Biochemical Pharmacology, Vol. 53, pp. 637–641, 1997.



ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(96)00865-9

# Glutathione Regulation in Rat Hepatic Stellate Cells

## COMPARATIVE STUDIES IN PRIMARY CULTURE AND IN LIVER INJURY *IN VIVO**

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**ABSTRACT.** Lipid peroxidation accompanies many types of liver injury and is believed to promote liver fibrosis. Cellular antioxidants are likely to play an important role in modulating this process; however, little is known about antioxidants in hepatic stellate cells, the major collagen-producing cells of liver. In this study, we measured glutathione homeostasis in stellate cells isolated from rat liver. Glutathione, measured by HPLC in stellate cell homogenates, increased significantly when the cells were plated in primary culture. The rise in glutathione coincided with pretranslational up-regulation of the synthetic enzyme γ-glutamylcysteine synthetase (GCS). Additional experiments were performed to determine whether stellate cell glutathione and GCS are similarly altered during liver injury *in vivo*. Two types of hepatic insults, namely, bile duct ligation (8 days) and carbon tetrachloride treatment (4 weeks), failed to provoke an increase in either stellate cell glutathione or GCS. This disparate behavior of stellate cells in culture and *in vivo* is unusual; the data suggest that stellate cells might respond variably to oxidants depending on their glutathione status. BIOCHEM PHARMACOL 53;5:637–641, 1997. © 1997 Elsevier Science Inc.

**KEY WORDS.** liver; fibrosis; oxidant stress; antioxidant; lipid peroxidation

Oxidant stress plays an important role in many types of acute liver injury and is being increasingly implicated as a cause of liver fibrosis [1, 2]. The exact mechanism whereby oxidant stress leads to fibrosis is unknown; however, some suggest that oxidants or their by-products act directly upon liver cells to stimulate collagen synthesis [3, 4]. The principal collagen-producing cells of liver are hepatic stellate cells. They are of mesenchymal origin, and bear resemblance to capillary pericytes. Stellate cells from normal liver produce very little collagen, but in the setting of liver injury they transform into myofibroblast-like cells with a markedly increased capacity for collagen synthesis [5].

In an effort to determine whether compounds produced during oxidative liver injury have a direct effect on stellate cells, we have added various aldehydes and lipid hydroperoxides to stellate cells in primary culture and measured their ability to stimulate collagen synthesis and gene expression. We find that acetaldehyde and malondialdehyde have little or no effect on stellate cells, particularly during the first week of primary culture [6, 7]. This contrasts with other studies, in which these same compounds induce significant increases in collagen synthesis by cultured fibroblasts [8, 9]. One means by which stellate cells could resist the adverse effects of lipid hydroperoxides is through intracellular glutathione. The antioxidant is of particular importance in view of its central role in the detoxification of lipid hydroperoxides [10]. Glutathione has never been measured in stellate cells; the objective of this study was to monitor glutathione homeostasis in these specialized liver cells, and to determine whether glutathione regulation in stellate cell culture parallels its regulation during fibrotic liver injury *in vivo*.

## MATERIALS AND METHODS

### *Stellate Cell Isolation and Culture*

Stellate cells were isolated from the livers of adult male Sprague-Dawley rats (500–600 g) by *in situ* perfusion with pronase and collagenase. Crude cell suspensions were purified to 97% homogeneity by density gradient centrifugation [11]. In experiments calling for fresh cell isolates, washed cell pellets were used immediately for biochemical or molecular analysis. For culture studies, stellate cells were suspended in Medium 199 containing 10% calf serum and 10% horse serum and plated on uncoated tissue culture plastic at a density of $1.5 \times 10^5/cm^2$. Culture medium was replenished daily.

### *Induction of Fibrotic Liver Injury*

Two independent procedures were used to induce liver fibrosis in rats. One group of animals underwent laparotomy

* Portions of this work appeared earlier in abstract form (*Gastroenterology* **106:** A936, 1994).

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Received 7 August 1996; accepted 26 September 1996.

with complete ligation of the common bile duct. A second group of rats received carbon tetrachloride (0.5 mL/kg) by gavage twice weekly for 4 weeks. Bile duct-ligated rats were killed 8 days postoperatively for isolation of hepatic stellate cells. $CCl_4$-treated rats were killed for cell isolation at 5 weeks, 7 days after the last dose of toxicant.

