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REVIEW ARTICLE

Control of energy metabolism by iodothyronines

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ABSTRACT. One of the most widely recognized effects of thyroid hormones (TH) in adult mammals is their influence over energy metabolism. In the past, this has received much attention but, possibly because of the complex mode of action of thyroid hormones, no universally accepted mechanism to explain this effect has been put forward so far. Significant advances in our understanding of the biochemical processes involved in the actions of TH have been made in the last three decades and now it seems clear that TH can act through both nuclear-mediated and extranuclear-mediated pathways. TH increase energy expenditure, partly by reducing metabolic efficiency, with control of specific genes at the transcriptional level, being is thought to be the major molecular mechanism. However, both the number and the identity of the thyroid-hormone-controlled genes remain unknown, as do their relative contributions. The recent discovery of uncoupling proteins (UCPs) (in addition to UCP1 in brown adipose tissue) in almost all tissues in animals, including humans, has opened new perspectives on the understanding of the mechanisms involved in the regulation of energy metabolism by thyroid hormones. Other approaches have included the various attempts made to attribute changes in respiratory activity to a direct influence of thyroid hormones over the mitochondrial energy-transduction apparatus. In addition, an increasing number of studies has revealed that TH active in the regulation of energy metabolism include not only T_3, but also other iodothyronines present in the biological fluids, such as 3,5-diiodothyronine (3,5-T_2). This, in turn, may make it possible to explain some of the effects exerted by TH on energy metabolism that cannot easily be attributed to T_3.

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INTRODUCTION

The thyroid gland produces two main hormones: T_4 and T_3, T_4 being the major one since, under normal conditions, only a small amount of T_3 is secreted by this gland. However, T_4 is converted to T_3, its biologically more active metabolite, by the tissue-specific deiodinase enzymes widely distributed in the periphery. This process accounts for about 80% of daily T_3 production, the remainder being secreted by the thyroid (1, 2). Strictly speaking, evaluation of the thyroid status of an individual requires knowledge not only of the thyroidal production of the hormones, and of serum levels, but also of the intracellular transformations of these hormones within different tissues (3). In fact, the peripheral availability of iodothyronines depends mostly on local deiodination of T_4 and T_3 and, consequently, on the activity of deiodinase enzymes. For that reason, deiodination activity is crucial to the local homeostasis of thyroid hormones (TH).

TH play an important role in several physiological processes, such as differentiation, growth, cognitive development and metabolism. One of the most widely recognized effects of TH in adult mammals is the regulation of energy metabolism (4). Despite there being a substantial literature on this topic, the mechanisms by which TH actually regulate energy metabolism are still far from clear. The control of energy metabolism requires the involvement of a multitude of biochemical and molecular mechanisms that may involve the participation of several cellular compartments. Indeed, both nuclear-me-

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diated and extranuclear-mediated actions may underlie the control exerted by TH on energy metabolism. The nuclear-mediated pathway involves a control of gene transcription mediated by several T_3-binding transcription factors belonging to the nuclear receptor superfamily. Various aspects of the molecular mechanisms involved in this pathway have recently been discussed in two excellent reviews (5, 6), so they will not be further discussed in this article. In actual fact, the nuclear-mediated signaling pathway is universally accepted, while the extranuclear-mediated pathway is still very much a matter of debate.

The first line of evidence for a nuclear-mediated effect of T_3 on energy metabolism came from the studies performed by Tata *et al.* in the 1960s (7, 8). These Authors showed that administration of T_3 to hypothyroid rats induces an increase in their basal metabolic rate, while the simultaneous injection of T_3 in combination with actinomycin D completely abolishes the stimulatory effect of T_3, pointing towards the involvement of transcription. However, both the number and the identity of the thyroid-hormone-controlled genes remain unknown, as do their relative contributions. On the other hand, some other characteristics of the effects of T_3 on energy metabolism have been described. These effects have been shown to be: 1) independent of thyroid-hormone nuclear receptors; 2) independent of protein synthesis; and 3) evident in the short-term. These characteristics emphasize that, in addition to nuclear-mediated actions, thyroid hormones can stimulate cellular activities through interactions with extranuclear components (9).

Mitochondria are considered to be likely subcellular targets for thyroid hormones because of their central role in cellular energy-transduction (10, 11). In fact, both the activity and the number of these organelles are influenced by the thyroid state of the animal. In addition, T_3 plays an important role in the regulation of mitochondrial function in several metabolically very active tissues, such as skeletal muscle, heart, kidney and liver (10). Mitochondria extracted from the tissues of hyperthyroid rats display respiration rates substantially greater than those recorded for euthyroid controls, whereas mitochondria from hypothyroid animals have lower respiration rates. The effects of TH are often divided into: 1) short-term effects, occurring within minutes or a few hours; and 2) long-term effects, occurring over several hours or days. This being so, it is quite conceivable that both nuclear-mediated and extranuclear-mediated pathways are in operation at the cellular level mediating the effects exerted by TH on energy metabolism. The purpose of this review is: 1) to discuss these effects of TH in the broader context of the regulation of energy metabolism; and 2) to describe the various mechanisms by which TH may control energy metabolism.

MECHANISMS UNDERLYING THE CONTROL OF THE ENERGY METABOLISM BY THYROID HORMONES

In general terms, the cellular processes that have been proposed as candidates to explain the effects of TH on energy metabolism can be categorized as processes acting on: 1) the maintenance of transmembrane ionic gradients; 2) substrate cycles; 3) the glycerol-3-phosphate/NADH shuttle; or 4) the mitochondrial energy-transduction apparatus. The first three of these categories have been discussed in a number of recent reviews (10-13) and will be dealt with only briefly in this article, so d) will constitute the main part of this review.

Maintenance of transmembrane ionic gradients

As far as these ionic gradients are concerned, two mechanisms, the Na^+ and K^+ gradients across the cell membrane and the Ca^{++} gradients inside the cell, have received the bulk of the attention.

