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EFFECT OF MICROCYSTIN-LR ON CULTURED RAT ENDOTHELIAL CELLS

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INTRODUCTION

→ Microcystin-LR, a seven-amino acid cyclic polypeptide synthesized by the cyanobacterium *Microcystis aeruginosa*, induces hepatotoxicity in many species including man (GORHAM and CARMICHAEL, 1979; JACKSON et al., 1985). After administration to laboratory rodents, microcystin-LR rapidly induces severe liver hemorrhage, which is associated with centrilobular hepatocyte necrosis. (SCHWIMMER and SCHWIMMER, 1964; THEISS and CARMICHAEL, 1986). Microcystin-LR not only induces the rapid onset of liver damage in rodents *in vivo*, but also induces necrosis of cultured rat hepatocytes after several hours of incubation with the toxin (FOXALL and SASNER, 1981). The mechanism by which microcystin-LR induces hepatotoxicity is not known. Microcystin-LR effects do not appear to be mediated by the inhibition of macromolecular biosynthesis; i.e. protein, RNA or DNA synthesis (RUNNEGAR and FALCONER, 1981). Microcystin-LR does however, induce early changes in cultured hepatocytes, such as cell deformation (blebbing), rapid rise in intracellular calcium, increased phosphorylase-a activity, depletion of glutathione, (RUNNEGAR et al., 1987; FALCONER and RUNNEGAR, 1987) and the release of arachidonic acid metabolites, (NASEEM et al., 1988). These early events were followed by the leakage of adenine nucleotides and, cytosolic enzymes and eventually, the loss of cell viability. (MEREISH et al., in preparation).

Although microcystin-LR toxicity to cultured hepatocytes has been well documented, relatively little is known about its effects on other non-parenchymal liver cells, that is, sinusoidal endothelial and Kupffer cells. In the present study, we investigated the effects of microcystin-LR on cultured primary liver endothelial cells. We also investigated whether hepatocytes treated with a cytotoxic dose (4 μ M) of microcystin-LR release factors (mediators) that could induce changes, or cytotoxicity in endothelial

endothelial cells: morphological effects, etc.

monolayers. Some endothelial cells were pre-treated with the anti-oxidants, dithioerythritol (DTE) (CLELAND, 1964), or silymarin (SM) (FRAGA *et al.*, 1987), in order to determine if these agents could prevent changes induced by supernatants derived from microcystin-LR treated hepatocytes. [^{14}C]Adenine nucleotide release was used to monitor cell damage (SHIRHATTI and KRISHNA, 1985). Changes in endothelial cell viability have frequently been assessed by the leakage of $^{51}\text{chromium}$ and/or the cytosolic enzyme, lactate dehydrogenase (LDH), as well as trypan blue-dye exclusion. These methods suffer from the limitations of high, non-specific leakage and lack of sensitivity, respectively (CHOPRA *et al.*, 1987). Labeling endothelial cells with [^{14}C]adenine has advantages over the other methods in which, [^{14}C]adenine could be incorporated into the total adenine nucleotide pool. While the release of [^{14}C]adenine nucleotides from healthy endothelial cells is relatively high, it is a sensitive method for detecting small perturbations or metabolic disturbances in endothelial cells (PEARSON and GORDON, 1979). Microscopy and cell density assays were also used to detect changes in the endothelial cells.

MATERIAL AND METHODS

Materials

The following materials were obtained commercially from the indicated sources: silymarin (SM) (Aldrich Chemical Co. Inc., Milwaukee, WI), [^{14}C]adenine (60 mCi/mmol, New England Nuclear Corp., Boston, MA), tissue culture media and fetal bovine serum albumin (Gibco, Grand Island, NY), tissue culture ware (Becton-Dickinson Labware, Lincoln Park, NJ), collagen,

collagenase type IV, dithioerythritol (DTE), endothelial growth factor and heparin (Sigma Chem. Co., St. Louis, MO).

Male, WF.LEW inbred rats (G. Anderson, USAMRIID, Fort Detrick, Frederick, MD), weighing 250-300 g, were used for all experiments. Microcystin-LR, assessed as > 85% pure by high-performance-liquid-chromatography was obtained from Dr. W. Carmichael, Wright State University, Dayton, OH.