### *Measurement of Glutathione*

Stellate cell glutathione was measured by HPLC using a modification of previously described assays [12, 13]. Briefly, fresh isolates or cell culture monolayers were washed with L-15 salts, sonicated in ice-cold distilled water, and acidified with trichloroacetic acid to a final concentration of 0.4 M. Precipitated protein was removed by centrifugation (15,000 *g* for 3 min at 4°). Supernatants were derivatized with orthophthalaldehyde prior to HPLC separation. Total cellular glutathione (GSH and acid-soluble GSSX) was quantitated as described [14] and expressed as nanomoles glutathione per microgram of DNA or per milligram of protein.

### *Kinetics of Glutathione Synthesis*

At 2 and 7 days of primary culture, stellate cells were depleted acutely of glutathione by addition of 2 mM DEM[‖] (Sigma Chemical Co., St. Louis, MO). After 15 min, the cells were washed thoroughly and replenished with Medium 199 containing supplemental amounts of glutathione-precursor amino acids (5 mM each of L-glutamic acid, L-glycine, and L-cysteine). Stellate cell glutathione content was monitored at 2, 4, 6, and 24 hr after DEM washout.

### *Measurement of GCS Activity*

GCS activity was measured in stellate cell homogenates by HPLC. Fresh cell isolates or cell culture monolayers were sonicated in cold PBS; insoluble material was pelleted by centrifugation for 30 min at 16,000 *g*. GCS activity was determined by measuring the amount of γ-glutamylcysteine produced during incubation of the supernatant with 1 mM L-cysteine, 5 mM magnesium-ATP, 0.15 M Tris (pH 8.0) for 20 min. γ-Glutamylcysteine was quantitated as described for glutathione [14]. GCS activity was measured in Units (micromoles γ-glutamylcysteine generated per minute) and expressed in milliUnits per milligram of soluble protein.

### *Measurement of GCS mRNA*

Total RNA was extracted from stellate cells using guanidine isothiocyanate. GCS mRNA was measured by RNase protection, using α-[$^{32}$P]-labeled cRNA ([α-$^{32}$P]CTP, > 800 Ci/mmol, Amersham Corp., Arlington Heights, IL) transcribed from a 390-bp cDNA encoding rat GCS (gift from S. Lu, University of Southern California). Autoradiographic signals were quantitated by scanning densitometry (Hoefer Scientific Instruments, San Francisco, CA). Signals for GCS were normalized to a control RNA signal encoding the ribosomal protein S14 [15].

## RESULTS

Stellate cells isolated from normal liver contained 0.15 nmol glutathione/μg cellular DNA. This amount of glutathione is comparable to that found previously in other cells with myofibroblastic phenotype [16–18]. When stellate cells were plated in primary culture, their glutathione content increased significantly; at the end of 1 week in culture, stellate cell glutathione concentrations were 7 times higher than that measured in fresh cell isolates (Fig. 1). One finding of note was that glutathione increased progressively over a 6-day interval in culture (Fig. 1). This was true despite the presence of adequate glutathione precursors in the culture medium at all times.

Glutathione synthesis was measured in cultured stellate cells by first depleting glutathione stores with DEM and monitoring their reappearance after addition of precursor