Na^+/K^+ ATPase

TH increases Na^+/K^+ ATPase activity and it has been hypothesized that this effect might account for an important fraction of the thermogenic effect of TH (14). Some Authors, however, estimated that this mechanism could account for only a small fraction of the overall thermogenic effect of TH (15-17). In order to establish the contribution made by the Na^+/K^+-pump to thyroid thermogenesis, experiments have been carried out in Na^+-free media (to block the pump). These conditions, however, can also affect other mechanisms, such as the Na^+-H^+ and Na^+-Ca^{++} antiports, and together these mechanisms could represent both a substantial part of the energy expenditure attributed to Na^+/K^+ATPase (18) and a pathway for the action of T_3. Indeed, it has recently been shown that T_3 is able to stimulate the Na^+/H^+ antiport in L6 muscle cells (19). The primary event in the TH-induced activation of Na^+/K^+ATPase may be a reduction in the Na^+ and K^+ gradients, with activation of the Na^+/K^+-pump as a secondary effect. Some studies, however, seem to suggest that T_3 may directly influence the pump by increasing the expression of Na^+/K^+ATPase.

Calcium

Ca^{++} plays an important role in the regulation of a wide variety of cellular processes. While the extra-

cellular Ca^{++} concentration is in the mM range, the cytosolic concentration is kept much lower and within a narrow range (10^{-7}-10^{-8} M), and even minimal changes may have large effects on the metabolic processes within the cell, such as mitochondrial metabolism (20). The maintenance of the Ca^{++} gradient is largely the function of Ca^{++}-dependent ATPases in the cell membrane and in the endoplasmic or sarcoplasmic reticulum (SR) (18). It has been estimated that about 10% of the cell energy expenditure is needed simply for cytosolic Ca^{++} homeostasis, although it depends on the cell type and the prevailing physiological conditions. Most of the published studies on the effects of TH on intracellular Ca^{++} cycling and energy expenditure have been performed on skeletal muscle (17). TH can apparently influence Ca^{++} turnover by producing increases in both the volume of the SR and the density of Ca^{++}-pumps in the SR, via the regulation of the expression of the corresponding genes. In addition, TH can directly stimulate plasma-membrane Ca^{++}-ATPase activity in a way that does not rely on an upregulation of gene expression (9, 21).

Substrate cycling

TH influence the intermediary metabolism of carbohydrates, lipids and proteins. In essence, the mechanism underlying induced changes in substrate cycling are essentially the same as those underlying effects on ion cycling: an acceleration of a pathway (metabolic or ionic) by external stimuli to maintain a basically constant steady-state, consisting a "futile cycle". As to substrates, this involves an acceleration of their turnover without a change in their steady-state level. The contribution made by a given substrate cycle to the thermogenic effect of TH seems to be low (13), and among the other cycles protein turnover seems to involve a negligible energy cost (22). The substrate cycle that has therefore received most attention is "lipogenesis-lipolysis". TH, in fact, stimulate both lipogenesis and lipolysis (23).

Glycerol-3-phosphate/NADH shuttle

The NADH shuttle, with the associated cytosolic NAD-linked glycerol-3-phosphate dehydrogenase (α-GPDc) and FAD-linked mitochondrial α-glycerophosphate dehydrogenase (α-GPDm), has received considerable attention as a possible biochemical mechanism involved in TH-induced thermogenesis. More than 35 years ago, Lee *et al.* (24) demonstrated that TH stimulate α-GPDm activity in metabolically relevant tissues and that the increases correlate well with the increases in O_2 consumption. Recently, it has been shown that the mechanism by which T_3 stimulates α-GPDm could involve stimulation of the transcription of the corresponding gene (25). The increased α-GPDm activity leads to a faster rate of gluconeogenesis from glycerol and may contribute to increased thermogenesis since only two ATPs/oxidised molecules will be formed in this way. However, the real contribution of this shuttle to the thermogenic effect of TH has not been directly assessed.

EFFECTS OF TH ON MITOCHONDRIAL ENERGY-TRANSDUCTION APPARATUS

As cited in the Introduction, because of the central role known to be played by mitochondria in cellular energy-transduction, several reports concerning the effect of TH on mitochondrial activity can be found in the literature.

Mitochondria are the site of oxidative phosphorylation, a step-by-step process responsible for most ATP synthesis within the cells. Oxidative phosphorylation consists of mitochondrial ATP synthesis from ADP, which is driven by the electron flow from the reduced substrates (NADH and FADH) to oxygen. Mitchell proposed that the oxidation of the reduced substrate that takes place via the respiratory chain may be coupled to a pumping of protons from the mitochondrial matrix, thus generating an electrochemical proton gradient ($\triangle\mu H^+$) across the inner membrane. $\triangle\mu H^+$ provides the force driving the protons back to the matrix through ATP synthase, which couples proton transport across the membrane to the phosphorylation of ADP (Fig. 1A). As ATP synthesis is not perfectly compled to oxygen consumption, the system is not completely efficient, a leakage of protons across the mitochondrial membrane directly dissipating part of the proton electrochemical gradient as heat. An additional phenomenon that reduces the efficiency of oxidative phosphorylation is a failure of the proton pumps in the respiratory chain, called "redox-slip". The above two phenomena, which are responsible for a reduced oxidative phosphorylation efficiency, are represented schematically in Figure 1B.

The effects of TH on mitochondrial activity are all, in one way or another, related to the effects that they exert on cellular respiration (calorigenic effect). The first hypothesis concerning the mechanism underlying the calorigenic effect of TH dates back to the early 1950s, when Martius *et al.* (26) and Lardy *et al.* (27) found that the addition of T_4 to isolated mitochondria *in vitro* led to a decrease in the efficiency of mitochondrial respiration. They hypothesised that the major cellular mechanism underlying the calorigenic effect of TH is an "uncoupling of oxidative phosphorylation", measured as a reduced respiratory control ratio (RCR). The value of this parameter is obtained

