Effect of thyroid state on cyclic AMP-mediated induction of hepatic phosphoenolpyruvate carboxykinase

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Hepatic phosphoenolpyruvate carboxykinase (PEPCK) is significantly increased in the hyperthyroid starved rat, and moderately decreased in the hypothyroid starved rat. As tri-iodothyronine by itself has only a small and sustained effect on the induction of this enzyme, as was previously shown in the isolated perfused organ, the effect of hypo- and hyper-thyroidism on the increase in cytosolic PEPCK provoked by dibutyl cyclic AMP (Bt₂cAMP) was investigated *in vivo* and in the isolated perfused liver. Compared with euthyroid fed controls, in hypothyroid fed rats Bt₂cAMP provoked in 2 h only a small increase in translatable mRNA coding for PEPCK. In contrast, in hyperthyroid animals PEPCK mRNA as measured by translation *in vitro* was already increased in the fed state, and further enhanced by Bt₂cAMP injection to values as in euthyroid controls. Under all thyroid states a close correlation between PEPCK mRNA activity and PEPCK synthesis was observed. In the isolated perfused liver from the hyperthyroid fed rat, the increase in PEPCK provoked by Bt₂cAMP or Bt₂cAMP + isobutylmethylxanthine was considerably enhanced compared with those obtained in livers of hypothyroid rats. Also, adrenaline provoked a stimulated induction of PEPCK in hyperthyroid rats compared with hypothyroid rats. To summarize, our data indicate that the primary action of thyroid hormones on the synthesis of hepatic cytosolic PEPCK is to accelerate the cyclic AMP- or adrenaline-induction of the enzyme, acting primarily at a pretranslational level.

In the liver *in vivo*, thyroid hormones induce the synthesis of a series of proteins, including regulatory enzymes (for a review, see Oppenheimer & Samuels, 1983). Thus a thyroid-hormone-dependent induction has been demonstrated for enzymes favouring glucose utilization, e.g. glucokinase (Sibrowski *et al.*, 1981), pyruvate kinase (Johnson & Veneziale, 1980), pyruvate dehydrogenase (Weinberg & Utter, 1979), malic enzyme (Goodridge, 1983), acetyl-CoA carboxylase and fatty acid synthetase (Das, 1980), as well as for enzymes favouring glucose production, e.g. pyruvate carb-

oxylase (Weinberg & Utter, 1979) and PEPCK (Sibrowski *et al.*, 1982). However, a direct effect of T₃ on the synthesis of these enzymes cannot be deduced from these findings *in vivo*, as different thyroid states affect the circulating concentration of several hormones, including insulin and glucagon, as well as affinity, number and responsiveness of plasma-membrane receptor proteins, e.g. glucagon receptor adenylate cyclase system (Sperling *et al.*, 1980) and/or α - and β -adrenergic receptors (for a review see Bilezikian & Loeb, 1983). In fact, in isolated hepatocytes in culture, T₃ by itself showed only a minimal effect on the induction of malic enzyme, acetyl-CoA carboxylase and fatty acid synthetase, although T₃ amplified the insulin-mediated induction of these enzymes (Fischer & Goodridge, 1978). On the other hand, in similar cultured hepatocytes T₃ provoked a significant in-

Abbreviations used: PEPCK, phosphoenolpyruvate carboxykinase (EC 4.1.1.32); Bt₂cAMP, N⁶,O^{2'}-dibutyl cyclic AMP; MIX, 3-isobutyl-1-methylxanthine; poly(A)⁺ RNA, polyadenylated RNA; T₃, 3,3',5-tri-iodothyronine; SDS, sodium dodecyl sulphate.

crease in the activity of glucokinase (Spence & Pitot, 1979) and ATP citrate lyase activity (Spence *et al.*, 1979).

The aim of the present study is to demonstrate the effect of different thyroid states on the cyclic AMP-provoked synthesis of hepatic PEPCK *in vivo* and in the isolated perfused liver. Our results indicate that the primary action of thyroid hormones is to accelerate the induction of the enzyme by cyclic AMP, acting primarily at a pretranslational level.

