Rapid stimulation of calcium uptake into rat liver by L-tri-iodothyronine

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The short-term effect of L-tri-iodothyronine (T_3) on hepatic Ca^{2+} uptake from perfusate was compared with changes induced by T_3 on cellular respiration and glucose output in isolated perfused livers from fasted and fed rats. The same parameters were also studied after the addition of glucagon or vasopressin. T_3 ($1\ \mu M$) induced Ca^{2+} uptake from the perfusate into the liver within minutes, and the time course was similar to that for stimulation of respiration and gluconeogenesis in livers from fasted rats, and for the stimulation of respiration and glucose output in livers from fed rats. The effects were dose-dependent in the range $1\ \mu M$ – $0.1\ nM$. Similar changes in the same parameters could be observed with glucagon and vasopressin, but with a completely different time course. Also, the influence of the T_3 analogues L-thyroxine ($L-T_4$), 3,5-di-iodo-L-thyronine ($L-T_2$) and 3,3',5-tri-iodo-D-thyronine ($D-T_3$) on hepatic energy metabolism was examined. Whereas $D-T_3$ had practically no effect, $L-T_4$ and $L-T_2$ caused changes in Ca^{2+} uptake, O_2 consumption and gluconeogenesis in livers from fasted rats similar to those with T_3 . It is concluded that changes in mitochondrial and cytosolic Ca^{2+} concentrations are involved in the stimulation of respiration and glucose metabolism observed with T_3 , glucagon and vasopressin.

INTRODUCTION

Thyroid hormones not only act at the level of the cell nucleus, but also influence cellular energy metabolism independent of protein synthesis in a short-term fashion. Thus it is reported that O_2 consumption is stimulated in liver mitochondria (Sterling, 1979), isolated hepatocytes (Higuti & Rottenberg, 1986) and isolated perfused rat liver (Müller & Seitz, 1980) by L-tri-iodothyronine (T_3). Energy-consuming pathways such as gluconeogenesis and urea synthesis are also stimulated in perfused liver (Müller & Seitz, 1980). Moreover, the uptake of amino acids and 2-deoxyglucose in rat thymocytes is enhanced by T_3 (Adamson & Ingbar, 1967; Segal & Ingbar, 1979). Others report that T_3 stimulates mitochondrial adenine nucleotide translocation (Seitz *et al.*, 1985; Mowbray & Corrigal, 1984).

In the present work we were mainly interested in the influence of T_3 on Ca^{2+} uptake into rat liver. Previous studies indicate that Ca^{2+} metabolism is affected by T_3 . In rat thymocytes Ca^{2+} is increased transiently (Segal & Ingbar, 1984). Likewise, liver mitochondria of thyroidectomized rats accumulate Ca^{2+} on addition of T_3 , which induces ATP export from the mitochondria (Herd, 1978). Others showed that ATP synthase and adenine nucleotide transport are regulated by Ca^{2+} movements (Moreno-Sanchez, 1985). From this it appears that T_3 may influence cellular energy metabolism through changes in cellular Ca^{2+} concentration.

MATERIALS AND METHODS

Animals

Male Wistar rats (190–230 g) received a standard diet (Altromin, Lage, Germany) and water *ad libitum* or were

starved for 24 h before isolation of the liver under pentobarbital anaesthesia.

Haemoglobin-free liver perfusion

Livers from fed or starved euthyroid rats were perfused with haemoglobin-free Krebs–Henseleit bicarbonate buffer in a non-recirculating system (pH 7.4, $37^\circ C$, saturated with O_2/CO_2 , 19:1) (Sies, 1978). Substrates were added as indicated in the legends of the Figures and Tables. Thyroid hormones were dissolved in 0.1 M-NaOH, diluted and infused at a flow rate of $55\ \mu l/min$.

O_2 and Ca^{2+} concentrations in effluent perfusate were monitored with a Clark-type oxygen electrode and a Ca^{2+} -sensitive electrode respectively. Glucose production was determined in the perfusate by enzymic analysis (Bergmeyer, 1970), by taking samples at 5 min intervals. Metabolic rates were calculated from arteriovenous concentration differences.

Isolation and incubation of hepatocytes

Hepatocytes were isolated from livers from euthyroid fed rats as described by Berry & Friend (1969) with slight modifications (Sies *et al.*, 1977). For determination of intracellular Ca^{2+} concentration, hepatocytes were loaded with the fluorescent Ca^{2+} -indicator dye fura 2/AM. Hepatocytes (5 mg of protein/ml) were incubated for 30 min in a shaking water bath (shaking frequency $120\ min^{-1}$, $37^\circ C$) in Krebs–Henseleit bicarbonate buffer with $10\ \mu M$ -fura 2/AM and $0.7\ mM$ -pyruvate + $2.1\ mM$ -lactate as substrates. The suspension was washed twice to remove extracellular fura 2/AM. The hepatocytes were resuspended in medium saturated with O_2/CO_2 (19:1), and the fluorescence of hepatocyte suspensions was measured by using a Perkin–Elmer LS-5 Luminometer

Abbreviations used: T_3 , tri-iodothyronine; $L-T_4$, L-thyroxine; $L-T_2$, 3,5-di-iodo-L-thyronine.