Hepatocytes

Rat hepatocytes were isolated and cultured according to the method of ELLIGET and KOLAJA (1983). Hepatocytes were separated from non-parenchymal cells by low-speed centrifugation (500 X g) for 35-45 sec. After washing the hepatocyte pellet several times, the number of viable cells was determined by trypan blue exclusion. Hepatocytes were suspended at 5×10^5 viable cells per ml in Leibovitz's (L15) culture medium containing 17% fetal bovine serum (FBS) and seeded in collagen-coated, 35 mm, 6-well-plates by adding 1 ml of the cell suspension per well. The cells were allowed to attach for 30 min at room temperature and then incubated at 37°C with 5% CO₂ and 90% humidity for an additional 2 hr. After incubation, the majority of hepatocytes attached and established a monolayer. Non-attached cells were removed by aspiration and an additional 1 ml of culture medium was added to each well.

Sinusoidal endothelial cells

Endothelial cells were isolated according to the procedure of SMEDSRUD and PERTOFT (1985). Non-parenchymal cells isolated from *in situ* liver perfusion were layered over a two-step Percoll gradient and centrifuged for 15 min at 4°C at 800 X g. After centrifugation, sinusoidal endothelial cells were collected at the interface between the two Percoll gradients. The

endothelial cells were washed twice at 700 x g for 5 min in RPMI 1640 medium. The number of viable endothelial cells was determined by trypan blue exclusion. Endothelial cells were resuspended at 0.5×10^6 viable cells per ml in RPMI 1640, containing 10% FBS and 15 mg of endothelial growth factor, and seeded on collagen-coated, 35 mm, 6-well-plates by adding 1 ml of cell suspension per well. The cells were incubated overnight at 37°C with 5% CO₂ and 90% humidity. After incubation, the majority of endothelial cells had attached and established a monolayer. Non-attached endothelial cells were removed by aspiration and an addition 1 ml of culture medium was added to each well.

Treatment of hepatocytes with microcystin-LR

One ml of L15 medium containing 4 μ M of microcystin-LR or 1 ml of medium alone was added to hepatocyte monolayers 3 hr post plating. The cells were incubated for 16 hr (overnight) at 37°C in a humidified incubator in the presence of 5% CO₂. After incubation, cell supernatants were removed from both the treated and control cells and centrifuged at 500 X g for 4 min in an Eppendorf centrifuge, Model-5414, to remove cell debris. Supernatants from both treated and non-treated hepatocytes were pooled separately and stored at 4°C for a maximum of 2 hr before use.

Labeling and treatment of endothelial cells

After endothelial cells were incubated overnight, the culture medium from each well was replaced with 1 ml of RPMI 1640 containing [¹⁴C]adenine (1.4 μ M, 0.082 μ Ci). The cells were incubated for 2 hr at 37°C in a humidified incubator in the presence of 5% CO₂. After incubation, the [¹⁴C]adenine-containing medium was removed and the cells were washed with RPMI 1640. These

labeled cells were then incubated with 1 ml of L15 medium containing 4 μ M microcystin-LR, medium alone, or 1 ml of supernatants from control or microcystin-LR-treated hepatocytes for a total of 20 hr. In addition, some endothelial cells were pre-treated for 30 min with 2.5 mM DTE or 0.2 mM SM before receiving 1 ml of supernatants derived from microcystin-LR-treated hepatocytes or L15 medium.

In order to determine the amount of [14 C]adenine nucleotides released from control and treated cells during incubation, 0.1 ml of cell supernatants was removed from each well at selected time intervals and counted in 10 ml of Hydrofluor (National Diagnostic, Summerville, NJ) in a Beckman Scintillation Counter, Model LS5800 (Beckman Inst. Co., Fullerton, CA). After 20 hr of incubation, the cells were lysed by the addition of 1 ml of 0.05% digitonin in phosphate buffer to each well. An aliquot of each cell lysate was used to measure cellular [14 C]adenine nucleotides and protein content. Protein levels were determined by using Pierce protein reagent (Pierce, Rockford, IL) with bovine serum albumin as the standard.

Cell morphology during the incubation period was assessed by light microscopy with a Nikon Diphot inverted phase contrast microscope. Photographs were taken with a Nikon FE camera with Tungsten 50, 35-mm, color slide film.

RESULTS

The release of [14 C]adenine nucleotides from endothelial cells treated with 4 μ M microcystin-LR was not significantly different from control cells incubated with L15 medium alone (Fig. 1). Concentrations of up to 50 μ M

microcystin-LR did not induce significant release of nucleotides over control levels (data not shown).

