Pergamon

Biochemical Pharmacology, Vol. 49, No. 2, pp. 219–225, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(94)00466-8

INHIBITION OF REDUCED GLUTATHIONE SYNTHESIS BY CYANOBACTERIAL ALKALOID CYLINDROSPERMOPSIN IN CULTURED RAT HEPATOCYTES

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(*Received* 10 *June* 1994; *accepted* 31 *August* 1994)

Abstract—Cylindrospermopsin (CY) is a naturally occurring alkaloid produced by the cyanobacterium *Cylindrospermopsis raciborskii*, which has been linked to an outbreak of hepatoenteritis in humans. We previously showed that CY is cytotoxic to primary cultures of rat hepatocytes and that CY lowers cell reduced glutathione (GSH) at nontoxic doses. Lower cell GSH also potentiates CY-induced cytotoxicity (Runnegar *et al.*, *Biochem Biophys Res Commun* **201**: 235–241, 1994). Our current work examined the mechanism of the fall in cell GSH induced by CY. We excluded several possible explanations for the loss in GSH, namely increased formation of oxidized glutathione (GSSG), increased GSH efflux, hidden forms of GSH, decreased GSH precursor availability, or decreased cellular ATP level. To address whether the fall in GSH was due to decreased GSH synthesis or increased GSH consumption, we examined the rate of fall in total GSH after 5 mM buthionine sulfoximine (BSO, an irreversible inhibitor of GSH synthesis) treatment. The rates of fall in total GSH (nmol/10^6 cells/hr) were 8.2 ± 2.5, 6.0 ± 1.7 and 5.9 ± 1.3 for control, 2.5 μM and 5 μM CY-pretreated cells, respectively. This suggests that the fall in GSH induced by CY was due to the inhibition of GSH synthesis rather than increased consumption, because in the latter case the rate of fall in GSH would have been accelerated by CY pretreatment. Furthermore, excess GSH precursor (20 mM *N*-acetylcysteine), which supported GSH synthesis in control cells, did not prevent the fall in GSH or toxicity induced by CY. Treatment of cells with the cytochrome P450 inhibitor α-naphthoflavone protected partially from CY-mediated toxicity and from the fall in cell GSH. Thus, it is likely that cytochrome P450 is involved in the metabolism of CY, and the metabolite(s) that is generated may be more toxic and/or potent in inhibiting GSH synthesis. Inhibition of GSH synthesis is most likely an important factor in the cytotoxicity of CY.

Key words: cylindrospermopsin; GSH synthesis; cyanobacterial toxin

Cyanobacteria often form blooms (dense growths) in waters where the nutrient concentration is elevated. Cyanobacteria produce a wide range of novel, biologically active natural products. Some of these compounds are toxic to animals and humans. The best characterized hepatotoxic compounds produced by cyanobacteria are cyclic peptides: microcystins [1]. The toxicity of these peptides is due to their specific inhibition of protein phosphatases 1 and 2A [2]. The hepatotoxicity of the cyanobacterium *Cylindrospermopsis raciborskii* and its implication as the causative agent of a severe outbreak of hepatoenteritis were described a number of years ago [3, 4]. Recently, it has been shown that an unusual alkaloid, CY,† is the hepatotoxic principle of *C. raciborskii* [5]. The mechanism of action at the cellular level of CY remains to be established. We have shown previously that primary cultures of rat hepatocytes provide a suitable model to probe the mode of action of this toxin [6]. We showed that CY is cytotoxic at micromolar concentrations and that toxicity is preceded by profound GSH depletion. Here we describe experiments in primary cultured hepatocytes aimed at explaining the mechanism of cell GSH depletion by the alkaloid CY.

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† Abbreviations: CY, cylindrospermopsin; GSH, reduced glutathione; GSSG, oxidized glutathione; DEM, diethyl maleate; LDH, lactate dehydrogenase; DME, Dulbecco's modified Eagle's medium; SAF, sulfur amino acid-free; FBS, fetal bovine serum; mBCl, monochlorobimane; CySSG, cysteine-GSH mixed disulfide; BSO, DL-buthionine-*S*-*R*-sulfoximine; NAC, *N*-acetylcysteine.

