclc^{h/h}* mice demonstrates that a major effect of NAC is the dramatic improvement of mitochondrial ultrastructure and *in vitro* function. Although mitochondrial energy coupling in NAC-treated *Gclc^{h/h}* mice is still somewhat impaired, membrane potential is remarkably improved and importantly hepatic ATP levels are largely maintained. The improvement of mitochondrial morphology and function afforded by NAC is reflected in a drastic increase in mitochondrial GSH. NAC is known to replenish GSH by providing cysteine for GSH biosynthesis (12). In the hepatocytes from *Gclc^{h/h}* mice, loss of the *Gclc* gene abolishes *de novo* GSH biosynthesis because γ -GC cannot be made. γ -GC may, however, be synthesized by the salvage pathway in which γ -glutamyltransferase (GGT) utilizes cystine as the acceptor to receive the γ -glutamyl moiety from GSH exported from non-hepatocyte cell types (20). This reaction produces γ -glutamylcystine, which can be reduced intracellularly to γ -GC. Interestingly, compared to controls, hepatic GGT activity in NAC-rescued *Gclc^{h/h}* mice was elevated 21- and 27-fold at PND28 and PND120, respectively (not shown). Thus, formation of γ -GC by the action of GGT represents a likely mechanism for GSH synthesis in *Gclc^{h/h}* hepatocytes and remains a testable idea for future studies. Compared with a 2-fold increase in cytoplasmic GSH, NAC-mediated restoration of mitochondrial GSH (>3-fold) in *Gclc^{h/h}* mice is much greater, accounting for >50% of total hepatic GSH pool. Such preferential increase in mitochondrial GSH following NAC treatment has been observed before (21;22) and is supported by considerable evidence demonstrating that mitochondria represent a separate and high-affinity pool for GSH (23). This effect of NAC is seemingly explained, at least in part, as consequent to increased active transport of cytoplasmic GSH into mitochondria, which is supported by the induction of *Slc25a10* and *Slc25a11* transporter genes in the current study. NAC, by mechanisms yet to be elucidated, favors mitochondrial GSH accumulation and may be important in the protective effects of NAC in a number of experimental systems (21; 22).

With NAC supplementation, *Gclc^{h/h}* mice continue to show depleted hepatic GSH and develop steatosis, suggesting that mitochondrial function *in vivo* may still be defective contributing to increased accumulation of lipids in hepatocytes. Moreover, these mice exhibit hepatic fibrosis and cirrhosis with age and show abnormalities in plasma liver biochemical functions. This finding may reflect chronic oxidative stress in NAC-treated *Gclc^{h/h}* mice. Indeed, despite of having >40% of normal mitochondrial GSH and greatly improved mitochondria, livers from NAC-rescued *Gclc^{h/h}* mice exhibit a persistent oxidant stress measured both as oxidized lipids and gene expressions. This observation is puzzling and may suggest that the bulk of oxidant stress measured as a consequence of GSH depletion in NAC-treated hepatocytes is not of mitochondrial origin. This idea may be supported by following findings: (i) H₂O₂ liberated from NAC-treated mitochondria was at the control level (data not shown), and (ii) hepatic CYPs 2E1 and 4A, which are reported inducers of lipid peroxidation in models of steatohepatitis (24;25), were upregulated in NAC-treated *Gclc^{h/h}* mice by 3~14-fold (data not shown). With regard to this report, most important is the demonstration that a persistent oxidative stress exists in our mouse model and that this may contribute to the development of

observed chronic liver pathologies. Future experiments applying other antioxidants to correct hepatic oxidative stress in this model will be valuable to dissect the role of GSH deficit in the pathogenesis of chronic liver diseases.