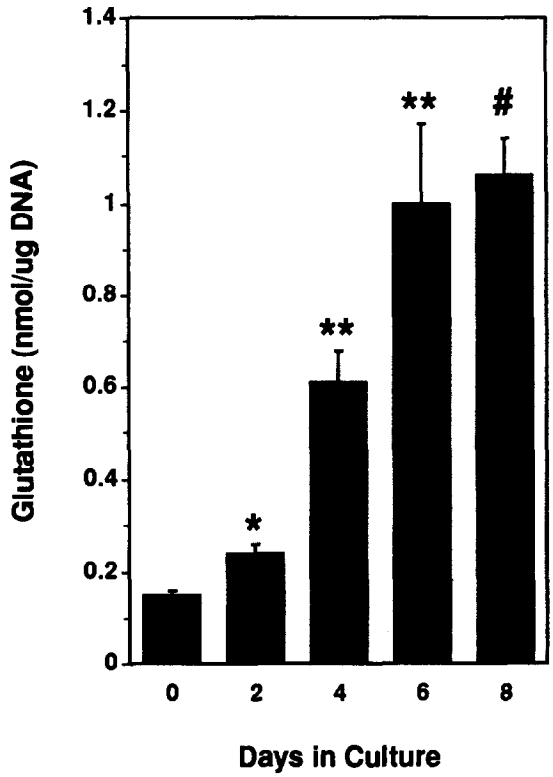


**FIG. 1. Stellate cell glutathione as a function of time in culture. Stellate cells were isolated from normal rat liver and plated in primary culture for various intervals. Total glutathione was measured in cell homogenates by HPLC as described in Materials and Methods. Data represent means ± SEM for N = 4. Key: (*)$P < 0.05$ vs day 0; (**) $P < 0.01$ vs day 0; (#) $P < 0.001$ vs day 0.**

‖ *Abbreviations:* DEM, diethylmaleate; and GCS, γ-glutamylcysteine synthetase.

amino acids. Figure 2 illustrates that at both 2 and 7 days of culture stellate cells resynthesized their basal levels of glutathione within 4 hr. Cells in early culture, however, exhibited a slower rate of glutathione synthesis and achieved lower plateau levels of glutathione than cells in later culture; this strongly suggested that during the 1-week culture interval, stellate cells increased their capacity for glutathione synthesis. We investigated this possibility by measuring GCS in stellate cell homogenates before and after 7 days of culture. GCS activity measured 1.55 ± 0.44 mU/mg protein on day 0, and increased to 7.23 ± 1.90 mU/mg protein on day 7 ($P < 0.05$; N = 3). This 4.7-fold rise in enzyme activity was accompanied by a 4-fold increase in GCS mRNA over the same interval (Fig. 3).

The fact that stellate cell glutathione and GCS activity increased significantly during primary culture led us to question whether similar changes occur during liver injury *in vivo*. This postulate was based on numerous previous reports that stellate cell alterations in primary culture mimic those of "activation" in fibrotic liver disease (reviewed in Ref. 5). To address this question, we first induced liver injury in rats by bile duct ligation or $CCl_4$ treatment (see Materials and Methods). Both insults induce lipid peroxidation [19, 20] and lead to liver fibrosis [21, 22]. After 8 days of bile duct ligation or 4 weeks of carbon tetrachloride, all rats had liver fibrosis; however, when glutathione and GCS activity were measured in stellate cells isolated from these fibrotic organs, neither was increased above control levels (Table 1). GCS activity actually decreased in stellate cells from bile duct-ligated rats (Table 1); this reduction in enzyme activity was not paralleled by a decrease in GCS mRNA (Fig. 4). Indeed, GCS mRNA remained constant in stellate cells after both bile duct ligation and carbon tetrachloride administration (Fig. 4).

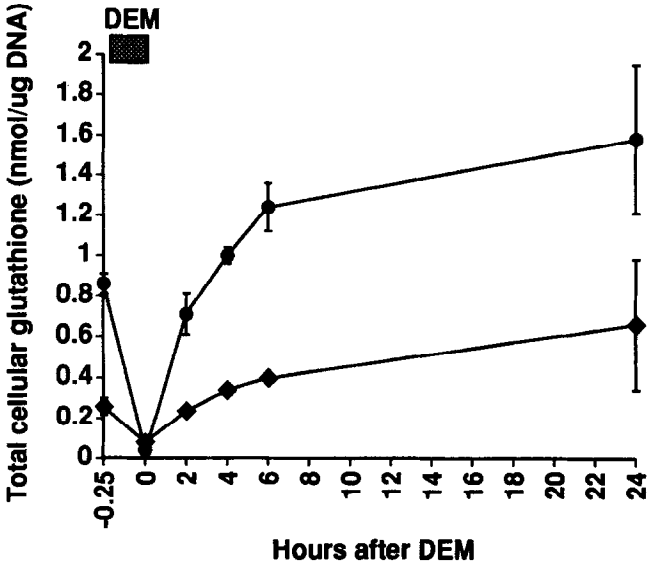


**FIG. 2. Glutathione synthesis by stellate cells after DEM treatment. Stellate cells on day 2 (♦) or day 7 (●) of primary culture were incubated with 2 mM DEM for 15 min (shaded box). The DEM was then removed and replaced with culture medium containing precursor amino acids for glutathione synthesis. Graph depicts total glutathione in stellate cells before and after DEM treatment.**