MATERIALS AND METHODS

Materials. GSH, collagenase (type IV), bovine serum albumin, NADPH, 5,5′-dithiobis (2-nitrobenzoic acid), GSH reductase, hydrocortisone, insulin, FBS, glutathione-*S*-transferase (GST), α-naphthoflavone, cimetidine, BSO, NAC and HEPES were purchased from the Sigma Chemical Co. (St. Louis, MO). SKF525A was a gift of Smith, Kline & French. DME/F12 medium and custom-made SAF

DME/F12 medium were purchased from Irvine Scientific (Irvine, CA). L-[^{35}S]Methionine (1120 Ci/mmol) was obtained from the Amersham Corp. (Arlington Heights, IL). mBCl was purchased from Molecular Probes, Inc. (Eugene, OR). All other reagents were of analytical grade and were obtained from commercial sources.

CY toxin preparation. *C. raciborskii* was grown in culture as previously described [4], and the aqueous extract was fractionated by successive gel filtration on Toyopearl HW40F with 1:1 MeOH:H_2O and reverse phase HPLC on C8 or C18 columns in 5% MeOH in H_2O to give white microcrystals of the cycloguanidine alkaloid CY. The isolation and chemical characterization of CY used in the work presented here were done by Dr. David Burgoyne in the laboratory of Dr. Richard Moore in the Chemistry Department of the University of Hawaii [5].

Animals. Male Sprague–Dawley rats (Harlan Laboratory Animals, Inc., San Diego, CA), weighing 260–350 g, were maintained on Purina rodent chow (Ralston Purina Co., St. Louis, MO) and water *ad lib.*

Cell culture. Isolation of hepatocytes was done aseptically according to the method of Moldeus *et al.* [7]. Initial cell viability was ≥90% as determined by 0.2% Trypan blue exclusion. The plating medium was DME/F12 containing high glucose (3151 mg/L), 10% FBS, insulin (1 μg/mL) and hydrocortisone (50 nM) and supplemented with excess methionine (1 mM). Cells (1–2 × 10^6) in 1.5 to 3.0 mL medium were plated on 6-well cluster plates (35 mm) and 60 × 15 mm dishes, precoated with rat tail collagen, and then incubated at 37° in 5% CO_2, 95% air. Two to three hours after plating, the medium was changed to remove FBS and dead, unattached cells. Cell attachment averaged ~60%.

The timing of CY addition for the various studies was such that all cell samples were processed for GSH and LDH measurements 20–22 hr after initial plating.

Measurement of GSH. Cultured cells were processed as previously described [6], and GSH was measured by the recycling method of Tietze [8]. GSH was expressed as nanomoles/10^6 cells.

Measurement of toxicity. Toxicity (cell lysis) due to CY was measured by the release of LDH from the cytosol into culture medium as previously described [6]. Percentage LDH release was the LDH activity in the medium as a percentage of total LDH (cellular + medium).

GSH accumulation experiments in cultured hepatocytes. The protocol for measuring GSH accumulation was similar to methods described previously [9]. Rat hepatocytes were cultured using 6-well cluster plates in standard DME/F12 medium. Dead and unattached cells were removed with a medium change 2 hr after plating. Subsequently, cells were treated with CY (2.5 and 5.0 μM) or vehicle for 12 hr, at which time GSH accumulation rates were measured. To measure GSH accumulation rates, cells were first treated with DEM (0.3 mM) to acutely deplete cellular GSH. Thirty minutes after DEM treatment, cells were washed free of DEM, replenished with fresh SAF medium supplemented with excess GSH precursors, methionine (1 mM), and serine (1 mM). Total GSH (cell + medium) was measured by the GSH recycling assay of Tietze [8] at 0, 30, 60 and 120 min after this change in medium. The rate of accumulation of total GSH (as determined by linear regression) after acute depletion with DEM was the GSH accumulation rate. Toxicity was measured by LDH release in the medium at the start of GSH accumulation experiments as well as during GSH accumulation.

GSH efflux. GSH efflux was estimated by using the percent of total GSH accumulated in the medium after 2 hr of incubation during the GSH accumulation experiment in the presence of SAF culture medium (see above). In some experiments, the molecular forms of intracellular and extracellular GSH, namely, GSH, GSSG, and CySSG, were determined by HPLC according to the method of Fariss and Reed [10].