Liver fibrosis is a reparative response to prolonged liver injury and it may advance into cirrhosis following sustained insult. Key mediators involved in the process of hepatic fibrogenesis include inflammatory cytokines, ROMs and growth factors (26). In addition, hepatic inflammatory milieu created by a certain insult has great impact on proliferative response by either hepatocytes or oval cells (27). In this study, NAC-rescued *Gclc^{h/h}* mice exhibit ongoing hepatocyte death and persistent hepatic oxidative stress, accompanied by (starting at PND28) elevated levels of hepatic cytokines including TNF- α (mRNA), IL-6 (mRNA) and TGF- β (immunostaining). Consistent with these observations, hepatic fibrosis occurs in these mice by PND50 and progresses to cirrhosis by PND120. Furthermore, elevated expression of IFN- γ and LT- β accompanies proliferation of oval cells in the cirrhotic liver, which is in line with other studies suggesting a specific role of these cytokines in oval cell-mediated liver regeneration (27). This observation also agrees with our mouse model, in which mature hepatocytes experience persistent GSH depletion due to albumin-driven-Cre-mediated deletion of *Gclc* gene and may be compromised in their replicative capacity, although the possibility of hepatocytes replication can not be excluded. The precise signals that direct the expansion of oval cells are not clearly understood, but it is suggested that the signals may be derived from inflammatory cells or activated HSCs (28;29). Furthermore, oxidant stress is believed to be a signal activating HSCs (26). Consistent with this observation, both activated HSCs and oval cells are rare in NAC-treated *Gclc^{h/h}* livers at PND30; by PND50, however, both are readily observed in all hepatic sections and their appearance is further increased in cirrhotic nodules at PND120. It should be mentioned that oval cell proliferation has been suggested to be associated with an increased risk of developing hepatocellular carcinoma (30). At PND120, even with hepatic cirrhosis, NAC-treated *Gclc^{h/h}* mice are seemingly healthy. It will be interesting to determine whether these mice develop hepatocellular carcinoma, as they grow older.

Past studies using GCLC inhibitors (31;32) as well as *Gclc^{h/h}* mice indicate that the pathological consequences of GSH deficit are correlated most closely with mitochondrial GSH depletion. This notion is supported herein by the demonstration that NAC-bolstered GSH repletion which is specific to mitochondria averts hepatic failure by preserving mitochondrial function and ultrastructure. NAC-mediated restoration of mitochondrial function likely provides necessities (such as ATP) for critical events involved in repairing liver damage, for instance the induction of key cytokine mediators of fibrogenesis. NAC-rescued *Gclc^{h/h}* mice continue to display a chronic oxidant stress response. They develop steatosis, which progresses to nodular cirrhosis, a condition that is similar to the human disease. The proposed mechanisms for the protective effects by NAC and for hepatic cirrhotogenesis in our mouse model are depicted in Fig. 8. Future studies are required to test these hypotheses and to identify principal signaling pathways involved. Importantly, hepatocytes-specific ablation of GSH synthesis accompanied with partial repletion of GSH using NAC offers many possibilities for understanding the cellular function of GSH and disease states that present upon its chronic depletion.

Abbreviations

GSH	glutathione
NAC	<i>L</i> -N-acetylcysteine
ROMs	reactive oxygenated metabolites

GCLC	glutamate-cysteine ligase catalytic subunit
γ-GC	γ -glutamylcysteine
PND	postnatal day
GSH-EE	GSH ethylester
DIC	dicarboxylate carrier
OGC	oxoglutarate carrier
ALT	alanine aminotransferase
AST	aspartate aminotransferase
MDA	malondialdehyde
HSCs	hepatic stellate cells
α-SMA	α -smooth muscle actin
ER	endoplasmic reticulum
HMOX1	heme oxygenase-1
MT1	metallothionein-1
ALB	albumin
AFP	α -fetoprotein
GGT	γ -glutamyltranspeptidase
TNF-α	tumor necrosis factor- α
IL-6	interleukin-6
TGF-β1	transforming growth factor- β 1
IFN-γ	interferon- γ
LT-β	lymphotoxin- β

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Reference List

1. Kretzschmar M. Regulation of hepatic glutathione metabolism and its role in hepatotoxicity. *Exp Toxicol Pathol* 1996;48:439–446. [PubMed: 8765689]
2. Cederbaum AI. CYP2E1--biochemical and toxicological aspects and role in alcohol-induced liver injury. *Mt Sinai J Med* 2006;73:657–672. [PubMed: 16878272]
3. Okanoue T, Mitsuyoshi H. [Non-alcoholic steatohepatitis. 3. Oxidative stress and NASH]. *Nippon Naika Gakkai Zasshi* 2006;95:51–56. [PubMed: 16499208]
4. Chen Y, Yang Y, Miller ML, Shen D, Shertzer HG, Stringer KF, et al. Hepatocyte-specific Gclc deletion leads to rapid onset of steatosis with mitochondrial injury and liver failure. *Hepatology* 2007;45:1118–1128. [PubMed: 17464988]
5. Pessayre D, Fromenty B, Mansouri A. Mitochondrial injury in steatohepatitis. *Eur J Gastroenterol Hepatol* 2004;16:1095–1105. [PubMed: 15489566]

