

GCN2 kinase is a key regulator of fibrogenesis and acute and chronic liver injury induced by carbon tetrachloride in mice

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General control nonderepressible 2 (GCN2) is a highly conserved cytosolic kinase that modulates a complex response for coping with the stress owing to lack of amino acids. GCN2 has been recently shown to be involved in the regulation of metabolic balance and lipid degradation rate in the liver. We hypothesized that GCN2 could have a role in in hepatic fibrogenesis and in the response to acute or chronic liver injury. Activation of GCN2 in primary or immortalized human hepatic stellate cells by incubation with medium lacking the essential amino acid histidine correlated with decreased levels of collagen type I protein and mRNA, suggesting an antifibrogenic effect of GCN2. *In vivo* studies with *Gcn2* knock-out mice (*Gcn2*^{-/-}) showed increased susceptibility to both acute or chronic liver damage induced by CCl₄, as shown by higher alanine aminotransferase and aspartate aminotransferase activities, increased necrosis and higher inflammatory infiltrates compared with wild-type mice (WT). Chronic CCl₄ treatment increased deposition of interstitial collagen type I more in *Gcn2*^{-/-} mice than in WT mice. *Col1a1* and *col1a2* mRNA levels also increased in CCl₄-treated *Gcn2*^{-/-} mice compared with WT mice. These results suggest that GCN2 is a key regulator of the fibrogenic response to liver injury. *Laboratory Investigation* (2013) **93**, 303–310; doi:10.1038/labinvest.2012.173; published online 14 January 2013

KEYWORDS: collagen type I; fibrosis; hepatic stellate cells

GCN2 (general control nonderepressible 2), is a cytosolic kinase present in all cell types that becomes activated by the accumulation of uncharged tRNAs,^{1,2} triggering a highly conserved and well-orchestrated pathway leading to changes in protein expression and helping to cope with the stress caused by nutrient deficiency.^{3,4} The effects of GCN2 activation were initially described to modulate processes related to protein metabolism or amino acid transport. However, recent studies have demonstrated the role played by GCN2 in other cellular responses, such as sensitivity to RNA viral infection⁵ or T-cell differentiation.⁶ Although GCN2 is typically activated by amino acid deprivation, UV exposure^{7,8} or high levels of urea⁹ can also lead to GCN2 activation. These data suggest that GCN2 could act as a key regulator to the cellular response under stress conditions.

Previous work indicates that in the liver GCN2 contributes to the control of protein balance during deprivation of

essential amino acids¹⁰ and has also been shown to regulate hepatic fatty-acid metabolism¹¹ and hepatic insulin sensitivity.¹² *Gcn2*^{-/-} mice are more sensitive to oxidative stress induced in the liver by an imbalanced diet and present decreased levels of glutathione peroxidase in the liver.¹³ Most of the studies have focused on stress caused by amino acid deprivation, yet the role of GCN2 in liver injury and fibrogenesis has not been studied. Work from our group suggested that GCN2 could be a regulator of the biology of hepatic stellate cells (HSC), the cell type mainly responsible for collagen deposition in the liver. Both in primary and in immortalized HSC, activation of GCN2 induced by either by amino acid restriction or by proteasome inhibition caused a fast and sustained decrease in intracellular hydrogen peroxide levels.¹⁴ This result pointed to GCN2 as a potential regulator of fibrogenesis, as hydrogen peroxide has been shown to induce collagen production and to mediate the profibrogenic action of TGF- β on HSC.^{15–17}

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Received 9 July 2012; revised 12 October 2012; accepted 30 October 2012

In the present paper we explored the role of GCN2 on the regulation of collagen type I expression in HSC and in the response to acute and chronic liver damage. Using *in vitro* and *in vivo* approaches, we demonstrate that GCN2 has a key role in collagen type I production by HSC and protects from liver injury and fibrosis in mice.

MATERIALS AND METHODS

Cell Culture and Treatments

Cell culture experiments were carried out using primary human HSC and the human HSC line LX-2. This non-tumoral cell line was obtained after immortalization in low-serum media culture of HSC isolated from normal primary human HSC¹⁸ and was kindly provided by Dr SL Friedman, Mount Sinai School of Medicine, New York. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 1% fetal bovine serum, penicillin/streptomycin and non-essential amino acids for 36 h. The medium was replaced with serum-free Dulbecco's modified Eagle's medium for 12 h, after which treatments were carried out.

Primary human HSC were kindly provided by Dr F Hong, Mount Sinai School of Medicine, New York. Cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin for 36 h. The medium was replaced with serum-free MEM for 12 h, after which treatments were added.

Cells were treated by replacing the media with MEM without histidine for 4, 8 and 24 h. Control cells were incubated with complete MEM, containing histidine 270 μ M. Cell culture reagents were obtained from Gibco BRL (Grand Island, NY, USA).

Western Blot

The antibodies used on western blot analysis were: total GCN2, phospho-specific GCN2, LC3I and II from Cell Signaling Technology (Beverly, MA, USA), collagen type I from Millipore; β -actin and p62 from Sigma (St Louis, MO, USA). Following incubation with primary antibodies, membranes were washed and incubated with a secondary polyclonal (Bio-Rad, Hercules, CA, USA) or monoclonal (Amersham Life Science, Arlington Heights, IL, USA) antibodies, conjugated to horseradish peroxidase. Immunoreactive proteins were detected by enhanced chemiluminescence system (Roche Molecular Biochemicals, Lewes, UK). Figures are representative of at least three independent experiments.