from the ratio of two mitochondrial respiration states: that in which the synthesis of ATP is at maximal rate (State 3), and that in which ATP synthesis is either absent or extremely low (State 4). This hypothesis was subsequently discarded and regarded as not physiologically relevant since a large concentration of T_4 and T_3 was required and, moreover, because the effects seen *in vitro* could not be demonstrated *in vivo*. Actually, most papers have reported that mitochondria in different thyroid states show no differences in RCR values. This absence of a change in RCR, however, does not exclude the possibility that changes in State 3 and/or State 4 may occur. In fact, a concomitant increase in (i) proton-leak kinetic, which is mostly responsible for State 4 respiration, and (ii) the phosphorylating-machinery kinetics (ATP synthase, adenine-nucleotide translocator, phosphate transporter), which are responsible for State 3 respiration, can lead to an unchanged set-point for the coupling ratio without excluding the possibility that an uncoupling ratio may take place (28). Moreover, the interpretation of these results obtained *in vitro* on isolated mitochondria is complicated because *in vivo* respiratory control is between States 3 and 4, and may change depending on the metabolic state of the cell. In addition, the proton-leak and the phosphorylating machinery compete for the same driving force, and the question arises as to whether the contribution of proton-leak to the metabolic rate remains significant at higher rates of metabolism, when the H^+ flux through ATP synthase must increase. It has recently been shown that the proton-leak remains an important component of the rat's metabolic rate even at higher metabolic rates. The published data suggest that the proton-leak accounts for about 20% of rat standard metabolic rate (SMR), making the proton-leak the largest individual contributor to the SMR (29). The questions as to whether thyroid-hormone-induced uncoupling is: 1) a physiological phenomenon; 2) a non-physiological response seen only with high levels of the hormones; or 3) an *in vitro* artifact have continued for almost half a century. However, when compared to those from euthyroid animals, mitochondria isolated from hypothyroid animals display lower State 4 and State 3 respiratory rates, while mitochondria from hyperthyroid rats show higher values. The mitochondrial proton-motive force, on the other hand, decreases in the order hypothyroid > euthyroid > hyperthyroid (30).

Effects of TH on State 3 and State 4 mitochondrial respiration

TH affect mitochondrial oxygen consumption by influencing both State 3 and State 4 respiration. Each of these influences may be mediated, at least in part, by a regulation of the expression of nuclear-encoded as well as mitochondrially-encoded respiratory genes (25), since the mitochondrion is a unique cellular organelle containing a distinct and separate genome. We have already discussed some aspects of the expression of nuclear-encoded and mitochondrially-encoded respiratory genes in a recent review (11).

A variety of experimental approaches have been used to analyze the mechanisms underlying TH-dependent alterations in mitochondrial function. In particular, in order to examine the extent to which the components of the respiratory chain limit respiration, metabolic control analysis has been used. This technique permits to determine the proportional control that individual steps in a complex pathway exert on the flux through that pathway (31). Groen *et al*. (32) first applied this approach to oxidative phosphorylation and, in recent years, Harper *et al*. (33) have refined and adapted it to examine how the thyroid state influences the control exerted on mitochondrial respiration by individual steps. These latter Authors introduced top-down elasticity analysis as an approach to identify blocks of reactions that have a changed kinetic response (*i.e.* changed elasticity) to a common intermediate as the sites of effector action which are significant in changing the flux through the intermediate (33, 34).

In this approach, oxidative phosphorylation can be considered a system consisting of a few components or blocks of reactions. These are: 1) the reactions that are involved in the oxidation of substrates and that generate $\triangle\mu H^+$: substrate translocase, substrate dehydrogenase and the components of the electron transport chain (Fig. 1A); 2) the overall reactions that dissipate $\triangle\mu H^+$ for the synthesis and export of ATP: ATP synthase, the phosphate transporter and the adenine nucleotide translocator (AdNT) (Fig. 1A); and 3) the reactions that dissipate $\triangle\mu H^+$ without ATP synthesis, represented by the cation cycles and the leak of protons across the inner membrane, which together are called the "proton leak" (Fig. 1B).

The components described above control both the respiration associated with ATP synthesis and that not associated with ATP synthesis in different ways, which implies that a change in the activity of a single block will have different effects on States 4 and 3. Actually, State 4 is controlled principally by the leak of protons across the mitochondrial inner membrane (responsible principally for mitochondrial uncoupling) and to a lesser extent by the activity of the respiratory chain. On the other hand, the control over State 3 is exerted via changes in both the activity of the respiratory chain and that of the phosphorylating system (*i.e.*

