Rapid Effect of Triiodothyronine on the Mitochondrial Pathway in Rat Liver in vivo

Abstract. *Intravenous injections of minute doses of triiodothyronine were administered to thyroidectomized rats 30 minutes before they were killed. Hepatic mitochondria were isolated rapidly and formation of adenosine triphosphate and consumption of oxygen were assessed by a 2-minute incubation. Hormone injection enhanced formation of adenosine triphosphate 114 to 217 percent over control values, with a proportionate increase in consumption of oxygen. The ratio of phosphate to oxygen was about 2.0, signifying tightly coupled oxidative phosphorylation. Stimulation was not abolished by injection of cycloheximide, puromycin, actinomycin D, or chloramphenicol 1 hour before the rats were killed. This signifies direct mitochondrial stimulation by triiodothyronine in the absence of protein synthesis.*

In view of the evidence suggesting direct action by thyroid hormone on mitochondria (*1-3*), it seemed important to undertake an experiment that would provide evidence for a rapid action in vivo by the mitochondrial pathway. This report describes a rapid effect of intravenous triiodothyronine (T_3) on the thyroidectomized rat and a lesser effect on the normal rat.

Materials and methods. Thyroidectomized and normal Sprague-Dawley rats (Taconic Farms and Hormone Assay Laboratories) (*4*) were used. All thyroidectomized rats were maintained for at least 1 month on a low-iodine diet (Teklad) with 0.2 percent $CaCl_2$ added to their drinking water. Hypothyroidism was confirmed by radioimmunoassay showing diminished thyroxine and virtually undetectable T_3 in the serum (*2*). Stable solutions of T_3 were made in 0.067*N* sodium hydroxide, diluted in 0.1 percent human serum albumin (~ 200 to ~ 6 ng of T_3 per milliliter), and injected into the tail veins of the rats under light sodium pentobarbital anesthesia (2.5 mg per 100 g of body weight, given intraperitoneally 1/2 hour before the injection of T_3). Control animals were given an analogous volume of the vehicle in the tail vein. The intravenous injections of T_3 were monitored by [^{125}I]T_3. The rats were killed 30 minutes later and liver mitochondria were obtained by centrifugation at 4°C (*1, 2*). At least three animals were used in each experimental and control group. The rats were matched for body weight, experimental and control rats were handled in alternating order, and the livers in each category were pooled.

After protein concentration was determined by the biuret reaction (*5*), the mitochondrial suspension was diluted with Hepes buffer to a protein concentration of 5 mg/ml. Oxygen consumption of an equal portion of mitochondria from each group was then determined in duplicate or triplicate with a Clark oximeter (Yellow Springs Instrument). A mitochondrial suspension of 0.5 ml was added to 2.4 ml of incubation buffer [0.05*M* tris-HCl, 0.001*M* phosphate, 0.075*M* sucrose, 0.05*M* KCl (*p*H 7.0), and 5 m*M* succinate as substrate]. Oximetry was carried out at 30°C both in the absence and presence of 1.7 m*M* adenosine diphosphate (ADP). Addition of the ADP during incubation permitted the determination of respiratory control ratios. In the incubations in the presence of ADP, inorganic phosphate was measured (*6*) at the beginning and end of a 2-minute period of oximetry to permit calculation of phosphate/oxygen (P/O) ratios (usually about 2.0, with a range from 1.3 to 3.6).

The formation of adenosine triphosphate (ATP) was determined by a 2-minute incubation in the same buffer, with nonradioactive ADP (1.7 m*M*) and [^{32}P]phosphate added to the 1 m*M* phosphate of the buffer. The incubation was terminated by addition of 0.4 ml of incubation mixture to the organic solvent containing perchloric acid. This is part of the Pullman (*7*) procedure for isolation and determination of ATP, either as such or as "trapped" ATP with its ^{32}P transferred to glucose-6-phosphate [obtained by adding 0.03*M* glucose and 90 units of hexokinase (Sigma) before incubation].

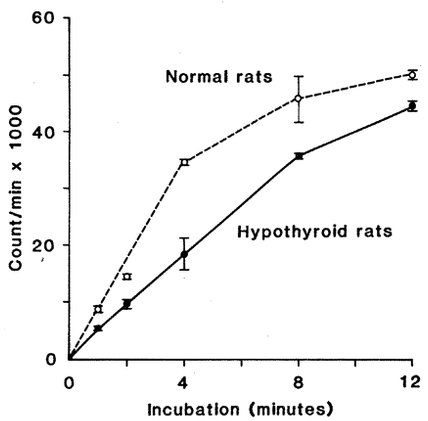

Fig. 1. Typical time course of ATP production in mitochondria from normal and hypothyroid rats. The 2-minute point was selected for all experiments as an optimum from the standpoint of convenience. The results typify the findings for 12 groups of normal rats and six groups of hypothyroid rats run in pools of three livers each.