Fate of labeled intracellular GSH. To follow the fate of the GSH, the protocol for GSH accumulation was adapted. After cells were treated with CY (5 μM) or vehicle for 12 hr and depleted of intracellular GSH with DEM as above, they were then incubated with SAF medium supplemented with 1 mM methionine and serine and [^{35}S]-methionine (1.5 to 2 μCi/mL, 2 mL/well). Cell and supernatant GSH mass as well as the molecular form of GSH were assayed by radio-HPLC according to the method of Fariss and Reed [10]. Total GSH mass was also checked by the GSH recycling assay of Tietze [8]. In general, the two methods were in close agreement, within 15% of each other.

Uptake of methionine. Cultured hepatocytes were treated with CY toxin (2.5 μM) or vehicle for 12 hr. The technique used for measuring sulfur amino acid uptake was adapted from Takada and Bannai [11] and described in detail previously [12]. Uptake was expressed as nanomoles of methionine per milligram of protein per minute.

Measurement of enzyme activities of GSH synthesis using mBCl. To measure GSH synthesis in cell lysates, cells were treated with CY (2.5 μM) or vehicle for 12 hr. Cells were then treated with DEM (0.3 mM) for 30 min to deplete cell GSH (from our previous experience this treatment will deplete cell GSH to 10–30% of original levels). This is to avoid a high background with mBCl and to eliminate feedback inhibition exerted by pre-existing GSH. At the end of a 30-min incubation with DEM, cells were washed and treated with digitonin (50 μM) for 20 min to permeabilize cell membrane. Digitonin-treated cell-free extract was obtained by pooling scraped cells from several plates and centrifuging at 1500 *g* for 20 min. The GSH synthesis rate from the precursors cysteine (0.1 mM), glutamate (10 mM) and glycine (10 mM) was measured in cell-free conditions according to previously described methods [12].

Cysteine assays. To measure cysteine concentrations from cultured hepatocytes, approximately 10^6 cells were collected by detaching cells with trypsin–EDTA and were centrifuged in a microfuge for 30 sec in order to remove the trypsin–EDTA solution. Cells were then treated with 5% perchloric acid to precipitate the proteins and

Table 1. Effects of CY on GSH accumulation in cultured rat hepatocytes

Treatment	Cell GSH before DEM (nmol/10^6 cells)	Cell GSH after DEM (nmol/10^6 cells)	GSH accumulation rate (nmol/10^6 cells/min)	LDH release* (% of total)
Control	131 ± 22	36 ± 7	0.49 ± 0.02	8.2 ± 1.5
2.5 μM CY	121 ± 26	30 ± 7	0.37 ± 0.01†	7.4 ± 1.5
5.0 μM CY	86 ± 17	18 ± 3†	0.27 ± 0.02‡	10.8 ± 2.3

Results are means ± SEM from 5–6 experiments. Cells were treated with CY for 12 hr and acutely depleted of GSH with DEM before accumulation of GSH was measured (see Materials and Methods). LDH released before the accumulation experiment was 11.1 ± 1.1, 10.9 ± 1.7 and 13.0 ± 1.6% of total for the control, 2.5 μM CY, and 5 μM CY treatment, respectively.

* LDH release during the 2-hr accumulation experiment.

† $P < 0.05$ vs control by ANOVA.

‡ $P < 0.05$ vs control and 2.5 μM CY by ANOVA.

were then centrifuged for 1 min in a microfuge. Supernatant cysteine levels were determined according to the method of Gaitonde [13].

ATP assay. Cellular ATP levels were determined by HPLC according to the method of Jones [14].

Statistical analysis. For cultured cells, each cell preparation was derived from one animal, and duplicate plates were used for each condition as well as time point. The mean of each duplicate from one experiment was considered N = 1, and the means of multiple experiments for a single treatment and control group were compared by paired Student's *t*-test (two comparisons). Two-tailed *t*-tests were used unless otherwise noted. When multiple comparisons were required (most of the results presented), one-way ANOVA was applied followed by Fisher's test. The criterion for significance was $P < 0.05$. Results are shown as means ± SEM of the number (N) of experiments indicated.