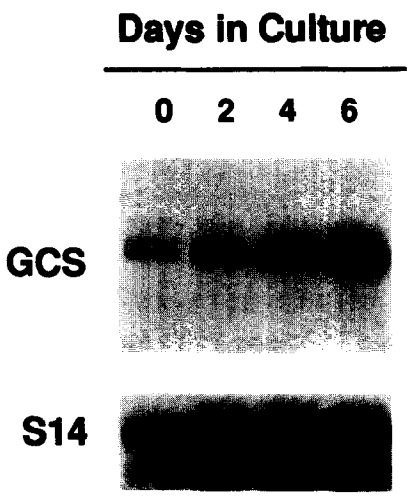


**FIG. 3. GCS mRNA in stellate cells. Stellate cells were isolated from normal rat liver and plated in primary culture for various intervals as shown. GCS mRNA was measured by RNase protection, along with S14 as an internal control. The autoradiograms indicate a progressive increase in GCS mRNA in stellate cells from day 0 to day 6 of culture. Densitometry readings for GCS mRNA, normalized to S14 and expressed as relative values, measured 1.0, 1.2, 1.9, and 3.9 for days 0, 2, 4, and 6, respectively.**

## DISCUSSION

Glutathione plays a central role in cellular defense against oxidant stress. This non-protein thiol reduces both hydrogen peroxide and organic peroxides, in reactions catalyzed by glutathione peroxidase and glutathione-S-transferases [23]. In the liver, studies of glutathione have focused primarily on hepatocytes because of their major role in glutathione synthesis and export [24]. In the current study, we examined glutathione homeostasis in hepatic stellate cells, because they are intimately involved in the pathogenesis of liver fibrosis and because fibrogenesis is being increasingly linked to hepatic oxidant stress [1, 2, 25, 26].

We found that certain conditions promote striking regulation of stellate cell glutathione. In particular, when the cells were plated in primary culture, their glutathione stores increased 7-fold above those in the intact liver. Stellate cell

**TABLE 1. Stellate cell glutathione and GCS activity***

| | Glutathione (nmol/mg protein) | GCS activity (mU/mg protein) |
|---|---|---|
| Normal (N = 4) | 9.3 ± 1.9 | 1.09 ± 0.25 |
| Bile duct-ligated (N = 6) | 9.6 ± 1.8 | 0.64 ± 0.12† |
| $CCl_4$-treated (N = 3) | 11.9 ± 1.6 | 1.71 ± 0.50 |

* Values are means ± SEM.
† $P < 0.05$, vs normal stellate cells.

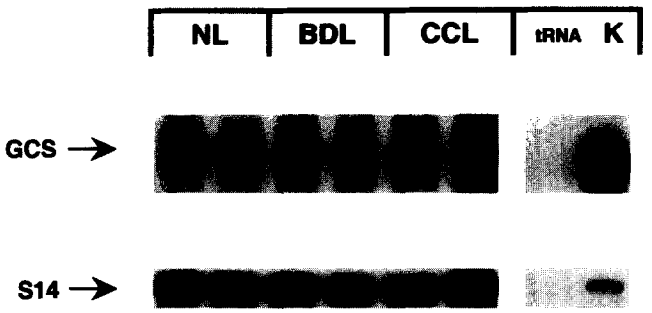


**FIG. 4. GCS mRNA in stellate cells from normal and injured liver *in vivo*. Autoradiograms depict GCS and S14 mRNA in stellate cells freshly isolated from normal rat liver (NL) or from livers injured by bile duct ligation (8 days; BDL) or carbon tetrachloride treatment (4 weeks; CCL). GCS mRNA in rat kidney is shown at the right as a positive control. Stellate cell signals were generated from 15 μg total RNA; those from kidney were generated from 5 μg total RNA. Densitometry readings for GCS mRNA, normalized to S14, measured 2.8 ± 0.2 for NL, 2.2 ± 0.1 for BDL, and 2.3 ± 0.04 for CCL ($P$ = NS; N = 3).**

