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other classes of phospholipids or neutral lipids. A 10% increase in phosphatidylcholine in hepatocytes treated with microcystin-LR may have resulted from conversion of phosphatidylethanolamine to phosphatidylcholine via the N-methylation pathway. These results indicate that microcystin-LR has important effects on the regulation of inflammatory mediator synthesis in hepatocytes. (AW)

TOXIN-INDUCED ACTIVATION OF RAT HEPATOCYTE PROSTAGLANDIN SYNTHESIS AND PHOSPHOLIPID METABOLISM

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Key Words: Microcystin-LR - Arachdonic acid - Phospholipid - Prostaglandin - Metabolism - Inflammation - Hepatocytes - Toxins

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SUMMARY

The effects of microcystin-LR, a trichothecene (T-2 toxin) and saxitoxin on membrane lipid mediators of inflammatory processes were evaluated in cultured rat hepatocytes. Microcystin-LR significantly stimulated the release of prostacyclin (6-keto $F_1\alpha$ 38%,$p<0.01$) and thromboxane B_2 (TxB_2, 50%,$p<0.001$) in a concentration-dependent manner. The trichothecene toxin, T-2, enhanced the release of prostaglandin $F_2\alpha$ ($PGF_2\alpha$) by 24% ($p<0.05$) and arachidonic acid by 29% ($p<0.05$); while saxitoxin failed to cause the release prostaglandins or arachidonic acid. Incorporation of arachidonic acid into the lipid pool was reduced to 47% ($p<0.025$) by 1 μM microcystin-LR. Changes in phospholipid classes indicated that prostaglandin formation induced by microcystin-LR was due to the release of arachidonic acid from the phosphatidylinositol pool. No statistically significant effect of toxin was observed on other classes of phospholipids or neutral lipids. A 10% increase in phosphatidylcholine in hepatocytes treated with microcystin-LR may have resulted from conversion of phosphatidylethanolamine to phosphatidylcholine via the N-methylation pathway. These results indicate that microcystin-LR has important effects on the regulation of inflammatory mediator synthesis in hepatocytes.

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INTRODUCTION

Free arachidonic acid is the precursor of an array of potent lipid mediators of inflammation and modulators of immunity (1-3). The release of arachidonic acid from cellular phospholipids is the rate-limiting step in prostaglandin synthesis, which is controlled by balancing deacylating and reacylating activities (4,5). Several natural toxins and bacterial endotoxins are potent vasodilators and powerful nociceptive agents which may induce inflammatory changes by generating arachidonic acid products (6-10). This notion is supported by reversal of their effects by agents such as indomethacin (8) or glucocorticoids (11,12) which inhibit arachidonic acid release or prostanoid synthesis. Trichothecene(T-2)-and microcystin-LR-induced lethality is significantly reduced by treatment with glucocorticoids (11,12). Hong and Levine (13) have suggested that the therapeutic effect of glucocorticoids may be due to impairment of release of both prostaglandin and lipooxygenase products.

The mechanism of action of microcystin-LR on inflammatory processes has not been fully evaluated. We have examined the effect of microcystin-LR on the arachidonic acid cascade and have attempted to correlate the release of prostaglandins with phospholipid metabolism. Microcystin-LR-induced release of prostaglandins by hepatocytes was also compared with that of T-2 toxin, a known inflammatory agent in various non-hepatic tissues, and saxitoxin, which is not yet known to cause inflammatory reactions in any tissue.

MATERIAL AND METHODS

Hepatocyte cultures: Hepatocytes were isolated from 200-280 g male Fisher rats (Charles River, Wilmington, MA) and cultured in 35 x 10 mm culture plates by the procedure described by Elliget and Kolaja (14) using Liebovitz's medium containing 18% fetal calf serum (FCS). Attached cells formed a uniform monolayer and had the characteristic polygonal shape of hepatocytes.

