Vol. 153, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1988 Pages 307-312

EFFECT OF INSULIN ON THE INDUCTION BY DEXAMETHASONE OF GLUCOSE-6-PHOSPHOHYDROLASE AND TRANSLOCASE ACTIVITIES IN CULTURED HEPATOMA CELLS

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Received April 19, 1988

The ten-fold increase in glucose-6-phosphatase, previously reported, in 2S FAZA hepatoma cells exposed to dexamethasone, is completely blocked by low concentrations of insulin. At $3x10^{-10}$M insulin, the activity induced by 10^{-6}M dexamethasone is reduced by half. The activity of intact microsomes, which reflects translocation of cytoplasmic glucose 6-phosphate into the endoplasmic reticulum, is induced by dexamethasone, but to a lesser extent than the hydrolase. Insulin also prevents this induction.

The enzyme, glucose-6-phosphatase, releases glucose derived from glycogen and gluconeogenesis into the general circulation in liver and kidney. Its location on the inner surface of the endoplasmic reticulum membrane, which is impermeable to charged molecules, requires a means of access of the substrate, glucose 6-phosphate (G6P), to the enzyme. Arion and others, on the basis of compelling kinetic (2), genetic (3) and physico-chemical evidence (4), postulated the existence of a specific translocase (T_1) which would carry this substrate from the cytoplasm to the lumen of the endoplasmic reticulum. Previously it was reported that the synthetic glucocorticoid, dexamethasone, can induce a ten-fold increase in glucose-6-phosphatase levels in a strain (2S FAZA) of hepatoma cells in tissue culture (1). Whether the translocase was also increased was not clear. To clarify this point, it was necessary to subtract the contribution of any disrupted microsomes in order to measure only the hydrolase activity mediated by the proposed carrier. Since mannose 6-phosphate (M6P) is a substrate for the hydrolase but not the translocase, it has been used to determine the proportion of intact microsomal membranes (5). The total hydrolase activity can be conveniently measured in the presence of low concentrations of detergents which, by disrupting the membrane, allow direct access of substrate to the enzyme.

This study was undertaken to determine the effect of dexamethasone on T_1 and to see to what extent insulin, which is known to lower glucose-6-phosphatase activity in rat liver , affects the

Abbreviations: G6P, glucose 6-phosphate; M6P, mannose 6-phosphate; T_1, glucose 6-phosphate translocase.

0006-291X/88 $1.50

activity of both the hydrolase and the translocase under control and inducing conditions in these hepatoma cells. For this, tissue culture has the advantage over intact animals that the levels of glucocorticoid and insulin can be easily controlled and independently varied.

EXPERIMENTAL PROCEDURES

Enzyme assays: Glucose 6-phosphatase was assayed as described previously (6), except 10 mg/ml bovine serum albumin and 0.06 M imidazole-HCl, pH 6.8, were included in all assay mixtures. The substrate concentration was 20 mM. Glucose 6-phosphate and mannose 6-phosphate were purchased from Sigma, and the glucose 6-phosphate was further purified by a previously described method (6). Free inorganic phosphate levels in assays with either preparation were well below the K_is for the hydrolase activities found with control or detergent-treated microsomes (7).

Cell culture: The initial stock of 2S FAZA cells was provided by Dr. Salome Gluecksohn-Waelsch. They were grown and harvested as described earlier (1). McCoy's 5A medium was supplemented with 15% fetal calf serum (both from Gibco) or 15% defined fetal calf serum (HyClone). Microsomes were prepared by homogenizing saline-washed cells from each 75 cm^2 flask in two ml 0.25M sucrose, 0.5mM EDTA, 5mM Tris-acetate, pH 7.4 , and centrifuging at 30,000g, 3^oC, for one hour. Pellets were resuspended by homogenization in one ml of the same buffer and stored at -80^o.

Hormone treatments: Dexamethasone and insulin (both from Sigma) were dissolved respectively in ethanol and 1mM acetic acid at a concentration of 10^{-3}M and diluted serially to the required concentration in medium.

