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Inhibition of protein phosphatases activates glucose-6-phosphatase in isolated rat hepatocytes

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Incubation of hepatocytes in the presence of microcystin-LR, okadaic acid, calyculin A (inhibitors of protein phosphatases PP1 and PP2A) or microcystin-RR (a specific inhibitor of PP2A) activated glucose-6-phosphatase both in the supernatant and in intact or disrupted microsomes. Puromycin, an inhibitor of protein synthesis, totally suppressed this activating effect, suggesting the involvement of protein phosphatases in the regulation of glucose-6-phosphatase synthesis.

Hepatocyte; Glucose-6-phosphatase; Protein phosphatase; Microcystin

1. INTRODUCTION

Liver plays a major role in the homeostasic regulation of blood glucose level. Gluconeogenesis and glycogenolysis are the two metabolic pathways producing glucose, and glucose-6-phosphatase (EC 3.1.3.9) catalyzes the terminal step of both pathways. Despite the physiological importance of this enzyme, little is known about it's regulation. There are been numerous reports of compounds modifying glucose-6-phosphatase activity in vitro ([1–3] for reviews) but no report of acute regulation of the enzymatic activity.

In the present work, we report acute activation of glucose-6-phosphatase in rat hepatocytes incubated with microcystin-LR, an inhibitor of protein phosphatases (PP1 and PP2A) [4]. Our results suggest that glucose-6-phosphatase may be regulated by phosphorylation at the level of protein synthesis.

2. MATERIALS AND METHODS

2.1. *Materials*

Glucose-6-phosphate (monosodium salt), mannose-6-phosphate (disodium salt), histone 2A, sodium cacodylate, microcystin-LR, microcystin-PR, okadaic acid, calyculin A and puromycin, were purchased from Sigma. Other biochemical reagents were of analytical grade.

2.2. *Preparation of hepatocytes*

Hepatocytes were isolated as described previously [5] from 24 h fasted male Wistar rats (200–220 g). The isolated hepatocytes (60–80 mg wet wt./ml) were shaken (165 cycles/min) in stoppered scintillation vials at 37°C for 45 min, except where otherwise stated. The incubation medium was a Krebs–Henseleit bicarbonate buffer at pH 7.4 in equilibrium with a gas phase of O_2/CO_2 (19:1).

2.3. *Preparation of microsomes*

At the end of incubation, cells 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 volume of buffer A in a Teflon–glass homogenizer (20 strokes) and centrifuged for 10 min at 10,000 × *g*. An aliquot of the supernatant was assayed immediately and the remaining supernatant centrifuged for 60 min at 105,000 × *g*. The resulting microsomal pellet was resuspended in 3 ml/g wet wt. of original cells in buffer A. The proportion of intact microsomes was ascertained by measuring the latency of low K_m mannose-6-phosphatase activity [6]. In all the preparations, microsomes were more than 90% intact; moreover addition of the different effectors did not significantly change the number of intact microsomes (data not shown).

2.4. *Assays*

Glucose-6-phosphatase and mannose-6-phosphatase activities were assayed as in [7] except that the inorganic phosphate released was measured according to Fiske and Subarrow [8]. Glucose-6-phosphatase activity was routinely measured in the presence of 20 mM glucose-6-phosphate. For kinetic studies the glucose-6-phosphate concentrations used were 0.4, 0.8, 1.25, 2.5, 5, 10 and 20 mM. K_m and V_{max} values were determined from Lineweaver–Burk plots. In experiments using disrupted microsomes, histone 2A (1 mg/ml) was added to the assay mixture. Protein concentration was determined by the method of Lowry [9].

2.5. *Results*

Glucose-6-phosphatase and mannose-6-phosphatase activities were expressed as nmol of Pi released/min per mg of protein. Statistical analysis was performed using Student's *t*-test for paired data.

