

Thyroid hormone stimulates expression of 6-phosphofructo-2-kinase in rat liver

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The activity of liver 6-phosphofructo-2-kinase (PFK-2), the enzyme that catalyses the synthesis of fructose 2,6-bisphosphate, was markedly decreased in hypothyroid rats and partially restored after 3 days of treatment with triiodothyronine. The changes in PFK-2 activity were accompanied by parallel changes in enzyme content measured by immunotitration and in PFK-2 mRNA determined by dot blot and Northern blot hybridization with cDNA probes. It is concluded that thyroid hormone stimulates liver PFK-2 gene expression by a pre-translational mechanism.

Phosphofructo-2-kinase, 6-; Fructose 2,6-bisphosphate; Thyroid hormone; mRNA; (Rat liver, Rat heart)

1. INTRODUCTION

Fructose 2,6-bisphosphate is a potent stimulator of glycolysis in mammalian tissues. It mediates the stimulation of phosphofructokinase by hormonal and metabolic signals [1]. Its synthesis from fructose 6-phosphate and ATP is catalyzed by 6-phosphofructo-2-kinase (PFK-2; EC 2.7.1.105), which occurs in several isozymic forms [1]. The liver (L) isozyme of PFK-2 is phosphorylated on Ser-32 by the cyclic AMP-dependent protein kinase and this covalent change decreases PFK-2 activity. The muscle (M) isozyme does not contain this phosphorylation site. The mRNA for the M-isozyme is found in rat liver, but at a much lower concentration than the L-type mRNA [2]. PFK-2 activity is also regulated by translational and transcriptional mechanisms. In diabetic or starved rats liver PFK-2 content, but not mRNA, is decreased [3], whereas with insulin injection or refeeding, respectively, PFK-2 content and mRNA levels are increased [4]. Rat liver PFK-2 activity decreases after adrenalectomy and is restored by glucocorticoid hormone replacement. This has been ascribed to altered rates of transcription of the PFK-2 gene, resulting in changes in content of PFK-2 mRNA and protein [5]. Gualberto et al. [6] have reported decreased PFK-2 activity in the heart of hypothyroid rats.

We have recently developed antibody and cDNA probes for PFK-2 [3,7]. Since liver glycolysis is impaired in hypothyroid rats [8], we have now used these probes to examine the effect of thyroid status on liver PFK-2.

2. EXPERIMENTAL

Male Wistar rats (120 g) were made hypothyroid by feeding an iodine-deficient diet (25 µg iodine/kg food) (UAR, Villemoisson-sur-Orge, France) with a 1% NaClO₄ solution as drinking water. Control rats were fed normal chow (diet no. A04 from UAR). After 40 days, control and hypothyroid rats were injected i.p. with vehicle (0.9% NaCl, 0.1% NaOH) or triiodothyronine (T₃) (Sigma; 10 µg/100 g body wt per day) for 3 days at 10.00 h. They were killed by a Nembutal injection at 14.00 h on the third day, followed by removal of the tissues. Livers and hearts were freeze-clamped in liquid nitrogen and blood samples were taken from the thoracic aorta. Liver samples (about 100 mg) were homogenized (Teflon-glass Potter) in 10 vols of an ice-cold buffer containing 50 mM KCl, 2 mM EDTA, 5 mM MgCl₂, 20 mM KF, 0.1 mM fructose 6-phosphate, 0.3 mM glucose 6-phosphate, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 µg/ml of pepstatin, leupeptin, and aprotinin, and 20 mM Hepes, pH 7.5. Cytosols were prepared by taking the supernate after centrifugation at 100 000 × g for 20 min. Liver PFK-2 was measured at 30°C and pH 8.5. This gives the total activity, which is insensitive to the phosphorylation state of the enzyme [9]. Heart PFK-2 activity, which is insensitive to cyclic AMP-dependent protein kinase, was measured at pH 7.1 [10] in cytosols prepared as described for liver. Immunotitration was carried out as described [3] using an antibody (BCL-2) raised against purified chicken liver PFK-2.

