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Triiodothyronine Rapidly Stimulates Mitochondrial Respiration in Isolated Hepatocytes

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Isolated hepatocytes from euthyroid or hypothyroid (thyroidectomized) rats showed a 20 to 30% stimulation of respiratory activity when treated with triiodothyronine (T_3). Maximal stimulation was obtained in the presence of the uncoupler carbonylcyanide *m*-chlorophenylhydrazone (CCCP) suggesting a direct effect on the respiratory chain. Physiological concentrations of T_3 (10^{-11}—10^{-9}) induced maximal stimulation after a short incubation (75% of maximum in 3 min) and the effect persisted for over 1 h of incubation. Mitochondria, rapidly isolated from T_3-treated hepatocytes, retained stimulated respiratory activity in both coupled and uncoupled states. These results are consistent with the hypothesis that the mitochondrion is a primary target for thyroid hormones, and the present system appears to be suitable for the systematic investigation of these effects.

Keywords——triiodothyronine; isolated hepatocyte; hypothyroid rat; mitochondria; respiration

Thyroid hormones regulate the rate of catabolism in target tissues, thereby affecting oxygen consumption and heat production. The major pathway, which is relatively well understood, is *via* nuclear receptors that control the translation and synthesis of key enzymes.[2-6)] A second pathway which causes a rapid increase in respiration and other activities, not associated with *de novo* protein synthesis, is still obscure.[7-10)] Short-term stimulation of respiration in mitochondria isolated from hypothyroid rats shortly after injection of triiodothyronine (T_3) was reported by Sterling *et al.*[10)] and Breton *et al.*[11)] However, since T_3 modulates the actions of other hormones which affect mitochondrial activities[12)] it is not clear if the effects observed after injection are due to a direct effect of T_3 on the mitochondria.

It was also shown recently that injection of T_3 into perfused isolated rat liver increased oxygen consumption and adenosine triphosphate (ATP) synthesis.[13,14)] However, this stimulation may also be due to indirect effects. Sterling and his colleagues[15,16)] claimed that mitochondria from target organs contain specific thyroid hormone receptors and can be stimulated directly *in vitro*. Despite extensive efforts we could not confirm the latter claim. However, we succeeded in demonstrating that mitochondrial respiration can be stimulated by T_3 in isolated hepatocytes and that this stimulation is retained by the isolated mitochondria.

Experimental

Parenchymal hepatocytes were isolated from the liver of normal male Sprangue-Dawley rats (200—250 g) and thyroidectomized rats by the method of Berry and Friend[17)] and Seglen,[18)] as described below. Liver was preperfused for 5 min with a medium containing 137 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_4, 0.649 mM $MgSO_4$, 10 mM Hepes buffer (pH 7.4), and 1 mM ethyleneglycol bis-(2-aminoethylether)-*N,N*-tetraacetate (EGTA) at a flow rate of 6 ml per min at 36 °C. Then the liver was perfused for an additional 30 min by using a Collagenase Medium (pH 7.6) containing 66.8 mM NaCl, 6.7 mM KCl, 4.76 mM $CaCl_2$, 100 mM Hepes buffer, and collagenase (9000 units/180 ml) at a

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flow rate of 12 ml per min. Hepatocytes were released from the perfused liver into a Washing Medium (pH 7.5) containing 142 mM NaCl, 6.7 mM KCl, 1.2 mM $CaCl_2$, and 10 mM Hepes buffer at 2 °C. The hepatocytes were filtered through 6 layers of gauze. The filtrate was adjusted to 50 ml with the Washing Medium and left for 1 min in a 50 ml centrifuge tube. The supernatant was transferred, adjusted to 100 ml with the Washing Medium and left for 10 min in 50 ml centrifuge tubes. The precipitated cells were suspended in 50 ml of the same medium and centrifuged at 300 rpm for 1 min. The cells were washed again, then suspended in a chilled incubation medium containing 120 mM NaCl, 4.69 mM KCl, 0.72 mM $MgSO_4$, 10 mM Hepes buffer, 13.9 mM glucose, 1 mM KH_2PO_4, 2 ml of MEM amino acids per 100 ml of the Incubation Medium, 2 mM L-glutamine, 1.28 mM $CaCl_2$, and 1 mg of bovine albumin (fatty acid-free) per ml at pH 7.4. Viability of the cells was measured by trypan blue exclusion (final concentration of the dye: 0.04%).