Cell Viability

To evaluate cell viability *in vitro*, trypan blue staining was used for determination of dead cells by dye exclusion. For these assays 1×10^6 LX-2 cells were seeded in 100 mm culture dishes and treated during 24 h either with histidine-free medium or complete medium. The cells were trypsinized, incubated with trypan blue (0.2% in phosphate-buffered saline) and counted within 3 min under light microscopy

using a hemocytometer. Triplicate samples from two independent experiments were examined for each data point. When indicated, cells were pretreated for 30 min with either chloroquine or 3-methyl adenine, obtained from SIGMA Chemical Company.

Quantitative Real-Time RT-PCR

Sybrgreen quantitative real-time RT-PCR was performed in a Roche Lightcycler 480 using the following PCR primers: human *COL1A1* forward 5'-ACGCTAACCCCTCCCCAGC-3' and human *COL1A1* reverse 5'-CGGTGGCCGCTAAGAGGAGC-3'; human *COL1A2* forward 5'-CGGCAGCAGGAGGTTTCGGC-3' and human *COL1A2* reverse 5'-GTCGCA GAGCCCCTGGGTCA-3'; mouse *COL1A1* forward 5'-GCGGTAACGATGGTCTGTT-3' and mouse *COL1A1* reverse 5'-CTTACCCTTAGCACCAAC-3'; mouse *COL1A2* forward 5'-TTGTGCGCCAGTGAG-3' and mouse *COL1A2* reverse 5'-CTGGTCTGCTGGT-3'. All values were normalized to glyceraldehyde-3-phosphate dehydrogenase levels. Results were expressed as relative fold change.

Animal Study Design

Homozygous mice for the GCN2.KO4 mutant locus and their WT littermates were used (The Jackson Laboratory, Bar Harbor, ME, USA). Mice received humane care in compliance with the guidelines of the National Institutes of Health and Animal Care Committee at the Mount Sinai School of Medicine. Acute liver injury was induced by injecting intraperitoneally 0.05 ml of CCl₄/kg of body weight (25% v/v in mineral oil). Chronic liver injury was induced by injecting intraperitoneally 0.05 ml of CCl₄/kg of body weight (25% v/v in mineral oil) twice a week for 4 weeks. Control groups were injected with mineral oil. Mice were maintained at 22 °C with 12 h light/dark cycle, were fed standard chow diet, had free access to water, and were killed under ketamine/xylazine anesthesia 48 h after the last dose of CCl₄.

Serum Transaminases

Blood was collected from the retro-orbital vein under anesthesia, centrifuged at 3000 r.p.m. for 3 min, and serum was separated to assay for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using kits from Pointe Scientific (Canton, MI, USA).

Liver Histology and Immunohistochemistry

Liver samples were fixed in 10% buffered formalin and embedded in paraffin. Five micrometer sections were dehydrated and stained with hematoxylin and eosin (H&E) and were evaluated by a liver pathologist. The sirius red/fast green staining was carried out as according to López-De León and Rojkind.¹⁹ Collagen type I immunostaining was carried out using a monoclonal antibody (Millipore, Darmstadt, Germany) and ImmunoCruz-staining system.

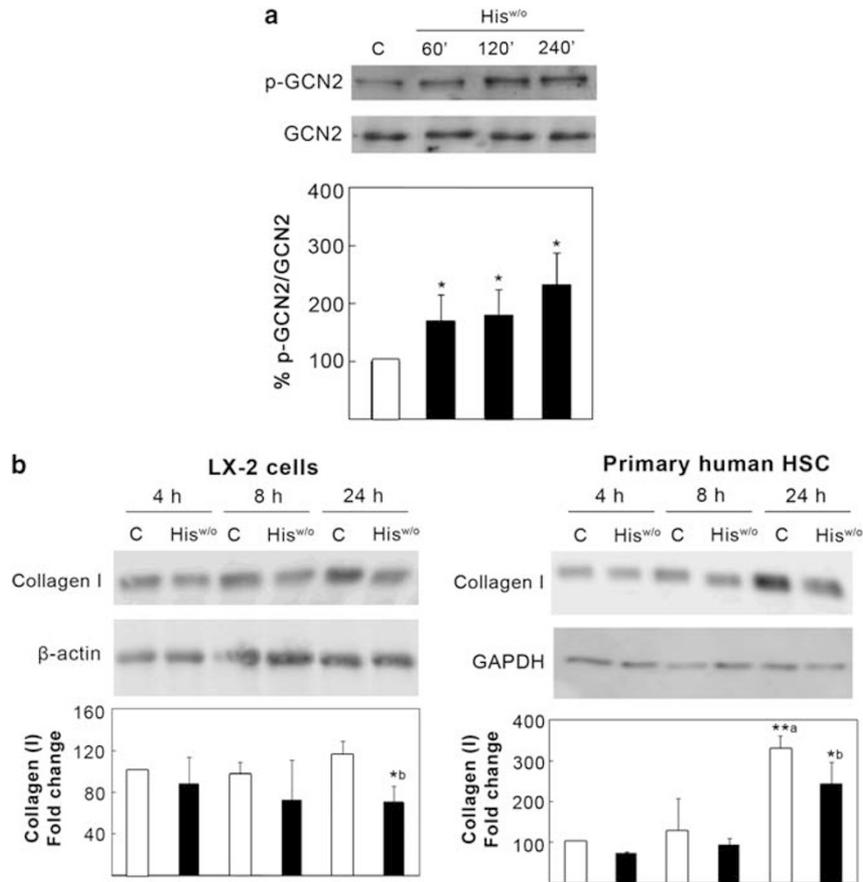


Figure 1 Time course analysis of GCN2 (general control nonderepressible 2) phosphorylation and collagen type I protein levels in hepatic stellate cells (HSC) treated with histidine-free medium. **(a)** The cell line LX-2 was treated from 30, 60, 120 and 240 min with medium without histidine and total and phospho-active GCN2 levels were determined by western blot. **(b)** LX-2 and primary human HSC were treated from 4 to 24 h with medium without histidine and collagen type I production was determined by western blot. Upper panels, representative immunoblots. Lower panels, densitometry values referred to the loading control. Each bar represents the mean \pm s.d. of protein fold change compared with control of four independent experiments ($*P < 0.05$; $**P < 0.01$, a vs 4 h control; b vs its own control; C control; His^{w/o}, histidine-free medium).