cDNA probes [2,7] specific for the L-type (22c2-*Sau*3A) and the M-type (5c2-*Dde*I) PFK-2 mRNA, and one common to both isozymes (22c1), were multiprimer-labelled with [α -³²P]dCTP to a specific activity of 2–4 × 10⁹ cpm/µg. A chicken β -actin probe was also used as an internal control. Total RNA was extracted by a modified [11] guanidine thiocyanate method [12]. Dot blot [13] RNA-cDNA hybridization was performed on RNA denatured with formaldehyde/formamide, using prehybridization and hybridization conditions as described [3]. Filters were washed for 30 min at room temperature, then for two periods of 30 min at 50°C in 2 × SSC (SSC

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Abbreviations: PFK-2, 6-phosphofructo-2-kinase; T₃, triiodothyronine; T₄, thyroxine

is 0.15 M NaCl, 0.015 M Na citrate), 0.1% SDS. Intensity of hybridization was measured by counting the radioactivity of the RNA spots, with radioactivity of blank filter subtracted as background. For Northern blot analysis [2], total RNA was resuspended in a denaturing loading buffer, heat denatured, and electrophoresed [14] in the presence of 1 μ g/ml ethidium bromide. The gel was photographed under UV light to record migration of RNA molecular weight markers (BRL) and to monitor the quantity of RNA loaded by densitometry of 28 S and 18 S rRNA. RNA was transferred to a nylon membrane (Hybond N, Amersham) in 10 \times SSC for at least 12 h, then fixed onto the membrane by UV irradiation for 5 min. Filters were prehybridized and hybridized as described [2] and washed twice in 200 ml 2 \times SSC, 0.1% SDS for 30 min at room temperature, and then twice in 200 ml 0.2 \times SSC, 0.1% SDS for 30 min at 50°C. Autoradiographs were scanned (Joyce-Loebl densitometer) and the values obtained were corrected for the amount of RNA loaded determined as described above.

Serum thyroxine (T_4) and T_3 concentrations were determined by radioimmunoassay, with the Magic kit of Corning for T_3 and the canine double antibody kit from Diagnostic Products Co. for T_4 . The latter can detect T_4 concentrations below 1.25 μ g/dl. Protein was measured [15] using bovine serum albumin as standard. Statistical analysis was carried out using Student's *t*-test for unpaired data or Mann-Whitney U-test with significance level chosen as $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Effect of the thyroid status on PFK-2 activity and content

Serum levels of thyroid hormones were significantly decreased in hypothyroid versus control rats (table 1). T_3 treatment normalized serum T_4 levels in hypothyroid animals. Heart PFK-2 activity, whether expressed per mg of protein (table 1) or DNA (not shown), was not significantly decreased in hypothyroid rats. The earlier demonstration [5] of decreased PFK-2 activity in hypothyroid rat heart may be related to the longer and more severe hypothyroid state of animals in that study. Liver PFK-2 activity in hypothyroid rats was about 25% of that in control rats (table 1). This reflects a decrease in total activity, and not simply an inactivation by phosphorylation of the enzyme, since the activity was measured under conditions that are insensitive to the phosphorylation state of liver PFK-2. Total PFK-2 activity in hypothyroid animals was partially restored after 3 days of T_3 treatment. There was no inhibitor of

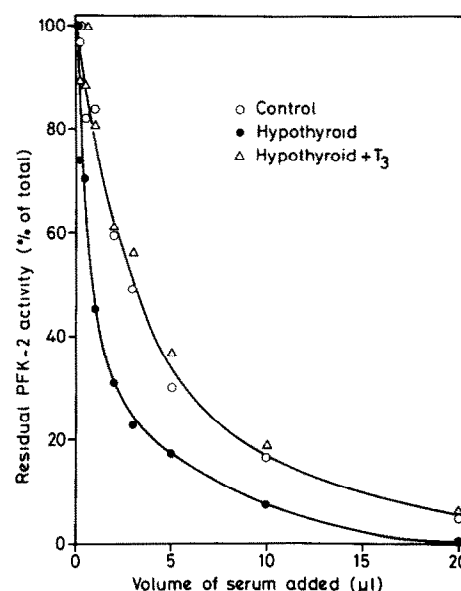


Fig.1. Immunotitration of liver PFK-2 measured as total activity remaining after precipitation with the quantities of antiserum indicated. Cytosol protein concentration in the incubations (0.2 ml) was 2 mg/ml. PFK-2 activity (μ U/mg protein) in absence of antiserum was 33.6 for the control, 9.3 for the hypothyroid, and 17.2 for the hypothyroid T_3 -treated rat.

