Inhibition of Microcystin-Induced Release of Cyclooxygenase Products from Rat Hepatocytes by Anti-Inflammatory Steroids (43153)

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Abstract. **We showed previously that exposure to microcystin causes eicosanoid release. That study was extended further to test the effect of glucocorticoids on microcystin-induced release of [^{14}C]arachidonic acid and its metabolites from rat hepatocytes previously treated with [^{14}C]arachidonic acid. Release of total radioactivity was 4-fold greater from hepatocytes after 2-hr incubation with 1 μM microcystin than after incubation with control medium. Fluocinolone pretreatment decreased the microcystin-induced synthesis and release of prostacyclin by 24 ± 2.6% ($P < 0.05$) and thromboxane B_2 by 39 ± 3% ($P < 0.025$). Treatment of hepatocyte cultures with either microcystin (1 μM) or steroids had no effect on cell viability or total cell protein. Total radioactivity released into the incubation medium was not affected by glucocorticoid alone. Under these conditions, the quantities of both prostaglandin $F_{2\alpha}$ and prostaglandin E_2 released were not significantly different when control and microcystin-treated cultures were compared. The half-maximal inhibition (IC_{50}) values obtained from the dose-response data for the inhibition of arachidonic acid release by steroids were comparable with normal cortisol levels in humans. Dose-response curves gave the following rank order of inhibitory potency: fluocinolone > dexamethasone > hydrocortisone. These results suggest that glucocorticoid therapy might be beneficial in microcystin toxicosis.**

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Microcystis aeruginosa algal extracts contain a toxic, potentially lethal cyclic peptide, microcystin. Microcystin is said to be a hepatotoxin, but the mechanism of interaction of microcystin with the liver is not understood. Sensitivities of nonhepatic tissues to microcystis extracts have also been reported (1). Biochemical and morphologic changes have been observed in livers of animals exposed to microcystin. Pathologic changes in the liver include congestion, edema, and leukocyte infiltration (2–4). The liver is often involved in the pathogenesis of a number of inflammatory diseases and in the defense against exogenous toxins (5, 6). Hepatocytes can synthesize arachidonic acid metabolites (7–9). We have shown that microcystin stimulates the cyclooxygenase pathway of arachidonic acid metabolism (10).

Steroids are used in the treatment of inflammatory and immune disorders. Glucocorticoids exert anti-inflammatory effects by inhibiting phospholipase A_2 activity (11–13) and by reducing the release of arachidonic acid metabolites, or reducing tissue prostaglandin synthesis through inhibition of prostaglandin synthetase or acceleration of the breakdown of prostaglandins (14). It is suggested that impairment of prostanoid synthesis by steroids reduces lethality in microcystin-challenged rats (4, 15).

We report that treatment of rat hepatocytes with microcystin promotes a rapid release of prostanoids into the incubation medium, and that the release can be blocked by pretreatment of these cells with glucocorticoids.

Materials and Methods

Hepatocyte Cultures. Hepatocytes from 200- to 280-g male Fisher rats (Charles River, Wilmington,

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MA) were isolated and cultured in Liebovitz's medium containing 18% heat-inactivated fetal calf serum (FCS) in 35- × 10-mm culture plates (16). After overnight culture at 37°C in 5% CO_2 and 95% air, only plates with ≥90% cells attached were used. Attached cells formed a uniform monolayer and had the characteristic polygonal shape of hepatocytes.

Strain, Purity, and Stability of Microcystin. Microcystin-LR (>95% pure), isolated from *M. aeruginosa*, strain PCC-7820, was supplied by Dr. Wayne W. Carmichael (Wright State University, Dayton, OH). Purity of microcystin was checked by both high-performance liquid chromatography and high-performance thin-layer chromatography systems (17). Stability of toxin was also tested in both biologic fluids and salt solutions and was found to be stable at least for 72 hr at 37°C (J. Pace, personal communication).