RESULTS

Mechanism of CY-induced fall in cell GSH. Our previous work showed that the effect of CY on cell GSH was concentration and time dependent [6]. At 2.5 μM CY, cell GSH fell significantly after 16 hr of incubation, but no toxicity was detected up to 18 hr. In contrast, 5.0 μM CY depleted cell GSH significantly after 10 hr of incubation, but significant toxicity was delayed for 6 more hours. Decreased cell GSH can result from increased GSH efflux, decreased GSH synthesis, increased GSH utilization (i.e. oxidant stress, conjugation), or a combination of these. Furthermore, there may be hidden forms of GSH (mixed disulfides such as CySSG) that would not be detected by the recycling assay [8]. To critically evaluate these possible explanations for the fall in GSH caused by CY, the rate of accumulation of total GSH was measured following 12 hr of treatment with CY (2.5 and 5 μM) or vehicle. GSH accumulation rates were measured following acute depletion of intracellular GSH with DEM, as described in Materials and Methods. As shown in Table 1, CY caused a concentration-dependent inhibition in GSH accumulation. This occurred in the absence of any sign of toxicity, as indicated by LDH release. There was no effect on GSH efflux (GSH efflux rate in percent of total GSH effluxed/hr: control = 4.1 ± 0.99, 2.5 μM CY = 3.43 ± 0.67 and 5.0 μM CY = 4.68 ± 1.36; results are means ± SEM from 3–4 experiments). To follow the fate of GSH and to examine the molecular forms of GSH in these accumulation experiments, in three separate experiments [^{35}S]methionine (1.5 to 2 μCi/mL, 2 mL/plate) was also added in the medium in order to label the intracellular GSH pool. Radio-HPLC was then used to examine the molecular forms of GSH intracellularly and in the supernatant at 0 and 2 hr. Cells that were pretreated with CY (5 μM) for 12 hr had lower intracellular and extracellular GSH mass in comparison to controls. The specific activities of total GSH after 2 hr of incubation with [^{35}S]-methionine were: controls = 3530 ± 290, CY pretreated = 2510 ± 460 cpm/nmol GSH (results are means ± SEM from 3 experiments, $P < 0.03$ between control and CY-pretreated cells). No increases in GSSG, CySSG or any extra peaks were detected following CY treatment in the cell or supernatant in comparison to controls. Thus, these experiments excluded the explanations that loss of GSH occurred as a result of increased GSH efflux, oxidation to GSSG, or formation of CySSG. However, if GSH had formed GSH conjugates with CY or its metabolite(s), they may not have been detected with the HPLC method of Fariss and Reed [10] under our experimental conditions.

To address whether it was decreased GSH synthesis or increased utilization that caused the decrease in GSH accumulation following CY treatment, parameters that are important in GSH synthesis in cultured rat hepatocytes were measured. In the GSH accumulation experiments, these parameters are (1) cysteine availability, which is determined largely by uptake of methionine and the activity of the enzymes involved in the *trans*-sulfuration pathway, (2) ATP level, and (3) the activity of the enzymes involved in the GSH synthesis pathway [15–18]. As shown in Table 2, these parameters, assessed by methionine uptake, cysteine levels, ATP levels, and enzyme activities of the GSH synthesis pathway (measured as GSH synthesis in cell-free extract with mBCl in the presence of excess

Table 2. Effect of CY on parameters of GSH synthesis in cultured rat hepatocytes

Parameters	Control	CY (2.5 μM)	N
Methionine uptake (nmol/mg/min)	3.0 ± 0.70	3.10 ± 0.56	3
Cysteine level (nmol/10^6 cells)	1.99 ± 0.30	1.80 ± 0.29	6
ATP level (nmol/10^6 cells)	26.02 ± 0.66	26.13 ± 2.61	4
GSH synthesis rates (nmol/mg protein/min)	0.52 ± 0.07	0.61 ± 0.07	4

Results are means ± SEM for N, the number of experiments. Cultured rat hepatocytes were treated with CY (2.5 μM) for 12 hr for measurement of methionine uptake, cysteine level and GSH synthesis rate and for 18 hr for measurement of ATP level. See Materials and Methods for details.

substrates cysteine, glutamate and glycine) were not altered by 2.5 μM CY, which inhibited GSH accumulation without causing toxicity (Table 1). Cystine uptake was not an issue since the medium contained only methionine as the sole sulfur amino acid precursor. Thus, there was no limitation in precursor or cofactor availability and in the enzymatic capacity to synthesize GSH. However, a concentration-dependent inhibition of GSH synthesis by CY and/or its metabolite(s) would be missed since there was a dilution factor of the cell-free protein extract (and therefore of CY) of ~500-fold in the assay (cytosolic proteins obtained from 5×10^5 cells were brought to a final assay volume of 2.5 mL, which represents a dilution of 500-fold, assuming 10^8 cells = 1 mL water content).