PFK-2 in hypothyroid rat liver since mixtures of liver cytosols from untreated and hypothyroid rats contained the expected PFK-2 activities.

To determine whether these changes resulted from changes in enzyme content, PFK-2 was immunotitrated in the same liver samples. Representative immunotitration curves are shown in fig.1. The decrease in total PFK-2 activity in liver from hypothyroid rats was accompanied by a two-fold decrease in enzyme content. While PFK-2 activity was not completely restored by T_3 treatment in hypothyroid rats, enzyme content was normalized (fig.2). The discordance between PFK-2 activity and content in these experiments suggests that the immunotitrable enzyme was less active in hypothyroid than in normal rats. This defect was not corrected by T_3 and could depend on unknown factor(s) whose response to T_3 treatment differs from that of PFK-2.

Table 1
Effect of hypothyroidism and T_3 on thyroid hormones in serum and on PFK-2 activity

	Serum concentration		PFK-2 activity (μ U/mg protein)	
	T_4 (μ g/dl)	T_3 (ng/dl)	Liver	Heart
Control (8)	5.36 \pm 0.47	68 \pm 4.0	28.4 \pm 1.9	7.86 \pm 2.2
T_3 (9)	4.21 \pm 0.33 ^b	1290 \pm 30 ^{a,b}	26.7 \pm 1.7	5.24 \pm 0.9
Hypothyroid (9)	1.15 \pm 0.07 ^a	25 \pm 1 ^a	6.94 \pm 0.6 ^a	4.00 \pm 0.6
Hypothyroid + T_3 (9)	7.29 \pm 0.36 ^b	1500 \pm 12 ^{a,b}	14.7 \pm 0.9 ^{a,b}	5.22 \pm 0.5

^a Significantly different from untreated controls

^b Significantly different from untreated hypothyroid

The values are means \pm SE for the number of animals given in parentheses

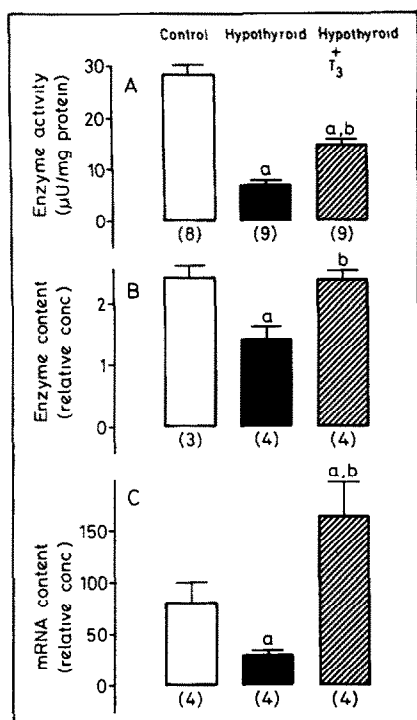


Fig.2. Effect of hypothyroidism and T₃ on PFK-2 activity (A), content (B) and mRNA (C) in rat liver. PFK-2 content is expressed as μ l of antiserum required to precipitate 50% of the activity measured in absence of antiserum. Data were calculated from logit-log transformations of plots such as those shown in fig.1. Data for mRNA are from dot blot experiments such as those in fig.3. For each RNA sample the slope of the linear relationship between cpm hybridized and μ g of RNA spotted was calculated for the PFK-2 probe (22c1) and for the actin probe. The ratio ($\times 10^2$) of these slopes for each sample gave the values shown. Values are means \pm SEM for the number of animals shown in parentheses. (a) Significantly different from untreated controls; (b) significantly different from untreated hypothyroid rats.

