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THE ROLE OF GLUTATHIONE IN THE TOXICITY OF A NOVEL CYANOBACTERIAL ALKALOID CYLINDROSPERMOPSIN IN CULTURED RAT HEPATOCYTES

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Cylindrospermopsin (CY) is a newly isolated alkaloid produced by the cyanobacterium *Cylindrospermopsis raciborskii*, which has been linked to an outbreak of hepatoenteritis in man. The current work examined the suitability of primary cultures of rat hepatocytes as an *in vitro* model for studying the cytotoxicity of CY. We found that CY (3.3-5.0 μM) caused significant cell death (40-67% of cells by LDH release) in cultured hepatocytes after 18 hr incubation. While investigating possible mechanisms for CY toxicity, we found that lower, nontoxic doses of CY (1.6-2.5 μM) decreased cell glutathione (GSH) to about 50% of control. For toxic doses (5μM), the loss of GSH preceded the onset of toxicity by six hr. Lowering cell GSH predisposed cells to CY toxicity. In conclusion, cultured hepatocytes are a suitable model for studies of CY cytotoxicity and GSH is involved in the detoxification of CY.

In 1979 an outbreak of hepatoenteritis occurred in the Palm Island Community, Australia. The illness began as a hepatitis like syndrome, lasted between four and 26 days and required the hospitalization of most of the 148 cases (1). The causative agent implicated in the outbreak was identified as the cyanobacterium *Cylindrospermopsis raciborskii* (*C. raciborskii*). Just preceding the outbreak this microorganism had formed a dense bloom (scum) on the dam that is the water supply for the community (2). An isolate of *C. raciborskii* from Palm Island was grown in culture and shown to be severely hepatotoxic in mice (2). This same isolate was used as the source for the

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ABBREVIATIONS:

GSH, reduced glutathione; DME/F12, Dulbecco's modified Eagle's/Ham's F12 medium 1:1 mix; PPG, propargyl glycine; CY, cylindrospermopsin; LDH, lactate dehydrogenase.

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cylindrospermopsin (CY) used in this study (3). *C. raciborskii* is regularly found in the plankton in temperate waters containing relatively high concentrations of organic matter, it often is the dominant species in blooms in tropical environments. Occurrences of gastrointestinal disease of unknown etiology are common in these areas.

CY, isolated from *C. raciborskii* is a novel alkaloid of polyketide origin: a sulfate ester of cycloguanidine substituted uracil (3). *In vivo* experiments indicated that CY was hepatotoxic in mice with symptoms that could not be distinguished from those originally observed with extracts of *C. raciborskii* (2). The LD_{50} at 24 hr for CY was found to be 2.1 mg/kg, with lower doses of CY (0.2 mg/kg) death was delayed occurring 5-6 days after injection (3). Although other organs were also involved (the kidney in particular), it has been clearly established that *C. raciborskii* and CY are primarily hepatotoxic. Histologically the primary lesion is that of coagulative hepatocyte necrosis, which is centrilobular at lower doses and panlobular at higher doses (3).

The aim of this study was to establish a convenient model for the investigation of the toxicity of CY, and of its mode of action as a toxin. Rat hepatocytes were used here as the model for CY hepatotoxicity, since these cells appear to be one of the primary targets of CY *in vivo*. Preliminary experiments showed that 100 μM CY did not cause any detectable cell toxicity when added to freshly isolated hepatocytes over a period of four hr, we therefore employed primary cultures of these cells to establish and characterize the cytotoxicity of CY.

METHODS

The alkaloid CY was isolated from cultures of *C. raciborskii* as as in (3). All other chemicals were from routine commercial sources.

Preparation of Cultured Hepatocytes. Hepatocytes were isolated from fed male Sprague-Dawley rats (260-350 g) as previously described (4). After attachment to collagen coated 6-well cluster plates hepatocytes were incubated at 37°C in 5% CO_2, 95% air in DME/F12 medium containing high glucose (3151 mg/L), insulin (1 μg/ml) and hydrocortisone (50 nM) and supplemented with excess methionine (1 mM) as described (5). 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 detached by trypsin-EDTA (0.05%, 0.02%, respectively) for cell counting by both Coulter counter and hemocytometer. Cellular GSH was measured on the 10% trichloroacetic acid supernatant by the recycling method of Tietze (6). GSH was expressed as nmol/10^6 cells.