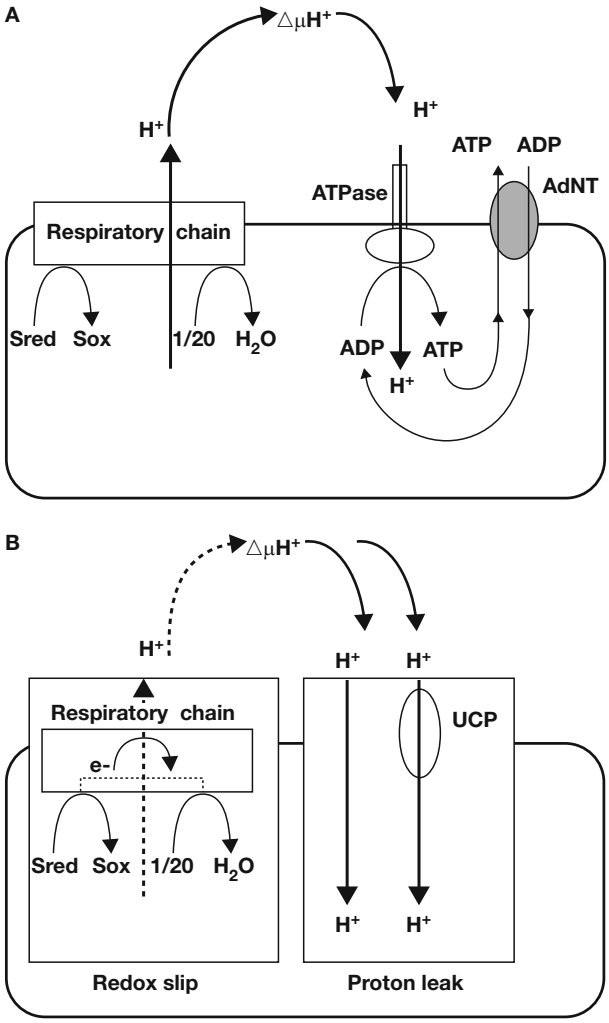

Fig. 1 - *Schematic representation of mechanisms linking respiration to ATP synthesis (A) and of mechanisms underlying reduced mitochondrial efficiency (B). Only the inner mitochondrial membrane is depicted. The bold black line represents the lipid bilayer. The respiratory chain works as a H^+ pump, generating an electrochemical proton gradient ($\triangle\mu H^+$). $\triangle\mu H^+$ is used by ATPase to phosphorylate ADP. ATP and ADP are then exchanged by AdNT (depicted in A). Alternatively, H^+ can enter the mitochondrial matrix by mechanisms not coupled to ATP synthesis:* i.e. *by leaking across the lipid bilayer or by protein-mediated H^+ transport (UCP, depicted in B). This mechanism decreases $\triangle\mu H^+$, activates respiration and increases reoxidation and thermogenesis (uncoupled respiration). Incomplete coupling of respiration can also be due to a redox slip (depicted in B) in the respiratory chain: fewer protons are pumped per oxygen consumed, causing reduced $\triangle\mu H^+$ production. In (B) the dotted lines represent decreased proton pumping.*

all the reactions involved in the synthesis and the export of ATP) (34).

Hafner *et al*. (35), by using top-down elasticity analysis, showed that, by comparison with the situation in the euthyroid controls, the kinetics of the $\triangle\mu H^+$ generators are unchanged in liver mitochondria from hypothyroid animals, while the kinetics of the $\triangle\mu H^+$ consumers are altered. This supports the idea that the important effects of TH on State 3 are actually exerted on ATP synthase or AdNT. Indeed, AdNT activity is affected by the thyroid state (36) and the injection of T_3 into hypothyroid rats induces a two-fold increase in the mitochondrial AdNT content and a three-fold increase in its activity (37). The notion that a higher AdNT activity is responsible for the increase in State 3 respiration also fits well the data of Seitz *et al*. (38) who showed that the cytosolic and mitochondrial ADP/ATP ratios are affected by thyroid state.

When the same analysis, however, was employed to investigate the effect of hyperthyroidism on State 3 respiration, no significant change in the kinetics of the $\triangle\mu H^+$ consumers was detected, although a significant change in the kinetics of the $\triangle\mu H^+$ producers was reported (39). Thus, these findings do not seem to match with the previous conclusions, although they do seem to suggest that different mechanisms may be operative in the hypothyroid and hyperthyroid states. In addition, the increased flow through the respiratory chain elicited by T_3 can be accomplished either by increasing the amounts of the components of the respiratory chain or by increasing their reduction state (40). Furthermore, since hyperthyroid mitochondria have a higher Ca^{++} content than euthyroid ones, State 3 respiration could be increased by T_3 via a rise in mitochondrial calcium uptake, which in turn may induce an increase in mitochondrial dehydrogenase activity (41). In State 4, the mitochondrial respiration rate is largely controlled by the proton-leak across the inner mitochondrial membrane, and TH could conceivably act predominantly by altering this leak (which indeed is depressed in hypothyroid mitochondria and stimulated in hyperthyroid mitochondria) (42-44).

TH and mitochondrial efficiency

Many efforts have been made to elucidate the molecular basis of the metabolic inefficiency induced by TH in mitochondria. Two main mechanisms have been proposed: 1) a classic uncoupler mechanism based on the action of TH at the level of the proton-leak; and 2) a slipping mechanism with more localized effects at the level of the redox proton pumps. A "slipping" of the respiratory chain can be defined as a failure of the proton pumps, a situation in which they transfer electrons with a reduced extrusion of protons out across the membrane; this implies that a higher respiratory rate, is necessary for a given level of ATP synthesis (45-47) (Fig. 1B).

TH and redox-slip

About 20 years ago, the concept of the redox-slip was a new unveiled uncoupling mechanism, and since then it has been shown to be partially responsible for the basal respiration in mitochondria (42, 46, 47). Moreover, just such a type of uncoupling has been proposed as an explanation for stimulatory effects exerted by TH on respiration. Brand *et al.* (42) concluded that the action of TH is not primarily concerned with the quantity of respiratory chain components or of mitochondria but, rather, with the leak/slip characteristics of mitochondria. Subsequently, however, Hafner *et al.* (48) rejected the existence of slip and its role in mediating hormonal effects. Schmehl *et al.* (49), on the other hand, demonstrated the occurrence of the slip reaction mainly at the level of the cytochrome oxidase complex, although Brand *et al.* (50) reported that no permanent functional alteration in the constitutive oxidase was induced by hyperthyroidism (by comparison with the situation in euthyroid mitochondria), indicating that the hormonal effects do not involve an inherent change in the properties of the enzyme. However, the c-oxidase (COX) complex has a very dynamic structure and, during the isolation procedure and the reconstitution of the enzymes, some changes (*i.e.* conformational, phosphorylation-dephosphorylation) can take place. It is also known that changes in the lipid environment, surface charge or pH alter the kinetics of the complex. It has been postulated that, *in vivo*, cytochrome c oxidase (and possibly other enzyme complexes of the respiratory chain) are in a steady state between the phosphorylated and dephosphorylated forms (51, 52). The dephosphorylated enzyme is no longer controlled by the intramitochondrial ATP/ADP ratio: it is controlled by the $\triangle\mu H^+$, and it shows an increased activity in the presence of a high ATP/ADP ratio, thus leading to an increased $\triangle\mu H^+$. An increased $\triangle\mu H^+$, consequently, would decrease the efficiency of the complex causing a slip of proton-pumping in cytochrome c oxidase (*i.e.* decreased H^+/e^- stoichiometry). It has been reported that there is a specific and tight association between cytochrome c oxidase and cardiolipin, which is functionally important for the maximal activity of this enzyme (53). TH induce a change in the phospholipid composition of the inner membrane and stimulates cardiolipin synthase activity through a rise in the mitochondrial phosphatidylglycerol pool (54). The increased amount of cardiolipin stimulates several mitochondrial carriers and enzyme activities (55). Removal of cardiolipin from isolated cytochrome oxidase enzyme leads to a decrease of about 60-70% in the electron transport, indicating that a modification of the immediate lipid environment of the protein causes it to malfunction. Canton *et al.* (56) suggested that, because of the marked temperature-dependence of the proton-leak processes, different contributions of redox-slip and proton-leak to basal respiration may be observed depending on the temperature at which mitochondria are incubated. The contribution made by the proton-leak to the basal respiration is reduced at low temperatures (25 C) and this reduction is accompanied by a higher contribution of redox-slip to resting respiration. At 37 C, the actual physiological temperature, the proton-leak makes the predominant contribution to basal respiration. In order to show an effect of T_3 on redox-slip, Luvisetto *et al.* (57) performed experiments at an incubation temperature of 25 C; at this temperature they showed that hyperthyroidism is able to increase both proton-leak and redox-slip. However, as the same Authors did not perform the same experiment at 37 C, it is not clear whether T_3 affects redox-slip only at non-physiologically low temperatures.