The isolated hepatocytes were incubated for 30 min (unless otherwise indicated) in the Incubation Medium containing an indicated amount of T_3 at the concentration of 10^6 cells per ml (total valume 5—15 ml) at 36 °C in a 50 ml Nalgene Erlenmeyer flask with a cap. Before the incubation, oxygen was supplied for about 1 min. After the incubation the mixtures were cooled with ice water and air was supplied for about 4 min. The activity of the respiration was measured polarographically with a Yellow Springs, model YSI-53, oxygen monitor in the absence and presence of 10 μM carbonylcyanide *m*-chlorophenylhydrazone (CCCP) at 36 °C.

The method of the preparation of mitochondria from the isolated hepatocytes was a modification of the method of Zuurendonk and Tager.[19] The isolated hepatocytes were incubated for 30 min in the Incubation Medium ($\pm$ 1 nM T_3) in a total volume of 40 ml in a 200 ml Nalgene Erlenmeyer flask with a cap as described above. The cells were centrifuged at $120 \times g$ for 2 min and suspended in 10 ml of Medium A (pH 7.2) containing 0.25 M sucrose, 20 mM potassium morpholinopropane sulfonate, and 3 mM ethylenediaminetetraacetic acid (EDTA) at about 2 °C. After addition of 10 ml of Medium A with 0.5 mg/ml of purified digitonin, the mixture was homogenized for 10 s at 240 rpm in a 50 ml glass homogenizer. Immediately, 60 ml of Medium A was added and the whole was centrifuged at $755 \times g$ for 5 min. The pellet was suspended with 0.25 M sucrose by using a 15 ml glass homogenizer and centrifuged at $755 \times g$ for 10 min. At this stage the complete disruption of the cells was confirmed by using a microscope. The supernatant was centrifuged at $12000 \times g$ for 10 min and suspended with 0.25 M sucrose.

The contents of the mitochondria from each batch of isolated hepatocytes ($\pm$ T_3) were estimated from the cytochrome a + a_3 content measured as the absorbance of dithionite-reduced minus oxidized mitochondrial suspension at pH 7.4, at the wavelength pair of 605—630 nm in an Aminco spectrophotometer, model DW-2, using a value of 16.0 for ε_{mM}.[20] Protein was determined by a cyanide biuret procedure.[21] Collagenase (type I), digitonin, CCCP, and T_3 were products of Sigma. Digitonin was purified as previously reported.[22] T_3 was solubilized with 0.1 N NaOH at the concentration of 1 mM and diluted with deionized water. MEM amino acids and L-glutamine were products of Grand Island Biological Co., Grand Island, New York. Surgically thyroidectomized rats obtained from Charles River Laboratories were given 0.2% $CaCl_2$ in tap water and low iodine diet for at least 1 month before the experiment.

Results and Discussion

Isolated hepatocytes from hypothyroid rats were incubated for 30 min (36 °C) with the hormone T_3 in the concentration range of 10^{-11} to 10^{-7} M. After completion of the incubation, the cell suspension was stored on ice and the rate of oxygen consumption of the cells was assayed. Figure 1 shows typical results from several such experiments. In these cells the uncoupler CCCP at 10 μM stimulated respiration 2—3 fold. Uncoupled respiration was stimulated at all hormone concentrations with maximal effect at 10^{-9} M. The T_3-induced stimulation of respiration varied from 20 to 50% from preparation to preparation. Preparations from both euthyroids and hypothyroid rats were stimulated, but the stimulation of hypothyroids was more reproducible. Coupled respiration was also stimulated but the magnitude of the effect was smaller and less reproducible. These data suggest that the effect, in isolated hepatocytes, is mainly on the respiratory chain itself and is best observed when the activity is measured with minimal control by cytoplasmic or energetic factors.

The effect of 10^{-10} M T_3 on coupled respiration in isolated hepatocytes from thyroidectomized rats reached 75% of maximal stimulation after only 3 min of incubation. At 15 min the effect was maximal. This very fast response clearly indicates that the effect is not mediated by protein synthesis. T_3 is rapidly accumulated in isolated hepatocytes,[23] which is consistent with the rapid effect observed on mitochondrial respiration in this experiment.

