Microcystin-LR induced an inhibition of protein synthesis in isolated rat hepatocytes

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The effect of microcystin-LR, an inhibitor of protein phosphatases PP1 and PP2A, was studied on protein synthesis by measuring the incorporation of labelled amino acid into protein in isolated rat hepatocytes. Microcystin-LR inhibited protein synthesis in the first minutes of the incubation period, and half-maximum effect was obtained at about 60 nM. Such an inhibition was also observed in the presence of different protein phosphatase inhibitors, i.e. okadaic acid, calyculin A and

microcystin-RR. This effect was observed in whole hepatocytes, in the supernatant of the post-mitochondrial fraction and in the microsomal fraction. It was independent of a substrate supply and of the labelled amino acid used. Furthermore, this inhibition preceded the previously reported glucose-6-phosphatase activation induced by microcystin-LR [Claeysens, Chédeville and Lavoinne (1993) FEBS Lett. **315**, 7–10].

INTRODUCTION

We have recently reported that nanomolar concentration of microcystin-LR (an inhibitor of protein phosphatases PP1 and PP2A [1]) increased the specific activity of the microsomal glucose-6-phosphatase in isolated rat hepatocytes [2]. This effect was suppressed by addition of puromycin, a protein-synthesis inhibitor, suggesting that the glucose-6-phosphatase activation induced by microcystin-LR might be mediated through a stimulation of protein synthesis.

In the present work, we investigated the possibility that microcystin-LR might stimulate protein synthesis. The results obtained clearly demonstrated that microcystin-LR did not stimulate, but by contrast inhibited, protein synthesis in the first minutes of the incubation period in isolated rat hepatocytes. Moreover, this inhibition preceded the glucose-6-phosphatase activation induced by microcystin-LR.

MATERIALS AND METHODS

Materials

Glucose-6-phosphate (monosodium salt), sodium cacodylate, microcystin-LR, okadaic acid, calyculin A, cycloheximide and emetin were purchased from Sigma. L-[³H]Leucine, L-[¹⁴C]proline and L-[³⁵S]methionine were from Amersham (France). Other biochemical reagents were of analytical grade.

Preparation of hepatocytes

Hepatocytes were isolated and prepared as described previously [3] from 24 h-fasted male Wistar rats (200–220 g). The incubation medium was Krebs–Henseleit bicarbonate buffer at pH 7.4 in equilibrium with a gas phase of O₂/CO₂ (19:1). The isolated hepatocytes (50–70 mg wet wt./ml) were shaken (165 cycles/min) in stoppered scintillation vials at 37 °C for 45 min, except where otherwise stated.

Preparation of subcellular fractions

All steps of preparation of subcellular fractions were conducted at 4 °C as previously described [2]. At the end of incubation, hepatocytes were diluted in an equal volume of ice-cold 0.25 M sucrose/5 mM Hepes buffer, pH 7.4 (buffer A), and spun down rapidly (1 min at 500 g). The cell pellet was homogenized in the same previous volume of buffer A with a Teflon/glass homogenizer (20 strokes, ensuring the disruption of a similar number of control and treated hepatocytes as indicated by similar lactate dehydrogenase release) and centrifuged for 10 min at 10000 g. A portion of the post-mitochondrial supernatant was centrifuged for 60 min at 105000 g, and the resulting pellet was resuspended in buffer A to give the microsomal fraction. The proportion of intact microsomes was ascertained as previously described [2]. In all the preparations, microsomes were more than 90 % intact, and addition of different effectors did not significantly change their intactness.

Determination of protein synthesis

Protein synthesis was studied by measuring the incorporation of added labelled amino acids into protein. L-[³H]Leucine was added to the incubation medium at 10 μM (10 mCi/mmol), except in experiments with unlabelled amino acid mixture, where it was added to the incubation medium at 0.37 mM (270 mCi/mol). L-[¹⁴C]Proline was added to the incubation medium at 25 μM (250 mCi/mmol) and L-[³⁵S]methionine at 20 nM (1000 Ci/mmol). Labelled amino acids were added to the incubation medium at the start of the incubation period of hepatocytes, except for L-[¹⁴C]proline, which was added 12 min before the end of the incubation period. After the incubation, 2 ml samples of the whole hepatocytes, of the supernatant of the post-mitochondrial fraction or of the microsomal fraction were precipitated with an equal volume of 4 % (v/v) HClO₄ and left at 4 °C for at least 15 min. The precipitate was then washed with 3 × 4 ml of cold 2 % (v/v) HClO₄ by resuspension and centrifugation, and the last pellet was dissolved in 0.5 ml of 0.3 M NaOH and assayed for radioactivity.