3.2. Effect of the thyroid status on liver PFK-2 mRNA

To investigate the mechanism of decreased liver PFK-2 content in hypothyroid rats, we measured PFK-2 mRNA with cDNA probes. Dot blot analysis of total liver RNA using probe 22c1 (fig.3) demonstrated that PFK-2 mRNA content of hypothyroid rat liver was about a third of that in control rats (fig.2). T₃ treatment of hypothyroid rats resulted in an approximate 6-fold increase in PFK-2 mRNA, to a level greater than that seen in untreated controls. Northern blot analysis confirmed that liver PFK-2 mRNA was indeed altered to the same extent as shown by dot blot (fig.4). Only one major hybridizing band at 2.1 kb (L-type mRNA) was seen with probe 22c1 under our experimental conditions. Since this probe is capable of hybridizing also with the 1.9 kb M-type mRNA [2], we conclude that the thyroid status did not shift the pattern of expression from the L-type towards the M-type mRNA. This was confirmed by dot blot analysis with the cDNA probe 5c2-*Dde*I which is specific for the M-type mRNA. Virtually no signal could be detected with this probe on total liver RNA from any of the groups (not shown).

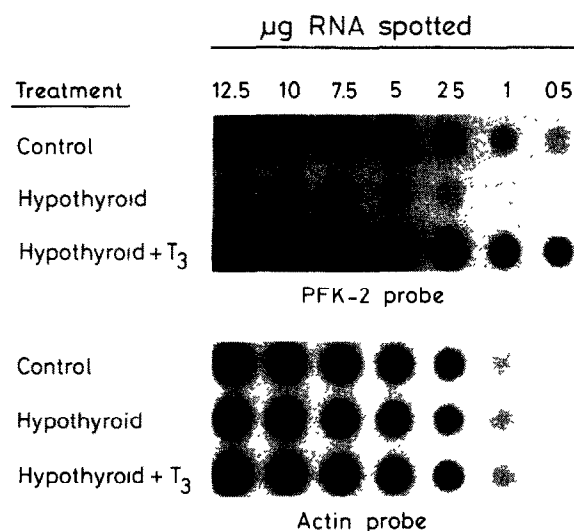


Fig.3. Dot blot hybridization of liver RNA with PFK-2 (22c1) and actin cDNA probes.

These data indicate that the L-type PFK-2 mRNA is specifically regulated by T₃ through mRNA stabilization or (and) by a direct transcriptional effect. The latter mechanism is consistent with the presence of nucleotide sequences containing putative thyroid hormone responsive elements in the 5' flanking region of the PFK-2 gene [16]. In this study, T₃ treatment of hypothyroid animals resulted in PFK-2 mRNA levels which were greater than those seen in controls, whereas enzyme content was restored only to control levels. This may be due to the turnover of PFK-2 being slower than that of its RNA [17]. Our results also suggest that heart PFK-2 is less sensitive to hypothyroidism than liver

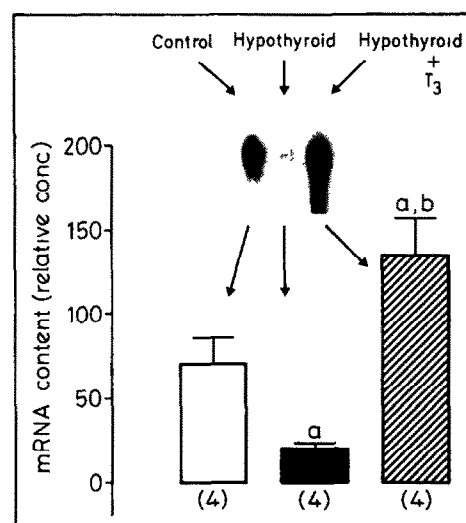


Fig.4. Northern blot analysis of liver RNA hybridized to PFK-2 cDNA probe 22c1. After 4 days of exposure, the 2.1-kb bands of the autoradiographs (a representative experiment is shown in the inset) were scanned. The integrated area for each sample was corrected for total RNA loaded as described. Symbols are as in fig.2.

PFK-2. This may be related to the fact that the concentration of thyroid hormones in hypothyroid rats decreases much more slowly over the weeks in heart tissue than in liver [18]. The stimulation of liver lipogenesis by thyroid hormones might involve a higher provision of C-3 units through increased glycolysis [8]. The stimulation of PFK-2 gene expression by T_3 described here is likely to contribute to this mechanism.

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