TH and proton-leak

As cited above, the proton-leak is the major process controlling mitochondrial oxygen consumption when cellular ATP requirements are low, and the size of this leak has been measured in mitochondria from liver, brain, kidney and skeletal muscle (34). This non-productive proton-conductance pathway is physiologically important: 1) because it accounts for a high proportion of cellular metabolic rates [*e.g.* 25% and 52% of the resting respiration rates of isolated rat hepatocytes and perfused rat skeletal muscles, respectively (58)] and 2) because an increased mass-specific metabolic rate related to thyroid-hormone levels is linked to an increased mitochondrial proton-leak (42). The mechanism by which the proton-leak occurs is unclear. Some evidence suggests that the phospholipid composition of the inner membrane is correlated with the proton-conductance of the membrane (59). However, variation in the phospholipidcomposition of mitochondrial-membrane phospholipids reconstituted in liposomes does not appear to account for the difference in proton-conductance (60). In addition, experiments on liposomes made from inner-membrane phospholipids suggest that the proton-conductance of a protein-free membrane would support only 5% of the total proton-leak in the rat (61). Therefore, it is clear that other factors, such as specific proteins in the inner membrane, could be responsibile of most of the mitochondrial proton-leak (62).

With regard to liver mitochondria, several studies have indicated that AdNT is a candidate able both

to uncouple oxidative phosphorylation and to interact with fatty acids (FA). Skulachev *et al.* (63) showed that AdNT mediates the uncoupling effect of FA in mitochondria and proteoliposomes. In fact, AdNT operates as a transmembrane carrier for FA anions by facilitating the H^+-conducting FA cycle: protonated FA diffuse to the mitochondrial matrix through the membrane while anions are electrophoretically expelled in the opposite direction via AdNT (63). As reported before, TH increase the mitochondrial AdNT content in the liver two-fold when administered to hypothyroid rats (36). Thus, the thyroid-hormone-induced uncoupling seen in the liver can be explained by a hormone-induced increase in the AdNT concentration, entailing an elevation of mitochondrial proton-conductance mediated by FA. In tissues other than liver, in addition to the effect on AdNT, the thyroid-hormone-induced decrease in energy coupling may be due to an induction of proteins belonging to the family of uncoupling proteins (UCPs).

UCPS AND TH

An increase in the proton-conductance may be associated with the presence of UCPs. Uncoupling protein-1 (UCP1) cloned in 1985, and called UCP until 1997, is an inner-mitochondrial-membrane protein expressed exclusively in the brown adipocyte. It dissipates the mitochondrial protoin-gradient generated by the respiratory chain, producing heat instead of ATP (64). Two major hypotheses have been put forward to explain the mechanism by which UCP1 exerts this effect: 1) according to the proton-buffering model, introduced by Klingenberg (65), UCP1 conducts protons through a hydrophilic pathway lined with FA head groups, which buffer the protons as they move across the membrane whereas; 2) according to the FA protonophore model, introduced by Garlid (66), UCP1 does not conduct protons at all, but it is rather an anion carrier able to transport FA anions. In each case, there would be a net transport of H^+, and there are arguments for and against both models (67, 68). The requirement for FA for the purposes of UCP1-mediated H^+ transport is generally believed to be absolute, although it has been questioned recently (69). It has been suggested that the activators of UCP1 are most likely not the FA themselves, but a FA metabolite (70). Echtay *et al.* (71) inferred that coenzyme Q is an obligatory co-factor for UCP function on the grounds that when CoQ10 was added to reconstituted UCP1 from inclusion bodies, FA-dependent H^+ transport reached the same rate as that seen with native UCP1. The authors concluded that the molecular role of CoQ probably resides in cooperation with FA (on the basis that there is physical contact between these two components at the membrane-UCP1 interface) (71).

The presence of UCP1 in brown adipose tissue (BAT) together with the finding of a high density of nuclear T_3 receptors in this tissue have, not surprisingly, focused attention on a possible role for this hormone in modifying the function of UCP1. In fact, UCP1-gene transcription is stimulated by T_3, which acts via its receptor on a discrete sequence of the gene well upstream in the 5' flanking region of the gene (72). In addition, two thyroid response elements are delineated within this sequence, which is separated from the sequence on which norepinephrine (NE), via cAMP, stimulates gene- expression (73). A synergism between T_3 and NE has been demonstrated both *in vivo* and *in vitro* (73) as well as in rat cultured brown adipocytes (74), and it has been suggested that T_3 plays a permissive role in stimulating the BAT response to cold. Moreover, it has been demonstrated that local production of T_3 by the deiodination of T_4 via 5'D-II activity is essential for the induction of UCP1-gene transcription (75, 76). For this reason, conditions that inhibit 5'D-II activity (*e.g.* T4 administration to hypothyroid rats) will lead to a reduced local generation of T_3 and a blunted response to BAT. Very recent studies, however, have indicated that T_3 alone vigorously stimulates UCP1 expression under conditions in which there is minimal interference with the sympathetic nervous system (SNS), pointing to a role of T_3 that is independent of NE (77).

