THYROID HORMONE AND METABOLITES: CENTRAL VERSUS PERIPHERAL EFFECTS

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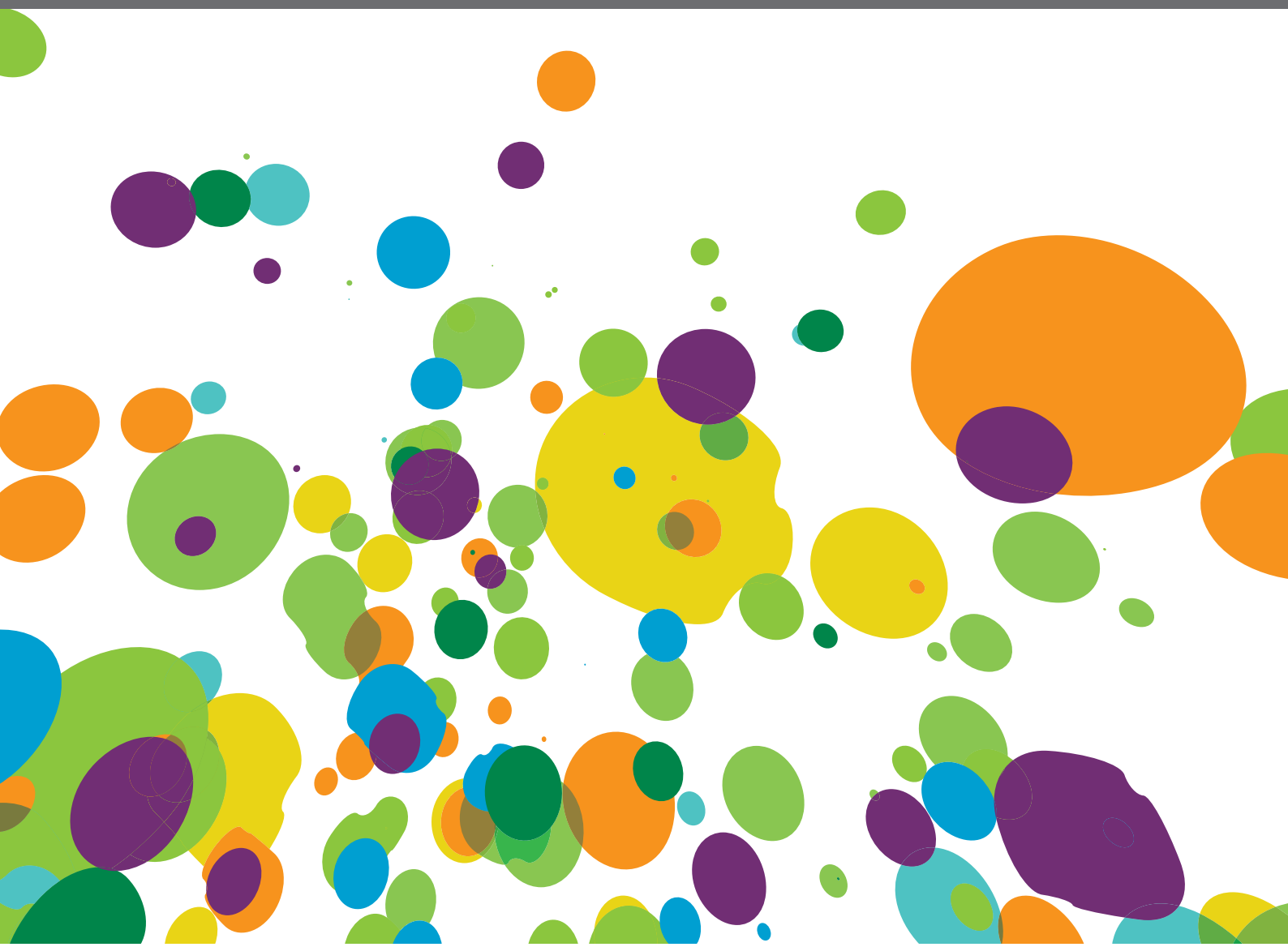

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THYROID HORMONE AND METABOLITES: CENTRAL VERSUS PERIPHERAL EFFECTS

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EDITORIAL
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Editorial: Thyroid Hormone and Metabolites: Central Versus Peripheral Effects

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Editorial on the Research Topic