Materials and methods

Animals and treatments

Male Wistar rats (SPF, Hagemann, Hannover, West Germany) weighing 200–220 g were housed under controlled conditions. A protein-free high-carbohydrate diet (Altromin C-1004) was fed to all animals 2 days before the experiments in order to minimize alterations in PEPCK activity owing to circadian variations (Tiedgen & Seitz, 1980). This feeding regimen was sufficient to attain equilibrium values of PEPCK and resulted in low PEPCK activity (see the Results section). All experiments were performed between 09:00 and 10:00 h; the nutritional status was in addition monitored by measurement of blood glucose concentration ($>110\text{ mg}\%$ in all thyroid states). Hypothyroidism was induced by intraperitoneal injection of Na^{131}I (Amersham-Buchler, Braunschweig, West Germany) ($250\text{ }\mu\text{Ci}/100\text{ g body wt.}$) at least 28 days before the experiments. Hyperthyroidism was produced by daily intraperitoneal injections of L-thyroxine (E. Merck, Darmstadt, West Germany) ($50\text{ }\mu\text{g}/100\text{ body wt.}$) for 7 days. The hypo- and hyper-thyroid states were monitored by measurement of serum L-thyroxine (<8 and $>200\text{ ng/ml}$ respectively) and hepatic malic enzyme activity (<2 and $>40\text{ munits/mg of protein}$). The data indicate that the induced thyroid states were comparable with those reported by others (Oppenheimer *et al.*, 1977). In experiments *in vivo*, 2 mg of Bt_2cAMP (Boehringer, Mannheim, West Germany) and 2 mg of theophylline (Serva, Heidelberg, West Germany) per 100 g body wt. were injected intraperitoneally 2 h before the animals were killed. Increasing Bt_2cAMP to $5\text{ mg}/100\text{ g body wt.}$ gave no further increases in PEPCK activity in all thyroid states.

Liver perfusion technique

Livers were isolated and perfused as described previously with some modification, with a fully synthetic perfusion medium (total volume 150 ml), which consisted of Fluorocarbon 43 emulsified in Pluronic F 68 and suspended in buffered saline (Krone *et al.*, 1974). The first 60 ml of the perfusate

to flow through the liver was discarded. Perfusate was then recirculated (zero time) with addition of 10% iodothyronine-free serum (prepared from native pig serum as described by Samuels *et al.*, 1979) and amino acids at 4 times the concentration found in rat plasma. It was shown previously that the amino acid supply given in these experiments was sufficient to support hormonal PEPCK induction (Seitz *et al.*, 1980). Adrenaline (Sigma, St. Louis, MO, U.S.A.), Bt_2cAMP and/or MIX (Sigma) were added at zero time (for concentrations see Figure and Table legends). The functional state of the livers was monitored by continuous measurement of flow ($3.5\text{--}4.5\text{ ml/min per g liver wet wt.}$), pH (7.35), release of glutamate:oxaloacetate transaminase (EC 2.6.1.1) activity, the linearity of glucose output and urea production (cf. Krone *et al.*, 1974). The last three parameters were measured by routine enzymic procedures. In control experiments it was confirmed that adrenaline (Fig. 1) or Bt_2cAMP (results not shown) provoked a linear increase in PEPCK activity up to 4 h in livers from hypo-, and hyper-thyroid rats. All further measurements were done at 3 h perfusion time. With respect to the protein-synthetic capacity of the isolated perfused liver, control experiments with livers from fed euthyroid rats revealed that Bt_2cAMP stimulated the relative rate of PEPCK synthesis about 3-fold within 4 h, which was in the range observed by Gunn *et al.* (1975) in Reuber H-35 cells.

Determination of enzyme activities

After the animals were killed by decapitation, or after the end of perfusion, parts of the livers were immediately homogenized in a Potter-Elvehjem homogenizer at 1000 rev./min for 45 s in 5 vol. of the following ice-cold buffer: 50 mM-Hepes , 0.25 M-sucrose , $2.5\text{ mM-dithiothreitol}$, 2.5 mM-EDTA , 100 mM-KCl , pH 7.4. The homogenate was centrifuged within 20 min at 150000 g for 30 min, and PEPCK activity was determined after dilution of the clear supernatant with 0.15 M-KCl as described by Seubert & Huth (1965). Enzyme activity is expressed in units of $\mu\text{mol of oxaloacetate converted into phosphoenolpyruvate/min}$ at 37°C under conditions of the assay. Malic enzyme activity was estimated from the clear supernatant as described by Hsu & Lardy (1969). A unit of enzyme activity is the amount required to reduce $1\text{ }\mu\text{mol of NADP}^+/\text{min}$ at 37°C . Protein was measured by the biuret method with bovine serum albumin as standard. DNA was measured by the method of Burton (1956), with herring sperm DNA (Sigma) as standard.