glutathione rose gradually during primary culture; the slow pace of the increase is noteworthy, and provides some clue regarding its mechanism. Most cells are capable of replenishing acutely depleted glutathione stores within hours (Fig. 2). Stellate cell glutathione rose progressively over 6 days, which argues against replenishment of depleted stores and in favor of an induction of the enzymes that control glutathione synthesis. Specific experiments confirmed the latter hypothesis; they demonstrated that the glutathione synthetic enzyme GCS increased significantly in stellate cells during primary culture. Up-regulation of GCS in stellate cell culture occurred at the level of mRNA, and closely paralleled the rise in stellate cell glutathione.

In addition to alterations in glutathione and GCS, stellate cells undergo a number of other phenotypic changes during primary culture. They spread and proliferate [27], release a significant proportion of their vitamin A stores [28], express surface receptors for cytokines and growth factors [29, 30], acquire smooth muscle markers [31] and significantly increase their collagen synthesis and gene expression [27]. These culture-induced alterations are of special interest because they mimic changes that take place in stellate cells during fibrotic liver injury *in vivo* [5]. In the case of glutathione, however, culture-induced alterations were not reproduced during hepatic fibrogenesis *in vivo*; two independent hepatic insults, both of which caused histologic fibrosis, failed to induce any change in stellate cell glutathione content. We cannot exclude the possibility that in rats with liver injury, some stellate cell glutathione might have been consumed to detoxify endogenously produced lipid hydroperoxides. However, if this were the case, glutathione consumption should have been paralleled by an increase in glutathione synthesis. Our data do not confirm this; in fact, our measurements of GCS mRNA and activity strongly suggest that stellate cell glutathione synthesis either remains constant or decreases during experimental liver injury. The reason for the marked discrepancy in stellate cell glutathione regulation in cell culture and in fibrotic liver injury *in vivo* remains uncertain. However, in the case of bile duct ligation, the post-transcriptional decrease in GCS activity may be due to allosteric feedback inhibition of the enzyme by excess tissue glutathione, which has been postulated in whole liver [32].

The marked difference in glutathione homeostasis between stellate cells in culture and *in vivo* is itself noteworthy. It represents the first documentation of a culture-induced change in stellate cell phenotype that is not recapitulated *in vivo* by experimental liver injury. The findings are also of potential relevance to liver fibrosis, in view of its close association with hepatic lipid peroxidation [25, 26]. The precise role of lipid peroxidation in the pathogenesis of liver fibrosis has been debated, in part because cell culture studies have documented only modest increases in stellate cell collagen synthesis in response to aldehydes and lipid hydroperoxides [6, 7]. The present study sheds new light on this issue by suggesting that cultured stellate cells, because of their relatively high glutathione concentrations, may respond less intensely to exogenous oxidants than their counterparts *in vivo*. It will be critical to determine the degree to which glutathione modulates the stellate cell response to exogenous oxidants; studies of this nature are currently underway in our laboratory.

In summary, the present study indicates that hepatic stellate cells contain glutathione in amounts commensurate with other cells of myofibroblastic phenotype. Stellate cell glutathione content increased during primary culture, but did not increase during liver injury *in vivo*, even following insults that provoked oxidant stress and led to fibrosis. The discrepancy between glutathione regulation in culture and *in vivo* is unique; more importantly, the data suggest that studies of oxidant-induced stellate cell collagen synthesis, which have been performed in culture, may not accurately reflect the potential for these compounds to induce liver fibrosis *in vivo*. Further studies are required to determine the degree to which glutathione influences the stellate cell response to oxidant stress.

*This work was supported by grants from the U.S. Public Health Service (AA07810, DK26743, and DK50178) and the Alcoholic Beverage Medical Research Foundation. The authors thank John Chung, G. Benson Fenn, and Lisa Wells for technical assistance.*

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