RESULTS

Activation of GCN2 Decreases Collagen Type I Expression in HSC

The first experiments were aimed to establish the effect of GCN2 activation on collagen type I expression by HSC. GCN2 was activated by incubation with histidine-free medium, and phosphorylated GCN2 (the active form of the enzyme) and protein levels of collagen type I were analyzed by western blot. As shown in Figure 1a, incubation of LX-2 cells with histidine-free medium increased phosphorylated GCN2 time-dependently up to 4 h. Both in LX-2 cells and in primary human HSC, collagen-I protein expression decreased after incubation with histidine-free medium up to 24 h (Figure 1b).

To determine whether changes in collagen type I protein expression in response to GCN2 activation were due to transcriptional inhibition, the *COL1A1* and *COL1A2* genes mRNA levels were analyzed. As shown in Figure 2a, in control LX-2 cells, which were incubated in complete medium, *COL1A1* and *COL1A2* mRNAs increased after 24 h. In

contrast, both mRNAs remained unchanged in cells cultured with histidine-free medium. The same effects were observed in primary human HSC (Figure 2b).

Autophagy is Not Induced By GCN2 Activation in HSC

GCN2 has been described in different cell types to mediate autophagy induced by amino acid starvation or by tRNA accumulation, suggesting a possible role of autophagy in the response of HSC to GCN2 activation. We first evaluated the effect of autophagy inhibitors chloroquine and 3-methyl adenine on the viability of HSC in response to GCN2 activation. As shown in Supplementary Figure 1, both autophagy inhibitors caused in LX-2 a decrease on cell viability, which was more significant for chloroquine than for 3-methyl adenine. GCN2 activation by histidine deprivation enhanced LX-2 viability both in control cells and in cells pretreated with autophagy inhibitors.

To analyze whether GCN2 activation does in fact induce autophagy in HSC, western blot analysis of LC3I, II and of

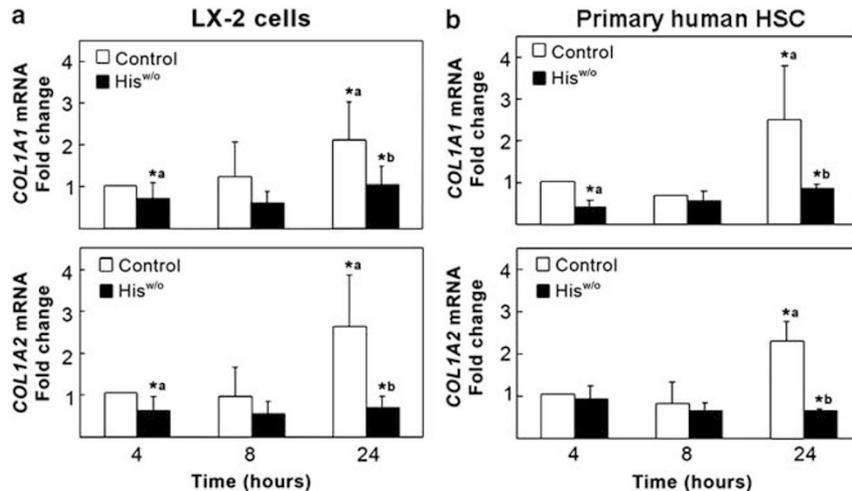


Figure 2 Time course analysis of collagen type I mRNA levels in hepatic stellate cells (HSC) treated with histidine-free medium. Analysis of *COL1A1* and *COL1A2* mRNA levels by quantitative PCR were carried out in the cell line LX-2 (a) or in primary human HSC (b) treated from 4 to 24 h with histidine-free medium. Values are referred to basal levels of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA. Control represents non-treated cells and was given the value of one. Each bar represents the mean \pm s.d. of mRNA fold change compared with control of at least triplicate data from four independent experiments (* $P < 0.05$; ** $P < 0.01$, a vs 4 h control; b vs its own control; His^{w/o}, histidine-free medium).

p62 was carried out in LX-2 incubated with histidine deprived medium. GCN2 activation did not alter p62 levels and failed to enhance LC3I and II levels (Supplementary Figure 2).

Acute CCl₄ Injection Causes More Liver Injury To *Gcn2*^{-/-} than to WT Mice

We had previously demonstrated that GCN2 activation causes an increase in HSC viability and a decrease in intracellular hydrogen peroxide levels.¹³ These results as well as the antifibrogenic action described above, suggested that GCN2 could have a role in regulating the response to liver damage. To evaluate this possibility we studied the effects of acute and chronic liver injury in *Gcn2*^{-/-} mice.

Acute CCl₄ treatment caused increased ALT and AST activities by 4.1 and 3.9-fold, respectively, in *Gcn2*^{-/-} mice compared with WT mice (Figure 3a).

Hematoxylin and eosin (H&E) staining showed more necrotic areas in CCl₄-treated *Gcn2*^{-/-} mice compared with WT mice, although there was more inflammation in acute CCl₄-treated WT than in *Gcn2*^{-/-} mice (Figure 3b). Pathological scores based on the Brunt classification validated these findings. The necrosis score was 2.5 ± 0.26 and 1.5 ± 0.34 for CCl₄-treated *Gcn2*^{-/-} mice and WT mice, respectively. The inflammation score was 1.1 ± 0.22 and 2.0 ± 0.35 for CCl₄-treated *Gcn2*^{-/-} and WT mice, respectively.