Endothelial cells treated with supernatants from untreated hepatocytes (Fig. 2) released less [^{14}C]adenine nucleotides than control cells incubated in L15 medium alone (Fig. 1). These healthy, control hepatocytes probably released into the medium "conditioning factors" which were beneficial to endothelial cells. Endothelial cells, however, incubated with supernatants derived from hepatocytes treated with 4 μM microcystin-LR, released significantly more [^{14}C]adenine nucleotides than cells incubated with control supernatants (Fig. 2). The difference in [^{14}C]nucleotide release between the treated and untreated cells was observed as early as 3 hr post-exposure and continued for 20 hr. Endothelial cells treated with 2.5 mM DTE and then incubated with supernatants derived from microcystin-LR-treated hepatocytes released the same amount of [^{14}C]adenine nucleotides as did cells treated with microcystin-LR supernatants. However, cells pre-treated with SM, then with microcystin-LR supernatants, released statistically significantly lower levels of [^{14}C]adenine nucleotides than were induced by microcystin-LR supernatants alone (Fig. 2).

The morphology of endothelial cells after 4 hr exposure to microcystin-LR, or hepatocyte supernatants is shown in Figure 3. Endothelial cells incubated with L15 medium alone, microcystin-LR or supernatants derived from untreated hepatocytes (Fig. 3a) displayed spindle-shape cells bodies with extended, cytoplasmic processes to neighboring cells. Endothelial cells, however, incubated with supernatants derived from microcystin-LR-treated hepatocytes (Fig. 3b), displayed rounded, more contracted cell bodies with fewer extensions.

Endothelial cell densities were determined by protein levels (in culture wells) measured 20 hr after incubation. There was no significant difference in protein content between endothelial cell monolayers treated with L15 medium alone, 4 μ M microcystin-LR or supernatants derived from control and treated hepatocytes (Table 1).

DISCUSSION

The release of [14 C]adenine nucleotides from control endothelial cells incubated in either L15 medium or with supernatants from untreated hepatocytes was similar to that released from porcine aortic endothelial cells labeled with [3 H]adenosine (LEROY *et al.*, 1984; PEARSON *et al.*, 1978). Endothelial cell monolayers incubated directly with the toxin exhibited the same morphological features, and cell density, and released the same amounts of [14 C]adenine nucleotides as control cells treated with L15 medium alone. These observations indicate that microcystin-LR did not directly injure primary sinusoidal liver endothelial cells. However, supernatants derived from microcystin-LR-treated hepatocytes did induce significant changes in cultured endothelial cell monolayers. These changes include significant increase in [14 C]adenine nucleotide release, rounded or contracted cell shape, and a small reduction in cell density. The release of [14 C]nucleotides combined with the changes in cell shape (which occurred during approximately the same time) suggests that supernatants derived from microcystin-LR-treated hepatocytes induced an increase in the permeability of endothelial cells. Permeability changes in endothelial cells are thought to be due to changes in cell shape mediated by a calcium-dependent contraction of actin-myosin microfilaments combined with the activation of actin-severing proteins, i.e., gelsolin, fragmin and villin (SHASBY *et al.*, 1982; SAVION *et al.*, 1982; WEEDS,

1982). The contraction of cytoskeletal microfilaments induces the loss of cell-to-cell junctions and creates gaps between adjacent cells (MAJINO *et al.*, 1967; FANTONE *et al.*, 1980).

It is possible that supernatants derived from microcystin-LR-treated hepatocytes contain either active microcystin-LR metabolite(s) or cell products that affect the permeability of endothelial cells. In fact, microcystin-LR has been shown to induce the release of arachidonic acid metabolites in cultured hepatocytes early after exposure (NASEEM *et al.*, 1988). Arachidonic acid is known to stimulate prostaglandin synthesis in cultured endothelial cells (BORDET *et al.*, 1986). Microcystin-LR-treated hepatocytes have also been shown to release adenine nucleotides (ATP, ADP, AMP) and adenosine (MERELISH *et al.*, in preparation), which act as local hormones in increasing the permeability of the microvasculature (PEARSON and GORDON, 1979). The release of adenine nucleotides from damaged hepatocytes could therefore play a role in the initiation of liver hemorrhage followed by permeability changes in endothelial cells, thereby creating red cell extravasation.