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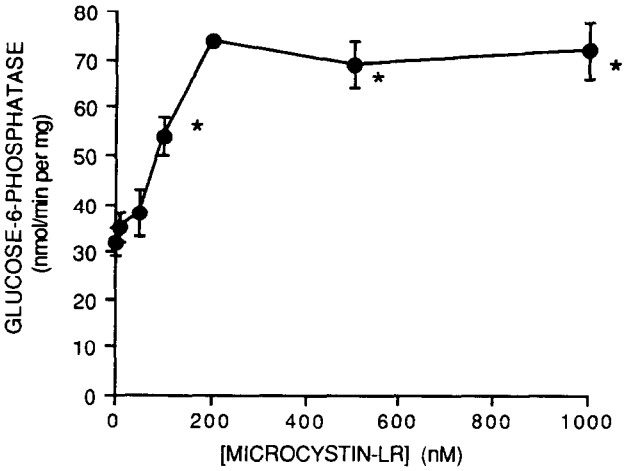

Fig. 1. Influence of various microcystin-LR concentrations on glucose-6-phosphatase activity in hepatocytes. The values are mean ± S.E.M. for four cell preparations (except for 200 nM microcystin-LR, $n = 2$). *Significantly different ($P < 0.05$) from values in the absence of microcystin-LR.

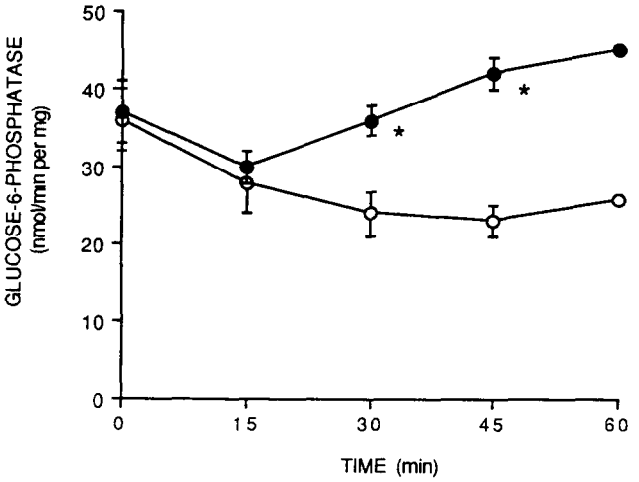

Fig. 2. Time-course of activation of glucose-6-phosphatase by microcystin-LR in hepatocytes. Hepatocytes were incubated with 10 mM dihydroxyacetone and in the absence (○) or in the presence of 100 nM microcystin-LR (●) for the indicated periods of time. The values are mean ± S.E.M. for four cell preparations. *Significantly different ($P < 0.05$) from values in the absence of microcystin-LR.

3. RESULTS AND DISCUSSION

3.1. *Microcystin-LR activates glucose-6-phosphatase*

As shown in Table I, glucose-6-phosphatase activity in supernatants was 1.9-fold higher in isolated hepatocytes incubated for 45 min in the presence of 100 nM microcystin-LR than in control cells. The increase in glucose-6-phosphatase activity was also observed in intact microsomal structures, although to a lesser extent than in the supernatant. Addition of microcystin-LR to hepatocytes caused a significant increase in the V_{max} value of glucose-6-phosphatase without altering the K_m value (Table I).

Glucose-6-phosphatase activity in intact microsomal structures has been proposed to be a measure of combined rates of glucose-6-phosphatase enzyme and of three transport proteins, T1, T2 and T3, which transport glucose-6-phosphate, phosphate and glucose, respectively, across the microsomal membrane [2,3,10]. To determine whether microcystin-LR was activating the glucose-6-phosphatase enzyme or one of the transport proteins, glucose-6-phosphatase activity was measured after microsomal vesicles had been fully disrupted. Microcystin-LR increased glucose-6-phosphatase activity at the same extent in intact and disrupted microsomes (Table I); moreover, in disrupted microsomes, like results obtained using intact microsomes, microcystin-LR significantly increased the V_{max} value despite a low but significant increase in the K_m value. Therefore, microcystin-LR seemed to induce an activation of glucose-6-phosphatase enzyme rather than a modification of the transport proteins. As shown in Fig. 1, half-maximum and maximum effects were obtained with 100 and 200 nM microcystin-LR, respectively.