Chronic CCl₄ Injection Causes More Liver Injury to *Gcn2*^{-/-} than to WT Mice

Chronic CCl₄ treatment increased ALT and AST activities 3.3 and 3.2-fold, respectively, in *Gcn2*^{-/-} compared with WT mice (Figure 4a).

H&E staining showed more necrosis and inflammation in *Gcn2*^{-/-} compared with WT mice (Figure 4b). The necrosis scores were 2.9 ± 0.1 and 2.4 ± 0.4 for CCl₄-treated *Gcn2*^{-/-} and WT mice, respectively; the inflammation scores for CCl₄-treated *Gcn2*^{-/-} and WT mice were 2.1 ± 0.7 and 1.7 ± 0.2 , respectively.

To evaluate whether the higher sensitivity of *Gcn2*^{-/-} mice to liver injury was owing to different levels of CYP2E2, protein levels of this enzyme were determined by western blot in liver samples of WT and *Gcn2*^{-/-} mice. No difference was found (data not shown).

Chronic CCl₄ Treatment Increases Collagen-I in *Gcn2*^{-/-} Compared with WT Mice

As shown in Figure 5a, *Gcn2*^{-/-} control mice had increased basal collagenous proteins compared with WT mice. Liver sections from CCl₄-treated *Gcn2*^{-/-} mice showed more collagenous proteins deposition compared with WT mice.

Collagen type I deposition was greater in CCl₄-treated *Gcn2*^{-/-} mice compared with WT mice (Figure 5b). Finally, mRNA levels for *Col1a1* and *Col1a2* were analyzed. Both *col1a1* and *col1a2* mRNAs showed a significant increase in CCl₄-treated *Gcn2*^{-/-} mice compared with WT mice (Figure 6).

DISCUSSION

Liver injury triggers a pathological response characterized by tissue damage and inflammatory infiltration leading to hepatic fibrosis. Collagen type I production by activated HSC contributes to the development of liver fibrosis. TGF- β ,¹⁵ reactive oxygen species^{16,17} and acetaldehyde¹⁷ regulate collagen type I production by HSC via transcriptional and translational mechanisms. Amino acid levels also exert