Labeling of lipids and toxin treatments of hepatocytes: After overnight incubation at 37°C under 5% CO2 and 95% air, medium was removed, and cells were washed twice with Hank's balanced salt solution (HBSS). Cellular lipids were labeled by incubating cultures for 16 hr with 1 ml per well of Medium 199, containing 10% FCS and 10 μmoles 1-^{14}C-arachidonic acid (specific activity of 52.7 mCi/mole, New England Nuclear, Boston, MA). The cells were then washed with HBSS, further incubated for 60 min with Medium-199 containing 10% FCS to remove unincorporated ^{14}C-arachidonic acid, and washed again three times with HBSS. Toxins (concentrations indicated in texts and figures) in HBSS containing 0.1% bovine serum albumin were added to cell culture and incubated for 2 hr at 37°C. The effect of toxins on radiolabeled arachidonic acid incorporation into phospholipid was studied by incubating washed hepatocyte cultures with toxins for 2 hr at 37°C. Cells were washed three times with HBSS to remove toxins and labeled with ^{14}C-arachidonic acid as described above.

Extraction of prostaglandins and lipids: After incubating hepatocytes with toxin, incubation medium was removed, cells were washed with 1 ml of HBSS, and the wash was pooled and extracted as described by Rouzer et al.(15) for prostaglandin assays. Cells were scraped with a rubber policeman, 1 ml methanol was added, and cells were extracted with chloroform:methanol (2:1, v/v) containing 0.005% butylated hydroxytoluene (Sigma Chem. Co., St. Louis, MO). The final ratio of solvents was chloroform:methanol:water (2:2:1.8, v/v/v). The chloroform phases were pooled, dried under nitrogen, and stored under nitrogen at -20°C.

Determination of released prostaglandins: Aliquots of lipid extracted from the medium, along with prostaglandin and arachidonic acid standards (Sigma Chem. Co. St. Louis, MO), were applied to prewashed and heat-activated (100°C) silica gel-60 thin-layer chromatography (TLC) plates (E. Merck, Scientific Products, Columbia, MD). The plates were developed in ethylacetate and formic acid (80:1, v/v) to separate arachidonic acid and its prostaglandin metabolites. Radioactive lipid bands, identified by co-migration with standards (Sigma Chem. Co., St. Louis, MO), were scraped and quantified by scintillation counting. The Rf values were: prostacyclin (6-keto $F_1\alpha$), 0.24; prostaglandin $F_2\alpha$ ($PGF_2\alpha$), 0.36; thromboxane (TxB_2), 0.57; prostaglandin E_2 (PGE_2), 0.63; and arachidonic acid, 0.95.

Determination of cellular phospholipid and neutral lipid: Cellular lipids, dissolved in chloroform: methanol (2:1,v/v),

were applied to a silicic acid column (5 x 1 cm) to separate neutral lipids and phospholipids and eluted with 10 ml each of chloroform (for phospholipid separation) and methanol (for neutral lipid separation); both solvents contained 0.005% butylated hydroxytoluene. Phospholipid classes were further separated by TLC into phosphatidylcholine (PC, Rf 0.34), phosphatidylinositol (PI, Rf 0.58), phosphatidylethanolamine (PE, Rf 0.62), phosphatidylserine (PS, Rf 0.73), and phosphatidic acid (PA, Rf 0.91). The TLC solvent system was composed of chloroform:propionic acid:1-propanol:water (30:30:45:10, v/v). Neutral lipids were separated as described earlier (16). Separated and identified compounds were scraped and counted for radioactivity in a scintillation counter. Protein was measured by the Lowry method (17).

Statistical analysis: Differences among control and various treatment groups were evaluated for statistical significance by analysis of variance for intergroup comparisons and Student's "t" test.

Toxins: Microcystin-LR was obtained from Dr. Wayne Carmichael, Wright State University, Dayton, OH; T-2 toxin [3α-hydroxy-4ß,15-diacetoxy-8α-(3-methylbutyryloxy)-12, 13-epoxytrichothec-9-ene] was purchased from Romer Labs, St. Louis, MO. and saxitoxin was obtained from Dr. Samuel Page, Food and Drug Administration, Washington, D.C. Each toxin was tested for purity by HPLC and TLC.