Thyroid Hormone and Metabolites: Central Versus Peripheral Effects

The organism's metabolic state is under tight control and an efficient balance between glucose and lipid metabolism is crucial for maintenance of energy homeostasis, which allows the body to function optimally. Metabolic homeostasis is under control of hormones as well as the sympathetic/parasympathetic nervous systems. Particularly thyroid hormone (TH) plays an important role in the regulation of energy expenditure. The action of metabolically active TH metabolites involves a plethora of avenues which have been and are still being unraveled from the previous century to the present day. At the cellular level, the classic, biologically active TH, 3,5,3′-triiodo-L-thyronine (T3), predominantly acts through thyroid hormone receptors (THRs), which are predominantly localized in the nucleus and, upon binding of T3, act as ligand-dependent transcription factors that regulate gene transcription through binding to thyroid hormone response elements (TREs). The THRs, though, are also known to act within the cytoplasm. In addition, T3 acts at the cell membrane level. All these effects, which are either "genomic" (involving gene transcription) or "non-genomic" (involving activation of pathways through resident proteins in the cell) are mediated directly by TH within the cell and are referred to in this Topic as "peripheral effects." In addition, as highlighted in this Research Topic, thyroid hormones can activate the central and autonomic nervous system, which, by acting through their appropriate membrane receptors, regulate cellular responses, not necessarily involving the intracellular response to the hormone itself. The TH-mediated effects of the autonomous and central nervous system are referred to in this Topic as "central" effects. This intriguing issue is presented and discussed here in 3 original research papers, 3 reviews, and 4 minireviews, in which the central as well as peripheral effects are highlighted. They address the mechanisms of action of the metabolically active THs T3, 3,5-diiodo-L-thyronine (T2), 3-iodothyronamine (T1AM), and their action on various tissues including liver, skeletal muscle, heart, bone, brain, and white and brown adipose tissue. Although the field is in rapid progress, the contributions successfully cover the current state-of-the-art and clearly present the vast versatility of action of hormones which directly or indirectly originate from the thyroid gland. A clear example of the combined action of T3 on the nervous system and the target cell itself is the induction of thermogenesis in brown adipose tissue, elegantly reviewed by Cioffi et al. BAT thermogenesis requires increased transcription of both uncoupling protein 1 (UCP1), inducing dissipation of energy through heat at the mitochondrial level, and of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), triggering transcription of genes involved in mitochondrial

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duplication and thermogenesis. UCP1 transcription is under direct control of T3 within the brown adipocyte through THR beta, whereas PGC-1alpha expression is under control of Norepinephrine (NE), a neurotransmitter of the sympathetic nervous system (SNS). Direct proof that both the nervous and the intracellular response are under control of T3 came from studies showing that T3 triggers SNS-mediated BAT activation upon hypothalamic injection. Since it has recently been shown that T2, one metabolically active thyroid hormone metabolite, is also able to activate BAT associated with increased UCP1 activity and NE-mediated signaling, a currently unresolved question is whether the effect of T3 on BAT is partly due to the conversion of T3 into T2. Interestingly, using the same technique revealed that T3, by interacting with the parasympathetic nervous system (PSNS), induces hepatic lipogenesis through de-phosphorylation of the AMP-activated protein kinase (AMPK), key to the induction of fatty acid oxidation in target tissues. Further evidence for central effects of T3 comes from an interesting study by Martins et al. showing that T3 acts on the SNS to control bone remodeling. Treatment of mice with depletions of the α2A or α2C adrenoreceptors with a supraphysiological dose of T3 alleviates the negative effects of the resulting thyrotoxicosis on distinct bone compartments. Also the central nervous system (CNS) is a target of TH metabolites. As reviewed by Laurino et al. T1AM stimulates metabolism and behavior in rodents, and its levels are critically regulated in the brain. Behavioral effects related to T1AM in the brain presumably involve trace amine-associated receptor 1, the histaminergic system, and mitochondrial monoamine oxidases. Peripheral effects of TH on metabolism in different contexts are reviewed by Kowalik et al.; Duntas and Brenta, and Louzada and Carvalho. Kowalik et al. highlight the effect of thyromimetics vs. TH metabolites on liver diseases ranging from non-alcoholic fatty liver disease to hepatocellular carcinoma in cell and rodent models, whereas Duntas and Brenta focus on lipid metabolism by TH in humans, and Louzada and Carvalho discuss to what extent the effects of TH metabolites mechanistically overlap in metabolically active tissues. Cicatiello et al. review why local conversion of T4 into T3 by type 1 and type 2 iodothyronine deiodinases, and TH inactivation type 3 iodothyronine deiodinase, is of crucial importance to render metabolically active tissues capable to promptly respond to various metabolic demands posed by for instance cold exposure or physical exercise without perturbing systemic TH levels. One TH metabolite, T2, mainly acts through THR-independent ways, as reviewed by Senese et al. who further highlight that in rodent diet-induced obesity models, T2 restores insulin sensitivity in skeletal muscle, a crucial tissue for the maintenance of normoglycemia. In this light, the study by Sacripanti et al. elegantly shows that in the isolated rat heart perfused with low concentrations of T2 (0.1 or 1.0 mM) significant increases in glucose uptake are observed (by 24 and 35%, respectively), without an alteration of cardiac output. Since T3 and T4 did not increase glucose consumption, direct cellular action of T2 may proof promising in increasing the response to glucose and, eventually, the efficient management of disturbances in glycemia related to diabetes type 2. Finally, Lorenzini et al. present an elegant method based on mass spectrometry (MS) that enables to precisely quantify 3,5-T2 and 3,3′-T2 in human serum. Overall, the original articles and reviews of this research topic clearly demonstrate that thyroid hormone action not only comprises an intricate network of distinct cellular pathways, differentially activated by each metabolite present in the cell, but also nervous signaling, which involves both the CNS, the PSNS, and the SNS. All these pathways converge to provide the final metabolic phenotype dictated by the thyroid. Future research is necessary to unravel these pathways, which will, as we hope, be triggered based on the information provided by the contributions to this research topic, and the relevant literature cited therein.