Abbreviation used: RER, rough endoplasmic reticulum.

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Assays

The activities of glucose-6-phosphatase and mannose-6-phosphatase were assayed as previously described [2] and lactate dehydrogenase activity was measured as described in [4]. The protein concentration was determined by the Lowry method [5].

Electron microscopy

Isolated hepatocytes were fixed in 2.0% (v/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 (60 min), washed in buffer at 4 °C and post-fixed in 1% (v/v) osmium tetroxide (60 min), dehydrated in graded alcohols and embedded in Epon. Then 70 nm-thick ultrathin sections were cut on a Reichert ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a Philips EM 310 electron microscope.

Results

Glucose-6-phosphatase activity was expressed as nmol of P_i released/min per mg of protein. Radioactivity was expressed as c.p.m./mg of protein, and represented net incorporation for the indicated period of incubation. Statistical analysis was performed by using Student's *t* test for paired data.

RESULTS AND DISCUSSION

Microcystin-LR inhibited protein synthesis

Microcystin-LR (200 nM) inhibited the incorporation of [3 H]leucine into protein in isolated hepatocytes incubated for 45 min (86% inhibition; $n = 4$; $P < 0.05$). As shown in Figure 1(a), this effect appeared in the first minutes of the incubation period, and half-maximum effect was obtained at about 60 nM (Figure 1b). Incorporation of [3 H]leucine was also studied incubating the hepatocytes for 45 min in the presence of a non-labelled amino acid mixture at 4-fold the normal amino acid concentration as described in [6] (and consequently in the presence of an approx. 40-fold higher leucine concentration than that used in Figure 1). Such experimental conditions did not modify the pattern of the observed inhibition of [3 H]leucine incorporation induced by microcystin-LR; for example, after 45 min of incubation, the values were 166 ± 5 c.p.m./mg of protein in the control, and 12 ± 1 c.p.m./mg of protein in the presence of 200 nM microcystin-LR ($n = 4$, $P < 0.05$). This demonstrated that the inhibition of [3 H]leucine incorporation induced by microcystin-LR did not result from a dilution of the specific radioactivity by unlabelled amino acids originating from protein degradation. This also suggested that microcystin-LR did not inhibit protein synthesis by decreasing the supply of endogenous amino acids provided by protein degradation.

The effect of microcystin-LR on [3 H]leucine incorporation into protein was also studied in the microsomal fraction. Indeed, glucose-6-phosphatase, which has been shown to be activated by microcystin-LR [2], is a microsomal protein whose synthesis is dependent on a specific system involving ribosomes associated with the rough endoplasmic reticulum (RER) [7,8]. Moreover, electron microscopy revealed that, beside the changes usually reported in cells incubated in the presence of phosphatase inhibitors (i.e. disappearance of pili from the cell surface and deformations of the plasma membrane [9–11]), microcystin-LR induced a redistribution of the RER to the cell periphery (results not shown). Thus microcystin-LR might affect differently the protein synthesis associated with RER. As shown in Table 1, microcystin-LR inhibited [3 H]leucine incorporation into protein in the microsomal fraction. Although the rate of amino acid incorporation was higher in the microsomal fraction than in the

Figure 1 Influence of microcystin-LR on [3 H]leucine incorporation in isolated rat hepatocytes

Hepatocytes were incubated in the presence of 10 mM glucose and [3 H]leucine (10 μ M; 10 mCi/mmol) for the indicated period of time (a) or for 45 min (b). At the end of the incubation, samples were taken and incorporation of radioactivity into acid-precipitable material was measured. (a) Time course of [3 H]leucine incorporation in the absence (○) or in the presence of 200 nM microcystin-LR (●). (b) [3 H]leucine incorporation in the presence of various microcystin-LR concentrations. The values represent net incorporation for the indicated period of incubation and are expressed as means $\pm$ S.E.M. for four independent experiments: * significantly different ($P < 0.05$) from control values.