Labeling and Stimulation of Hepatocytes. After 24 hr of incubation, culture medium was removed and cells were washed with Hanks' balanced salt solution (HBSS). Fresh medium (1 ml) with 10% FCS was then added along with the test steroid (Sigma Chemical Co., St. Louis, MO) and 10 μM [^{14}C]arachidonic acid (52.7 mCi/mmol; New England Nuclear, Boston, MA). Sixteen hours later, the cells were washed and incubated for 60 min with Medium 199 containing 10% FCS to remove unincorporated [^{14}C]arachidonic acid, then washed three times with HBSS. Finally, microcystin in HBSS containing 0.1% bovine serum albumin was added, and cells were incubated for 2 hr.

Extraction of Prostaglandins. Arachidonic acid and its metabolites were extracted from the incubation medium with 6 ml of chloroform:methanol (2:1, v/v) containing 0.005% butylated hydroxy toluene (Sigma). Cells were then washed with 2 ml of chloroform. The chloroform phases were combined, the total lipid extract was evaporated to dryness under nitrogen, and the samples were stored under nitrogen at −20°C. Lipid residues were dissolved in 100 μl of chloroform:methanol (2:1, v/v) and aliquots were counted for total radioactivity released from hepatocytes. Arachidonic acid and its metabolites were resolved on thin-layer chromatography plates precoated with silica gel-60 (E. Merck, American Scientific Products, Columbia, MD). A solvent system of ethyl acetate-formic acid (80:1, v/v) was used. Spots that migrated with authentic standards of prostaglandins (Sigma) were identified by exposure to iodine vapor, scraped, eluted with methanol, and their radioactivity assessed by scintillation counting. Protein was measured by the Lowry method (18).

Data Analysis. Raw data were expressed as dpm/mg protein. Effects of glucocorticoids were determined by comparing changes in radiolabeled phospholipids, arachidonic acid, or arachidonate metabolites between steroid-pretreated and untreated cells after microcystin challenge. The arithmetic average of data from duplicate cultures in each treatment group was used for statistical analyses. Differences between control, microcystin-treated, and steroid-treated groups were compared by the paired t test.

Results

Microcystin-Induced Hepatocyte Arachidonic Acid Uptake and Release. Changes in phospholipid total radioactivity and arachidonic acid release were measured after exposure to microcystin (1 μM) for 2 hr. Microcystin-treated hepatocytes showed a decrease ($P < 0.005$) in phospholipid radioactivity (116,665 ± 3,265 dpm/mg protein; $n = 12$) as compared with control hepatocytes (138,314 ± 3,327 dpm/mg protein, $n = 13$). Total radioactivity released from control hepatocytes was 7% of the activity in hepatocyte phospholipids compared with 27% from microcystin-treated hepatocytes (Table I). Approximately 50% (control) and 63% (microcystin-treated) of total released radioactivity was arachidonic acid (Table I, column B versus C). Treatment of hepatocyte cultures with either microcystin (1 μM) or steroids had no effect on cell viability or total cell proteins.

Arachidonic Acid Metabolism. Microcystin treatment not only reduced activity in cellular phospholipid, but also stimulated total radioactivity release by 338% and free arachidonic acid release by 430% as compared with control cultures (Table I). Prostacyclin (6-keto $F_1\alpha$) and thromboxane (TXB_2) were major cyclooxygenase metabolites synthesized by rat hepatocytes in response to microcystin challenge. Microcystin-induced changes in release of radiolabeled arachidonic acid and its metabolites from hepatocytes are illustrated in Figure 1. Compared with control values, microcystin treatment (1 μM for 2 hr) stimulated the release of 6-keto $F_1\alpha$ by 19 ± 1.2% ($P < 0.05$) and TXB_2 by 52 ± 1.5% ($P < 0.001$). Under these incubation conditions, prostaglandin (PG) $F_2\alpha$ and PGE_2 release was not affected (data not shown).