To directly examine whether the fall in GSH is due mainly to inhibition of GSH synthesis or increased GSH consumption, we compared the rate of fall in total GSH (cells plus medium) in control and CY-pretreated (2.5 and 5 μM for 12 hr) cells following treatment with BSO, an irreversible inhibitor of γ-glutamylcysteine synthetase, which is the rate-limiting step in GSH synthesis. After 12 hr of pretreatment with CY or vehicle, GSH levels (nmol/10^6 cells) were 101 ± 17, 87 ± 14, and 65 ± 8 for controls, 2.5 μM and 5 μM CY, respectively. As we had shown previously [6], 2.5 μM CY did not lower cell GSH at 12 hr but 5 μM CY did. There was no significant difference in cell lysis between the three groups. The medium was then changed to SAF + 1 mM methionine with or without 5 mM BSO for up to 5 hr. CY was not added back to the medium. If the GSH loss caused by CY is due largely to increased GSH consumption, we would expect the rate of fall after BSO to accelerate in cells pretreated with CY. As shown in Fig. 1, in the absence of BSO, total GSH (nmol/10^6 cells) remained unchanged in controls, whereas in cells pretreated with CY it fell by the end of the 5-hr incubation (control = 101 ± 5, 2.5 μM CY = 78 ± 3, 5 μM CY = 49 ± 1; $P < 0.05$ for both CY values as compared with control, by ANOVA). The rates of fall in the CY-pretreated cells in the absence of BSO were 1.8 ± 2.1 and 3.1 ± 1.5 nmol/10^6 cells/hr for 2.5 and 5 μM CY, respectively. After BSO treatment, the rate of fall in total GSH in control cells was

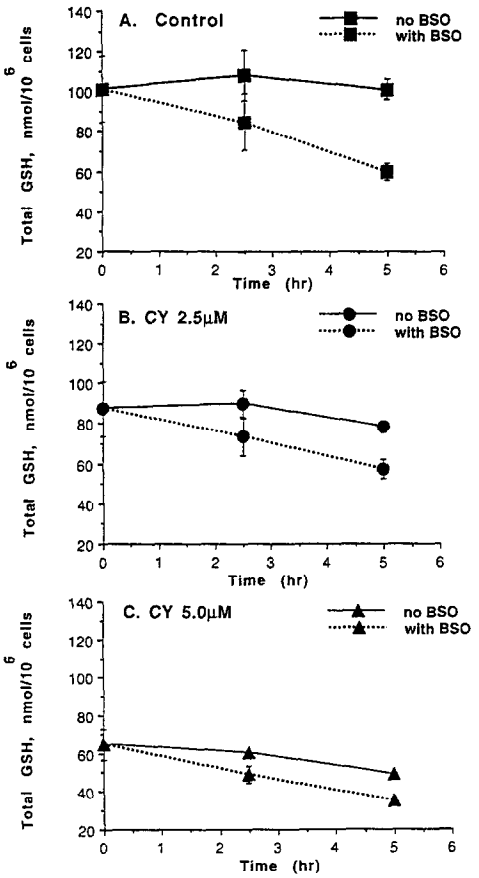

Fig. 1. Effect of BSO on the fall of total GSH in control cells (**A**), and cells pretreated with 2.5 μM CY (**B**), or 5 μM CY (**C**). Results are means ± SEM from 3 experiments. Cells were pretreated with CY (2.5 or 5 μM) or vehicle for 12 hr; then the medium was changed to SAF + 1 mM methionine with or without BSO (5 mM) for up to 5 hr. Total GSH (cell plus medium) was measured at time 0 (time of medium change), 2.5 and 5 hr. The rate of change in total GSH (nmol/10^6 cells/hr) was determined by linear regression.