supernatant of the post-mitochondrial fraction as previously described by Siekevitz [12], microcystin-LR inhibited [3 H]leucine incorporation with a similar intensity in both fractions. Indeed, the ratio of the [3 H]leucine incorporation with and without microcystin-LR was not different in the supernatant of the post-mitochondrial fraction and in the microsomal fraction (0.42 ± 0.03 and 0.43 ± 0.05 , respectively; data from Table 1). Moreover, addition of glucose to the incubation medium did not significantly modify the intensity of the microcystin-LR effect on protein synthesis (the ratios were 0.45 ± 0.04 in the supernatant of the post-mitochondrial fraction and 0.40 ± 0.03 in the microsomal fraction; data from Table 1). Therefore, microcystin-LR inhibited protein synthesis independently of an energetic-substrate supply and affected the general process of protein synthesis in the cell.

Furthermore, the incorporation into protein of [14 C]proline, whose transport system in hepatocytes is known to be different from that of leucine [13], was inhibited by microcystin-LR in both the supernatant of the post-mitochondrial fraction (26.7×10^3 c.p.m./mg of protein for control; 4.5×10^3 c.p.m./mg of protein for 100 nM microcystin-LR; $n = 2$) and in the microsomal fraction (55.0×10^3 c.p.m./mg of protein for control; 10.8×10^3 c.p.m./mg of protein for, 100 nM microcystin-LR; $n = 2$). Moreover, such an inhibition was also observed when

Table 1 Influence of microcystin-LR on [^3H]leucine incorporation in the supernatant of the post-mitochondrial fraction, and in the microsomal fraction in isolated rat hepatocytes

Hepatocytes were incubated for 45 min in the absence or in the presence of 100 nM microcystin-LR with or without 10 mM glucose. [^3H]leucine (10 μM ; 10 mCi/mmol) was added to the incubation medium. At the end of the incubation, samples were taken for cellular fractionation, and the incorporation of radioactivity into acid-precipitable material was measured in the supernatant of post-mitochondrial fraction and in the microsomal fraction. The values represent net incorporation for the period of incubation and are expressed as means $\pm$ S.E.M. for four independent experiments: * significantly different ($P < 0.05$) from values in the absence of microcystin-LR; † significantly different ($P < 0.05$) from values in the absence of glucose.

	[^3H]Leucine incorporation (c.p.m./mg of protein)			
	No glucose		+ Glucose	
	Supernatant	Microsomes	Supernatant	Microsomes
Control	732 $\pm$ 200	1394 $\pm$ 300	1057 $\pm$ 278†	1826 $\pm$ 395†
+ Microcystin-LR	296 $\pm$ 66*	647 $\pm$ 186*	468 $\pm$ 105†	736 $\pm$ 176†

Table 2 Influence of various inhibitors of protein phosphatases on [^3H]leucine incorporation in isolated rat hepatocytes

Hepatocytes were incubated for 45 min in the presence of 10 mM glucose and [^3H]leucine (10 μM ; 10 mCi/mmol) (control) and in the presence of 200 nM microcystin-LR, 1 μM okadaic acid, 1 μM calyculin A or 2 μM microcystin-RR. At the end of the incubation, samples were taken and incorporation of radioactivity into acid-precipitable material was measured. The values represent net incorporation for the period of incubation and are expressed as means $\pm$ S.E.M. for four independent experiments: *significantly different ($P < 0.05$) from control values.

	[^3H]Leucine incorporation (c.p.m./mg of protein)
Control	933 $\pm$ 73
+ Microcystin-LR	107 $\pm$ 14*
+ Okadaic acid	211 $\pm$ 36*
+ Calyculin A	50 $\pm$ 1*
+ Microcystin-RR	532 $\pm$ 78*

Table 3 Influence of emetin on the microcystin-LR effect on glucose-6-phosphatase activity in isolated rat hepatocytes

Hepatocytes were incubated for 45 min in the presence of 200 nM microcystin-LR ($\mu\text{-LR}$) and/or 1 mM emetin as indicated. At the end of the incubation, glucose-6-phosphatase activity was measured in the supernatant of the post-mitochondrial fraction and in the microsomal fraction. Values are expressed as means $\pm$ S.E.M. for five independent experiments: *significantly different ($P < 0.05$) from control values; †significantly different ($P < 0.05$) from values in the presence of microcystin-LR but in the absence of emetin.