Measurement of PEPCK synthesis

Antibody to PEPCK was raised in sheep by subcutaneous application of the enzyme purified

Fig. 1. Effect of adrenaline on the induction of PEPCK in the isolated perfused liver from (▲) hypo-, (●) eu- and (■) hyper-thyroid rats

Experimental conditions: initial concentration of adrenaline $10\mu\text{M}$. Data are given as means $\pm$ S.E.M. ($n = 4-6$). For details see the Materials and methods section.

essentially as described by Colombo *et al.* (1978) and emulsified in complete Freund's adjuvant. Quantitative precipitation of immune complexes was obtained when $100\mu\text{l}$ of PEPCK antiserum was incubated with an amount of liver cytosol containing PEPCK activity in the range of 50–200 munits. Specificity of the antigen/antibody reaction was demonstrated by disc-gel electrophoresis (Laemmli, 1970) of the immunoprecipitate from labelled cytosol. A sharp radioactive peak co-migrating with purified PEPCK was observed, representing over 80% of the radioactivity of the immune complex. Labelling *in vivo*

was performed by intraperitoneal injection of $25\mu\text{Ci}$ of L-[^3H]leucine (140Ci/mmol)/100g body wt. (New England Nuclear Corp. Boston, MA, U.S.A.) 30 min before the rats were killed. In the isolated perfused liver $75\mu\text{Ci}$ of L-[^3H]leucine was added to the perfusate 30 min before liver samples were taken. Parts of the livers were immediately homogenized in a Potter-Elvehjem homogenizer at 1100 rev./min for 45 s in 5 vol. of ice-cold buffer as described above. The homogenate was centrifuged within 20 min at $150000g$ for 30 min at 4°C . An amount of homogenate containing 100–150 munits of PEPCK activity was mixed with $100\mu\text{l}$ of PEPCK antiserum and a solution of 5% (w/v) bovine serum albumin in phosphate-buffered saline (10 mM-sodium phosphate/150 mM-NaCl, pH 7.4) added to a final volume of $400\mu\text{l}$. After 30 min incubation at 37°C , the samples were stored at 4°C overnight. Non-specific binding was determined by addition of $100\mu\text{l}$ of pre-immune serum, obtained from the same sheep before the first antigen injection instead of PEPCK antiserum. Tubes were centrifuged for 1 h at $10000g$ and 4°C , and supernatants removed by aspiration. Immunoprecipitates were washed with $3 \times 500\mu\text{l}$ of 0.9% NaCl, dissolved in $500\mu\text{l}$ of Protosol (New England Nuclear Corp.) and finally transferred to scintillation vials and assayed for radioactivity in 10 ml of Rotiszint (Roth, Karlsruhe, W. Germany).

Radioactivity incorporated into total soluble protein was estimated by precipitation with trichloroacetic acid as described previously (Müller *et al.*, 1982). Relative rate of synthesis is expressed as [specific radioactivity (c.p.m) in PEPCK immunoprecipitate/radioactivity incorporated into total protein] $\times 100$. A close correlation between the rate of PEPCK synthesis and the increase in PEPCK activity after induction with Bt_2cAMP , adrenaline and/or MIX was observed in the isolated perfused livers from rats in the different thyroid states ($r = 0.86$, $n = 70$).