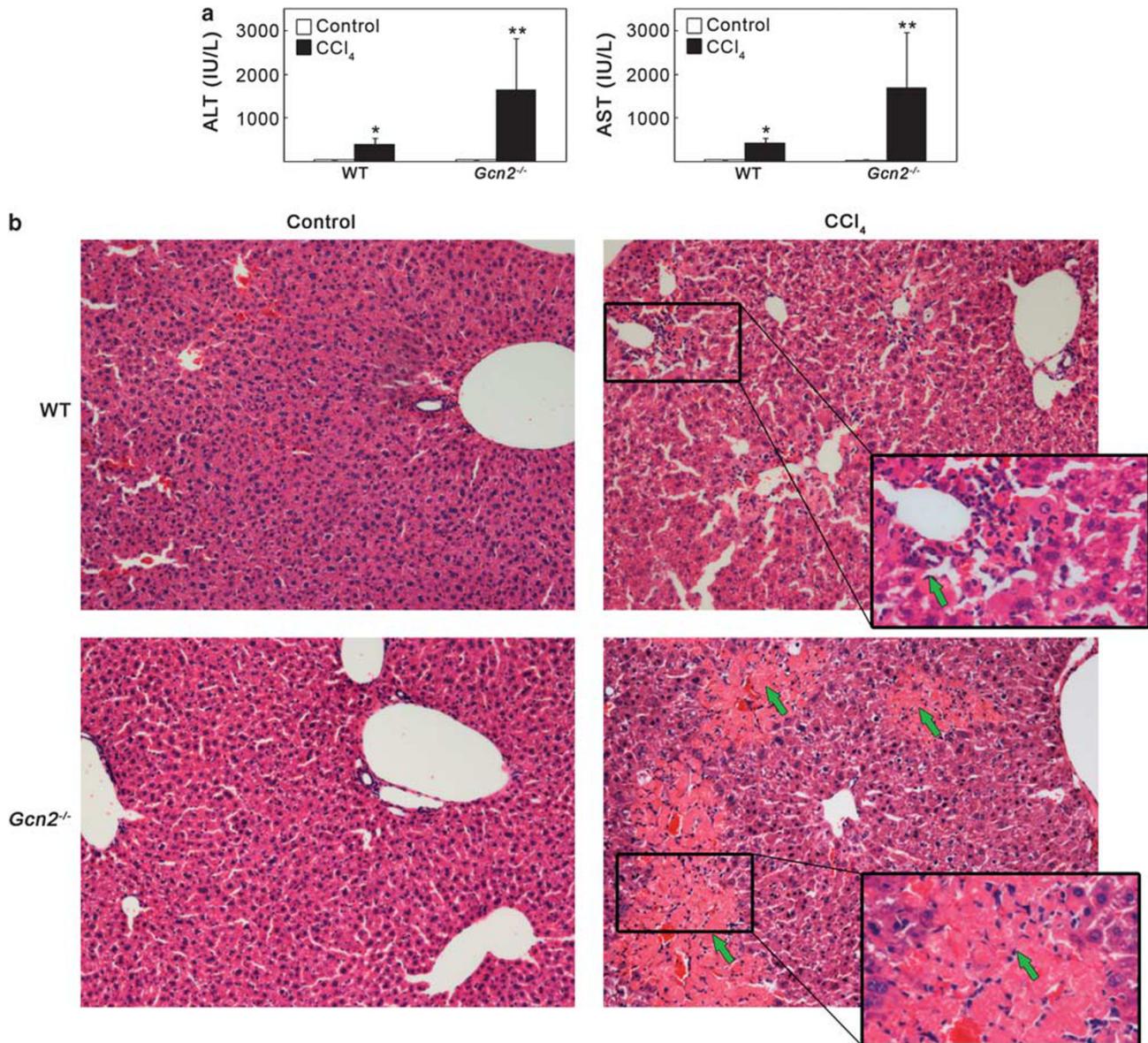


Figure 3 Analysis of the effects of acute carbon tetrachloride (CCl₄) treatment on *Gcn2*^{-/-} mice. Wild-type (WT) or *Gcn2*^{-/-} mice were injected with CCl₄ (0.05 ml/kg of body weight) or with mineral oil (MO) for the controls, and killed 48 h later. (a) Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in blood samples collected from retro-orbital vein 24 h after treatment. Each bar represents the mean ± s.d. of IU/l compared with control of at least triplicate data from four independent experiments (**P* < 0.05; ***P* < 0.01). (b) Liver sections of WT and *Gcn2*^{-/-} mice treated with either MO or CCl₄ and stained with hematoxylin and eosin. Green arrows point to necrosis (× 400).

important regulatory effects in HSC. Studies from our group demonstrated that increased levels of the essential amino acid leucine had a profibrogenic effect on HSC, stimulating selective translation of *coll1a1* via ROS production and eIF4E phosphorylation.^{20,21} Previous results obtained in fibroblasts indicated that amino acid deprivation could be antifibrogenic,^{22,23} and our own data in HSC suggested this possibility, as histidine deprivation decreased hydrogen peroxide,¹⁴ a key activator of collagen type I production.

In the present study we analyzed the effects of GCN2 activation on collagen type I production by HSC. On the

basis of data from other groups and our own, GCN2 activation was induced by incubation of HSC in the absence of histidine, an essential amino acid. There was a decrease in both collagen type I protein and mRNA levels of *COL1A1* and *COL1A2* mRNAs in response to GCN2 activation by histidine deprivation. As changes in metalloproteinase (MMP) activity or in the levels of its inhibitor TIMP1 could be involved in diminishing collagen-I deposition, protein levels of MMP1, MMP2, MMP9 and MMP13 and TIMP1 were analyzed in primary human HSC treated with histidine-free medium. None of these proteins changed in response to GCN2 activation (data not shown).

