EFFECTS OF STARVATION, CORTICOTROPIN INJECTION AND ETHANOL FEEDING ON THE ACTIVITY AND AMOUNT OF PHOSPHATIDATE PHOSPHOHYDROLASE IN RAT LIVER

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Received 3 March 1981

1. Introduction

Phosphatidate phosphohydrolase has an important regulatory role in hepatic glycerolipid synthesis, especially in facilitating an increased synthesis of triacylglycerols [1,2]. Many of the changes observed in this activity are brought about by glucocorticoids [3–5], but other mechanisms also seem to be involved [5–10]. This paper describes the preparation of an antibody against the soluble phosphatidate phosphohydrolase of rat liver. It was used to investigate the extent to which some changes in this phosphohydrolase activity resulted from alterations in its concentration. To the best of our knowledge, this is the first report of this type of experiment with phosphatidate phosphohydrolase. This activity decreased after starving rats for 24 h, but the amount of the phosphohydrolase relative to DNA was unchanged. The amount of the phosphohydrolase increased by ~3.5-fold at 400 min after injecting corticotropin, whereas the activity increased by 2.5-fold. At 400 min after feeding with ethanol the phosphohydrolase activity and amount were increased by 7- and 6.3-fold, respectively. The increases in phosphohydrolase concentration were probably mediated by corticosterone. It also appears likely that the phosphohydrolase is capable of being activated and inactivated since changes in amount were not necessarily paralleled by changes in activity.

2. Materials and methods

2.1. *Materials*

The source of these has been described [11,12].

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1,2-Diacyl-*sn*-[^{3}H]glycerol 3-phosphate was prepared enzymically by using 0.4 mM each of palmitate and oleate as acyl-donors [12].

2.2. *Animals*

Thirty two male Wistar rats weighing 240 ± 2 (SEM) g were divided into 4 groups. One of the these was starved for 24 h and the control group was fed ad libitum. The other groups were fed with 5 g ethanol/kg body wt [13], or they were injected intramuscularily with 1 unit β1–24 corticotropin (Synacthen)/kg body wt. These 2 groups were then deprived of food and killed 400 min later. The Synacthen was from Ciba Lab., Horsham, West Sussex and it was diluted to 1 unit/ml with sterile 0.16 M NaCl before use. The animal room was lit from 8:00–20:00 h. All rats were allowed free access to water and they were killed between 15:30–16:30 h.

2.3. *Partial purification of soluble phosphatidate phosphohydrolase*

Rats were fed with 5 g ethanol/kg body wt 6 h before they were killed to increase the phosphohydrolase activity [13]. Livers were perfused in situ with ice cold 0.16 M NaCl to remove plasma proteins and they were homogenized with a teflon–glass homogenizer in 3 vol. 0.25 M sucrose containing 0.5 mM dithiothreitol and adjusted to pH 7.4 with $KHCO_3$ (sucrose medium). This and all subsequent procedures were at 4°C. Particle-free supernatant was prepared by centrifuging the homogenate for 90 min at 76 000 × *g* (r_{av} = 7.62 cm). This was loaded onto a column of DEAE-cellulose (OH^- form) and the column was washed with sucrose medium containing 70 mM potassium phosphate buffer (pH 7.4). About 92% of

the protein, but negligible phosphohydrolase activity was recovered. The phosphohydrolase was obtained by elution with sucrose medium containing 0.3 M potassium phosphate buffer (pH 7.4) and then precipitated with a 50% saturation of ammonium sulphate. The pellet was suspended in sucrose medium containing 20 mM phosphate buffer (pH 7.4) and dialysed overnight. This was loaded onto a column of spheroidal hydroxyapatite (British Drug Houses), and the column was washed with sucrose medium containing 40 mM phosphate buffer (pH 7.4). The phosphohydrolase was eluted with a linear gradient of sucrose medium containing 40–600 mM phosphate buffer (pH 7.4) and fractions containing 200–600 mM phosphate buffer were pooled. The phosphohydrolase was precipitated with a 50% saturation of ammonium sulphate and the pellets were stored at −20°C. When required this precipitate was dissolved in sucrose medium containing 10 mM Tris–HCl buffer, (pH 7) and dialysed overnight against the same buffer. The solution was passed through a column of octyl-Sepharose which was then washed with the same buffer containing 200 mM Tris–HCl (pH 7.4) and the combined eluate were retained. The increases in specific activity of the phosphohydrolase after chromatography on DEAE, hydroxylapatite and octyl-Sepharose were 1.8-, 40-, and 52-fold, respectively. However, these are minimum values since the enzyme was unstable at all stages of purification.