In order to ascertain whether the stimulation of respiration is a direct and persistent effect on the respiratory chain itself, we prepared mitochondria from T_3-treated hepatocytes

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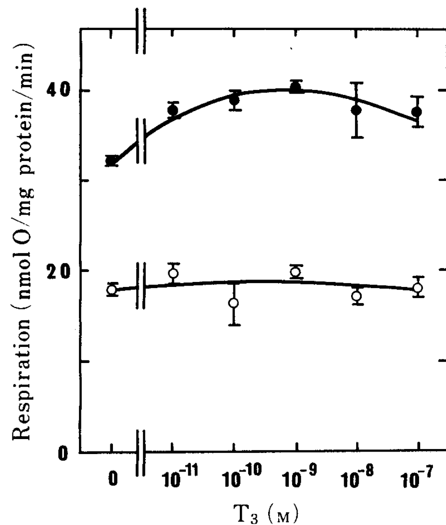

Fig. 1. Effect of T_3 on the Respiratory Activity in the Isolated Hepatocytes

The hepatocytes isolated from thyroidectomized rats were incubated for 30 min in the Incubation Medium with the indicated amount of T_3. Each system contained 10^6 cells per ml (2.9 mg protein/ml) in a total volume of 6 ml at 36 °C. Other conditions were as described under Experimental. The values are means from two experiments and the bars represent the standard deviation. Viability of the cells was 92%. (○) Endogenous respiration; (●) CCCP-stimulated respiration.

TABLE I. T_3-Induced Increase of the Respiratory Activities in Isolated Hepatocytes

	O_2-Uptake			
	Control (nmol O/mg protein/min)		+CCCP (10 μM)	
Control	10.9±0.3	100%	29.3±1.7	100%
$+T_3$	13.2±0.9	121%	38.4±3.0	131%

The hepatocytes isolated from normal rats were incubated for 30 min in the Incubation Medium (±1 nM T_3, at the concentration of 10^6 cells per ml (3.58 mg protein/ml)). The other conditions were as described under Experimental. Values are means from three experiments, with the standard deviation. Oxygen uptake was measured at 36 °C. Viability of the cells was 81%.

TABLE II. T_3-Induced Increase of the Respiratory Activities in Mitochondria Isolated from T_3-Treated Hepatocytes

	O_2-Uptake					
	State 4		State 3 (nmol O/0.263 nmol cytochrome $a+a_3$/min)[a]		+CCCP (0.1 μM)	
L-Glutamate (5 mM) L-Malate (5 mM)						
Control	46.7±9.1	100%	133± 2.4	100%	195± 4.9	100%
T_3-Treated	50.4±0.5	108%	171± 2.5	129%	261± 0.8	134%
Succinate (5 mM)						
Control	91.1±1.6	100%	247± 3.5	100%	333±63.6	100%
T_3-Treated	99.1±6.5	109%	287±14.6	116%	410± 0.0	123%

Mitochondria were prepared from the isolated hepatocytes used in Table I as described under Experimental. Values are means from three experiments, with the standard deviation. Oxygen uptake was measured at 36 °C. ADP, when added (state 3), was 100 μM. *a*) The amount of cytochrome $a+a_3$ (0.263 nmol) corresponds to the amount of intact mitochondria (1 mg protein).

and from untreated hepatocytes of the same preparation. The results are summarized in Tables I and II. In these experiments with hepatocytes from a euthyroid rat, T_3 stimulated the coupled respiration by 20% and uncoupled respiration by 30%. Mitochondria prepared from these treated cells showed increased respiration at all metabolic states when compared to

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controls from untreated cells. In this case, the effects were greater in uncontrolled respiration either when stimulated by adenosine diphosphate (ADP) (state 3) or when stimulated by the uncoupler CCCP. It appears that the effect is greater on reduced nicotinamide adenine dinucleotide (NADH)-dependent respiration (glutamate+malate) than on succinate respiration. The increase in respiratory activity of uncoupler-stimulated NADH-dependent respiration of mitochondria prepared from T_3-treated hepatocytes was practically identical to the increase of uncoupler-stimulated respiration in T_3-treated hepatocytes.

This finding indicates that the effect in the hepatocytes is entirely due to increased activity of the respiratory chain itself and does not depend on substrate availability, redox potential, pH or other cytoplasmic factors that control the rate of respiration. Nevertheless, the fact that we and others[24] could not observe an *in vitro* effect in isolated mitochondria or submitochondrial particles suggests that a cellular factor may be required to mediate the hormone effect on mitochondria. However, once the mitochondria are activated they retain their activated state even after isolation and separation from the cytoplasm. The results of this study indicate that the present system is suitable for the study of the rapid effect of thyroid hormones on mitochondrial respiration, and may eventually lead to the development of a cell free system.

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References and Notes

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