Table I

Effect of microcystin-LR on activity and kinetic constants of glucose-6-phosphatase in hepatocytes

			Control	+ Microcystin-LR
Supernatant		(nmol/min per mg) (5)	23 ± 5	43 ± 6*
Microsomes	intact	(nmol/min per mg) (9)	139 ± 12	199 ± 15*
	disrupted	(nmol/min per mg) (9)	250 ± 23	353 ± 32*
Microsomes	intact	K_m (mM) (6)	3.36 ± 0.63	2.96 ± 0.67
		V_{max} (nmol/min per mg) (6)	199 ± 17	272 ± 13*
	disrupted	K_m (mM) (4)	1.15 ± 0.14	1.56 ± 0.09*
		V_{max} (nmol/min per mg) (4)	395 ± 37	537 ± 43*

Hepatocytes were incubated with 10 mM dihydroxyacetone in the presence of 100 nM microcystin-LR as indicated. The values are mean ± S.E.M. for the number of cell preparations indicated in parentheses. *Significantly different ($P < 0.05$) from the control values.

Table II

Influence of various inhibitors of protein phosphatases on the glucose-6-phosphatase activity in hepatocytes

		Control	+ Microcystin-LR	+ Okadaic acid	+ Calyculin A
Supernatant	(nmol/min per mg)	36 ± 7	61 ± 7*	61 ± 8*	55 ± 12*
Microsomes	intact (nmol/min per mg)	138 ± 19	178 ± 20*	177 ± 12*	174 ± 22*
	disrupted (nmol/min per mg)	228 ± 34	296 ± 31*	316 ± 39*	299 ± 34*

Hepatocytes were incubated in the presence of 100 nM microcystin-LR, 1 μM okadaic acid or 1 μM calyculin A as indicated. The values are mean ± S.E.M. for five cell preparations. *Significantly different ($P < 0.05$) from the control values.

3.2. *Inhibition of protein phosphatases activates glucose-6-phosphatase*

The microcystin effect on glucose-6-phosphatase activity seemed to be related to inhibition of protein phosphatases during the incubation of hepatocytes. Indeed, two other chemically different inhibitors of PP1 and PP2A (okadaic acid and calyculin A) [11,12] also increased glucose-6-phosphatase activity, both in the supernatant and in intact or disrupted microsomes (Table II). Moreover, microcystin-RR, a specific inhibitor of protein phosphatase 2A [13], was also able to significantly activate glucose-6-phosphatase in the supernatant (control 29 ± 5 nmol/min per mg; + 2 μM microcystin-RR-treated hepatocytes 46 ± 5 nmol/min per mg; $P < 0.05$, $n = 4$) and in intact or disrupted microsomes (control 133 ± 13 or 241 ± 18 nmol/min per mg, respectively; + 2 μM microcystin-RR-treated hepatocytes 160 ± 12 or 283 ± 15 nmol/min per mg, respectively; $P < 0.05$, $n = 4$). Since enzymatic activity itself was unaffected by inclusion in assays of any of these compounds (not shown), it could be concluded that the observed activation of glucose-6-phosphatase enzyme may be related to an inhibition of protein phosphatases and, more probably, of protein phosphatase 2A. Moreover, these results also suggested the involvement of a phosphorylation mechanism in this phenomenon.

3.3. *Puromycin suppresses microcystin effect on glucose-6-phosphatase activation*

Two main mechanisms may participate in the activation of an enzyme by phosphorylation: firstly, a direct phosphorylation of the enzyme by a protein kinase and (or), secondly, an increase in its synthesis by a phosphorylation mechanism [14]. Thus, we tested the influence of puromycin (an inhibitor of protein synthesis) on the effect of microcystin on glucose-6-phosphatase activation. Incubation of hepatocytes in the presence of puromycin totally suppressed the activation of glucose-6-phosphatase by microcystin-LR both in the supernatant and on intact or disrupted microsomes (Table III); similar results were obtained in hepatocytes treated with 200 nM microcystin-LR (not shown). These results suggested an acute regulation of the glucose-6- phosphatase enzyme by phosphorylation at the level of protein synthesis. As shown in Fig. 2, activation of glucose-6-phosphatase by microcystin-LR significantly appeared after 15 min incubation: this lag-time was also consistent with such an interpretation. Moreover, incubation of hepatocytes in the presence of puromycin totally suppressed the activation of glucose-6-phosphatase by microcystin-RR both in the supernatant and on intact or disrupted microsomes (Table III). All these results suggested the involvement of protein phosphatases, most probably

Table III

Influence of puromycin on the microcystin effect on glucose-6-phosphatase activity in hepatocytes