8.2 ± 2.5 nmol/10^6 cells/hr versus 6.0 ± 1.7 and 5.9 ± 1.3 nmol/10^6 cells/hr for cells pretreated with 2.5 or 5 μM CY, respectively (results are means ± SEM from 3 experiments; neither CY value was significantly different from control by ANOVA). Lysis was increased significantly ($P \leq 0.05$) at the end of the 5-hr incubation in the 5 μM CY group with BSO (without BSO: controls = 23 ± 4.7%, 2.5 μM CY = 24 ± 5.3%, 5 μM CY = 31 ± 4.8%; with BSO: controls = 23.3 ± 4.6%, 2.5 μM CY = 25 ± 5.5%, 5 μM CY = 37.7 ± 5.3%). To avoid the possibility that the effect of CY required its continuous presence, the above experiments were repeated in the presence of 5 μM CY during the 5-hr incubation with or without BSO. Similar results were obtained as before [starting GSH level (nmol/10^6 cells) for control = 84 ± 5, CY = 59 ± 7; after BSO treatment the rate of fall in GSH (nmol/10^6 cells/hr) for control = 5.3 ± 0.4, CY = 3.2 ± 0.5; results are means ± SEM from 4 experiments; the rates of fall following BSO were significantly different between the two by paired *t*-tests]. Since CY did not accelerate the rate of fall in GSH following BSO, it is most likely that both agents (CY and BSO) inhibit GSH synthesis. GSH efflux as determined by the percentage of total GSH that accumulated in the medium per hour was not altered by CY pretreatment (data not shown).

To further support inhibition in GSH synthesis as the predominant mechanism for the fall in GSH, we examined the effect of excess GSH precursor, 20 mM NAC, on the CY-induced fall in GSH and toxicity. Following 12 hr of treatment with 5 μM CY, NAC did not prevent the fall in GSH or toxicity induced by CY (Fig. 2). In these experiments, the addition of NAC did not raise the control cell GSH. This is because the medium already contained 1 mM methionine and the control cells were repleted of GSH. In fact, this concentration of NAC was capable of supporting GSH synthesis. In two experiments, control cells were depleted of GSH by DEM (0.5 mM for 30 min) and then medium was changed to SAF alone or SAF plus 20 mM NAC for 5 hr. Before DEM depletion, cell GSH levels (nmol/10^6 cells) were 133 and 92 (mean of duplicates); after DEM treatment and medium change to SAF alone, cell GSH levels were 40 and 24, and with NAC added they were 70 and 75. To exclude the possibility that the uptake of NAC was inhibited by CY, we measured total non-protein thiols by the Ellman assay, as described in Ref. 19, following incubation with 20 mM NAC. Total thiols increased by 200 nmol/10^6 cells in both controls and cells pretreated with CY (5 μM) after 30 min of incubation with 20 mM NAC. This amount of increase correlates with the ~20 mM concentration of NAC that was used (assuming 1 mL = 10^8 cells). Thus, the lack of effect of NAC in preventing the continuing fall of cell GSH in CY-pretreated cells can only be explained by a block in the GSH synthesis pathway exerted by CY.

Effect of inhibition of cytochrome P450. One common pathway of xenobiotic metabolism involves oxidation through the P450 pathway [20]. To see if this pathway was involved in the metabolism and toxicity of CY, cells were pretreated with 10 μM α-naphthoflavone, an inhibitor of P450 [21, 22]. As shown in Fig. 3, 10 μM α-naphthoflavone partially protected from the fall in cell GSH and toxicity induced by CY. Other P450 inhibitors (1 mM cimetidine or 15 μM SKF525A) also had similar effects (results not shown). Thus, the cytochrome P450 pathway is likely to be involved in the metabolism of CY, and the metabolite(s) generated may be more toxic and/or more potent as an inhibitor of GSH synthesis than the parent compound.