mRNA preparation

Total RNA was prepared as described by Chirgwin *et al.* (1979). Poly(A)⁺ RNA was purified by using oligo(dT)-cellulose (Type 7; Pharmacia P-L Biochemicals, Uppsala, Sweden). Total RNA from 0.8–1.2 g of liver (3–6 mg) was dissolved in 1 ml of sterile water and heated to 65°C for 5 min. The solution was diluted with 1 ml of 40 mM-Tris/HCl/1.0 M-LiCl/2 mM-EDTA/0.2% SDS, pH 7.6, and applied to a column packed with 0.5 g of oligo(dT)-cellulose. The flow-through fraction was collected, again heated to 65°C and re-applied to the column. After washing with 10 vol. of 20 mM-Tris/HCl/0.5 M-LiCl/1 mM-EDTA/0.1% SDS, pH 7.6, followed by 10 vol. of the same buffer but containing 0.1 M-LiCl, poly(A)⁺ RNA was

eluted with 4 ml of 10 mM-Tris/HCl/1 mM-EDTA/0.05% SDS, pH 7.5. The yield was 3–5% of the total RNA applied to the column. RNA and poly(A)⁺ RNA were determined spectrophotometrically by assuming $A_{260}^{1\%}/A_{280}^{1\%} = 200$ at 260 nm. The A_{260}/A_{280} ratios were in the range of 1.9–2.1.

Translation assay *in vitro*

Activity of mRNA coding for PEPCK was measured with a rabbit reticulocyte translation kit obtained from New England Nuclear Corp. Assays were carried out in a total volume of 25 μ l, containing final concentrations of 80 mM-K⁺ and 0.4 mM-Mg²⁺. To each reaction mixture 50 μ Ci of L-[³⁵S]methionine and 0.2–0.4 μ g of poly(A)⁺ RNA were added. A parallel increase of incorporation of radioactive amino acid into total protein and PEPCK protein was attained with 0.1–0.5 μ g of poly(A)⁺ RNA. After 30 min incubation at 37°C, the reaction was stopped with 175 μ l of ice-cold 20 mM-Tris/HCl/5 mM-L-methionine/5 mM-dithiothreitol/5 mM-EDTA/0.1 mM-phenylmethanesulphonyl fluoride, pH 7.4, and centrifuged for 1 h at 10000g, 4°C. Incorporation of radioactivity (total c.p.m.) was determined by precipitation with trichloroacetic acid. To 10 μ l samples from the supernatants 500 μ l ice-cold 10% trichloroacetic acid was added and after centrifugation the precipitates were dissolved in 100 μ l of 1 M-NaOH. The protein was again precipitated by addition of 1000 μ l of 10% trichloroacetic acid, centrifuged and washed with 2 $\times$ 500 μ l of 10% trichloroacetic acid. The pellets were then solubilized in 0.5 ml of Protosol, transferred into scintillation vials and assayed for radioactivity in 10 ml of Rotiszint. For quantitative immunoprecipitation, samples from the supernatants (50–80 μ l) were mixed with freshly prepared liver cytosol from starved rats containing 80–120 munits of PEPCK activity, 100 μ l of PEPCK antiserum and 220 μ l of 5% bovine serum albumin in phosphate-buffered saline (see above) containing 0.1 mM-phenylmethanesulphonyl fluoride. Incubation and washing were performed as described for measurement of PEPCK synthesis, except that a solution of 0.9% NaCl, 0.5% Triton X-100 and 0.5% sodium deoxycholate was used. The precipitate was heated with 30 μ l of sample buffer (0.1 M-sodium phosphate/6 M-urea/1% SDS/1% mercaptoethanol) to 95°C for 15 min and subjected to disc-gel electrophoresis (Laemmli, 1970). To quantify the radioactivity incorporated into PEPCK protein, the gel tracks were cut in 2 mm slices, eluted with 0.5 ml of Protosol (2 h, 60°C) and assayed for radioactivity in 10 ml of Rotiszint. For qualitative purposes the slab gels were stained with Serva Violet (0.2% in 25% propan-2-ol/10% acetic acid), destained with 25% ethanol/8% acetic acid, soaked for 30 min in

ENLIGHTNING (New England Nuclear Corp.) and finally dried on filter paper. Autofluorographs were obtained after 4 days exposure to Kodak X-OMat AR film at –70°C. A sharp band co-migrating with authentic PEPCK was seen in the region of 70000 Da (Fig. 2). Other bands frequently observed in the range below 50000 Da did not affect the quantification of the incorporation of radioactivity into PEPCK protein. Results for