			Control	+ Microsystin-LR	Control	+ Microcystin-RR
Supernatant	(nmol/min per mg)	Control	40 ± 6	64 ± 3*	36	50
		+ Puromycin	46 ± 3	41 ± 3**	37	40
Microsomes	intact	Control	151 ± 3	190 ± 3*	148	174
	(nmol/min per mg)	+ Puromycin	159 ± 4	157 ± 9**	153	158
	disrupted	Control	207 ± 5	273 ± 4*	259	297
	(nmol/min per mg)	+ Puromycin	229 ± 5	229 ± 10**	259	258

Hepatocytes were incubated in the presence of 100 nM microcystin-LR, 2 μM microcystin-RR or 1 mM puromycin as indicated. The values are mean ± S.E.M. for five (microcystin-LR) or two (microcystin-RR) cell preparations. *Significantly different ($P < 0.05$) from values in the absence of microcystin-LR; **Significantly different ($P < 0.05$) from values in the presence of microcystin-LR but in the absence of puromycin.

PP2A, in the regulation of glucose-6-phosphatase synthesis. This was consistent with the proposed role for PP2A in the regulation of protein synthesis [15,16].

A direct dephosphorylation of the enzyme might explain the lesser extent of glucose-6-phosphatase activation in the presence of protein phosphatase inhibitors observed in microsomes than in the supernatant (Tables I–III). However, total suppression of the microcystin-LR effect by puromycin observed in the supernatant (Table III) ruled out a possible contribution of an activation of glucose-6-phosphatase by direct phosphorylation.

In conclusion, our results clearly demonstrate an acute activation of the hepatic glucose-6-phosphatase enzyme by inhibition of protein phosphatases, most probably by PP2A. These results also suggest the involvement of a phosphorylation mechanism in the regulation of the glucose-6-phosphatase enzyme at the level of protein synthesis.

REFERENCES

[1] Nordlie, R.C. (1971) in: The Enzymes, vol. IV (Boyer, P.D. ed.) 3rd edn., pp. 543–609, Academic Press, New York.
[2] Nordlie, R.C. and Sukalski, K.A. (1985) in: The Enzymes of Biological Membranes (Martonosi, A.N. ed.) 2nd edn., pp. 349–398, Plenum Press, New York.
[3] Burchell, A. and Waddell, I.D. (1991) Biochim. Biophys. Acta 1092, 129–137.
[4] Mac Kintosh, C., Beattie, K.A., Klumpp, S., Cohen, P. and Codd, G.A. (1990) FEBS Lett. 264, 187–192.
[5] Marchand, J.C., Lavoinne, A., Giroz, M. and Matray, F. (1979) Biochimie 61, 1273–1282.
[6] Arion, W.J., Ballas, L.M., Lange, A.J. and Wallin, B.K. (1976) J. Biol. Chem. 251, 4901–4907.
[7] Burchell, A., Hume, R. and Burchell, B. (1988) Clin. Chim. Acta 173, 183–192.
[8] Fiske, C.H. and Subbarow, Y. (1925) J. Biol. Chem. 66, 375–400.
[9] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[10] Arion, W.J., Lange, A.J., Walls, H.E. and Ballas, L.M. (1980) J. Biol. Chem. 255, 10396–10406.
[11] Haystead, T.A.J., Sim, A.T.R., Carling, D., Honnor, R.C., Tsukitani, Y., Cohen, P. and Hardie, D.G. (1989) Nature 337, 78–81.
[12] Ishihara, H., Martin, B.L., Brautigan, D.L., Karaki, H., Ozaki, H., Kato, Y., Fusetani, N., Watabe, S., Hashimoto, K. and Uemura, D. (1989) Biochem. Biophys. Res. Commun. 159, 871–877.
[13] Matsuchima, R., Yoshizawa, S., Watanabe, M.F., Harada, K., Furusawa, M., Carmichael, W.W. and Fujiki, H. (1990) Biochem. Biophys. Res. Commun. 171, 867–874.
[14] Cohen, P. (1988) Proc. R. Soc. 234, 115–144.
[15] Cohen, P. (1989) Annu. Rev. Biochem. 58, 453–508.
[16] Morley, S.J. and Thomas, G. (1991) Pharmacol. Ther. 50, 291–319.