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(340 nm excitation and 500 nm emission wavelength) in 1 cm-light-path quartz cuvettes with continuous magnetic stirring. The cytosolic Ca^{2+} concentration was calculated from eqn. (1):

$$[\text{Ca}^{2+}] = K_d(F - F_{\min.}) / (F_{\max.} - F) \quad (1)$$

(Gryniewicz *et al.*, 1985), where $F_{\max.}$ represents the fluorescence of the Ca^{2+} -saturated dye and was obtained by digitonin lysis of the hepatocytes, and $F_{\min.}$ was obtained by addition of 2 mM-EGTA (final concn.) to digitonin-lysed cells (10 μM -digitonin) and represented the fluorescence of Ca^{2+} -free fura 2. F , $F_{\max.}$ and $F_{\min.}$ were corrected for autofluorescence of control hepatocyte suspensions. K_d was taken as 224 nM (Gryniewicz *et al.*, 1985).

Enzymes and coenzymes were purchased from Boehringer (Mannheim, Germany) and Sigma (München, Germany); sodium pentobarbital was from Abbott (Neuilly-sur-Seine, France). All other chemicals were from Merck (Darmstadt, Germany) and were of the highest purity available.

RESULTS

Tri-iodothyronine-mediated changes

Infusion of T_3 into isolated livers from euthyroid fasted rats caused a decrease in effluent perfusate Ca^{2+} , which corresponded to an increase in O_2 consumption and gluconeogenesis (Fig. 1). The effects were dose-dependent (Table 1). A decrease in perfusate Ca^{2+} was also observed in livers from fed rats, where it corresponded to an increase in O_2 uptake and glucose output (Table 2). With 10 μM extracellular Ca^{2+} T_3 did not induce any changes in perfusate Ca^{2+} or in O_2 consumption and gluconeogenesis (Table 2).

Effects of T_3 analogues

In livers from euthyroid fasted rats, L- T_4 and L- T_2 induced the same changes in perfusate Ca^{2+} , O_2 consumption and gluconeogenesis as T_3 , whereas D- T_3 had no significant effect (Table 3).

Comparison with other hormones

Since it is known that several hormones exert their rapid metabolic changes via Ca^{2+} as third messenger, the time course of metabolic changes was studied in perfused livers under the influence of glucagon and vasopressin (Figs. 2a and 2b). As with T_3 , both hormones caused an increase in Ca^{2+} and O_2 uptake and in gluconeogenesis. The time course, however, was completely different. Whereas T_3 reached its maximum effect after about 20 min, vasopressin released Ca^{2+} from the liver within the first 1 min, followed by an uptake within the following 2–3 min which was accompanied by an increase in O_2 consumption and gluconeogenesis. Within the following 1 min a new steady state was established, which was elevated over the control. When the hormone was removed, O_2 consumption and glucose production returned slowly to the initial values within 15 min, whereas Ca^{2+} was still taken up.

Glucagon had similar kinetics, with a somewhat slower time course. However, O_2 consumption increased nearly continuously to a new steady state, and the oscillations seen with Ca^{2+} and gluconeogenesis under the influence of the hormone were hardly observable.

The decrease in perfusate Ca^{2+} after addition of the three hormones is also reflected by an increase in cytosolic Ca^{2+} as measured in fura 2/AM-loaded hepatocytes (Table 4). T_3 increased cytosolic Ca^{2+} by about 35 nM, whereas vasopressin and glucagon raised the concentration by about 50 nM. Similarly to the perfusion

Fig. 1. Time course of changes induced by 1 μM - T_3 in metabolism in perfused livers from euthyroid fasted rats with 1 mM-pyruvate and 10 mM-lactate as substrates

Results are means $\pm$ S.E.M. ($n = 8$). Controls showed a slight decrease in gluconeogenesis and no changes in other parameters after the same perfusion time.

Table 1. Concentration-dependence of short-term effect of T_3 on metabolism in liver from fasted rats

Perfusate $[Ca^{2+}]$ was 1.25 mM. Results are means $\pm$ S.E.M. ($n = 4-8$). Control values were: O_2 uptake, 2.46 μ mol/min per g of liver; glucose synthesis, 0.725 μ mol/min per g of liver.