Measurement of Toxicity. Toxicity to CY was measured by release of lactate dehydrogenase (LDH) from the cytosol into culture medium (kit from Sigma Chemical Co.: Procedure No. DG1340-K). Washed cells were scraped off the culture plates in phosphate buffered saline and this suspension used for cellular LDH measurements after sonication. Other wells were extracted with 0.1% Triton X-100 in phosphate buffered saline. Both methods gave statistically indistinguishable values for cellular LDH activity. Percentage LDH release was the LDH activity in the medium as percentage of total LDH (cellular+medium).

Statistical Analysis. For cultured cells, each cell prep 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 mean ± SE of n, the number of experiments.

RESULTS

Toxicity of Cylindrospermopsin (CY). Pilot studies of cultured rat hepatocytes incubated with CY (10-100 μM) for 18-20 hr showed that the toxin caused essentially complete cell death. This observation led to a more detailed examination of the dose and time course of toxicity.

Fig. 1 shows the dose dependence of CY toxicity. Cultured rat hepatocytes were incubated in various doses of CY for 18 hours and toxicity was assayed by LDH release as described in Methods. 3.3 μM CY caused significant cell lysis (LDH = 39±4%, an increase of 19% over control). The toxicity window was remarkably narrow, since with 5 μM CY release of 67±7% of LDH was seen.

Pilot experiments had shown that there was no measurable cell lysis within the first 12 hr of incubation of hepatocytes with CY, with only slight rounding and no detachment of the cells. Fig. 2 illustrates the time course of toxicity of 5μM CY. Significant elevation in LDH (40±5%) only occurred after 16 hours of incubation. As in the pilot experiments, CY caused some rounding of the cells, first seen at 12 hr and becoming more pronounced at 16 hr.

Mechanism of CY Toxicity. Since GSH is one of the most important defense against many toxins, we examined the effect of CY on GSH metabolism. Cultured rat hepatocytes were incubated

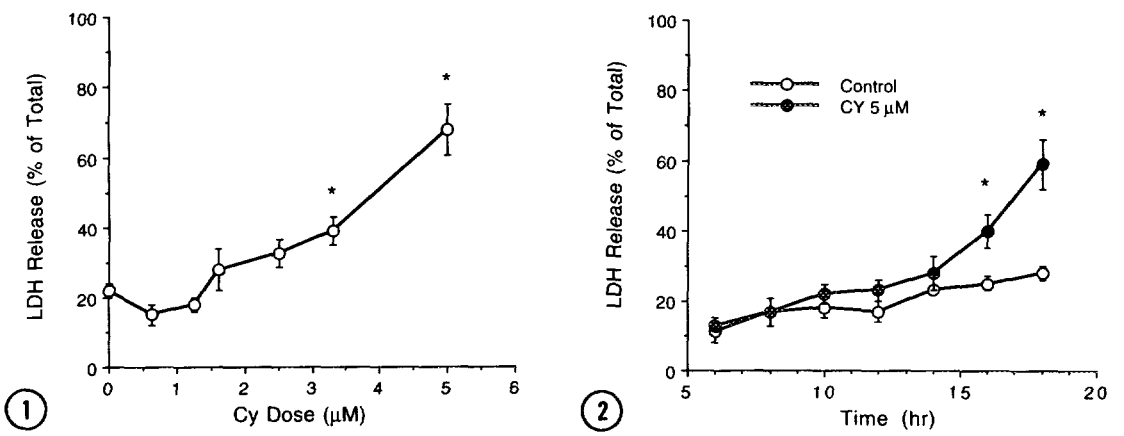
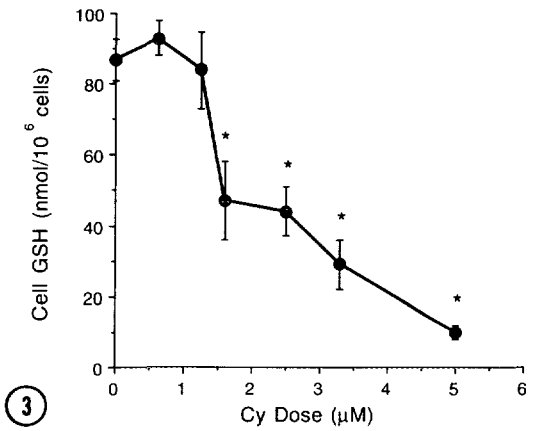