Since 1997, several genes that encode proteins closely related to the UCP1 found in brown adipose tissue have been discovered (64, 78). To date, five genes have been identified in mammals: for UCP1, UCP2, UCP3, BMCP1 (brain mitochondrial carrier protein 1) or UCP5 and UCP4 . The expression pattern of these genes, based on mRNA detection, has been evaluated in various papers and reviewed (64, 78, 79). Briefly, UCP3 mRNA is found in skeletal muscle, in heart and also in brown adipose tissue. BMCP1/UCP5 and UCP4 are predominantly expressed in brain, while UCP2 is more widely expressed. Based on the relatively high similarity between UCP2 and UCP3, on the one hand, and UCP1 on the other, it was predicted that these new UCP homologues would have uncoupling activity. When expressed in yeast, murine UCP2 and UCP3 both lower the potential of the mitochondrial membrane, raise the respiration rate and reduce sensitivity to uncouplers (80). These results, although obtained in a non-physiological system, are indicative

of an uncoupling property of these proteins. Moreover, recent studies show that mice overexpressing UCP3 are hyperphagic and lean, with a decreased mitochondrial efficiency (81), while mice lacking UCP3 show a reduced mitochondrial proton-conductance (82, 83). Administration of T_3 to rodents has been reported to increase the expression of the mRNA for UCP2 and UCP3 in heart and skeletal muscle (84-87). These results are very important and suggest a hypothetical molecular mechanism by which T_3 increases energy expenditure partly by reducing metabolic efficiency. We showed that the expression of UCP3 mRNA in skeletal muscle increased strongly during the transition from hypothyroidism to hyperthyroidism, and an evaluation of the proton-leak in skeletal muscle mitochondria revealed that those from hyperthyroid rats display a greater proton-leak than those from their euthyroid or hypothyroid counterparts (87). Jucker *et al.* (88), who assayed mitochondrial energy coupling in skeletal muscle *in vivo* by a non-invasive method (NMR spectroscopy), confirmed that injection of T_3 into hypothyroid rats led to a mitochondrial uncoupling in skeletal muscle.

In a study in our laboratory on rat skeletal muscle mitochondria, clear-cut changes in proton-leak kinetics that accompanied the transition from hypothyroidism to hyperthyroidism disappeared when BSA was included in the incubation medium (87). An interpretation of these results could be that the difference in proton-leak between the hypo- and hyperthyroid states is related to differences in the endogenous content of free fatty acids (FFA), which are known mitochondrial uncouplers. BSA chelates FFA and therefore abolishes the effect. However, FFA may be important cofactors for UCP3-uncoupling activity, and the results may also be explained on the basis of the crucial need for the presence of these putative cofactors. In fact, this hypothesis seems to be confirmed by the recent findings of Echtay *et al.* (89), who reported that FFA are important factors for the activation by CoQ of UCP2 and UCP3 expressed in *E.coli.* In skeletal muscle, physiological states associated with enhanced fat metabolism are correlated with increased UCP3 expression. Such situations include starvation (90), and fed animals given Intralipid plus heparin, which raises circulating FFA levels (91). As hyperthyroidism is accompanied by high levels of FFA, the effect of TH on the UCP3 level could be mediated, at least in part, by FFA [which may directly regulate the transcription of UCP3 mRNA by interacting with transcriptional factors such as peroxisome-proliferator activated receptor (PPAR)]. The UCP3 gene, in fact, contains response elements for both TH and PPARs (92), and agonists of PPARs have been shown to regulate the expression of both UCP2 and UCP3 (93).

In a very recent study we obtained evidence that UCP3 has the potential to be the molecular determinant for the regulation of resting metabolic rate by T3 (94).

TH CONTROL OF MITOCHONDRIAL ENERGY METABOLISM: THE MITOCHONDRIAL-DEPENDENT PATHWAY

As stated above, the question of a direct action of TH on mitochondria has long been controversial, even though a large amount of evidence has accumulated supporting the putative direct effects of TH. For example, when T_3 is injected into hypothyroid rats the respiration rate of mitochondria isolated only 20-30 min later is found to be raised, and this activation is unaffected by the use of a protein-synthesis inhibitor (95, 96). Oxygen consumption in isolated hepatocytes is stimulated by T3, independently of any effect on protein synthesis (97, 98), and this increase persists in uncoupled hepatocytes and subsequently isolated mitochondria (98). Perfusion of liver with T_3 increases respiration within 30 min (99-102) and this stimulation correlates with observed increases in the uptake of Ca^{++} and amino acids from the perfusate (101, 103). Nevertheless, the most important finding confirming a direct mitochondrial pathway would be the identification of specific binding sites for T_3 in mitochondria. In fact, high-affinity binding sites for T_3 were identified in the mitochondrial inner membrane as long ago as 1975 by Sterling *et al.* (104).

Although two other groups then confirmed the existence of such sites, the site-characterizations given in these later reports differed from each other as well as from that in the initial report (105, 106). In a further study, adenine nucleotide translocase was claimed to be the mitochondrial receptor for T_3 (107) but this has not been confirmed by other investigators (108). The conflicting results referred to above and the lack of a chemical identity for these sites raised some doubts about their physiological relevance and indeed their very existence.

Quite recently, however, the existence of specific mitochondrial binding sites for T_3 has received strong support from the work of Wrutniak *et al.* (109) and Morel *et al.* (110). The latter group showed by quantitative electron-microscopic autoradiography that after an injection of radio-labelled T_3, specific binding sites were displayed by five cell compartments, including mitochondria. Wrutniak