Effect of Glucocorticoids on Arachidonic Acid Uptake. To determine whether glucocorticoids alter the uptake of radiolabeled arachidonic acid, we treated hepatocytes with 1 μM fluocinolone, dexamethasone, and hydrocortisone for 16 hr during incubation with [^{14}C]arachidonic acid. This hormone treatment did not alter cell viability and there was no resultant difference in cell protein content. Mean total cellular radioactivity for control hepatocytes was 14.2 ± 0.98 × 10^4 dpm/mg protein ($n = 9$). Hepatocytes cultured under identical conditions but treated with various glucocorticoids (1 μM) for 16 hr showed no significant change in radiolabel uptake. Mean cellular radioactivity was 13.9 ± 1.67, 13.1 ± 0.96, and 12.7 ± 1.3 × 10^4 dpm/mg protein for fluocinolone, dexamethasone, and hydrocortisone, respectively.

Effect of Glucocorticoids on Microcystin-In-

Table I. Effect of Microcystin and Fluocinolone on Uptake and Release of ^{14}C-Labeled Radioactivity by Hepatocytes[a]

	A: Cellular phospholipid radioactivity (dpm/mg)	B: Total radioactivity released (dpm/mg)	C: Arachidonic acid radioactivity released (dpm/mg)
Control (n = 13)	138,314 ± 3,307[b]	9,273 ± 1,050	4,573 ± 761
Fluocinolone (n = 9)	137,085 ± 2,630	8,719 ± 1,032	5,641 ± 528
Microcystin (n = 12)	116,665 ± 3,265 (<0.005)[c]	31,415 ± 6,307 (<0.001)	19,688 ± 1,468 (<0.005)
Microcystin + fluocinolone (n = 6)	124,990 ± 4,718 (<0.001)	24,839 ± 2,342 (<0.001)	13,629 ± 874[d] (<0.05)

[a] Hepatocytes were pretreated with 1 μM fluocinolone for 16 hr. Prelabeled cells were exposed to 1 μM microcystin for 2 hr. Cellular radioactivity (from arachidonic acid uptake) associated with phospholipid was determined by washing monolayers with HBSS to remove radioactivity associated with cell surface. Cells were digested with 1 N NaOH and an aliquot counted for cellular radioactivity (Column A). Culture medium was collected and counted (Column B); medium was extracted and arachidonic acid release was determined by thin-layer chromatography (Column C).
[b] Mean ± SE.
[c] Numbers in parentheses, P values.
[d] $P \leq 0.025$ significant for difference from microcystin-treated cultures alone.

duced Arachidonic Acid Metabolism. Fluocinolone pretreatment of hepatocytes produced no effect on cellular uptake of radioactivity or release of total radioactivity (Table I). Treatment of cultures with fluocinolone for 2, 4, 8, and 16 hr produced, respectively, 3.6, 5.6, 20.7, and 42.7% inhibition of radiolabeled arachidonic acid release in response to microcystin treatment (1 μM). Dexamethasone and hydrocortisone produced similar results (data not shown). In all subsequent experiments, 16-hr preincubation with glucocorticoids was used when measuring arachidonic acid metabolism.

As shown in Figure 1, fluocinolone significantly suppressed microcystin-induced release of free arachidonic acid ($P < 0.025$). Pretreatment with steroids also inhibited synthesis and release of radiolabeled 6-keto $F_1\alpha$ and TXB_2. The degree of inhibition of TXB_2 appeared to be greater (39 ± 3%) than the degree of inhibition of 6-keto $F_1\alpha$ (24 ± 2.6%). Under these experimental conditions, the quantities of $PGF_2\alpha$ and PGE_2 release did not differ significantly when control and experimental cultures were compared (data not shown). Under the same conditions, dexamethasone treatment inhibited the release of arachidonic acid from phospholipid by 38 ± 3.1% ($P < 0.005$), and hydrocortisone reduced the release by 26 ± 2.9%. As would be expected, dexamethasone and hydrocortisone pretreatment, under the same conditions, also inhibited TXB_2 release by 37 ± 1.4% and 29.0 ± 2.1% ($P < 0.05$), respectively. Dexamethasone and hydrocortisone did not significantly reduce microcystin-induced release of 6-keto $F_1\alpha$ (Fig. 1).