Figure 1. Dose response for the release of lactate dehydrogenase (LDH) by cultured rat hepatocytes exposed to cylindrospermopsin (CY) for 18 hr. Values represent mean ± SE, n≥ 6 experiments. * Significantly different from control.

Figure 2. Time course for the release of lactate dehydrogenase (LDH) by cultured rat hepatocytes exposed to 5 μM cylindrospermopsin (CY). Values represent mean ± SE, n ≥ 3 experiments. * Significantly different from the respective controls.



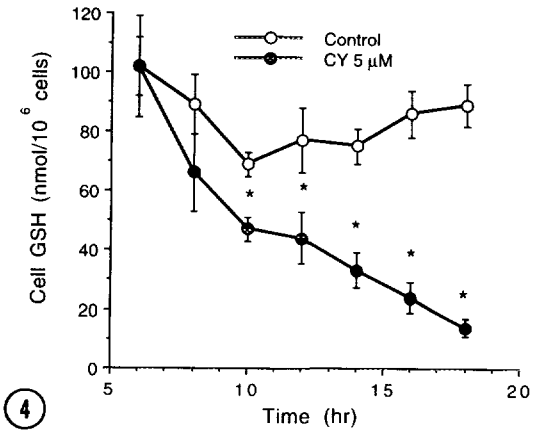


Figure 3. Dose response for the loss of cell GSH in cultured rat hepatocytes exposed to cylindrospermopsin (CY) for 18 hr. Values represent mean ± SE, n ≥ 6 experiments. * Significantly different from control.

Figure 4. Time course for the loss of cell GSH in cultured rat hepatocytes exposed to 5 μM cylindrospermopsin (CY). Values represent mean ± SE, n ≥ 3 experiments. *Significantly different from the respective controls.

with varying doses of CY (0.8-5.0 μM) for 18 hr and cell GSH was measured as described in Methods. Fig. 3 shows that 1.6 μM CY caused a significant fall of nearly 50% in cell GSH. This dose of CY causes no significant toxicity (Fig. 1). Increasing doses of CY depleted GSH more profoundly: at 5 μM CY cell GSH was only 10 nmol/10^6 cells (12.5% of control). When the time course of the CY effect on GSH was examined, the fall in cell GSH preceded any increase in LDH release. For cells incubated with 5 μM CY (Fig. 4), cell GSH fell significantly after 10 hr of incubation whereas toxicity only become significant 6 hr later (Fig. 2). The fall in GSH following incubation with 2.5 μM CY became significant only after 16 hr of incubation (n=4, not shown).

To examine the role of GSH in the toxicity of CY, we decreased the cell GSH level by treatment of cells with propargylglycine (PPG), which blocks the utilization of methionine for GSH synthesis (inhibitor of transsulfuration) (7). As Fig. 5A shows, CY (2.5 μM) and PPG (0.5 mM) each by itself significantly lowered cell GSH while causing no significant increase in toxicity (Fig. 5B) after 18 hr of incubation. However, when cells were incubated with both agents, cell GSH fell further and significant toxicity was observed. Similar potentiation of CY toxicity was found when cell GSH was decreased by incubation in sulfur amino acid free medium containing limiting methionine: 0.01 mM instead of 1 mM (results not shown). Thus, lowering of cell GSH predisposed to CY toxicity.