2.4. *Preparation of antiserum against phosphatidate phosphohydrolase and purification of IgG*

The partially purified phosphohydrolase (23 mg protein) was mixed with Freund's adjuvant. This was injected into a sheep on 4 separate occasions as described for 6-phosphogluconate dehydrogenase [14] except that on the last 3 occasions, Freud's incomplete adjuvant was used. Control serum was prepared from the sheep before the first injection and antiserum was first detected after 12 weeks. IgG fractions were prepared from control serum and antiserum [15], except that the final ammonium sulphate precipitate was resuspended in 20 mM Tris–HCl buffer (pH 7.4) containing 0.15 M NaCl.

2.5. *Determination of the activity and concentration of phosphatidate phosphohydratase*

Livers were homogenized immediately in 3 vol. sucrose medium using a teflon–glass homogenizer. The sample used to prepare particle-free supernatant was adjusted to 2 mM with phenylmethylsulphonylfluoride to decrease protease activity before centrifuging for 90 min at 4°C and 76 000 × g (r_{av} = 7.62 cm).

Phosphohydrolase activity was determined in incubations with 0.25 ml final vol. and containing 100 mM Tris–HCl buffer, 1 mM dithiothreitol, 0.1 mM EGTA, 5 mM $MgCl_2$, 2 mg fatty acid poor bovine serum albumin/ml, 0.6 mM [^{3}H]phosphatidate (0.4 Ci/mol), 0.4 mM phosphatidylcholine and the equivalent of 10–190 μg protein from the particle-free supernatant. Incubations were for 20 min or for 60 min depending upon the activity of the samples. Reactions were stopped and lipids were extracted with chloroform/methanol [12]. The bottom chloroform phase was shaken with 0.75 ml of a suspension of Al_2O_3 (pH 9.5) in chloroform (1:2; v/v) then centrifuged. The supernatant was decanted into 0.75 ml of the same suspension and the process repeated to remove unreacted phosphatidate. The supernatant was decanted into a counting vial, the chloroform removed by evaporation and the diacyl [^{3}H]glycerol determined by scintillation counting [11]. The recovery of this product was 92% of that obtained after chloroform chromatography on columns of Al_2O_3, and the results were corrected by this factor.

Preliminary experiments showed that the IgG from the antiserum decreased the activity of the purified phosphatidate phosphohydrolase, but it did not inhibit, or precipitate the phosphodydrolase in the native particle-free supernatant. We cannot yet explain this observation, but purification appears to have exposed antigenic determinants. Samples (1 ml) of the particle-free supernatant from experimental animals were therefore treated with 0.3 ml of a 3% (w/v) suspension of calcium phosphate gel in the presence of 0.15% Tween 20 at 4°C. The samples were centrifuged and the gel was washed twice with 1 ml portions of sucrose medium containing 40 mM phosphate buffer (pH 7.4) and 0.15% Tween 20. The phosphohydrolase was then eluted from the gel by washing twice with 1 ml portions of sucrose medium containing 0.4 M phosphate buffer (pH 7.4) and 0.15% (w/v) of Tween 20. The specific activity of the phosphohydrolase was increased by ~10-fold and this enzyme preparation interacted with the antibody (fig.1). The addition of Tween 20 stabilized the activity and this was discovered after the partial purification of the enzyme for antibody preparation had been completed.

Samples (40 μl) of the combined eluate from the calcium phosphate gel were incubated at 37°C for 22 min in 154 μl final vol. in a medium containing

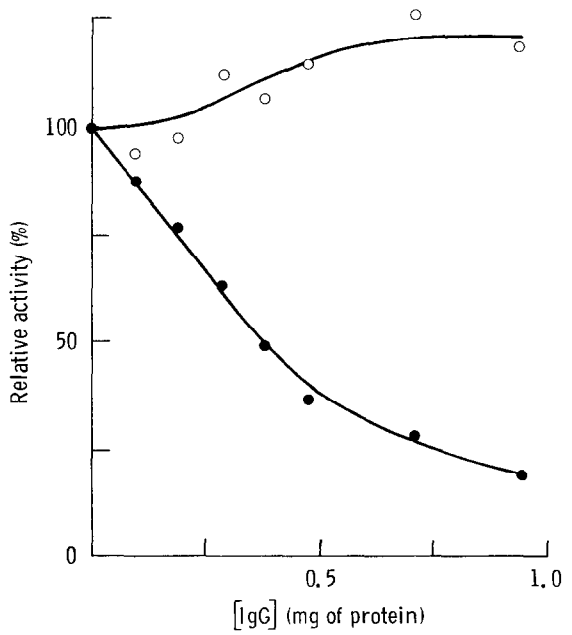

Fig.1. Effects of IgG from control serum (○) and antiserum (●) on the activity of the soluble phosphatidate phosphohydrolase of rat liver. A partially purified preparation of the soluble phosphohydrolase was treated with various concentrations IgG and the remaining phosphohydrolase activity was determined (section 2). The results from one of the rats described in table 1 is shown as an example.