RESULTS

Effect of toxins on arachidonic acid uptake: Toxin treatment for 2 hr did not alter cell viability as measured by trypan blue exclusion. Furthermore, there was no significant difference in the protein content of control (0.61 ± 0.13 mg/plate) and toxin-treated wells (0.69 ± 0.16 mg/plate, mean ± SD). Uptake of radiolabeled arachidonic acid by hepatocytes treated with varying concentrations of microcystin-LR was inhibited in a dose-dependent manner (Table I). The reduction in total cellular radioactivity uptake by microcystin-LR was specific. The other two toxins (T-2 and saxitoxin) under similar conditions of incubation and concentration did not inhibit the uptake and accumulation of radiolabeled arachidonic acid in hepatocytes (Table I).

Time course effect of microcystin-LR on arachidonic acid release: Maximum incorporation of ^{14}C-arachidonic acid into the phospholipid pool was obtained when hepatocytes were labeled for 16 hr (13.5 ± 0.53 x 10^4 dpm/mg protein). Addition of microcystin-LR (1 μM) in such cultures for 2 hr resulted in maximum release of radioactivity into the incubation medium (2.3 ± 0.2 x 10^4 dpm/mg protein), while untreated cultures released 0.60 ± 0.08 x 10^4 dpm/mg protein during the same period of time. Prolonged incubation with microcystin-LR resulted in a gradual decline in ^{14}C-arachidonic acid release (0.93 ± 0.09 x 10^4 dpm/mg protein after 4 hr of incubation). In all subsequent experiments, hepatocytes were prelabeled for 16 hr with ^{14}C-

arachidonic acid and treated with toxins for 2 hr.

Effect of toxins on prostaglandin release: Data in Table II demonstrate that saxitoxin failed to stimulate prostaglandin release from hepatocytes within 2 hr. T-2 toxin (1 μM) stimulated the release of $PGF_2\alpha$ by 24% ($p < 0.05$) and arachidonic acid by 29% ($p < 0.05$). Lower concentrations of T-2 were completely ineffective in inducing prostaglandin or arachidonic acid release (data not shown). Synthesis and release of both 6-keto $F_1\alpha$ and TxB_2 were most sensitive to microcystin-LR, although the effect at lower concentrations was minimal (Table III). $PGF_2\alpha$ and PGE_2 release was not affected at 1 μM microcystin-LR. The release of free arachidonic acid into the culture medium was significantly increased at the lowest concentration tested (0.01 μM for 2 hr) by almost 71% ($p < 0.05$).

Distribution of radioactivity in lipid classes: Cellular phospholipids were separated from total lipids and the distribution of radioactivity among different classes of phospholipids was determined (Table IV). No significant changes in PC, PS or PA were observed except that 1 μM microcystin-LR decreased PA significantly from 1.2 ± 0.06% in the control culture to 0.72 ± 0.04% in treated cultures ($p < 0.005$). Phosphatidylinositol and PE gradually declined with increasing concentrations of microcystin-LR (Table IV). Neutral lipids were also analyzed by TLC. Cholesterol and cholesterol ester fractions remained unaffected at all microcystin-LR concentrations tested (data not shown). The fatty acid ester

fraction showed a dose-dependent increase in ^{14}C-arachidonic acid incorporation. Microcystin-LR at 0.5 to 1.0 μM stimulated free fatty acid and triglyceride synthesis (Table V).

DISCUSSION

Few investigations concerned with the stimulation of the inflammatory cascade by toxins have been reported (6,7,11,12,18). However, several tissues and organs have been shown to be exquisitly sensitive to a number of toxins (11,12). Reaction to such exposures results in inflammation, necrosis or accumulation of inflammatory cells at the site of toxin-induced injury. We speculated that toxins activate phospholipase A_2 to release arachidonic acid, a precursor for the synthesis of prostaglandins, leukotrienes, and other immunoregulatory mediators. These substances are all known to cause inflammation and membrane damage by activation of protease enzymes (19).