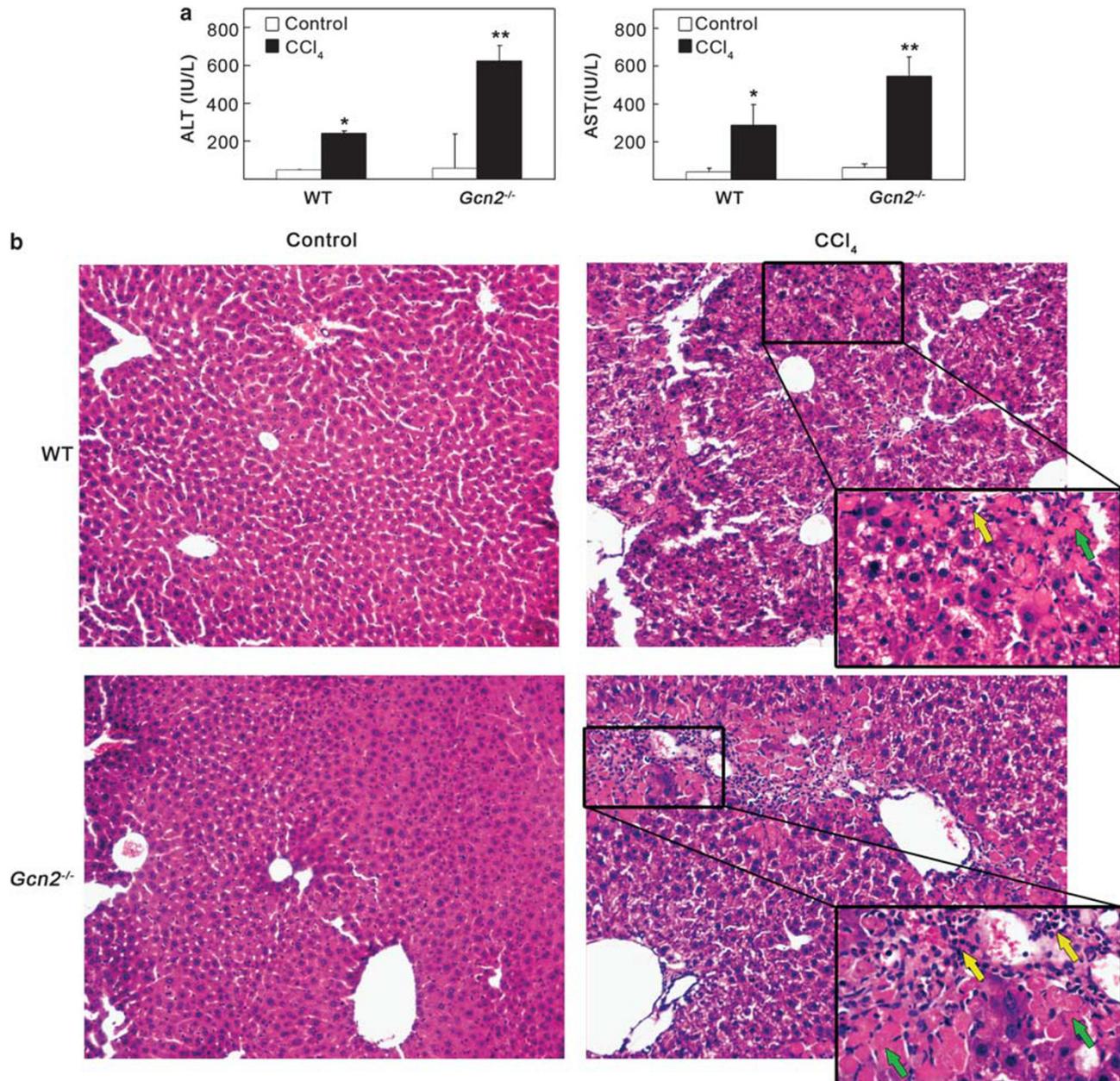


Figure 4 Analysis of the effects of chronic carbon tetrachloride (CCl₄) treatment on *Gcn2*^{-/-} mice. Wild-type (WT) or *Gcn2*^{-/-} mice were injected with either mineral oil (MO) for the controls or 0.05 ml/kg of body weight of CCl₄ twice a week for 1 month. Mice were killed 48 h after the last injection. Blood was collected from retro-orbital vein. **(a)** Serum analysis of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) of WT and *Gcn2*^{-/-} mice. Each bar represents the mean \pm s.d. of IU/l compared with control of at least triplicate data from four independent experiments (* P < 0.05; ** P < 0.01). **(b)** Liver sections of WT and *Gcn2*^{-/-} mice treated with either MO or CCl₄ stained with hematoxylin and eosin. Green arrows point to necrosis areas and yellow arrows point to inflammation areas, characterized by lymphocyte infiltration (\times 400).

GCN2 activation induced by amino acid restriction²⁴ or tRNA accumulation²⁵ has been shown to cause autophagy in different cell types. The role of autophagy in liver injury is complex. On the one hand, autophagy has been shown to protect against liver injury from alcohol and oxidative stress.^{26,27} On the other, autophagy contributes to activation of HSC, a key process leading to hepatic fibrosis in HSC.²⁸ In our system, activation of GCN by histidine

deprivation failed to induce autophagy in HSC and had a protective effect, counteracting the loss of viability caused by autophagy inhibitors. These results suggest that GCN2 exerts in HSC antifibrogenic and protective effects, which are unrelated to autophagy.

Although further research should establish in greater detail the molecular mechanisms involved in the downregulation of collagen type I by GCN2 activation, there are evidence from

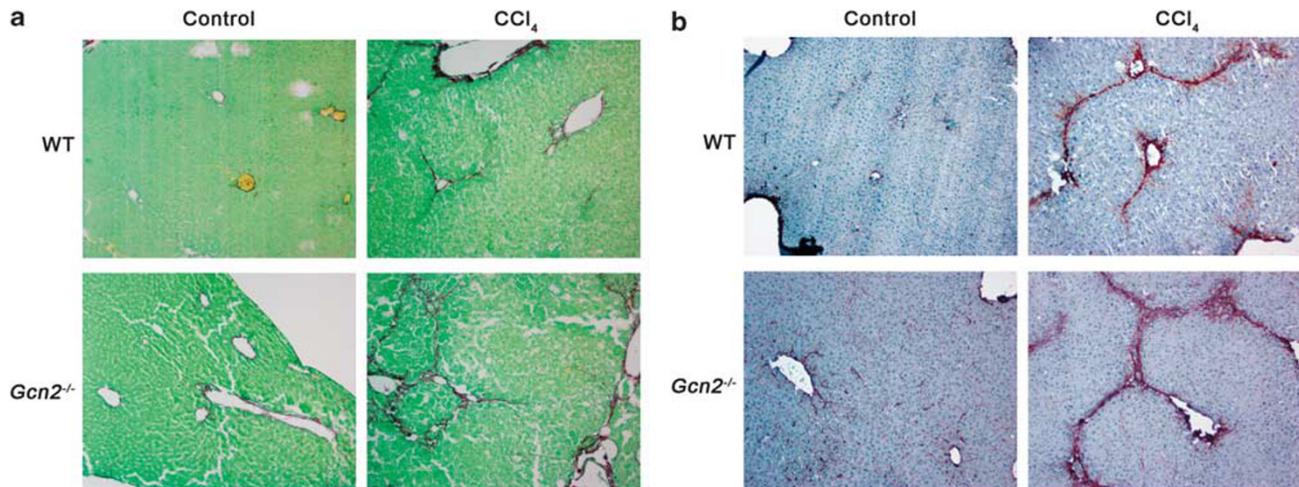


Figure 5 Collagen content on liver sections of chronic carbon tetrachloride (CCl₄)-treated *Gcn2*^{-/-} mice. Wild-type (WT) or *Gcn2*^{-/-} mice were injected with either mineral oil (MO) for the controls or 0.05 ml/kg of body weight of CCl₄ twice a week during one month. Forty-eight hours after the last injection mice were killed. (a) Liver sections stained with fast green/sirius red that specifically stains collagen fibers in red. (b) Collagen-I immunostaining carried out using a monoclonal antibody.

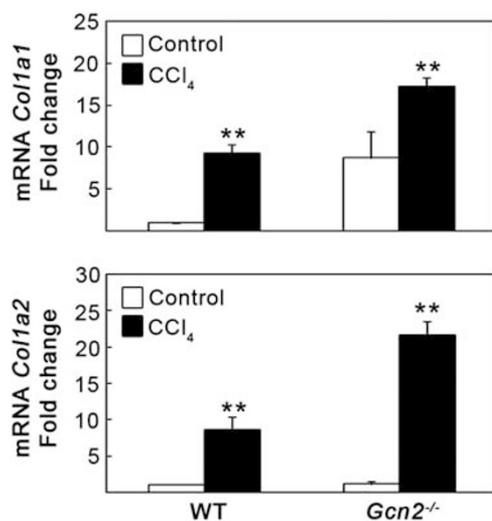


Figure 6 Collagen type I mRNA levels in chronic carbon tetrachloride (CCl₄)-treated *Gcn2*^{-/-} mice. Wild-type (WT) or *Gcn2*^{-/-} mice were injected with either mineral oil (MO) for the controls or 0.05 ml/kg of body weight of CCl₄ twice a week during 1 month. Mice were killed 48 h after the last injection. Analysis of *Col1a1* and *Col1a2* mRNA levels was carried out by quantitative PCR in liver homogenates. Values were referred to basal levels of *Gadph* mRNA. Controls represent non-treated mice and were given the value of one. Each bar represents the mean ± s.d. of mRNA fold change compared with controls (***P* < 0.01).