The reduction in the concentration of phosphate during the 2 minutes of oximetry was used for determining P/O ratios and ATP formation. All values were adjusted to a per minute basis. Coefficients of variation for replicates were within 5 percent.

To suppress possible effects via pathways requiring synthesis of protein, companion experiments were carried out in which inhibitory agents were administered intraperitoneally 30 minutes before the hormone injections, that is, 1 hour before the rats were killed. The inhibitory agents and their doses (per 100 g of body weight) were as follows: cycloheximide, 0.5 mg (*8*); puromycin, 10 mg; actinomycin D, 100 μg (*9*); and chloramphenicol, 42 mg. These doses produce almost complete suppression of protein or nucleic acid synthesis (*8, 9*).

Results. The production of ATP by liver mitochondria in vitro achieved a plateau value after approximately 10 minutes. The production was higher in mitochondria from normal rats than from hypothyroid rats (Fig. 1). In both instances, ATP formation was coupled to oxygen consumption.

The minimum effective dose of T_3 administered as a bolus was lower than expected for some groups of thyroidectomized rats. Pronounced stimulation of the mitochondria was achieved with T_3 doses of 50, 25, and 12.5 ng per 100 g of body weight. However, some of the hypothyroid rats from Hormone Assay Laboratories required lower doses of anesthetic, and in these animals, T_3 doses as low as 3 ng resulted in 114 to 217 percent stimulation of ATP synthesis over control values. A lower dose (1.5 ng) had no effect on ATP production by mitochondria from any of the rats. In a typical experiment, there was significant stimulation of ATP formation (145 percent) by mitochondria from hypothyroid rats given 5 ng of T_3; 8 ng caused even greater stimulation (171 percent). However, at 100 ng of T_3, ATP production was dimished (73 percent) (Fig. 2A).

In contrast, much larger doses (micrograms) of T_3 were required to produce significant changes in oxygen consumption and ATP formation by mitochondria from normal rats (Fig. 2B). Furthermore, the changes were always relatively small. In the control hypothyroid rats, cycloheximide and puromycin (inhibitors of protein synthesis), actinomycin

D (the inhibitor of messenger RNA synthesis), and even chloramphenicol (the inhibitor of mitochondrial protein synthesis), sometimes reduced ATP production and oxygen consumption, but all failed to abolish the stimulatory effect of T_3 (Table 1).

Discussion. These findings support an extranuclear action of thyroid hormone not requiring protein synthesis (*10–13*). In keeping with data indicating specific thyroid hormone receptors in the inner mitochondrial membrane (*1–3*), it is reasonable to envision a direct stimulation of mitochondrial oxidative phosphorylation.

This mechanism of T_3 action, although present in normal rats, is more pronounced in hypothyroid rats. A minute amount (several nanograms) of T_3 elicited a strong response by mitochondria from hypothyroid rats, whereas doses in the microgram range were required to stimulate mitochondria from normal rats. This is analogous to the effects of thyroid hormone on hypothyroid patients and normal subjects: one-eighth of one grain of thyroid may have more effect on the former than several grains on the latter.

Formation of ATP in vivo was tightly coupled to O_2 consumption. Uncoupling of oxidative phosphorylation, or so-called loose coupling, was not achieved even when 100 ng of T_3 was administered to thyroidectomized rats (Fig. 2). In that case, a reduction in ATP production occurred in 6 of 13 instances and may have been an acute toxic effect; in the remaining instances, production continued to rise or reached a plateau.