Fig. 2. SDS/polyacrylamide-gel electrophoresis of immuno-precipitated PEPCK synthesized in a rabbit reticulocyte lysate with mRNA isolated from a *Bt₂cAMP*-injected fed hyperthyroid rat

For details see the Materials and methods section. Lane (a), M_r -marker proteins: phosphorylase a (92000), bovine serum albumin (69000), ovalbumin (43000), carbonic anhydrase (30000), soya-bean trypsin inhibitor (20000). Lane (b), PEPCK immunoprecipitate.

mRNA activity coding for PEPCK obtained by translation *in vitro* are expressed as (radioactivity incorporated into PEPCK/radioactivity incorporated into total protein) $\times 100$.

Results

Effects of Bt₂cAMP on PEPCK synthesis and PEPCK mRNA activity in the different thyroid states

Hypo- and eu-thyroid rats fed on a high-carbohydrate diet showed low relative rates of PEPCK synthesis and low amounts of translatable PEPCK mRNA, whereas in hyperthyroid rats a significant increase in both parameters was observed (Table 1, controls). Injection of Bt₂cAMP resulted within 2 h in an increase in PEPCK mRNA activity in all thyroid states, followed by a corresponding increase (correlation coefficient $r = 0.97$; $n = 12$) in the rate of PEPCK synthesis. The effect of Bt₂cAMP in euthyroid rats was within the range observed by others (Beale *et al.*, 1982); however, in hypothyroid animals the increase in both PEPCK mRNA activity and PEPCK synthesis was impaired. Hyperthyroid animals injected with Bt₂cAMP showed similar values to those in euthyroid controls.

Induction of hepatic PEPCK in isolated perfused livers from different thyroid states

As expected, livers taken from hypo-, eu- and hyper-thyroid fed animals showed low values of PEPCK activity (16 ± 2 , 26 ± 2 and 43 ± 3 munits/mg of protein respectively; see Fig. 1). Perfusion without Bt₂cAMP or adrenaline up to 3 h resulted only in a minimal increase in PEPCK activity (Fig. 3) in livers from the different thyroid states. Addition of Bt₂cAMP or adrenaline to the perfusate led to a significant PEPCK induction (Fig.

3); however, the effect was more pronounced in livers of hyperthyroid rats.

As hypothyroidism is associated with an increase in hepatic low-K_m phosphodiesterase activity (Morgan *et al.*, 1982), probably increasing tissue cyclic AMP turnover rate, the experiments were repeated in the presence of MIX, an inhibitor of phosphodiesterases. This addition led to a con-

Fig. 3. Effect of Bt₂cAMP, adrenaline (Epi), Bt₂cAMP + MIX and adrenaline + MIX on the induction of PEPCK in isolated perfused livers of (□) hypo-, (▨) eu- or (■) hyper-thyroid fed rats

Experimental conditions: 3 h perfusion time, initial concentrations of Bt₂cAMP 0.2 mM, adrenaline (Epi) 10 μ M, MIX 0.5 mM. Basal values at zero time (see Fig. 1) were subtracted. Data are given as means $\pm$ S.E.M. for n rats.

Table 1. Effect of different thyroid states on the cyclic AMP-induced increase in the relative rates of PEPCK synthesis and PEPCK mRNA activities

For details see the Materials and methods section; data are mean values $\pm$ S.E.M. for the numbers of experiments in parentheses. Significance of Bt₂cAMP-induced increase: in the relative rate of PEPCK synthesis, hypo- versus eu-thyroid $P < 0.01$, hypo- versus hyper-thyroid $P < 0.025$; in PEPCK mRNA activity; hypo- versus eu-thyroid $P < 0.0125$, hypo- versus hyper-thyroid $P < 0.025$.

