Research Article

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

Ying Chen[1,2,*], Elisabet Johansson[1], Yi Yang[1], Marian L. Miller[1], Dongxiao Shen[1], David J. Orlicky[3], Howard G. Shertzer[1], Vasilis Vasiliou[2], Daniel W. Nebert[1], Timothy P. Dalton[1]

[1]*Department of Environmental Health, University of Cincinnati, Cincinnati, OH 45267, USA;* [2]*Department of Pharmaceutical Sciences, University of Colorado Denver, Aurora, CO 80045, USA;* [3]*Department of Pathology, University of Colorado Denver, Aurora, CO 80045, USA*

Background & Aims: Certain liver diseases have been associated with depletion of glutathione (GSH), the major antioxidant in the liver. A recent report about *Gclc*$^{h/h}$ mice with 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 functioning.

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 use of NAC supplementation, and survived until adulthood. NAC replenished the mitochondrial GSH pool and attenuated mitochondrial damage, with accompanying diminished hepatic steatosis; however, abnormal liver biochemical tests, hepatocyte death, and hepatic oxidative stress persisted in the rescued mice. At 50 days of age, 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 as well as the expression profile of hepatic cytokines indicated that the repopulation of hepatocytes in the cirrhotic nodules involved the expansion of oval cells.

Conclusions: Replenishment of mitochondrial GSH and restoration of mitochondrial function by NAC prevents mortality caused by the loss of hepatocyte GSH *de novo* synthesis, allowing steatosis to progress 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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*Corresponding author. Address: Department of Pharmaceutical Sciences, University of Colorado Denver, School of Pharmacy, Aurora, CO 80045, USA. Tel.: +1 303 724 3521; fax: +1 303 724 7266.

E-mail address: ying.chen@ucdenver.edu (Y. Chen).

Abbreviations: GSH, glutathione; NAC, *L-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-β_1, transforming growth factor-β_1; IFN-γ, interferon-γ; LT-β, lymphotoxin-β.

Introduction

Because hepatocytes play a unique role in drug, xenobiotic, and endogenous compound metabolism, they 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 of known importance 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 display steatosis and necroinflammation in the liver, which is first observable 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 allow us 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 avoid hepatic failure;

Table 1. Primers used in Q-PCR analysis.

mRNA	Forward	Reverse
SLC25A10	5' -TGGACCTGCTCAAGGTGCATCTAC-3'	5' -AGATTGCGAACCGAGTCAGAGAGT-3'
SLC25A11	5' -AGTCTGTCAAGTTCCTGTTTGGG-3'	5' -ACCAGCTGACAGCCCAGTGTAAAT-3'
HMOX1	5' -GCCACCAAGGAGGTACACAT-3'	5' -GCTTGTTGCGCTCTATCTCC-3'
MT1	5'- CACGACTTCAACGTCC-3'	5'- CAGCAGGAGCAGCAGCTCTTCTTGCAG
TNF-α	5' -AATTCGAGTGACAAGCCTGTAGCC-3'	5' -TGTCTTTGAGATCCATGCCGTTGG-3'
IL-6	5' -TGTCTTTGAGATCCATGCCGTTGG-3'	5' -TCTGCAAGTGCATCATCGTTGTTC-3'
TGF-β1	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' -AATGTGAGGCGGGTGGAACTG-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-β1*, transforming growth factor-β_1; *IFN-γ*, interferon-γ; *LT-β*, lymphotoxin-β; *B2M*, β2-microglobulin.

however, the rescued mice developed macronodular hepatic cirrhosis by PND120.

Materials and methods

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, 10 g/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.

Measurement of hepatic ATP and GSH levels

Fresh liver pieces were processed for ATP measurement using the ATP luminescence kit (Sigma) in accordance with the manufacturer's protocols. Whole liver homogenates and liver cytosolic fractions were prepared from frozen liver pieces. 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.

Mitochondrial isolation and in vitro mitochondrial analysis

Liver mitochondrial suspension was prepared from freshly excised liver as previously described [9]. Aliquots were subject to measurements of oxygen consumption [9], as well as membrane potential using 5,5′,6,6′,-tetracholoro-1,1,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Sigma) [7], and GSH levels using OPA [10], as described. Mitochondrial GSH as the percent of total hepatic GSH was calculated as: Percent (%) = $(GSH_{mit} \times V_{mit} \times 100)/(GSH_{liv} \times W_{liv})$, where: GSH_{mit} is the concentration of mitochondrial GSH (μmol/μl), V_{mit} is the total volume of mitochondrial suspension (μl), GSH_{liv} is the concentration of hepatic GSH (μmol/g liver), and W_{liv} is the wet weight of liver (g) used for the mitochondrial isolation.

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 was performed by the Department of Pathology at the University of Cincinnati, using standard procedures. Liver histopathology was examined and blindly scored by a pathologist (DJO) using the Brunt scoring system [11]. TUNEL stain was performed on paraffin sections using the In-Situ Cell Death Detection kit (Roche; Indianapolis, IN) in accordance with the manufacturer's protocol. Thin sections were stained with uranyl acetate and lead citrate. Images were obtained using a Nikon Eclips TE-300 microscope.

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 in accordance with the manufacturer's protocol (NEN Life Science Products, Boston, MA). IHF was performed in a similar manner, except that sections were

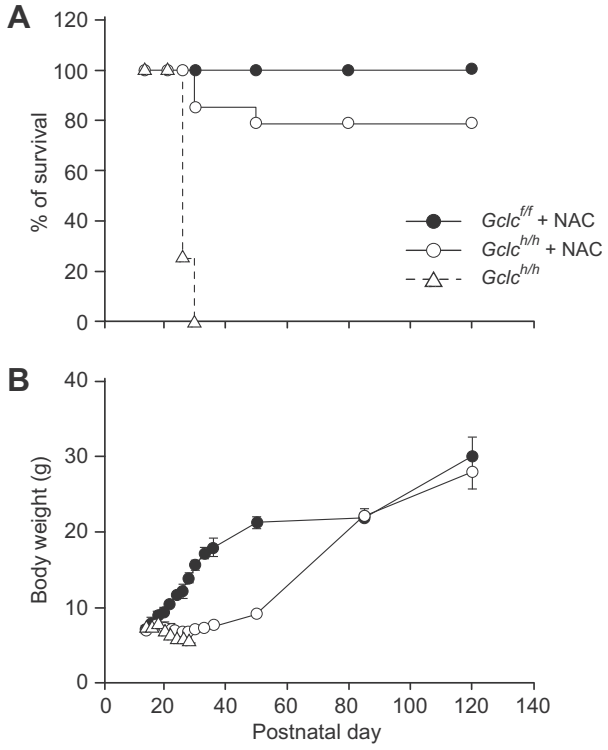

Fig. 1. Restoration of survival and growth of *Gclc*$^{h/h}$ mice by NAC supplementation. (A) Survival rates and (B) growth curves of *Gclc*$^{f/f}$ and *Gclc*$^{h/h}$ mice. NAC (10 g/L) was supplemented in the drinking water, starting at PND18. Data are reported as means ± SEM (N = 7–10 mice for each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