et al., using a photoaffinity-labelling technique, identified two T_3-binding proteins in a rat liver mitochondrial extract. One, of 43 kD (p43), was located in the matrix and the other, of 28 kD (p28), in the inner membrane. In fact, p43 binds to one of the five sequences highly related to the TH-response element in the D-loop region of the mitochondrial genome (the region containing the promoter of the mitochondrial genome). These results led to the hypothesis that p43 could function as a T_3-dependent mitochondrial transcription factor. The same group (109) later showed that p43 has an affinity for T_3 that is similar to that of the T_3 nuclear receptor, and that it is able to stimulate mitochondrial protein synthesis. Western blots and immunoprecipitation, using antibodies raised against the T_3-binding domain of the T_3 nuclear receptor c-Erb A α1 protein, indicated that p43 is at least related to an isoform of the T_3 nuclear receptor c-Erb A α1 (109). Wrutniak *et al.* (109) also showed that expression of the truncated form of the c-Erb A α1 nuclear receptor in CV1 cells was imported into mitochondria and was associated with a stimulation of mitochondrial activity. To examine more directly the ability of p43 to activate mitochondrial transcription, Casas *et al.* (111) performed in organello transcription experiments using isolated rat liver mitochondria and p43 proteins synthesized in rabbit reticulocyte lysate; they observed that p43 increases the levels of both precursors. Taken together, these results indicate that p43 may be a potent T_3-dependent transcription factor for the mitochondrial genome and strongly support the existence of a direct regulation by T_3 of mitochondrial gene expression. More recently, p43 has been shown to be able to heterodimerise with a truncated form of the PPARγ nuclear receptor (mtPPAR) found in the mitochondrial matrix (112). These results could help to clarify the mechanisms underlying the thyromimetic properties of PPARs. Be that as it may, the above data do at least seem to support the hypothesis that a direct mitochondrial pathway is involved in the action of T_3.

Diiodothyronines and mitochondria

Studies concerning the effects of iodothyronines at the cellular level have been mostly focused on T_3 but, in the last decade, a growing number of researchers have become excited by the possibility that iodothyronines other than T_3 and T_4 may be active in the regulation of energy metabolism. Studies from several laboratories have suggested that among these other iodothyronines, 3,5-T_2, a putative product of the deiodination pathway involved in T_3 metabolism, could be of biological relevance. Horst *et al.* (100), examining the rapid effects of iodothyronines on the respiratory capacity of perfused livers isolated from hypothyroid rats, showed that 3,5-T2, as well as T_3 and T_4, was able to produce a rapid stimulation of oxygen consumption. In the same study, the Authors showed that the effects elicited by T_3 and T_4 were completely abolished by inhibiting the deiodinase activity of the liver, while the effect elicited by 3,5-T_2 was unaffected by this inhibition. These results stimulated other groups to focus more deeply on a putative physiological role for 3,5-T_2.

Our group has shown that chronic administration of 3,5-T_2 to hypothyroid rats significantly enhances both the mitochondrial respiratory rate and cytochrome COX activity (113, 114). When 3,5-T_2 and T_3 were acutely injected, 3,5-T_2 had an earlier effect on respiratory parameters than T_3, the effect of the former being evident as soon as 1 h after its injection, while that of the latter became evident only after 24 h. Effects of 3,5-T_2 on respiratory parameters have also been demonstrated by others, in rats (115) and also in humans (116). O'Reilly *et al.* (115) reported that the rapid effect of 3,5-T_2 on mitochondrial respiration was independent of changes in protein synthesis (since it was also evident in the presence of cycloheximide). Interestingly, the direct effects of 3,5-T_2 on the mitochondrial metabolism have recently been confirmed in mitochondria isolated from the liver and red muscle of the goldfish (117), thus indicating that the influence of 3,5-T_2 is not restricted to mammals.

The hypothesis that 3,5-T_2 has the mitochondrion as its direct target is supported by our identification of specific binding sites for 3,5-T_2 at the mitochondrial level (118). The data, however, should be treated with some caution because of a limitation inherent in these studies: namely, that [3,5-^{125}I]T_2 was used at low specific activity (due to the inner-ring labeling procedure). Because of this, it was possible to perform binding studies only over a narrow range of concentrations and thus there may not be a particularly close correspondence between the values for binding capacity and affinity constant calculated following our experiments and the real ones. Some hypotheses have been put forward concerning the biochemical nature of these sites. By top-down elasticity analysis, we showed that 3,5-T_2 affects the overall kinetics of the reactions involved in the oxidation of substrates by acting on two different sites in the respiratory chain: namely, complex IV and the block of reactions involved in the oxidation of cytochrome c (119). Goglia *et al.* (120) found that, *in vitro*, 3,5-T_2 stimulated the activity of the COX complex isolated from bovine

heart. Later, Arnold *et al.* (121) used photoaffinity labelling to identify subunit Va of the COX complex as the binding site for 3,5-T_2. Specific binding of the hormone to subunit Va was then confirmed by the finding that a monoclonal antibody against subunit Va had an action that prevented the effect of 3,5-T_2. More recently, Kadenbach *et al.*, adding 3,5-T_2 to a reconstituted COX complex, showed a decrease in the respiratory control ratio of the complex (measured as the ratio of respiration in the presence of uncouplers over that in their absence). This *in vitro* effect was seen in the presence of intraliposomal ATP but not in the presence of intraliposomal ADP. On the basis of their data, the authors suggested that either under conditions of low energy-utilization (high ATP/ADP ratio) as well as at rest, 3,5-T_2 induces a redox-slip in cytochrome oxidase (122).