the literature pointing to the possibility of collagen-I being transcriptionally regulated. Amino acid deficiency typically activates ATF-4 and other members of the leucine-zipper family such as CHOP and C/EBP-β via GCN2.^{3,4} Some members of this family of transcription factors have been shown to participate either in the upregulation^{15,29} or in the downregulation^{30,31} of the *colla1* and *colla2* genes in rat HSC, and other cells.³² Therefore, downregulation of *colla1*

and *colla2* genes could be part of the modification of gene expression mediated by GCN2 to cope with stress situations.

The hepatoprotective role of GCN2 was further established when the effects caused by acute and chronic injury in mice lacking GCN2 were evaluated. Although *Gcn2*^{-/-} mice have been characterized in other experimental approaches such as their response to amino acid deficiency,¹⁰⁻¹² to our knowledge this is the first study evaluating the ability of these mice to react to stress not directly related to nutritional conditions. After acute and chronic CCl₄-treatment, *Gcn2*^{-/-} mice exhibited increased ALT and AST activities, more necrotic areas and, in the case of chronic treatment, enhanced inflammatory cell infiltration and collagen type I production. Control *Gcn2*^{-/-} mice showed greater collagen type I deposition compared with control WT animals, suggesting that lack of GCN2 induces by itself a fibrogenic effect that could be further enhanced by the response to chronic liver injury. Thus, GCN2 could be involved in the regulation of basal collagen type I production in the absence of liver injury.

The reason(s) why *Gcn2*^{-/-} mice are more sensitive to liver damage remain to be established. Recent studies have shown a regulation of hepatic cytochrome P450 (CYP) 3A because of activation of GCN2 by proteasome inhibition,³³ pointing to a possible alteration on members of the CYP family on *Gcn2*^{-/-} animals. However, analysis on WT and *Gcn2*^{-/-} mice of hepatic levels of CYP2E2, the main responsible for CCl₄ metabolism, did not show any difference. Data from our own group and others indicate that oxidative stress could be involved in the higher susceptibility of *Gcn2*^{-/-} mice to liver injury. *Gcn2*^{-/-} mice present decreased levels of glutathione peroxidase in the liver¹³ and we found GCN2 activation by histidine deprivation to have antioxidant effects on hepatocytes and HSC, causing a decrease in intracellular hydrogen peroxide levels.¹⁴

Therefore, GCN2-deficient mice would lack a protective pathway than can be triggered by different stimuli and this could contribute to their being more sensitive to liver damage.

In summary, our results indicate that GCN2 can be an important regulator of the liver injury and fibrosis in the acute and chronic setting. Thus, GCN2 may be a potential target for antifibrotic or hepatoprotective strategies.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

ACKNOWLEDGEMENTS

This work was supported by Fondo de Investigaciones Sanitarias (FIS) grant 00/0143 (to MI, EA, MRG and MJLZ). US Public Health Service Grants 5ROI AA 017733, 5ROI AA017733-0151, 5P20 AA017067, 5P20 AA017067-0151 and AA017067-0351 from the National Institute on Alcohol Abuse and Alcoholism (to NN). EA was supported by fellowships from Gobierno de Navarra and Bancaja.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