Relative Efficacy of Glucocorticoids as Inhibitors of Microcystin-Induced Arachidonic Acid Release. The rank order of fluocinolone, dexamethasone, and hydrocortisone potencies as inhibitors of microcystin-induced arachidonic acid release was determined. Cultures were pretreated with glucocorticoid concentra-

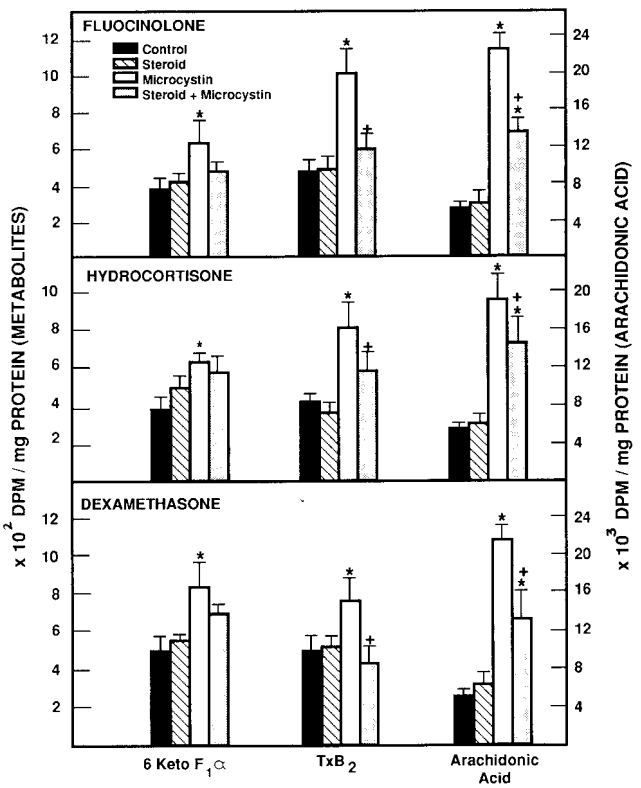

Figure 1. Effect of fluocinolone, dexamethasone, and hydrocortisone on hepatocyte arachidonic acid release and metabolism. Microcystin challenge (1 μM for 2 hr) resulted in radiolabeled arachidonic acid release and radiolabeled metabolite production. These parameters were inhibited by pretreatment for 16 hr with 1 μM steroids. Data points represent mean values from duplicate determinations of three to six separate experiments. Microcystin-induced changes in radioactivity were compared for fluocinolone, dexamethasone, and hydrocortisone-treated and untreated cells by means of a paired t test. *$P \leq 0.05$ from control. †$P \leq 0.05$ from microcystin-treated cultures.

tions for 16 hr. The inhibition of microcystin-induced release of free arachidonic acid was dependent on steroid concentrations and reached a maximum of 50% at 10^{-3} *M* or less. The half-maximal inhibitions (IC_{50}) of arachidonic acid release by fluocinolone, dexamethasone, and hydrocortisone were 3×10^{-8} *M*, 1×10^{-6} *M*, and 9×10^{-6} *M*, respectively (Fig. 2).

Discussion

In this study, we showed that microcystin, a potentially inflammatory toxin (4, 11), increased the release of arachidonic acid and its metabolites, TXB_2 and prostacyclin, from cultured rat hepatocytes. In addition, 16-hr pretreatment with glucocorticoids significantly reduced the microcystin-induced release of these eicosanoids.

Although the mechanism for the release of arachidonic acid metabolites is unknown, it has been suggested that inhibitors of sulfhydryl enzymes block the reuptake of free arachidonic acid into lipid pools, which results in an increase in prostaglandin synthesis (19). Reesterification of liberated arachidonic acid would thereby be a controlling factor in the balance of free arachidonic acid and production of its metabolites. We reason that microcystin may alter the sulfhydryl enzymes active in phospholipid reacylation. In this study, about 63% of radioactivity liberated by microcystin from [^{14}C]arachidonic acid-incubated hepatocytes was detected as free arachidonic acid, which apparently was not reincorporated into membrane phospholipid. Microcystin at a very low concentration (10 n*M*) caused these hepatocytes to produce 71% of its released radioactivity as free arachidonic acid, with no change in metabolite synthesis and release (unpublished data).