Microcystin-LR-treated hepatocytes may possibly induce the release of substances such as histamine, oxygen-free radicals, hydrogen peroxide, leukotrienes, lysolecithin, phosphatidic acid, and/or oxidized fatty acids. It is known that histamine and oxygen-free radicals enhance endothelial cell permeability (OLESEN, 1987; SHASBY *et al.*, 1985). Leukotrienes C4 and D4 have been shown to increase endothelial permeability in hamster cheek pouches (DAHLEN *et al.*, 1981), while oxidized fatty acids and phosphatidic acid have been shown to be calcium ionophores (SERHAN *et al.*, 1981; 1982).

A 30 min exposure to SM lessened the changes induced by supernatants from microcystin-LR-treated hepatocytes. Both DTE and SM have been shown to protect cultured hepatocytes against a variety of hepatotoxic agents including microcystin-LR (NICOTERA *et al.*, 1985; WAGNER, 1986; HIKINO *et al.*, 1984; MEREISH *et al.*, in preparation). SM provides protection through the combined action of scavaging free radicals (VALENZUELA *et al.*, 1986), inhibiting lipoxygenation and, therefore, leukotriene synthesis (BAUMANN *et al.*, 1980). It is possible that the continuous presence of DTE and SM might be required to reduce the changes induced in endothelial cells by the supernatants.

It could be argued that the permeability changes produced in endothelial cells by supernatants from microcystin-LR-treated hepatocytes are just the response of endothelial cells to the release of contents of dead hepatocytes. Experiments are now in progress to test this possibility. It is far more likely, however, that the factors responsible for producing permeability changes in endothelial cells are induced as a consequence of the interaction of microcystin-LR with hepatocytes. The increase of endothelial cell permeability induced by factors released by microcystin-LR-treated hepatocytes may contribute secondarily to the toxicity of microcystin-LR *in vivo*.

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TABLE 1. PROTEIN CONTENTS OF TREATED ENDOTHELIAL CELLS

Treatment A*		Treatment B†	
<u>Control</u>	<u>Microcystin-LR</u>	<u>Control</u>	<u>Microcystin-LR</u>
0.213	0.176	0.144	0.119‡
±0.041	±0.052	±0.019	±0.008

*Endothelial cells treated with L15 medium containing 4 μ M microcystin-LR or media alone

†Endothelial cells treated with supernatants from control or microcystin-LR (4 μ M) treated hepatocytes

Each value represents the mean of three determinations $\pm$ SD.

‡P < 0.1

FIG. 1. EFFECT OF MICROCYSTIN-LR ON THE PERCENT RELEASE OF [14 C]ADENINE NUCLEOTIDES FROM PRIMARY SINUSOIDAL ENDOTHELIAL MONOLAYERS. Endothelial cells (5×10^5 /ml) were incubated with [14 C]adenine (1.4 μ M, 0.081 μ Ci) for 1 hr at 37°C. The cells were then washed and reincubated in 1 ml L15 medium containing 4 μ M microcystin-LR (■-■) or medium alone (○-○), as described in the text, for 20 hr. At selected time intervals, supernatants were collected and the amount of [14 C]nucleotides released from endothelial cells were determined as described in the text. Each point represents the mean of three determinations + SD.

FIG. 2. EFFECT OF SUPERNATANTS DERIVED FROM MICROCYSTIN-LR-TREATED HEPATOCYTES ON THE PERCENT RELEASE OF [14 C]ADENINE NUCLEOTIDE FROM PRIMARY SINUSOIDAL ENDOTHELIAL MONOLAYERS. [14 C]Adenine endothelial cells were incubated with supernatants collected from hepatocytes incubated with 4 μ M microcystin-LR (■-■) or medium alone (○-○), as described in the text. Some endothelial cells were pretreated with either 2.5 mM DTE (■-■) or 0.2 mM SM (●-●) for 30 min before receiving supernatants from microcystin-treated hepatocytes. Endothelial cells were incubated for a total of 20 hr and the amount of [14 C]adenine nucleotides released was determined as described in figure 1. The results are the mean of three determination + SD.

FIG. 3. PHASE CONTRAST MICROGRAPH (X40) OF RAT PRIMARY SINUSOIDAL ENDOTHELIAL CELLS 4 HR AFTER EXPOSURE TO (A) L15 MEDIUM ALONE, 4 μ M MICROCYSTIN-LR IN L15 MEDIUM OR SUPERNATANTS DERIVED FROM UNTREATED HEPATOCYTES OR (B) 4 μ M MICROCYSTIN-LR TREATED HEPATOCYTES.

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