6. Day CP. Pathogenesis of steatohepatitis. *Best Pract Res Clin Gastroenterol* 2002;16:663–678. [PubMed: 12406438]
7. Shen D, Dalton TP, Nebert DW, Shertzer HG. Glutathione redox state regulates mitochondrial reactive oxygen production. *J Biol Chem* 2005;280:25305–25312. [PubMed: 15883162]
8. Chen Y, Shertzer HG, Schneider SN, Nebert DW, Dalton TP. Glutamate cysteine ligase catalysis: dependence on ATP and modifier subunit for regulation of tissue glutathione levels. *J Biol Chem* 2005;280:33766–33774. [PubMed: 16081425]
9. Senft AP, Dalton TP, Nebert DW, Genter MB, Hutchinson RJ, Shertzer HG. Dioxin increases reactive oxygen production in mouse liver mitochondria. *Toxicol Appl Pharmacol* 2002;178:15–21. [PubMed: 11781075]
10. Senft AP, Dalton TP, Shertzer HG. Determining glutathione and glutathione disulfide using the fluorescence probe o-phthalaldehyde. *Anal Biochem* 2000;280:80–86. [PubMed: 10805524]
11. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005;41:1313–1321. [PubMed: 15915461]
12. Sjodin K, Nilsson E, Hallberg A, Tunek A. Metabolism of N-acetyl-L-cysteine. Some structural requirements for the deacetylation and consequences for the oral bioavailability. *Biochem Pharmacol* 1989;38:3981–3985. [PubMed: 2597179]
13. Lash LH. Mitochondrial glutathione transport: Physiological, pathological and toxicological implications. *Chem Biol Interact* 2006;163:54–67. [PubMed: 16600197]
14. Davies RA, Knight B, Tian YW, Yeoh GC, Olynyk JK. Hepatic oval cell response to the choline-deficient, ethionine supplemented model of murine liver injury is attenuated by the administration of a cyclo-oxygenase 2 inhibitor. *Carcinogenesis* 2006;27:1607–1616. [PubMed: 16497703]
15. Evarts RP, Nagy P, Nakatsukasa H, Marsden E, Thorgeirsson SS. In vivo differentiation of rat liver oval cells into hepatocytes. *Cancer Res* 1989;49:1541–1547. [PubMed: 2466557]
16. Engelhardt NV, Factor VM, Yasova AK, Poltoranina VS, Baranov VN, Lasareva MN. Common antigens of mouse oval and biliary epithelial cells. Expression on newly formed hepatocytes. *Differentiation* 1990;45:29–37. [PubMed: 2292360]
17. Lowes KN, Croager EJ, Olynyk JK, Abraham LJ, Yeoh GC. Oval cell-mediated liver regeneration: Role of cytokines and growth factors. *J Gastroenterol Hepatol* 2003;18:4–12. [PubMed: 12519217]
18. Gressner AM, Dooley S, Weiskirchen R. TGF- β and the Smad Pathway in Liver Fibrogenesis. *Signaling Pathways in Liver Diseases*. Springer Berlin Heidelberg 2005;139(150)
19. Akhurst B, Matthews V, Husk K, Smyth MJ, Abraham LJ, Yeoh GC. Differential lymphotoxin-beta and interferon gamma signaling during mouse liver regeneration induced by chronic and acute injury. *Hepatology* 2005;41:327–335. [PubMed: 15660390]
20. Njalsson R, Norgren S. Physiological and pathological aspects of GSH metabolism. *Acta Paediatr* 2005;94:132–137. [PubMed: 15981742]
21. Circu ML, Rodriguez C, Maloney R, Moyer MP, Aw TY. Contribution of mitochondrial GSH transport to matrix GSH status and colonic epithelial cell apoptosis. *Free Radic Biol Med* 2008;44:768–778. [PubMed: 18267208]
22. Xiong Y, Peterson PL, Lee CP. Effect of N-acetylcysteine on mitochondrial function following traumatic brain injury in rats. *J Neurotrauma* 1999;16:1067–1082. [PubMed: 10595823]
23. Fernandez-Checa JC, Kaplowitz N. Hepatic mitochondrial glutathione: transport and role in disease and toxicity. *Toxicol Appl Pharmacol* 2005;204:263–273. [PubMed: 15845418]
24. Leclercq IA, Farrell GC, Field J, Bell DR, Gonzalez FJ, Robertson GR. CYP2E1 and CYP4A as microsomal catalysts of lipid peroxides in murine nonalcoholic steatohepatitis. *J Clin Invest* 2000;105:1067–1075. [PubMed: 10772651]
25. Ip E, Farrell GC, Robertson G, Hall P, Kirsch R, Leclercq I. Central role of PPARalpha-dependent hepatic lipid turnover in dietary steatohepatitis in mice. *Hepatology* 2003;38:123–132. [PubMed: 12829994]
26. Bataller R, Brenner DA. Liver fibrosis. *J Clin Invest* 2005;115:209–218. [PubMed: 15690074]
27. Santoni-Rugiu E, Jelnes P, Thorgeirsson SS, Bisgaard HC. Progenitor cells in liver regeneration: molecular responses controlling their activation and expansion. *APMIS* 2005;113:876–902. [PubMed: 16480456]