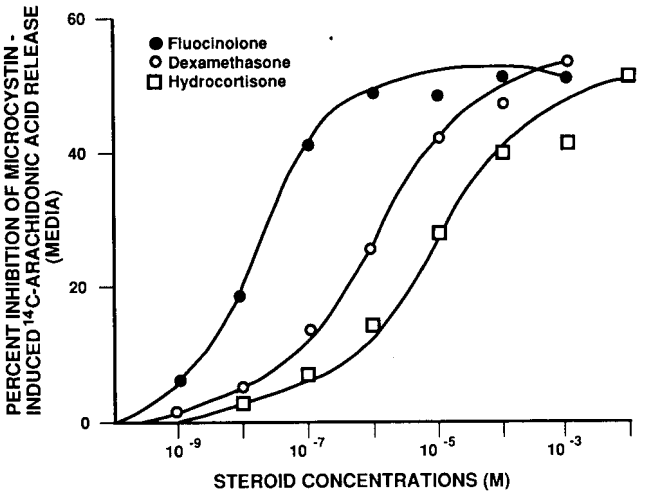

Figure 2. Dose-response relationships for glucocorticoid inhibition of microcystin-induced arachidonic acid release. The glucocorticoid pretreatment period was 16 hr. Data points represent mean values from duplicate determinations in three to five separate experiments. Standard deviation in these experiments was always within 10% of the mean.

This result suggests that reesterification of arachidonic acid is at least partially blocked, probably by inhibition of sulfhydryl arachidonyl CoA synthetase and acyl transferase activities.

Steroids are widely used as anti-inflammatory agents. They inhibit the hydrolytic release of arachidonic acid from phospholipids by inducing a phospholipase A_2 inhibitory protein, lipomodulin (12, 14). The protective effects of dexamethasone T-2 toxicosis (20) are probably mediated by suppression of PG synthesis and release, but the molecular basis for this protection remains obscure.

This study was initiated to determine the effects of glucocorticoids on the uptake and release of radiolabeled arachidonic acid by rat hepatocytes in response to microcystin exposure. Data show that microcystin stimulates the synthesis and release of arachidonic acid and its metabolites. The effect was partially blocked by pretreatment of cultures with glucocorticoids. Pretreatment of hepatocytes with glucocorticoids results in a moderate decrease in the uptake of radiolabeled arachidonic acid into cellular phospholipids. Steroid pretreatment also inhibits the release of arachidonic acid from microcystin-treated hepatocytes. The long duration of pretreatment required for the inhibition of arachidonic acid release, the persistence of the inhibition effect after steroid removal from the incubation medium, and the large differences in the potencies of the inhibitory effects of different steroid molecules suggest that the effect may be dependent on a receptor-mediated process involving new protein synthesis (21). The degree of inhibition of TXB_2 synthesis (42.8 ± 6.1%), compared with the inhibition of arachidonic acid release from cellular phospholipid (37.4 ± 3.9%), suggests that fluocinolone blocks both phospholipase A_2 and thromboxane synthetase. Similar findings have been reported in rat macrophages (22, 23). It was suggested that glucocorticoids may exert their effects on phospholipase A_2 levels and also at steps regulating PG synthetic and/or degradative enzymes. This hypothesis has not been tested for hepatocytes.

Dose-response data in Figure 2 illustrate a rank order of potency for the three steroids tested. Fluocinolone was the most potent in inhibiting arachidonic acid release induced by microcystin, followed by dexamethasone and hydrocortisone. These findings are similar to what has been observed for a variety of biologic effects of these hormones (24, 25). Furthermore, the half-maximal inhibition values calculated from the dose-response data for the inhibition of arachidonic acid release are comparable with normal cortisol levels in humans (hydrocortisone 10^{-7} *M*).

The clinical relevance of toxin-specific inhibition of arachidonic acid release by glucocorticoid-treated hepatocytes remains to be established *in vivo*. Arachi-

donic acid metabolism may be important in the pathogenesis of toxin-induced injury.

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