- Wek SA, Zhu S, Wek RC. The histidyl-tRNA synthetase-related sequence in the eIF-2 alpha protein kinase GCN2 interacts with tRNA and is required for activation in response to starvation for different amino acids. *Mol Cell Biol* 1995;15:4497–4506.
- Dong J, Qiu H, Garcia-Barrio M, *et al*. Uncharged tRNA activates GCN2 by displacing the protein kinase moiety from a bipartite tRNA-binding domain. *Mol Cell* 2000;6:269–279.
- Kilberg MS, Shan J, Su N. ATF4-dependent transcription mediates signaling of amino acid limitation. *Trends Endocrinol Metab* 2009;20:436–443.
- Kimball SR, Jefferson LS. Role of amino acids in the translational control of protein synthesis in mammals. *Semin Cell Dev Biol* 2005;16:21–27.
- Berlanga JJ, Ventoso I, Harding HP, *et al*. Antiviral effect of the mammalian translation initiation factor 2alpha kinase GCN2 against RNA viruses. *EMBO J* 2006;25:1730–1740.
- Sundrud MS, Koralov SB, Feuerer M, *et al*. Halofuginone inhibits TH17 cell differentiation by activating the amino acid starvation response. *Science* 2009;324:1334–1338.
- Deng J, Harding HP, Raught B, *et al*. Activation of GCN2 in UV-irradiated cells inhibits translation. *Curr Biol* 2002;12:1279–1286.
- Parker SH, Parker TA, George KS, *et al*. The roles of translation initiation regulation in ultraviolet light-induced apoptosis. *Mol Cell Biochem* 2006;293:173–181.
- Cai Q, Brooks HL. Phosphorylation of eIF2 α via the general control kinase, GCN2, modulates the ability of renal medullary cells to survive high urea stress. *Am J Physiol Renal Physiol* 2011;301:F1202–F1207.
- Anthony TG, McDaniel BJ, Byerley RL, *et al*. Preservation of liver protein synthesis during dietary leucine deprivation occurs at the expense of skeletal muscle mass in mice deleted for eIF2 kinase GCN2. *J Biol Chem* 2004;279:36553–36561.
- Guo F, Cavener DR. The GCN2 eIF2alpha kinase regulates fatty-acid homeostasis in the liver during deprivation of an essential amino acid. *Cell Metab* 2007;5:103–114.
- Xiao F, Huang Z, Li H, *et al*. Leucine deprivation increases hepatic insulin sensitivity via GCN2/mTOR/S6K1 and AMPK pathways. *Diabetes* 2011;60:746–756.
- Chaveroux C, Lambert-Langlais S, Parry L, *et al*. Identification of GCN2 as new redox regulator for oxidative stress prevention in vivo. *Biochem Biophys Res Commun* 2011;415:120–124.
- Arriazu E, Pérez de Obanos MP, López-Zabalza MJ, *et al*. Amino acid deprivation decreases intracellular levels of reactive oxygen species in hepatic stellate cells. *Cell Physiol Biochem* 2010;26:281–290.
- García-Trevijano ER, Iraburu MJ, Fontana L, *et al*. Transforming growth factor beta1 induces the expression of alpha1(I) procollagen mRNA by a hydrogen peroxide-C/EBPbeta-dependent mechanism in rat hepatic stellate cells. *Hepatology* 1999;29:960–970.
- Nieto N, Friedman SL, Cederbaum AI. Cytochrome P450 2E1-derived reactive oxygen species mediate paracrine stimulation of collagen I protein synthesis by hepatic stellate cells. *J Biol. Chem* 2002;277:9853–9864.
- Greenwel P, Domínguez-Rosales JA, Mavi G, *et al*. Hydrogen peroxide: a link between acetaldehyde-elicited alpha1(I) collagen gene up-regulation and oxidative stress in mouse hepatic stellate cells. *Hepatology* 2000;31:109–116.
- Xu L, Hui AY, Albanis E, *et al*. Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis. *Gut* 2005;54:142–151.
- López-De León A, Rojkind M. A simple micromethod for collagen and total protein determination in formalin-fixed paraffin-embedded sections. *J Histochem Cytochem* 1985;33:737–743.
- Pérez de Obanos MP, López-Zabalza MJ, Prieto J, *et al*. Leucine stimulates procollagen alpha1(I) translation on hepatic stellate cells through ERK and PI3K/Akt/mTOR activation. *J Cell Physiol* 2006;209:580–586.
- Pérez de Obanos MP, López-Zabalza MJ, Arriazu E, *et al*. Reactive oxygen species (ROS) mediate the effects of leucine on translation regulation and type I collagen production in hepatic stellate cells. *Biochem Biophys Acta* 2007;1773:1681–1688.
- Rishikof DC, Ricupero DA, Poliks CF, *et al*. Amino acid availability regulates type I procollagen accumulation in human lung fibroblasts. *J Cell Biochem* 1999;75:130–137.
- Krupsky M, Kuang PP, Goldstein RH. Regulation of type I collagen mRNA by amino acid deprivation in human lung fibroblasts. *J Biol Chem* 1997;272:13864–13868.
- Tallóczy Z, Jiang W, Virgin 4th HW, *et al*. Regulation of starvation- and virus-induced autophagy by the eIF2alpha kinase signaling pathway. *Proc Natl Acad Sci USA* 2002;99:190–195.
- Huynh LN, Thangavel M, Chen T, *et al*. Linking tRNA localization with activation of nutritional stress responses. *Cell Cycle* 2010;9:3112–3118.
- Ding WX, Li M, Chen X, *et al*. Autophagy reduces acute ethanol-induced hepatotoxicity and steatosis in mice. *Gastroenterology* 2010;139:1740–1752.
- Czaja MJ. Functions of autophagy in hepatic and pancreatic physiology and disease. *Gastroenterology* 2011;140:1895–1908.
- Hernández-Gea V, Ghiassi-Nejad Z, Rozenfeld R, *et al*. Autophagy releases lipid that promotes fibrogenesis by activated hepatic stellate cells in mice and in human tissues. *Gastroenterology* 2012;142:938–946.
- Attard FA, Wang L, Potter JJ, *et al*. CCAAT/enhancer binding protein beta mediates the activation of the murine alpha1(I) collagen promoter by acetaldehyde. *Arch Biochem Biophys* 2000;378:57–64.
- Greenwel P, Tanaka S, Penkov D, *et al*. Tumor necrosis factor alpha inhibits type I collagen synthesis through repressive CCAAT/enhancer-binding proteins. *Mol Cell Biol* 2000;20:912–918.
- Iraburu MJ, Domínguez-Rosales JA, Fontana L, *et al*. Tumor necrosis factor alpha down-regulates expression of the alpha1(I) collagen gene in rat hepatic stellate cells through a p20C/EBPbeta- and C/EBPdelta-dependent mechanism. *Hepatology* 2000;31:1086–1093.
- Ghosh AK, Bhattacharyya S, Mori Y, *et al*. Inhibition of collagen gene expression by interferon-gamma: novel role of the CCAAT/enhancer binding protein beta (C/EBPbeta). *J Cell Physiol* 2006;207:251–260.
- Acharya P, Engel JC, Correia MA. Hepatic CYP3A suppression by high concentrations of proteasomal inhibitors: a consequence of endoplasmic reticulum (ER) stress induction, activation of RNA-dependent protein kinase-like ER-bound eukaryotic initiation factor 2alpha (eIF2alpha)-kinase (PERK) and general control nonderepressible-2 eIF2alpha kinase (GCN2), and global translational shutdown. *Mol Pharmacol* 2009;76:503–515.