28. Knight B, Matthews VB, Akhurst B, Croager EJ, Klinken E, Abraham LJ, et al. Liver inflammation and cytokine production, but not acute phase protein synthesis, accompany the adult liver progenitor (oval) cell response to chronic liver injury. *Immunol Cell Biol* 2005;83:364–374. [PubMed: 16033531]
29. Lowes KN, Brennan BA, Yeoh GC, Olynyk JK. Oval cell numbers in human chronic liver diseases are directly related to disease severity. *Am J Pathol* 1999;154:537–541. [PubMed: 10027411]
30. Knight B, Yeoh GC, Husk KL, Ly T, Abraham LJ, Yu C, et al. Impaired preneoplastic changes and liver tumor formation in tumor necrosis factor receptor type 1 knockout mice. *J Exp Med* 2000;192:1809–1818. [PubMed: 11120777]
31. Martensson J, Meister A. Mitochondrial damage in muscle occurs after marked depletion of glutathione and is prevented by giving glutathione monoester. *Proc Natl Acad Sci U S A* 1989;86:471–475. [PubMed: 2911592]
32. Meister A. Mitochondrial changes associated with glutathione deficiency. *Biochim Biophys Acta* 1995;1271:35–42. [PubMed: 7599223]

Fig. 1. Restoration of survival and growth of *Gclc*^{h/h} mice by NAC supplementation. (**A**) Survival rates and (**B**) growth curves of *Gclc*^{ff/ff} and *Gclc*^{h/h} mice. NAC (10 g/L) was supplemented in the drinking water, starting at PND18. Data are reported as means $\pm$ S.E.M. (N = 7–10 mice for each group). NAC supplementation offered no growth advantage to *Gclc*^{ff/ff} or *Gclc*^{+/+} mice (not shown).

Fig. 2.

Liver cytosolic and mitochondrial GSH levels. (**A**) Cytosolic and mitochondrial GSH were determined using *o*-phthalaldehyde and reported as nmol/mg protein. Mitochondrial GSH as percent of total hepatic GSH was calculated as described in *Materials and Methods*. (**B**) Q-PCR analysis of liver mRNA for dicarboxylate carrier (SLC25A10) and oxoglutarate carrier (SLC25A11). Relative mRNA levels were reported as fold of control, after normalization with housekeeping gene β -2 microglobulin (B2M). Data are expressed as means $\pm$ S.E.M. (N = 4–6 mice for each group). * $p < 0.05$, when compared with age- and treatment-matched *Gclc^{f/f}* mice. † $p < 0.05$ when compared with untreated mice of the same genotype.

Fig. 3.