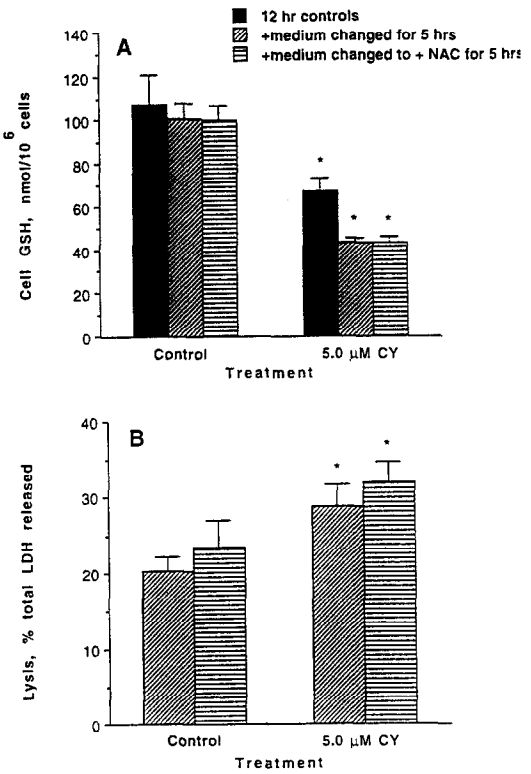

Fig. 2. Effect of NAC on (**A**) cell GSH and (**B**) lysis in controls and cells pretreated with CY. Results are means ± SEM from 4 experiments. Cells were pretreated with 5 μM CY or vehicle for 12 hr. The medium was then changed to DME/F12 plus 1 mM methionine with or without 20 mM NAC for 5 hr. Key: (*) $P < 0.05$ vs respective controls by ANOVA followed by Fisher's test.

DISCUSSION

GSH is the most abundant cellular non-protein thiol, and it plays a key role in cellular defense against oxidative damage and participates in the detoxification of many xenobiotics by serving as a substrate for GSH transferases and GSH peroxidase. GSH transferases catalyze the conjugation of GSH with electrophilic metabolites to form conjugates, and GSH peroxidase utilizes GSH as a reductant to reduce toxic peroxides [23]. Toxins and xenobiotics deplete GSH by several mechanisms: first, when conjugation exceeds the GSH synthetic capacity; second, when there is increased oxidant stress; and third, when GSH synthesis is inhibited as in the case

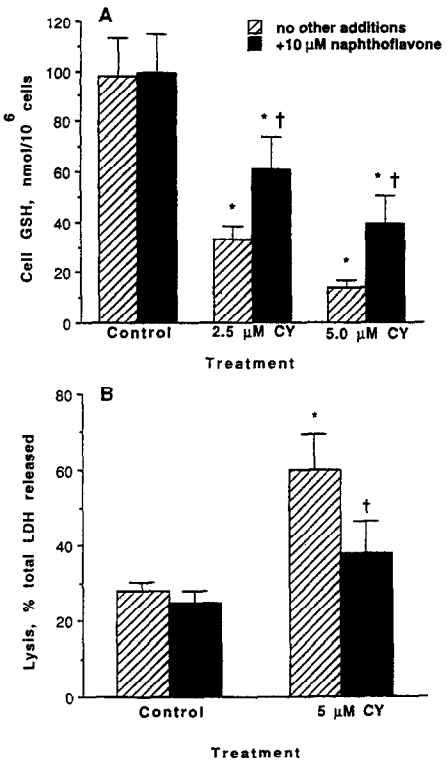

Fig. 3. Effect of α-naphthoflavone on (**A**) cell GSH levels and (**B**) lysis as measured by the percentage of total LDH release. Results are means ± SEM from 3–5 experiments. Cells were treated with 10 μM α-naphthoflavone 1 hr before CY (2.5 and 5 μM) or vehicle, and 18 hr later cell GSH and lysis were measured. Key: (*) $P < 0.05$ vs respective controls, and (†) $P < 0.05$ vs respective CY concentrations by ANOVA followed by Fisher's test.

of galactosamine hepatoxicity [24]. When the capacity of cells and tissues to maintain GSH homeostasis is lost, injury often follows [25]. Conversely, treatments that deplete cell GSH make tissues and cells more susceptible to potential electrophiles and toxins. In the case of CY, cell GSH fell prior to any sign of toxicity, and lower cell GSH potentiated CY-mediated cytotoxicity [6].

Decreased cell GSH can result from decreased synthesis or increased utilization (including export) or both. The synthesis of GSH from its constituent amino acids, L-glutamate, L-cysteine, and glycine, involves two ATP-requiring enzymatic steps: (1) formation of γ-glutamyl-L-cysteine from L-glutamate and L-cysteine, and (2), formation of GSH from γ-glutamyl-L-cysteine and glycine. The first step is catalyzed by γ-glutamylcysteine synthetase, the rate-limiting enzyme that is under negative feedback inhibition by GSH. Under normal physiologic conditions, the availability of its precursor, L-cysteine, governs the rate of GSH synthesis [15, 16]. In cultured rat hepatocytes, the major sources of cysteine are uptake of methionine and cystine since almost all cysteine is autoxidized to cystine in culture media [17, 18]. In our accumulation experiments, the only precursor sulfur amino acid present in the medium was methionine so cystine utilization was not a concern. CY treatment resulted in decreased GSH accumulation following acute depletion with DEM. This occurred prior to any evidence of toxicity. No increase in GSSG or CySSG was detected. The rate of GSH efflux also was not altered by CY. Thus, these experiments excluded increased GSH efflux, major oxidant stress or hidden forms of GSH as potential explanations for the CY-induced fall in GSH. The remaining possible explanations for the fall in GSH, namely inhibition of GSH synthesis and increased GSH utilization, were then explored further.