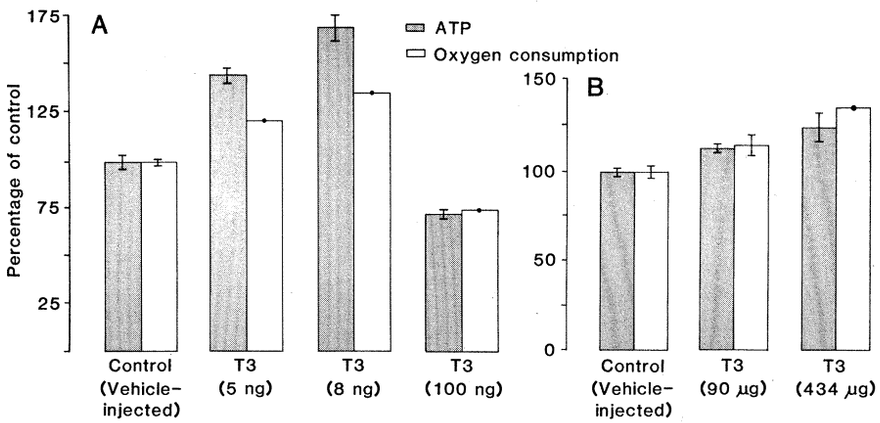

Fig. 2. (A) Production of ATP and oxygen consumption in mitochondria from livers of hypothyroid rats. The results typify the findings of 22 experiments. The results are standardized, with the control values set equal to 100 percent. The error bars indicate coefficients of variation; dots signify identical results that occasionally occurred with replicate oximetry determinations. Each histogram represents a group of three rats. (B) Formation of ATP and oxygen consumption in mitochondria from livers of normal rats. The results typify the findings for 12 rats. Note that the T_3 doses are in micrograms rather than nanograms.

The significant effect of T_3 on rat liver mitochondria 30 minutes after its intravenous injection is more rapid than the stimulation of protein synthesis by increased formation of messenger RNA due to hormone binding by receptors in the nuclear chromatin (*10–13*). The studies in which enhanced mitochondrial oxidative phosphorylation was unimpaired despite prior blockade of protein synthesis by four different inhibitors indicate a rapid, direct pathway that does not require increased protein formation for its initiation. The very early effects in hepatic mitochondria may seem more rapid than the increases in heart rate or oxygen consumption usually observed when large doses of T_3 are administered intravenously to hypothyroid rats. However, the caloric expenditure by the liver is a relatively minor proportion of the total; it is the carcass, including the skeletal musculature, that is the major contributor to total body oxygen consump-

Table 1. Mitochondrial oxidative phosphorylation by intact mitochondria isolated from the livers of rats killed 30 minutes after the intravenous injection of T_3 (with or without inhibitor) or vehicle. Data are given as micromoles per minute per 5 mg of protein. Abbreviations: Hypo., hypothyroid; Exp., experimental. Control rats were injected with vehicle; experimental rats, with hormone.

T_3 dose	Inhibitor	Thyroid status	ATP		Phosphate		Oxygen		Phosphate/oxygen (P/O)	ATP production (% of control)	Oxygen consumption (% of control)
			Control	Exp.	Control	Exp.	Control	Exp.			
5 ng		Hypo.	0.604	0.878	0.688	1.069	0.536	0.661	1.45	145	123
8 ng		Hypo.	0.604	1.034	0.688	1.234	0.536	0.736	1.48	171	137
100 ng		Hypo.	1.274	0.925	1.373	1.023	0.858	0.638	1.60	73	74
76 μg		Normal	0.815	0.891	0.701	0.820	0.525	0.600	1.35	109	113
90 μg		Normal	1.160	1.330	1.199	1.501	0.871	1.080	1.38	115	115
434 μg		Normal	0.711	0.839	0.701	0.875	0.525	0.710	1.29	118	135
3 ng	Cycloheximide	Hypo.	0.110	0.148	1.040	0.840	0.369	0.483	2.28	135	131
3 ng	Cycloheximide	Hypo.	0.079	0.136	0.810	1.040	0.195	0.343	3.59	172	176
3 ng	Cycloheximide	Hypo.	0.087	0.174	0.470	0.870	0.114	0.319	3.42	200	280
3 ng	Puromycin	Hypo.	0.232	0.284	0.520	0.750	0.370	0.430	1.58	122	116
6 ng	Actinomycin D	Hypo.	0.298	0.364	0.550	0.830	0.412	0.574	1.38	122	139
3 ng	Actinomycin D	Hypo.	0.342	0.370	0.522	0.667	0.250	0.331	2.05	108	132
26 ng	Cloramphenicol	Hypo.	0.208	0.285	0.264	0.290	0.170	0.237	1.39	137	139
3 ng	Chloramphenicol	Hypo.	1.040	1.296	1.170	1.436	0.756	0.912	1.56	125	121
5 ng	Chloramphenicol	Hypo.	0.654	1.058	0.608	1.058	0.536	0.699	1.32	162	130

tion; the mitochondria of the muscles may be stimulated more slowly than the hepatic mitochondria.