Liver histopathology. (A) Representative gross morphology at PND120 of livers from *Gclc^{ff}* (left) and *Gclc^{h/h}* (right) mice. Magnification: 6×. (B) Representative images of histological and histochemical analysis of liver sections from NAC-treated mice. H&E-stained liver sections (top row) from NAC-treated *Gclc^{h/h}* mice displayed macrovesicular and microvesicular steatosis at all ages (right insets); areas of focal hepatocyte necrosis surrounded by inflammatory infiltrate were most abundant at PND30 (lower right inset). Inflammatory infiltrate extended pericellularly at PND50. At PND120, liver showed nodular structures (left inset, *). At all time points, apoptosis of hepatocytes (red) was noted in NAC-treated *Gclc^{h/h}* mice by TUNEL stain (second row). Mason's trichrome stain for collagen (third row) shows regular staining (blue) in the vascular walls of *Gclc^{ff}* mice and NAC-treated *Gclc^{h/h}* mice at PND30; perisinusoidal fibrosis was visible in NAC-supplemented *Gclc^{h/h}* mice at PND50 and intensified at PND120, when fibrous septa became clearly visible (left inset, arrows). Activation of hepatic stellate cells identified by immunopositivity of α-smooth muscle actin (α-SMA; red, bottom row) was detected at PND50 and increased at PND120 in NAC-treated *Gclc^{h/h}* mice, whereas regular positive staining of α-SMA was observed in the vascular wall in *Gclc^{ff}* mice and NAC-treated *Gclc^{h/h}* mice at PND30. Magnifications: 100× (left insets) and 400× (right insets). (C) Brunt score of liver histopathology of NAC-treated *Gclc^{h/h}* mice. Data are reported as means ± S.E.M. (N = 7–8 mice for each group). Individual *p* values (< 0.05) for pair-wise comparisons are indicated.

Fig. 4.

Ultrastructure of hepatocytes and liver mitochondrial function *in vitro*. (A) Electron microscopy of hepatocytes from NAC-treated *Gclc*^{ff} and *Gclc*^{h/h} mice. Hepatocytes from NAC-treated *Gclc*^{h/h} mice at PND28 and PND50 revealed mitochondria with rarefaction of matrix, but lamellar cristae and proportional size were preserved; vesiculated rough endoplasmic reticulum was still present at these time points. Both mitochondria and rough endoplasmic reticulum recovered to normal at PND120. RER, rough endoplasmic reticulum; V, cytoplasmic vesicles; M, mitochondrion; F, fat droplet. Bar = 1 μ m. (B) Mitochondrial respiration indices, membrane potential and hepatic ATP levels were measured as described in the *Materials and Methods*. * $p < 0.05$, when compared with age-matched untreated *Gclc*^{ff} mice. [†] $p < 0.05$ when compared with age-matched NAC-treated *Gclc*^{ff} mice. Individual *p* values for pair-wise comparisons are indicated.

Fig. 5. Persistent oxidative stress and stress response in the liver from NAC-rescued $Gclc^{h/h}$ mice. (A) Hepatic lipid peroxidation was measured by malondialdehyde (MDA) assay. Results are expressed as nmol MDA/mg liver protein, according to a MDA-diacetal standard curve. * $p < 0.05$, when compared with age-matched $Gclc^{f/f}$ mice. (B) Q-PCR analysis of liver mRNA for heme oxygenase-1 (HMOX1) and metallothionein-1 (MT1). Relative mRNA levels were reported as fold of control, after normalization with housekeeping gene β -2 microglobulin (B2M). * $p < 0.05$, when compared with age-matched $Gclc^{f/f}$ mice. ** $p < 0.05$, when compared with age-matched $Gclc^{f/f}$ mice and with untreated $Gclc^{h/h}$ mice.

Fig. 6.

Detection of oval cells in the liver of NAC-rescued *Gclc^{h/h}* mice. Immunofluorescent co-localization analysis was performed using specific markers: A6 for identifying oval cells (red), AFP as a hepatocyte marker (green), and CK19 as bile duct epithelium marker (green); co-localization of two markers (orange) is presented in the overlay in which nuclei are stained with DAPI (blue). Magnification: 400 $\times$.

Fig. 7.

Upregulation of inflammatory cytokines in livers from NAC-rescued *Gclc^{h/h}* mice. **(A)** Liver mRNAs for inflammatory cytokines were determined by Q-PCR analysis. Relative mRNA levels were reported as fold of control, after normalization with housekeeping gene β -2 microglobulin (B2M). * $p < 0.05$, when compared with age-matched *Gclc^{ff/ff}* mice. **(B)** Immunohistochemical staining for TGF β on liver sections revealed negative staining in *Gclc^{ff/ff}* mice; on the contrary, livers from NAC-rescued *Gclc^{h/h}* mice revealed TGF β -immunopositive staining in a focal pattern at PND30 and diffusely at PND50 and PND120. Magnification: 400 $\times$.

Fig. 8.