	[L- T_3]... 10^{-6} M	10^{-8} M	10^{-10} M
Change in perfusate $[Ca^{2+}]$ (μ M)	12.4 $\pm$ 0.9	5.2 $\pm$ 1.2	0.1 $\pm$ 0.9
Change in O_2 uptake (% of control)	14.8 $\pm$ 3.5	6.3 $\pm$ 1.3	2.4 $\pm$ 0.9
Change in glucose output (% of control)	36.4 $\pm$ 2.9	22.5 $\pm$ 2.7	11.6 $\pm$ 4.1

Table 2. Changes in perfusate Ca^{2+} and metabolic rates at 10 μ M external Ca^{2+} and in livers from fed rats

Results are means $\pm$ S.E.M. ($n = 4-8$). Control values for livers from fasted rats were: O_2 uptake, 2.39 μ mol/min per g of liver; glucose synthesis, 0.82 μ mol/min per g of liver; for livers from fed rats: O_2 uptake, 1.5 μ mol/min per g of liver; glucose output, 0.60 μ mol/min per g of liver.

	Fasted, 10 μ M- Ca^{2+}	Fed, no substrates
Change in perfusate Ca^{2+} (μ M)	0.5 $\pm$ 0.05	10.9 $\pm$ 2.6
Change in O_2 uptake (% of control)	0.9 $\pm$ 0.02	7.4 $\pm$ 0.07
Change in glucose output (% of control)	2.3 $\pm$ 0.2	15.8 $\pm$ 0.1

experiments, the kinetics were different, the response being fastest with vasopressin and slowest with T_3 (Fig. 3).

DISCUSSION

It is well known that several hormones stimulate energy metabolism, e.g. respiration and gluconeogenesis in livers from fasted rats and respiration and glycogenolysis in livers from fed rats. Among these hormones are glucagon, acting via cyclic AMP, and α -adrenergic agonists such as vasopressin and adrenaline, mediating intracellular effects via inositol phosphates as second messengers (for reviews see Halestrap *et al.*, 1985;

Kraus-Friedmann, 1986; Soboll & Sies, 1988). In the search for a common agent for these uniform events, it was found that Ca^{2+} is increased in liver cytosol by both types of hormones (Exton, 1981; Williamson *et al.*, 1981), and that a redistribution of Ca^{2+} across plasma and mitochondrial membranes plays a central role whereby these hormones affect cellular energy metabolism (Prpic & Bygrave, 1980; Goldstone *et al.*, 1983; Reinhart *et al.*, 1984; Shears & Kirk, 1984; Kleineke & Söling, 1985).

Also for thyroid hormone it has been reported that respiration and gluconeogenesis from alanine is stimulated by T_3 within about 30 min in livers from fasted hypothyroid rats (Müller & Seitz, 1980). Stimulation of gluconeogenesis was not dependent on cyclic AMP, but cyclic AMP synergistically enhanced gluconeogenesis (Müller & Seitz, 1983). Here we have shown that T_3 stimulates respiration and gluconeogenesis from lactate and pyruvate also in livers from euthyroid fasted rats within minutes, and that these effects have a similar time course to the decrease in perfusate Ca^{2+} (Fig. 1). The metabolic changes are concentration-dependent and appear not to be due to uncoupling, since it has been shown that the increase in O_2 consumption is oligomycin-sensitive (Müller & Seitz, 1980) and that gluconeogenesis is enhanced. The rather high concentration of T_3 which was necessary to induce the metabolic changes is explainable from the use of an albumin-free open perfusion system, since a uniform distribution of thyroid hormone within tissues requires circulating thyroid-hormone-binding proteins, e.g. thyroid-hormone-binding globulin and thyroid-hormone-binding prealbumin (Mendel *et al.*, 1987). In a recirculating liver perfusion system, T_3 increased O_2 uptake already at concentrations of 1 pM (Horst, 1988).

That the decrease in perfusate Ca^{2+} is not only the result of Ca^{2+} binding, but also reflects an increase in cytosolic Ca^{2+} , is shown in fura 2/AM-loaded hepatocytes and is indicated by the fact that T_3 stimulates glucose output in livers from fed rats. The total amount of Ca^{2+} which disappeared from the perfusate on infusion of T_3 (100–1000 nmol/g of liver) is at least three orders of magnitude higher than the increase in free Ca^{2+} within the cell (~ 100 pmol/g of liver; Table 4). This was previously observed by Altin & Bygrave (1985) for vasopressin, and it was suggested that most of the Ca^{2+} taken up is accumulated in the mitochondria.