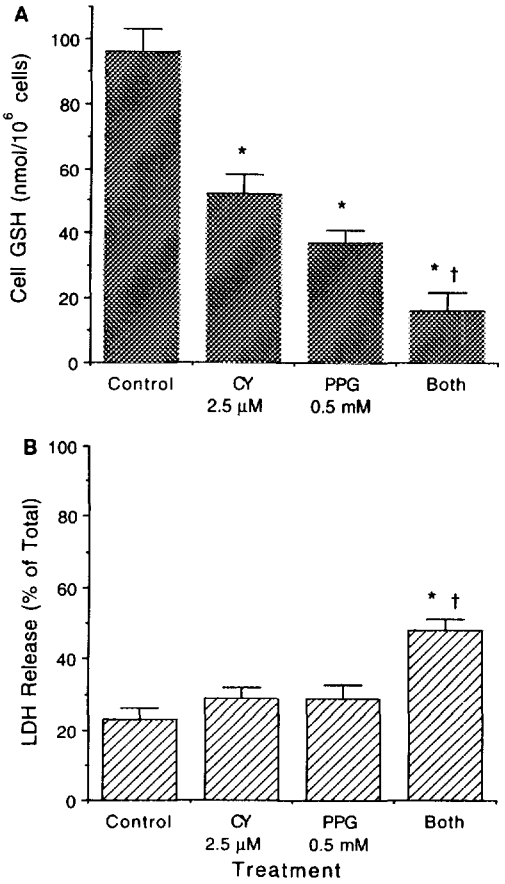


Figure 5. Effect of 0.5 mM propargylglycine (PPG) on (**A**) cell GSH levels and on (**B**) the release of lactate dehydrogenase (LDH) in cultured rat hepatocytes exposed to 2.5 µM cylindrospermopsin (CY) for 18hr. Values represent mean ± SE, n ≥ 6 experiments. * Significantly different from control, † significantly different from cells treated with 2.5 µM CY only.

DISCUSSION

CY, a newly described cyanobacterial alkaloid, is hepatotoxic *in vivo*, with death occurring at the earliest eight to ten hr after CY injection. The present studies in cultured hepatocytes establish that CY is directly cytotoxic (Fig. 1). The injury we observed in hepatocytes was also delayed and progressive. Injury to the monolayers first became apparent after twelve hr as morphological changes in the cells. When compared to controls, CY treated cells lacked hepatic cord structures, appeared more rounded but did not detach from the culture plates. Toxicity caused by 5 µM CY, as measured by LDH release, was progressive and by 16 hr it was significantly different from controls (Fig. 2).

When we examined the effect of CY on cell GSH, we found that in all cases CY toxicity was preceded by significant losses in GSH (Fig. 4). GSH is the most abundant cellular non-protein thiol,

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. Lowering of cell GSH may be due to increased utilization by conjugation with electrophilic metabolites or as a reductant to reduce toxic peroxides, or decreased GSH synthesis, or a combination of these. Severe depletion of GSH can then result in cell death or predispose the cell to various injuries (8). This was in fact the case in CY cytotoxicity as well, so that lower cell GSH potentiated CY-mediated cytotoxicity (Fig. 5). The precise mechanism for the fall in cell GSH remains to be elucidated.

The doses of CY required for cell lysis (3-5 μM) are of the same order of magnitude as the LD_{50} for CY in mice. CY (2.1 μg/g = 5 nmol/g) causes death by 24 hr *in vivo*, *in vitro* 3-5 nmol/ml are the amounts of CY that will cause significant release of cell LDH. The time to death in mice and the onset of toxicity in our cultured hepatocyte model are also similar. It is therefore reasonable to conclude that the toxicity we see in these cells reflects the *in vivo* hepatotoxicity of CY, and that the cultured hepatocyte is a good model for the study of the mechanism of CY toxicity. It follows that GSH is most likely to be involved in the detoxification of CY *in vivo*.

The microcystins (cyclic peptides) are the only other group of cyanobacterial hepatotoxins that has been well characterized. The effect of CY on hepatocytes differs from that of the microcystins. Unlike CY, a lethal dose of microcystins in mice will kill in one to two hr. Microcystin toxicity is due to the specific inhibition of protein phosphatases (9). CY therefore represents a completely new type of toxin produced by cyanobacteria. *C. raciborskii* , the organism that produces CY is widespread in occurrence, it is therefore likely that CY intoxication may explain many occurrences of gastrointestinal disease that appear to be water-borne.

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