Animals and treatments		Relative rate of PEPCK-synthesis (% of total soluble protein)	PEPCK mRNA activity (% of total mRNA activity)
Hypothyroid	+ NaCl	0.25 ± 0.14 (4)	0.072 ± 0.016 (3)
	+ Bt ₂ cAMP	1.57 ± 0.14 (4)	0.195 ± 0.016 (3)
Euthyroid	+ NaCl	0.30 ± 0.13 (4)	0.095 ± 0.027 (4)
	+ Bt ₂ cAMP	2.58 ± 0.27 (3)	0.301 ± 0.025 (4)
Hyperthyroid	+ NaCl	1.75 ± 0.24 (4)	0.187 ± 0.010 (4)
	+ Bt ₂ cAMP	2.78 ± 0.40 (4)	0.336 ± 0.045 (4)

siderable increase in the Bt_2cAMP - and adrenaline-provoked PEPCK induction rate (Fig. 3); however, it was not more pronounced in livers of hypothyroid rats. This indicates that the increases in low- K_m phosphodiesterase activity in hypothyroid rats did not significantly contribute to the impaired Bt_2cAMP effect on PEPCK synthesis.

Discussion

It was demonstrated that cyclic AMP induces hepatic PEPCK by rapidly increasing the specific mRNA coding for this enzyme (Lamers *et al.*, 1982; Beale *et al.*, 1982). By the use of a cDNA probe to PEPCK from rat liver cytosol, Beale *et al.* (1982) provided direct evidence for a cyclic AMP-mediated stimulation in the rate of transcription of the PEPCK gene. The close correlation between PEPCK mRNA quantified by DNA/RNA hybridization and by the reticulocyte-lysate translation assay shown by Beale *et al.* (1982) demonstrated that the translation system *in vitro* accurately reflects PEPCK mRNA concentration. Thus it is apparent from our data (Table 1) that the Bt_2cAMP -provoked availability of specific mRNA coding for PEPCK is enhanced in the presence of thyroid hormones under conditions *in vivo*: in euthyroid fed animals the injection of the cyclic nucleotide provoked within 2 h a dramatic increase in the relative rate of PEPCK mRNA activity; however, in hypothyroid animals only a small elevation was observed (Table 1). From the close correlation between the relative rate of PEPCK synthesis and PEPCK mRNA activity (Table 1) it is apparent that thyroid hormones exert their action primarily at a pretranslational level. Comparing the cyclic AMP-mediated effect on PEPCK induction in eu- and hypo-thyroid animals (Table 1), it is furthermore evident that thyroid hormones act at their physiological circulating concentration in the regulation of this enzyme.

Experiments in the isolated perfused liver (Müller *et al.*, 1982) and in hepatocytes in culture from hypothyroid fed animals provided direct evidence that T_3 by itself within 4–6 h had virtually no effect, and within 24 h had only a small effect, in increasing the rate of PEPCK synthesis (Süssmuth *et al.*, 1984) and PEPCK mRNA (W. Süssmuth, W. Höppner & H. J. Seitz, unpublished work). Yet the addition of Bt_2cAMP to the perfusion medium resulted in a significantly pronounced increase in PEPCK induction in livers from eu- and hyperthyroid animals compared with hypothyroid controls. Thus these findings *in vitro* support our conclusion that the primary action of thyroid hormones is to enhance the cyclic AMP- and adrenaline-stimulated synthesis of this enzyme (Fig. 3).

In hyperthyroid rats PEPCK synthesis and mRNA activity were already elevated in the fed state (Table 1). However, since this condition *in vivo* is associated with elevated concentrations of circulating glucagon (Müller *et al.*, 1983) and, accordingly, elevated hepatic cyclic AMP values (results not shown), it is reasonable to assume that the increase *in vitro* in PEPCK synthesis and mRNA activity compared with euthyroid rats is mainly due to the effect of the cyclic nucleotide.

Data on the action of thyroid hormones in stimulating hepatic enzyme synthesis favour the hypothesis that T_3 primarily acts as an amplifier of hormonal or nutritional signal systems (see Mariash & Oppenheimer, 1983). This was mainly deduced from a permissive effect of T_3 for the insulin-mediated increase in translatable mRNA coding for malic enzyme (Mariash & Oppenheimer, 1983) and for glucokinase (Sibrowski & Seitz, 1984) under conditions *in vivo*, and from a more than additive effect of T_3 and insulin in the induction of acetyl-CoA carboxylase and fatty acid synthetase in chick hepatocytes in culture (Siddiqui *et al.*, 1981). The data in the present paper extend this hypothesis to an important regulatory enzyme, induced by cyclic AMP.

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