20 mM Tris–HCl buffer (pH 7.4), 0.15 M NaCl, 0.175% (w/v) Tween 20 and various concentrations of IgG. The incubation was then continued for a further 40 h at 4°C. The samples were centrifuged at 4°C and 2100 × g (r_{av} = 35 cm) for 60 min and 60 μl samples were analysed for phosphohydrolase activity. The addition of 0.15% Tween 20 to the particle-free supernatants decrease the phosphohydrolase activity by 28 ± 7 (SEM) %. However, this activity was recovered after treatment with calcium phosphate gel and the total recovery of activity after incubating as above, but without IgG was 100 ± 5 (SEM) %. At no stage was there any significant difference in the recoveries of phosphohydrolase activity in different experimental groups.

Incubating the partially purified phosphohydrolase with higher concentrations of IgG from the control serum stimulated the activity by ~20% (fig.1). This could result from a direct action on the enzyme, or it may be an effect on the characteristics of the phosphatidate emulsion used as a substrate. The IgG from the antiserum decreased the phosphohydrolase activity by up to 87% (fig.1). The amount of IgG required to decrease this activity by 50% for the different rats was determined by linear regression analysis [16].

2.6. *Determination of protein and DNA concentrations*

The methods are described in [17].

3. Results and discussion

Compared with control rats, the starved animals showed 32% and 23% less phosphohydrolase activity when this was expressed relative to DNA, or soluble protein, respectively (table 1). However, the amount of the phosphohydrolase when expressed relative to DNA was not significantly altered, and it was increased when expressed relative to protein. This means that some of the phosphohydrolase appeared to be less active in these starved rats. Special care was taken to avoid undue stress when the rats were killed, and this may explain why our results show a decrease in phosphohydrolase activity, whereas previous work showed increases [18,19]. We also killed all rats at the same time of day since there is a diurnal variation in phosphohydrolase activity [20]. The present results are compatible with the observation that fatty acids are preferentially oxidized in the liver in starvation, but the capacity for triacylglycerol synthesis is retained [21,22].

Injection of corticotropin increased the activity and concentration of the phosphohydrolase by 2.5- and 3.5-fold, respectively. This is compatible with predictions that glucocorticoids stimulate the synthesis of phosphatidate phosphohydrolase [1–4], but the values in table 1 show that the phosphohydrolase was relatively less active than that of control rats.

Feeding rats with ethanol increased the phosphohydrolase activity by ~7-fold and its amount by ~6.3-fold. Corticosterone seems to be responsible for part of this change, since adrenalectomizing the rats and maintaining them with saline to drink abolishes ~85% of the increase in activity [5]. However, the phosphohydrolase appears to be more active ($P <$ 0.001) relative to its concentration when compared with the rats injected with corticotropin (table 1). Thus ethanol treatment may also cause the activation of existing phosphohydrolase. This action could explain some of the stimulations of the phosphohydrolase that

Table 1
Effect of starvation, corticotropin injection and ethanol feeding on the activity and relative concentration of the soluble phosphatidate phosphohydrolase in rat liver

Treatment	Diacylglycerol produced		Phosphohydrolase amount		Ratio of activity divided by amount (V)
	nmol . min^{-1} . mg DNA^{-1} (I)	nmol . min^{-1} . mg protein^{-1} (II)	Relative to DNA (III)	Relative to protein (IV)	
Control rats	71.8 ± 4.9	2.98 ± 0.20	1560 ± 200	65 ± 5.6	49.3 ± 6.1
Starved for 24 h	46.1 ± 1.2[a]	2.20 ± 0.05[b]	1880 ± 200	90 ± 9.4[b]	25.9 ± 2.4[b]
400 min after corticotropin injection	180 ± 13[a]	7.16 ± 0.35[a]	5500 ± 340[a]	220 ± 8.0[a]	33.1 ± 1.9[c]
400 min after ethanol feeding	516 ± 30[a]	20.9 ± 1.9[a]	9910 ± 740[a]	400 ± 45[a]	53.5 ± 4.4

Values that are significantly different from control results are indicated by: [a]$P < 0.001$; [b]$P < 0.005$; [c]$P < 0.02$. Results are mean ± SEM for 8 rats/group

Phosphatidate phosphohydrolase activity was determined in the original particle free supernatant in the absence of Tween 20. Its amount was measured after treatment with calcium phosphate and in the presence of Tween 20 (section 2). The concentration is expressed as μl antiserum (1 μl = 14 μg IgG protein) required to inhibit the total activity in the liver by 50%. This has been divided by the total mg of DNA (III) and the total mg soluble protein (IV) in order to compensate for differences in liver size. Values in (V) were obtained by taking the individual results from (I) × 10^3 and dividing by the corresponding value from (III)

have been observed with ethanol, but which cannot be related to the action of glucocorticoids [5,7,10].

Acknowledgements

We thank Dr R. J. Mayer for his help and advice in the preparation of the antibody and Mrs I. S. McKechnie for technical assistance. The work was supported by a project grant from MRC and a studentship to S. C. B. from SRC

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