Proposed mechanisms for the protective effects by NAC and for hepatic cirrhogenesis in rescued *Gclc^{h/h}* mice. Loss of hepocyte *Gclc* results in a depletion of hepatic GSH by 95% in *Gclc^{h/h}* mice, which precedes the depletion of mitochondrial GSH (<25% of control). *Gclc^{h/h}* mice reveal necroinflammation in the liver, loss of mitochondrial function and a dramatic decline in hepatic ATP, and die of liver failure within a month. Oral NAC supplementation rescues *Gclc^{h/h}* mice to adulthood. NAC specifically replenishes mitochondrial GSH pool (>40% of control), leading to partially restored mitochondrial function and morphology and sustained ATP levels. However, rescued *Gclc^{h/h}* mice still show ongoing hepatocytes death and persistent oxidative stress which, when supplied with preserved

mitochondrial function, signals the induction of key cytokine mediators for liver fibrosis and oval cell-mediated liver regeneration.

Table 1

Primers used in Q-PCR analysis

mRNA	Forward	Reverse
SLC25A10	5'-TGGACCTGCTCAAGGTGCATCTAC-3'	5'-AGATTGCGAACCGAGTCAGAGAGT-3'
SLC25A11	5'-AGTCTGTCAAGTTCCTGTTGGG-3'	5'-ACCAGCTGACAGCCCAGTGTAAT-3'
HMOX1	5'-GCCACCAAGGAGGTACACAT-3'	5'-GCTTGTGCGCTCTATCTCC-3'
MT1	5'-CACGACTTCAACGTCC-3'	5'-CAGCAGGAGCAGCAGCTCTTCTGCAG-3'
TNF- α	5'-AATTCGAGTGACAAGCCTGTAGCC-3'	5'-TGTCTTTGAGATCCATGCCGTTGG-3'
IL-6	5'-TGTCTTTGAGATCCATGCCGTTGG-3'	5'-TCTGCAAGTGCATCATCGTTGTTC-3'
TGF- β 1	5'-CAACTTCTGTCTGGGACCCT-3'	5'-TAGTAGACGATGGGCAGTGG-3'
IFN- γ	5'-TCTTCCTCATGGCTGTTTCTGGCT-3'	5'-TGCCAGTTCCTCCAGATATCCAAG-3'
LT- β	5'-TACTTCCCTGGTGACCCTGTTGTT-3'	5'-GACGGTTTGCTGTCATCCAGTCTT-3'
B2M	5'-CATGGCTCGCTCGGTGACC-3'	5'-AATGTGAGGCGGGTGGAAGT-3'

SLC25A10, dicarboxylate carrier; SLC25A11, oxoglutarate carrier; HMOX1, heme oxygenase-1; MT1, metallothionein-1; TNF- α , Tumor Necrosis Factor- α ; IL-6, interleukin-6; TGF- β 1, transforming growth factor- β 1; IFN- γ , interferon- γ ; LT- β , lymphotoxin- β ; B2M, β 2-microglobulin.

Table 2

Liver weight and plasma clinical pathology tests of liver function at PND120

Parameters	<i>Gclc^{f/f}</i>	<i>Gclc^{h/h}</i>
Body weight (g)	30 ± 2.5 (14.3 ± 0.7)	28 ± 2.3 (5.8 ± 0.5 [*])
Liver weight (% of body weight)	4.7 ± 0.7 (5.7 ± 0.6)	8.5 ± 0.4 [†] (5.5 ± 0.8)
ALT (IU/L)	28 ± 6 (37 ± 2)	428 ± 41 [†] (1720 ± 124 [*])
AST (IU/L)	107 ± 8 (122 ± 8)	853 ± 31 [†] (3400 ± 261 [*])
Unconjugated bilirubin (mg/dL)	<0.01 (0.0 ± 0.0)	0.6 ± 0.2 [†] (0.2 ± 0.0 [*])
Conjugated bilirubin (mg/dL)	<0.01 (0.0 ± 0.0)	11.3 ± 1.0 [†] (9.2 ± 0.6 [*])

Results are reported as means ± S.E.M. of 4–5 mice. Numbers in parentheses are data from mice without NAC supplementation at PND28 (4).

[†] $p < 0.01$ when comparing NAC-treated *Gclc^{f/f}* and *Gclc^{h/h}* mice.

^{*} $p < 0.01$ when comparing untreated *Gclc^{f/f}* and *Gclc^{h/h}* mice.