The present study was undertaken to evaluate the effect of microcystin-LR, T-2 toxin, and saxitoxin on the release of cyclooxygenase products. Microcystin-LR specifically induced the release of arachidonic acid metabolites in hepatocytes, neither T-2 toxin or saxitoxin, at the concentration tested, showed an effect. This is consistent with the general belief that microcystin-LR is a specific hepatotoxin, while T-2 toxin causes inflammation in other tissues, and saxitoxin is not yet known to induce inflammatory reaction in any tissue.

Hepatocytes exposed to microcystin-LR synthesized and

produced cyclooxygenase products, mediated via phospholipid metabolism. We observed a significant breakdown of PI in hepatocytes treated with microcystin-LR. Further steps in arachidonic acid liberation by this pathway have yet to be determined. Some loss of arachidonyl radioactivity from PE might be transferred to PC by the N-methylation pathway of PC synthesis (Table IV). ^{14}C-arachidonic acid radioactivity increased by 124% and 440% in free and esterified fatty acid, respectively. No notable change in radioactivity incorporation was observed in free and esterified cholesterol fractions of neutral lipid. Thus it appears that prostaglandin formation by microcystin-LR may be due to release of arachidonic acid exclusively from PI.

The source of arachidonic acid release from cellular membrane varies with the cell type and the donor for the arachidonic residue. In most cases, PC is the donor; however, other classes of phospholipid are also known to play important roles in arachidonic acid metabolism (20,21). Marshall et al.(21) reported the release of arachidonic acid upon the stimulation of PI breakdown, serving as second messenger to elicit a cellular response by activating calcium mobilization. Data from our experiments also showed that prostaglandin formation stimulated by microcystin-LR was due to release of arachidonic acid by PI metabolism.

Several toxic effects of microcystin-LR have been reported in the literature, but its mode of action at the cellular and molecular level in liver, the target organ, have not been clearly

identified and understood (22-25). Microcystin-LR significantly reduced the uptake and increased the release of arachidonic acid. These effects may be important in the mechanism of its toxicity, as they may involve changes in cell membrane structure, and perhaps, alterations in fatty acid transport and metabolism.

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TABLE I

EFFECT OF TOXINS ON UPTAKE OF ARACHIDONIC ACID IN CULTURED RAT HEPATOCYTES

Treatment (μM)	Uptake (x 10^4 dpm/mg protein)	% Change	P-Value
Control	12.74 ± 1.01	100.0%	--
0.01 Microcystin	10.71 ± 0.57	84.1%	NS
0.1 Microcystin	9.74 ± 0.37	76.5%	<0.05
0.5 Microcystin	7.34 ± 0.19	57.6%	<0.05
1.0 Microcystin	6.74 ± 0.54	52.9%	<.025
1.0 Saxitoxin	13.21 ± 0.72	103.8%	NS
1.0 T-2 Toxin	12.74 ± 1.35	101.6%	NS

Hepatocytes were incubated for 2 hr with the indicated concentration of toxins. Washed cells were then labeled with ^{14}C-arachidonic acid for 16 hr. Results represent mean ± SEM of three cultures in duplicate.

TABLE II

EFFECTS OF TOXINS ON THE RELEASE OF ARACHIDONIC ACID AND ITS METABOLITES IN CULTURED RAT HEPATOCYTES

Metabolites	Control	T-2	Saxitoxin	Microcystin-LR
6-Keto $F_1\alpha$	472 ± 33	514 ± 44	576 ± 31	623 ± 19*
$PGF_2\alpha$	419 ± 18	519 ± 33*	429 ± 20	439 ± 38
PGE_2	564 ± 30	587 ± 39	580 ± 33	635 ± 70
TxB_2	469 ± 13	517 ± 10	558 ± 47	739 ± 36*
AA	8750 ± 606	11337 ± 693*	10503 ± 1167	20680 ± 1380*

Hepatocytes were labeled with ^{14}C-arachidonic acid for 16 hr. Toxins at 1.0 μM concentration were added for 2 hr. Medium was extracted and arachidonic acid metabolites were separated by TLC. Results represent dpm/ mg protein (mean ± SEM for three separate determinations in triplicate. Statistical significance was determined by one-way analysis of variance. * $P \leq 0.05$.