	Glucose-6-phosphatase activity (nmol/mg of protein)			
	Supernatant		Microsomes	
	Control	+ $\mu\text{-LR}$	Control	+ $\mu\text{-LR}$
Control	48.6 $\pm$ 2.7	70.6 $\pm$ 4.0*	163 $\pm$ 10	228 $\pm$ 31*
+ Emetin	46.8 $\pm$ 3.4	45.4 $\pm$ 2.0†	179 $\pm$ 20	172 $\pm$ 25†

[^3S]methionine was used, suggesting that microcystin-LR did not inhibit protein synthesis by interfering with amino acid transport (results not shown).

Okadaic acid and calyculin A, two other chemically different inhibitors of protein phosphatases (PP1 and PP2A) [14,15], also inhibited [^3H]leucine incorporation (Table 2). Table 2 also showed that microcystin-RR, a structural analogue of microcystin-LR with an equal potency to inhibit PP1 and PP2A *in vitro*, but requiring higher concentrations for inducing increased protein phosphorylation in hepatocytes [16], also inhibited protein synthesis. Therefore, inhibition of protein phosphatases (PP1 and PP2A) appeared to be responsible for the observed inhibition of protein synthesis in isolated rat hepatocytes. Interestingly, it has been reported recently that 1 μM okadaic acid inhibited protein synthesis in isolated rat hepatocytes at the end of a 3 h incubation in the presence of a non-labelled amino acid mixture [17]. Our results extended this observation and demonstrated that, firstly, microcystin-LR inhibited the general process of protein synthesis in the cell and, secondly, this inhibition occurred rapidly in the first minutes of the incubation period. Such an inhibitory effect of protein phosphatase inhibitors had been reported previously in reticulo-cyte lysates [18].

Inhibition of protein synthesis preceded the activation of glucose-6-phosphatase induced by microcystin-LR

The fact that microcystin-LR inhibited protein synthesis, whereas puromycin, a protein-synthesis inhibitor inducing premature chain termination [19], suppressed the glucose-6-phosphatase activation induced by microcystin-LR [2], was paradoxical. We therefore re-investigated the effect of protein-synthesis inhibitors on the glucose-6-phosphatase activation induced by microcystin-LR. Emetin, a protein-synthesis inhibitor acting at the level of the 40 S subunit [19], also suppressed the glucose-6-phosphatase activation induced by microcystin-LR (Table 3). Moreover, 5 mM cycloheximide, a protein-synthesis inhibitor acting at the level of the 60 S subunit and inhibiting the peptidyl-transferase [19], also suppressed this effect (results not shown). These results confirmed our previous data obtained with puromycin [2]. However, a dose-response study of the effect of emetin showed that the concentration of emetin necessary to suppress the microcystin-LR effect on the glucose-6-phosphatase activity was approx. 10-fold higher (500 μM) than that necessary to inhibit protein synthesis (50 μM) (Figure 2). Both these results, (i) the shift between the concentration at which emetin inhibited protein synthesis and the concentration at which it suppressed the glucose-6-phosphatase activation induced by microcystin-LR and (ii) the inhibition of protein synthesis by microcystin-LR

Figure 2 Influence of various emetin concentrations on [³H]leucine incorporation and on the glucose-6-phosphatase activation induced by microcystin-LR in isolated rat hepatocytes

Hepatocytes were incubated for 45 min with various emetin concentrations in the absence (○, △) or in the presence (●, ▲) of 200 nM microcystin-LR. [³H]Leucine (10 μM; 10 mCi/mmol) was added in the experiments for measuring protein synthesis. At the end of the incubation, samples were taken for cellular fractionation, and the incorporation of radioactivity into acid-precipitable material (○, ●) and glucose-6-phosphatase activity (△, ▲) were measured in the supernatant of the post-mitochondrial fraction. The values of [³H]leucine incorporation represented net incorporation for the period of incubation and are expressed as means for two independent preparations.

itself, suggested that the glucose-6-phosphatase activation induced by microcystin-LR might not be mediated by a stimulation of its protein synthesis. However, the necessity for a high concentration of protein synthesis inhibitors to suppress the glucose-6-phosphatase activation induced by microcystin-LR might be explained by the requirement for a total inhibition of protein synthesis (more than 95%). In this regard, it may be suggested that the synthesis of glucose-6-phosphatase was controlled differently from that of most other proteins. Alternatively, it cannot be excluded that the suppression of the glucose-6-phosphatase activation may result from side-effects of these effectors. In this case, microcystin-LR may act on glucose-6-phosphatase without affecting protein synthesis. Therefore, the activation of glucose-6-phosphatase induced by microcystin-LR might be post-translational.