Mitochondria from many of the tissues possess saturable receptors—not only hepatic mitochondria but also mitochondria from the kidney, myocardium (atrial and ventricular), skeletal muscle, lung, small intestine, adipose tissue, and the brains of neonates (*2*). In contrast, no such receptors have been found in mitochondria from adult brain, spleen, or testes, organs that are calorigenically unresponsive to thyroid hormone (*14*).

Summary. It is probable that there is no single intracellular effector for thyroid hormone action; rather, several pathways may combine to give an integrated response. Early effects that are independent of protein synthesis have been described at the level of the plasma membrane (*15-23*). Binding by nuclear chromatin may be related to anabolic effects not evident until after a lag period; such effects may be essential to normal growth, differentiation, and cell maintenance, but are probably sustained, delayed effects rather than immediate or initiating actions. Activation of mitochondrial energy metabolism seems a likely mechanism behind the earliest effects, such as increased heart rate and oxygen consumption a few hours after intravenous injection of T_3 in myxedema patients (*24*).

KENNETH STERLING
MILTON A. BRENNER
TOSHIRO SAKURADA

Department of Medicine, Columbia University College of Physicians and Surgeons, and *Protein Research Laboratory, Bronx Veterans Administration Medical Center, Bronx, New York 10468*

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Lithium Reduces the Number of Acetylcholine Receptors in Skeletal Muscle

Abstract. *Extended treatment of rats with lithium inhibits the increase in the number of extrajunctional acetylcholine receptors that occurs in their denervated skeletal muscle. In normal muscle, lithium reduces the number of acetylcholine receptors at neuromuscular junctions. These changes appear to be a relatively specific effect of lithium on the turnover of receptors. Skeletal muscle provides an accessible system for analyzing the role of lithium (and other cations) in the regulation of cell surface receptors. This regulation may play a role in the mechanism by which lithium prevents recurrent manic-depressive episodes.*

The distribution and turnover of acetylcholine (ACh) receptors in mammalian skeletal muscles are regulated by intrinsic muscle cell mechanisms, which are largely influenced by motor nerves (*1*). In innervated muscles, most ACh receptors are located at the neuromuscular junctions, but after denervation (*1*) or pharmacological blockade of cholinergic transmission (*2*) there is a great increase in the density of extrajunctional ACh receptors. In the central nervous system, receptors for various neurotransmitters appear to be similarly regulated. For example, after surgical lesions of the nigrostriatal dopamine pathway (*3*) or pharmacological blockade of dopamine transmission (*4*), supersensitivity to dopamine and an increase in the number of dopamine receptor sites have been observed. It was recently reported that lithium treatment prevents these denervation-like changes occurring after blockade of dopamine transmission (*5*). The present study was undertaken to determine whether lithium alters the denervation-induced increase in the number of extrajunctional ACh receptors in skeletal muscle.

We injected 0.4*M* LiCl (1.0 ml, intraperitoneally) twice daily into female Sprague-Dawley rats weighing 230 to 270 g. Control animals were given injections of equal amounts of 0.4*M* NaCl (1.0 ml). After 1 to 2 days, the soleus muscle was denervated by avulsing the sciatic nerve in the thigh under cloral hydrate anesthesia (400 mg/kg). At 4 and 7 days after denervation, the soleus muscles were removed and ACh receptors were measured by a method that utilizes saturation binding of [^{125}I]α-bungarotoxin (α-BuTx) (*6*). The specific and irreversible binding of this snake venom fraction to ACh receptors allows quantitative measurement of the receptors (*7*). Junctional ACh receptors were measured in innervated muscles and extrajunctional ACh receptors were measured in both denervated and innervated muscles. The muscle fiber diameters were measured in soleus muscles pinned at resting length and quickly frozen in isopentane cooled with solid CO_2. Transverse sections 6 μm thick were cut in a crytostat and stained with hematoxylin and eosin, modified Gomori's trichrome, and adenosine triphosphatase (*p*H 4.3 and 9.4). A microscope with an ocular micrometer was used to measure the muscle fiber diameters; mean diameters were computed from at least 50 fibers in each muscle.

Lithium treatment markedly inhibited the increase in the number of extrajunctional ACh receptors in the denervated muscles. Seven days after denervation, the mean density of extrajunctional ACh receptors in the soleus muscles of lithium-treated animals was only 39 percent as high as that in saline-treated controls (Table 1) ($P < .001$). Similarly, 4 days after denervation, the density of extrajunctional ACh receptors in soleus muscles from the lithium-treated animals (70 ± 17 per square micrometer) was only 39 percent of the value for the saline-treated animals (185 ± 48) ($P < .02$). Lithium treatment also produced a significant ($P < .005$)

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