There are other mechanisms by which 3,5-T_2 might affect mitochondrial metabolism. Evidence from Hummerich *et al.* (100) suggested an influence of 3,5-T_2 over mitochondrial metabolism mediated by an increase in mitochondrial Ca^{2+} uptake, which is known to increase mitochondrial metabolism. In actual fact, what they showed was that perfusion of the euthyroid rat liver with T_3 or 3,5-T_2 led to an increase in Ca^{2+} uptake by the mitochondria. The effect of T_3, however, disappeared when the perfusion was performed in the presence of propylthio uracil (PTU), suggesting that the uptake was due to 3,5-T_2, not to T_3. The resulting increase in mitochondrial metabolism seems likely to be due to an increase in the activity of the mitochondrial dehydrogenases (41), which would lead to an increase in the amount of reduced substrate available for the respiratory chain. The above-mentioned effects of 3,5-T_2 on mitochondria led us to consider this diiodothyronine as a possible peripheral mediator of the effect of TH on the energy metabolism of the whole animal. In our laboratory, we have demonstrated that 3,5-T_2, as well as T_3, is able to enhance the resting metabolic rate (RMR) of hypothyroid rats, although their effects differ in terms of both time course and dependency on protein synthesis (123, 124). Thus, injection of T_3 enhances RMR via a nuclear-mediated pathway, so its effect takes some days to start and some days to stop and is blocked by the simultaneous injection of actinomycin D (124). 3,5-T_2 affects RMR more rapidly, its effect being already evident 6-16 h after a single injection, and in a manner that is independent of protein synthesis. In practice, the actions of 3,5-T_2 and T_3 probably do not occur independently of each other, they may rather cooperate in determining the final metabolic state of the animal. However, injection of 3,5-T_2 into euthyroid animals results in a slight or non-existent change in RMR. This could mean that in euthyroid animals 3,5-T_2 needs to be formed from a precursor to be effective in enhancing RMR. Indeed, very recently we have shown that T_3 administration to euthyroid rats induces an early effect on RMR that is independent of actinomycin D and attributable to the *in vivo* formation of 3,5-T_2 from T_3 (125). We believe that results obtained *in vivo* showing 3,5-T_2 enhancing the resting metabolic rate of hypothyroid rats via a nuclear-independent pathway may be indicative of a physiological role for this iodothyronine in the regulation of energy metabolism. Interestingly, some recent studies have shown that 3,5-T_2 may have significant effects in other species and on other biochemical and physiological parameters. They lead further support to the notion of a physiological role

Table 1 - *Summary of 3,5-T_2 effects both on mitochondrial and non-mitochondrial parameters.*

Effects on mitochondrial parameters	References
Increase in oxygen consumption of perfused rat liver	99
Increase in oxygen consumption of mononuclear blood cells	115
Increase in liver mitochondrial respiration	112-114
Increase in mitochondrial Ca^{++} uptake	100
Increase in α-glycerophosphate dehydrogenase activity	125
Increase in respiratory chain activity	118
Increase in cytochrome oxidase activity	112,119,120
Increase in β-oxidation	126
Effects on non-mitochondrial parameters	
Increase in malic enzyme activity	125, 127
Increase in glucose 6-phosphate-dehydrogenase activity	125
Inhibition of TSH secretion	128-130
Stimulation of GH secretion	125, 130
Increase in deiodinase activity	131
Increase in leucine metabolism	126
Increase in cold tolerance of hypothyroid rats	132
Increase in resting metabolic rate	122-124,126
Influence on lipid peroxidation	133
Increase in follicle cell volume in locusta migratoria	134
Inhibition of cancer cell proliferation	135

for 3,5-T_2. The effects so far attributed to 3,5-T_2 are summarised in Table 1 with the relative references.

CONCLUSIONS

Iodothyronines may influence energy metabolism, principally by affecting the mitochondrial energy-transduction apparatus. Both nuclear-mediated and mitochondrial-mediated pathways are involved in this effect. Some conflicting results may be found in the literature and different ideas are supported by different authors. This may be due to several factors, of which the wide variety of experimental methods and conditions used in the various investigations may be of some importance. Indeed, the most important of all, in our opinion, are: 1) the wide range of doses used both in *in vivo* and *in vitro* studies; 2) the different animal models used in studies on hypothyroid conditions (chemical or surgical thyroidectomy) and 3) the fact that most studies have been performed on liver cells (whose special tasks are in intermediary metabolism rather than in energy expenditure).

Recent reports, however, have advanced our understanding of the molecular processes that may be involved in the action of iodothyronines on the mitochondrial apparatus. In particular, it appears that by a nuclear-independent pathway, T_3 and 3,5-T_2 could help to adapt mitochondrial activity to different physiological conditions. In this pathway, T_3 - by binding to a specific mitochondrial receptor and affecting the transcription apparatus- may act in a coordinated manner with the T_3 nuclear pathway to regulate mitochondrial biogenesis and turnover. 3,5-T_2, on the other hand, seems likely to act directly on the energy-transduction apparatus by binding with some components of the respiratory chain, thus regulating mitochondrial respiration (Fig. 2).

The nuclear-mediated molecular mechanisms through which T_3, by regulating the transcription of specific genes, may influence energy metabolism have considerable impetus received from the discovery that it may regulate the expression of UCP2 and UCP3. In particular, the regulation of UCP3 expression in skeletal muscle seems to be very promising as a putative molecular mechanism underlying the regulation of metabolic rate by T_3. Taken together, these results direct our attention to other questions related to the regulation of energy metabolism. In particular, we need to know whether the iodothyronines change the metabolic rate in a quantitative (increase in the concentrations of enzymes or of components of the respiratory chain, etc.) or in a qualitative manner, or indeed both. Further, we should now realize that results obtained *in vitro* in isolated mitochondria or in isolated enzyme complexes must always be interpreted with caution. The low degree of reproducibility shown by the results so far obtained *in vitro* could be due, at least in part, to an alteration from the *in vivo* situation that could be due to a loss of essential metabolites or cofactors as well as to the absence of the features of the physiological environment (such as lipids in the membrane).

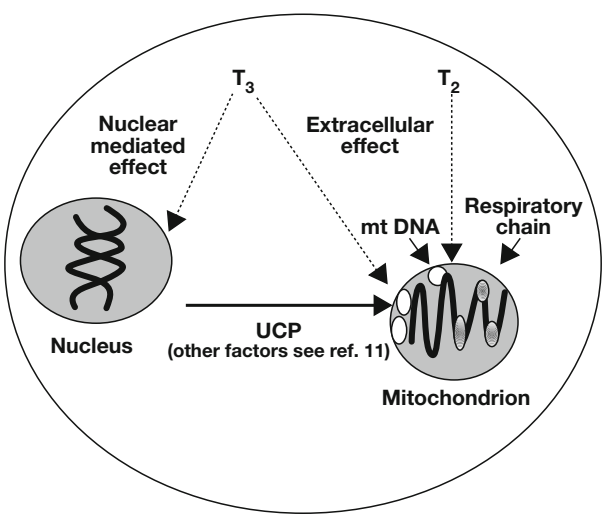

Fig. 2 - *Schematic representation of the mechanisms underlying the effect of iodothyronines on energy metabolism.*

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