RESULTS

The induction of glucose-6-phosphatase and mannose-6-phosphatase activities in two strains of 2S FAZA cells were compared. Strain 5A has high, and Strain 2, low (or no) inducibility for glucose-6-phosphatase by dexamethasone (Table I). Latency was calculated as the ratio of the enzyme activity not expressed in untreated microsomes to the total activity (that revealed by DOC treatment). With M6P as substrate, it represents the fraction of microsomes which are intact, i.e. which does not allow M6P access to the enzyme. With intact microsomes from rat liver, Arion (8) found that low (2mM or less) concentrations of M6P were necessary to test the intactness of microsomal membranes, since higher concentrations apparently allowed it to be transported by the putative translocase. However, with our preparations, the latency determined with 20 mM M6P was equal to or greater than with 2mM. For that reason and because it seemed preferable to compare activities with the same substrate level for M6P as well as G6P , 20 mM was used for this purpose. A method of calculating translocase-mediated activity , similar to that used by Arion (7), was adopted. The following notation is used for the calculations: G, hydrolase activity with G6P; M, hydrolase activity with M6P; (-), without DOC; (+), with DOC; L, latency; D, activity of the disrupted component; I, activity of intact microsomes; T, translocase activity, i.e. the calculated activity of the preparation if all microsomes were intact. Then, $L = (\boxed{M+} - \boxed{M-})/\boxed{M+}$; $D = \boxed{G+}(1-L)$; $I = \boxed{G-} - D$ and $T = I/L$. It must be emphasized that: (a) the physical nature of the "translocase" is not clear, but that its existence is inferred from evidence mentioned earlier and (b) the translocation step is assumed to be rate-limiting (see below). Measuring translocase activity in intact microsomes by the rate of G6P hydrolysis is similar to measuring an enzyme activity by coupling it to another enzyme system with a greater velocity.

TABLE I

Comparison of latency measured with 2mM and 20 mM M6P

Strain	Treatment	Activity (nmoles P_i/min/mg)					
		20mM G6P		20mM M6P		2mM M6P	
		-DOC	+DOC	-DOC	+DOC	-DOC	+DOC
2	Control	30	75	12	74	5	27
	Latency	.60*		.84		.81	
2	Control	29	60	10	64	6	31
	Latency	.52*		.84		.81	
2	$5x10^{-6}$ Dex	31	56	20	62	8	22
	Latency	.45*		.68		.64	
2	$5x10^{-6}$M Dex	79	145	53	162	31	66
	Latency	.46*		.67		.53	
5A	Control	24	39	11	42	8	16
	Latency	.38*		.74		.50	
5A	Control	20	33	13	40	7	17
	Latency	.39*		.68		.58	
5A	5x10-6M Dex	164	429	117	417	49	181
	Latency	.62*		.72		.73	
5A	$5x10^{-6}$M Dex	230	656	170	660	66	236
	Latency	.65*		.74		.72	

* Apparent latency; not corrected for disrupted component

Then the rate of product formation in the coupled system reflects the rate-limiting activity of the first enzyme.

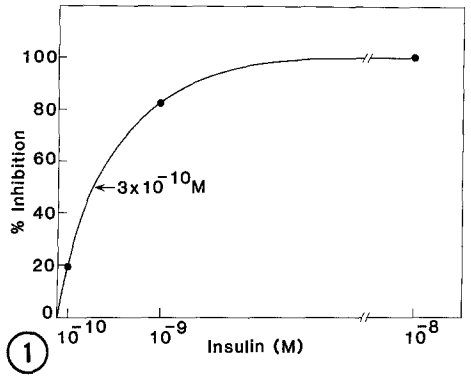
The results indicate that when dexamethasone induces glucose-6-phosphohydrolase activity, it also increases the activity of its translocase (Table II). With Strain 2 which responds poorly or not at all to dexamethasone induction of glucose-6-phosphatase, latency does not change upon treatment with the hormone. On the other hand, under the same conditions, latency with 5A is significantly increased. To put it another way, hydrolase activity is increased relatively more than the translocase in this strain.

Insulin is known to suppress the synthesis of hepatic gluconeogenic enzymes. Weber et al (9) showed that glucose-6-phosphatase and fructose 6-phosphatase from rat liver, both of which are more active in alloxan-induced diabetes and after triamcinalone injection, either fail to increase or actually decrease if insulin injections are given simultaneously. However, the circulating levels of

TABLE II

Enzyme and translocase activity calculated from the data in Table 1

Strain	Experimental condition	Activity (nmoles P_i/min/mg)			
		Enzyme	Translocase		Latency
			2mM M6P	20mM M6P	(%)
2	Control	75	19	21	72
2	Control	60	22	23	62
2	$5x10^{-6}$M Dex	56	16	19	66
2	$5x10^{-6}$M Dex	145	21	46	68
5A	Control	39	9	19	51
5A	Control	33	11	14	58
5A	$5x10^{-6}$M Dex	429	66	61	86
5A	$5x10^{-6}$M Dex	656	64	80	88



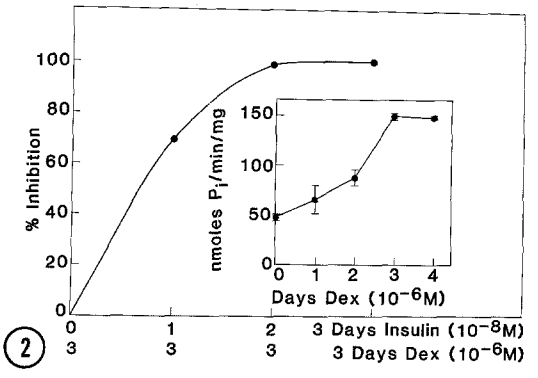


Figure 1. Inhibition by different insulin concentrations of glucose-6-phosphatase induction by dexamethasone(10^{-6}M). Microsomes were pretreated with 0.2% DOC before assay.