CY lowered cell GSH at micromolar concentrations; the total equivalent amount of CY per well was ≤5 nmol. This was at least 10-fold lower than the absolute amount of fall in cell GSH observed. Thus, it is unlikely that GSH loss was due to GSH conjugation.

Parameters that are important in GSH synthesis include cysteine availability, ATP level and activity of enzymes of the GSH synthesis pathway. Methionine uptake, cysteine and ATP levels were unaffected by CY. When GSH synthesis capacity was measured in cell-free extracts using mBCl, we were not able to detect any difference after CY pretreatment. However, in this assay the cellular cytosolic content is diluted ~500-fold, so that a concentration-dependent inhibition of the enzymes of GSH synthesis by CY and/or metabolite(s) would not necessarily have been detected.

Three lines of evidence point to inhibition of GSH synthesis being the predominant mechanism for the CY-induced fall in GSH. First, an excess of NAC, a sulfur amino acid precursor, did not protect from the fall in cell GSH. This occurred despite the fact that both control and CY-treated cells took up NAC, and NAC was able to support GSH synthesis in control cells. This suggests that the block in GSH synthesis is in the final common pathway, the GSH synthesis pathway, rather than *trans*-sulfuration, which NAC bypasses. Second, the rate of fall in GSH following BSO treatment was not accelerated by CY. If CY had increased GSH consumption, the rate of fall in GSH would have been accelerated. The possibility that CY interacted with BSO extracellularly, which then would have decreased the availability of BSO to the cell, was excluded since CY was removed during BSO treatment. Since similar results were seen with or without CY present during the 5 hr of incubation with BSO, this suggests that CY (a very hydrophilic compound) or its metabolite, once inside the cell, remains largely intracellular to exert its effect. Third, in experiments where cells were incubated with radioactive methionine for 2 hr to label the intracellular GSH, CY-pretreated cells had lower GSH specific activity as compared with controls. This can only be explained by a block in GSH synthesis. Since CY-treated cells were still able to reaccumulate GSH (Table 1), the inhibition on GSH synthesis was not complete under the experimental conditions. The exact mechanism of the inhibition exerted by CY is still unclear. Either γ-glutamylcysteine synthetase or GSH synthetase may be inhibited. Since no inhibition was observed

when the cellular cytosolic content was diluted, the nature of the inhibition is likely to be concentration dependent. Direct addition of CY to cytosolic content in order to approximate the maximal intracellular CY concentration is prohibitive at present due to the lack of sufficiently large quantities of CY.

Many cytotoxic xenobiotics are activated metabolically to reactive intermediates such as epoxides that are more toxic than parent compounds. These oxidative activations are catalyzed by P450 enzymes. Addition of P450 inhibitors, such as α-naphthoflavone, SKF525A and cimetidine, has been shown to protect partially from cytotoxicity induced by known xenobiotics and drugs [21, 22]. In the case of CY cytotoxicity, similar protection against toxicity was also found with α-naphthoflavone (Fig. 3). This compound, as well as SKF525A and cimetidine, also partially prevented the fall in cell GSH induced by CY. These results suggest the involvement of cytochrome P450 in generating a more toxic intermediate and/or the intermediate(s) may be a more potent inhibitor of GSH synthesis than the parent CY.

In summary, we have established previously that following CY treatment of cultured rat hepatocytes, cytotoxicity is preceded by a significant fall in cell GSH. This study showed that the fall in GSH is due to inhibition of the final common pathway of GSH synthesis. This inhibition in GSH synthesis contributes to CY cytotoxicity as lower cell GSH predisposed to CY toxicity.

Acknowledgements—This work was supported by NIEHS Grant ES05678 and NIH Grant DK45334. The authors thank Dr. Richard Moore of the Department of Chemistry of the University of Hawaii for providing purified cylindrospermopsin for these studies and Dr. Neil Kaplowitz for his helpful discussions and suggestions.

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