TABLE III

EFFECT OF MICROCYSTIN-LR CONCENTRATIONS ON THE RELEASE OF ARACHIDONIC ACID AND ITS METABOLITES IN CULTURED RAT HEPATOCYTES

Microcystin Conc. (μM)	6-Keto-$F_1\alpha$	$PGF_2\alpha$	PGE_2	TxB_2	Arachidonic Acid
0.00	510 ± 177	428 ± 35	507 ± 57	533 ± 35	6,08 ± 1,336
0.01	532 ± 79	462 ± 24	502 + 85	593 ± 16	10,404 ±* 1,012
0.10	596 ± 51	463 ± 30	522 ± 43	612 ±* 10	11,293 ±* 748
0.50	601 ± 32	498 ± 48	528 ± 75	657 ±* 18	14,893 ±* 2,432
1.00	727 ±* 46	479 ± 12	667 ± 69	759 ±* 53	18,432 ±* 761

^{14}C-arachidonic acid-labeled hepatocytes were incubated with varying concentrations of microcystin for 2 hr. Free arachidonic acid and metabolites were separated by TLC. Results represent dpm/ mg protein (mean ± SEM) of three separate experiments in duplicate. (*)P $\leq$0.05 from control.

TABLE IV

EFFECT OF MICROCYSTIN-LR ON THE PERCENT DISTRIBUTION OF RADIOACTIVITY IN CELLULAR PHOSPHOLIPID CLASSES

Microcystin Concentration (μM)	PC	PI	PE	PS	PA
	Percent Distribution				
Control	68.5 ± 1.9	6.9 ± 0.2	5.0 ± 0.3	18.4 ± 2.4	1.20 ± 0.06
0.01	70.0 ± 1.2	5.1 ±* 0.3	5.6 ± 0.2	18.2 ± 1.0	1.10 ± 0.11
0.10	76.1 ± 2.9	3.9 ±* 0.6	2.6 ±* 0.3	18.5 ± 0.1	0.95 ± 0.21
0.50	75.1 ± 1.7	3.6 ±* 0.3	2.5 ±* 0.1	17.7 ± 2.3	1.10 ± 0.09
1.00	75.5 ± 2.4	2.6 ±* 0.3	2.3 ±* 0.6	18.5 ± 0.6	0.72 ±* 0.04

Microcystin-LR at indicated concentrations was added to prelabeled cultures for 2 hr. Data represent percent distributions (mean ± SEM) of three separate hepatocyte cultures in duplicate. Percent distribution of radioactivity was calculated on the basis of total radioactivity in phospholipid pool. For abbreviation of phospholipid classes see "Materials and Methods."

(*) $P \leq 0.025$ from the control value.

TABLE V

EFFECTS OF MICROCYSTIN ON NEUTRAL LIPID SYNTHESIS IN RAT CULTURED HEPATOCYTES

Microcystin Concentration (μM)	Fatty Acid Free	Fatty Acid Ester	Triglyceride
	($x10^3$ dpm/mg Protein)		
Control	2.57 ± 0.09	1.63 ± 0.32	2.10 ± 0.39
0.01	2.80 ± 0.40	4.52 ± 0.50*	2.53 ± 0.58
0.1	2.90 ± 0.45	5.03 ± 0.42*	3.15 ± 0.64
0.5	4.83 ± 0.59*	5.93 ± 0.59*	3.97 ± 0.60*
1.0	6.01 ± 1.01*	8.87 ± 0.81*	5.04 ± 0.80*

Conditions were the same as described in Table IV. Neutral lipid was extracted and separated by TLC as described in methodology. Results represent mean ± SEM of three experiments in duplicate. (*)- P ≤0.05 from control.