Figure 2. Inhibition by insulin of dexamethasone induction of glucose-6-phosphatase ativity. 10^{-8}M insulin was added on different days to flasks of cells to which 10^{-6}M dexamethasone had been added at 0 time. (Inset) Time course of induction of glucose-6-phosphatase by 10^{-6}M dexamethasone. Data represent the mean +/- the rangeof two separate experiments.

glucocorticoid and insulin were not determined. In the following experiments , the effect of insulin on glucose-6-phosphatase induction was tested by adding different amounts to the medium simultaneously with dexamethasone (10^{-6}M). The hydrolase activity did not rise above control levels until the insulin concentration was dropped to 10^{-9}M or lower (Figure 1). The insulin concentration which inhibits full dexamethasone induction of the enzyme by 50% was approximately $3x10^{-10}$M. Physiological levels of plasma insulin in rats range from $3x10^{-11}$M during fasting to $9x10^{-10}$M after glucose administration (10). (The concentration of insulin in the medium unsupplemented by the hormone was $5x10^{-12}$M.) It is clear from Figure 1 that with these hepatoma cells, insulin, within the physiological range, can have a profound effect in blocking dexamethasone-stimulated enzyme activity.

In another experiment, 10^{-8}M insulin was added at different times to a culture being exposed for 3 days to 10^{-6}M dexamethasone (Figure 2). If added at 0 time, it completely prevented induction of the hydrolase (microsomes were exposed to 0.2% DOC before assay). If added 24 hrs after dexamethasone, new enzyme synthesis was blocked and, in two more days, the specific activity of previously induced levels (see inset) had decayed almost to control levels. When added after 2 days (i.e. cells were exposed to insulin for just one day), insulin suppressed the induction only to a modest extent.

To test the effect of insulin on both glucose-6-phosphatase and its translocase, hydrolase activity was measured with or without DOC, using glucose 6-phosphate or mannose 6-phosphate as substrate (Table III). The latency with 20 mM M6P was used as above to calculate the translocase activity of each preparation which would result if all membrane vesicles were intact. In both experiments the effect of insulin was to lower the dexamethasone-stimulated hydrolase activity proportionately more than that of the translocase, so that latency with G6P was diminished. It is also apparent that insulin alone decreased the constitutive levels of both the hydrolase and translocase. These results indicate that, when studying the induction by dexamethasone of glucose-6-phosphohydrolase and its translocase in the whole animal, it is necessary

TABLE III

Effect of 10^{-8}M insulin in blocking induction by 10^{-6}M dexamethasone of enzyme and translocase activity of microsomes from 2S FAZA cells

	Enzyme Activity[a]	Translocase activity[a]	Latency[b] (%)
Experiment I			
Control	22	6.5	70
10^{-8}M Insulin	6	2.6	57
10^{-6}M Dex	105	31.6	70
10^{-8}M Insulin & 10-6M Dex	10	5.4	46
Experiment II			
Control	25	11.4	54
10^{-8}M Insulin	16	9	44
10^{-6}M Dex	277	42	85
10^{-8}M Insulin & 10-6M Dex	11	7.4	33

a. nmoles P_i/min/mg

b."latency" here represents (G+ -T)/ G+ as calculated for intact microsomes.

to consider the circulating levels of both glucocorticoid and insulin. In fact, the observation that glucocorticoids seem to decrease latency *without* raising hydrolase activity, in rat liver microsomes (2), may be explained, in part, by the presence of insulin in the portal circulation of the animal.

DISCUSSION

The assumption that a translocase, specific for glucose 6-phosphate, is rate-limiting for hydrolysis of that substrate is based on a body of kinetic evidence cited by Arion (7). An important finding is that the glucose derivative, phlorizin, inhibits the glucose-6-phosphatase activity of intact microsomes in a linear manner, and without the lag which would be expected if P_i efflux were blocked. However, P_i inhibition of intact microsomes is also linear (although it does not inhibit G6P transport competitively). That is, plots of $1/V_{max}$ vs. P_i do not show the break which should occur if T_1 were the rate-limiting step (see reference 7, Figure 1). Perhaps the possibility that efflux of P_i from microsomes is energetically and/or mechanistically coupled to the hydrolysis of glucose 6-phosphate should be considered. In any case, future progress in analyzing the regulation of glucose-6-phosphatase is likely to depend on isolation and reconstitution of the catalytic and translocase components in an active system.

ACKNOWLEDGEMENT

I am grateful to Dr. Manfred L. Karnovsky and members of his laboratory, where this work was performed, for advice and support.

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