The T_3 -mediated metabolic changes are nearly abolished if the Ca^{2+} concentration is decreased from 1.25 mM to 10 μ M (Table 2). Thus it appears firstly that Ca^{2+} is indeed a prerequisite for the stimulation of mitochondrial metabolism, and secondly that Ca^{2+} stems from the extracellular space.

In contrast with this, α -adrenergic agonists mobilize

Table 3. Effect of analogues (1 μ M) on metabolism in liver from fasted rats

Results are means $\pm$ S.E.M. ($n = 4-8$). For conditions and control values see Table 1.

	L- T_3	L- T_2	L- T_4	D- T_3
Change in perfusate Ca^{2+} (μ M)	12.4 $\pm$ 0.9	11.3 $\pm$ 3.9	12.3 $\pm$ 1	2.6 $\pm$ 2.7
Change in O_2 uptake (% of control)	14.8 $\pm$ 3.5	11.5 $\pm$ 1.8	3.6 $\pm$ 0.7	0.7 $\pm$ 0.1
Change in glucose output (% of control)	36.4 $\pm$ 2.9	29.6 $\pm$ 2.8	13.2 $\pm$ 1.7	0.5 $\pm$ 0.2

Fig. 2. Time course of changes in metabolism in perfused livers induced by 10 nM-glucagon (a) or 10 nM-vasopressin (b)

Conditions were as in Fig. 1. Results are means $\pm$ S.E.M. ($n = 4$).

Table 4. Increase in cytosolic Ca^{2+} in isolated hepatocytes loaded with fura 2/AM after addition of T_3 , glucagon and vasopressin

Results are means $\pm$ S.E.M. ($n = 10-15$). The maximum values after addition of the hormone were taken (within 1-3 min).

	Cytosolic Ca^{2+} (nM)	
	Control	+ hormone
T_3 (1 μ M)	120 ± 8	155 ± 10
Glucagon (10 nM)	110 ± 7	150 ± 8
Vasopressin (10 nM)	126 ± 9	173 ± 9

Fig. 3. Time course of changes in cytosolic $[Ca^{2+}]$ in isolated hepatocytes from euthyroid fed rats induced by 1 μ M-NaOH (control, a), 1 μ M- T_3 (b), 10 nM-glucagon (c) and 10 nM-vasopressin (d)

Fluorescence changes from one representative experiment are shown. For conditions, see the Materials and methods section.

Ca^{2+} not only from the extracellular space but also from endoplasmic reticulum (Sies *et al.*, 1983; Reinhart *et al.*, 1984; Joseph *et al.*, 1985), and the same is suggested for glucagon (Kraus-Friedmann, 1986). Extracellular Ca^{2+} appears to be responsible for the sustained effects of the hormones, and therefore we may assume that the lack of the fast component in T_3 -mediated action on cellular Ca^{2+} is due to the fact that it only affects plasma-membrane Ca^{2+} transport. The difference in kinetics and extent of metabolic changes is in contrast with the similarity in the affected metabolic pathways, and could thus arise from the different pools where the hormones mobilize Ca^{2+} .

Interestingly not only T_3 , but also the analogues L- T_4 and L- T_2 , exerted the same effect on liver energy metabolism. D- T_3 stimulated Ca^{2+} uptake, but not significantly. The response to L- T_2 is slightly faster than that to L- T_4 and to T_3 . This opens the question as to whether L- T_2 has a distinctive role in regulating mitochondrial metabolism in a short-term fashion. Horst (1988) found that infusion of livers with propylthiouracil, an inhibitor of hepatic deiodinase, inhibits T_3 -mediated stimulation of respiration in livers from fasted hypothyroid animals completely, whereas it does not prevent stimulation of respiration by T_2 .

The question is still open how on a molecular basis hormones may stimulate mitochondrial metabolism. Since Ca^{2+} is the unique factor which is changed by the three types of hormones, e.g. glucagon, vasopressin and thyroid hormones, it is probable that it may directly influence mitochondrial metabolism, as suggested by Denton & McCormack (1985), by stimulating mitochondrial dehydrogenases through an enhancement of mitochondrial Ca^{2+} and thus of substrate supply to the respiratory chain. On the other hand, as shown in Table 1, addition of 0.1 nM- T_3 to the liver causes still a slight increase in O_2 consumption and gluconeogenesis, whereas no Ca^{2+} uptake could be detected. Further, D- T_3 caused a very slight uptake of Ca^{2+} from the perfusate (Table 3), with no effect on gluconeogenesis and on O_2 consumption. This could be ascribed to the scatter of measurements in the very low range of changes of the metabolic parameters, but it cannot be ruled out that, besides Ca^{2+} , other mechanisms are also effective in stimulating mitochondrial metabolism. Whether T_2 is directly involved in the modulation of mitochondrial metabolism has to be investigated in the future.

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