Although the concentrations of microcystin-LR necessary to

observe the half-maximum effect on protein synthesis (60 nM) and on glucose-6-phosphatase activation (100 nM) [2] were of the same order of magnitude, the effect on protein synthesis occurred more rapidly (less than 15 min) than the effect on glucose-6-phosphatase (more than 15 min) [2]. Therefore the inhibition of protein synthesis preceded the activation of glucose-6-phosphatase. If this activating effect is post-translational, the observed time lag may be due to the fact that the relevant kinase is not very active: the kinase is activated by microcystin-LR, and no effect can be seen on glucose-6-phosphatase until the kinase becomes sufficiently active.

In conclusion, microcystin-LR inhibited protein synthesis in the first minutes of the incubation period in isolated rat hepatocytes and affected the general process of protein synthesis in the cell. This inhibitory effect on protein synthesis preceded its activatory effect on glucose-6-phosphatase. However, the possibility of a link between the inhibition of protein synthesis and the activation of glucose-6-phosphatase remains to be established.

REFERENCES

- 1 Mackintosh, C., Beattie, K. A., Klumpp, S., Cohen, P. and Codd, G. A. (1990) *FEBS Lett.* **264**, 187–192
- 2 Claeysens, S., Chédeville, A. and Lavoinne, A. (1993) *FEBS Lett.* **315**, 7–10
- 3 Marchand, J. C., Lavoinne, A., Giroz, M. and Matray, F. (1979) *Biochimie* **61**, 1273–1282
- 4 Bergmeyer, H. U. and Bernt, E. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 574–579, Verlag Chemie, Weinheim, and Academic Press, New York and London
- 5 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- 6 Seglen, P. O. (1976) *Biochim. Biophys. Acta* **442**, 391–404
- 7 Pfeffer, S. R. and Rothman, J. E. (1987) *Annu. Rev. Biochem.* **56**, 829–852
- 8 Pryer, N. K., Wuestehube, L. J. and Schekman, R. (1992) *Annu. Rev. Biochem.* **61**, 471–516
- 9 Boe, R., Gjertsen, B. T., Vintermyr, O. K., Houge, G., Lanotte, M. and Doskeland, S. O. (1991) *Exp. Cell Res.* **195**, 237–246
- 10 Falconer, I. R. and Yeung, D. S. K. (1992) *Chem.-Biol. Interact.* **81**, 181–196
- 11 Eriksson, J. E., Paatero, G. I. L., Meriluoto, J. A. O., Codd, G. A., Kass, G. E. N., Nicotera, P. and Orrenius, S. (1989) *Exp. Cell Res.* **185**, 86–100
- 12 Siekevitz, P. (1959) *Exp. Cell Res.* **7**, 90–101
- 13 McGivan, J. D. and Pastor-Anglada, M. (1994) *Biochem. J.* **299**, 321–334
- 14 Cohen, P., Holmes, C. F. B. and Tsukitani, Y. (1990) *Trends Biochem. Sci.* **15**, 98–102
- 15 Ishihara, H., Martin, B. L., Brautigan, D. L., Karaki, H., Ozaki, H., Kato, Y., Fusetani, N., Watabe, S., Hashimoto, K., Uemura, D. and Hartshorne, D. J. (1989) *Biochem. Biophys. Res. Commun.* **159**, 871–877
- 16 Ericksson, J. E., Toivola, D., Meriluoto, J. A. O., Karaki, H., Han, Y.-G. and Hartshorne, D. (1990) *Biochem. Biophys. Res. Commun.* **173**, 1347–1353
- 17 Holen, I., Gordon, P. B. and Seglen, P. O. (1993) *Eur. J. Biochem.* **215**, 113–122
- 18 Redpath, N. T. and Proud, C. G. (1991) *Biochim. Biophys. Acta* **1093**, 36–41
- 19 Di Giambattista, M. and Cocito, C. (1990) *Médecine/Sciences* **6**, 46–54