AUTHOR CONTRIBUTIONS

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MINI REVIEW
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Effect of Iodothyronines on Thermogenesis: Focus on Brown Adipose Tissue

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Thyroid hormones significantly influence energy expenditure by affecting the activity of metabolic active tissues, among which, mammalian brown adipose tissue (BAT) plays a significant role. For a long time, the modulation of BAT activity by 3,3′,5-triiodo-L-thyronine (T3) has been ascribed to its direct actions on this tissue; however, recent evidence indicates that T3, by stimulating specific brain centers, activates the metabolism of BAT *via* the sympathetic nervous system. These distinct mechanisms of action are not mutually exclusive. New evidence indicates that 3,5-diiodo-L-thyronine (3,5-T2), a thyroid hormone derivative, exerts thermogenic effects, by influencing mitochondrial activity in metabolically active tissues, such as liver, skeletal muscle, and BAT. At the moment, due to the absence of experiments finalized to render a clear cut discrimination between peripheral and central effects induced by 3,5-T2, it is not possible to exclude that some of the metabolic effects exerted by 3,5-T2 may be mediated centrally. Despite this, some evidence suggests that 3,5-T2 plays a role in adrenergic stimulation of thermogenesis in BAT. This mini-review provides an overview of the effects induced by T3 and 3,5-T2 on BAT thermogenesis, with a focus on data suggesting the involvement of central adrenergic stimulation. These aspects may reveal new perspectives in thyroid physiology and in the control of energy metabolism.

Keywords: thyroid hormone, metabolism, 3,5-diiodo-L-thyronine, thermogenesis, brown adipose tissue

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INTRODUCTION

In mammals and in homeotherms, a tight control of heat production allows maintenance of a constant core temperature despite variations in environmental temperature. Heat production is customarily divided into obligatory and facultative/adaptative thermogenesis. Obligatory thermogenesis represents constitutive heat production, normally resulting from sustaining vital functions; it is sufficient to maintain body temperature of animals at thermoneutrality. When ambient temperature descends below thermoneutral temperature (that differs between mammal species), heat-saving and heat-producing mechanisms are activated. Indeed, heat-saving mechanisms (pilo-erection, vasoconstriction, adoption of a curled posture, immobility) are limited, and an additional heat is promptly produced by a large energy consuming process, such as shivering, and then substituted by a long-lasting activation of more efficient heat generating metabolic mechanisms, occurring principally in brown adipose tissue (BAT) (1).

Recently, emerging novel aspects have renewed the interest for BAT as a potential target for the treatment of human obesity and related diseases (2, 3) since functional BAT has been detected in adult human (4–6), where its amount correlates positively with resting metabolic rate and inversely with body mass index (7). BAT can utilize blood glucose and lipid, thus improving glucose metabolism and lipid profiles in some conditions (8, 9). Moreover, when specifically stimulated, BAT-precursor cells placed in white adipose tissue (WAT) can differentiate to beige/brite cells instead of white adipocytes (10, 11). Thus, studies on the mechanism underlying BAT activation as well as its hormonal regulation are still ongoing.

Thyroid hormones (TH) play a crucial role in stimulating both obligatory and adaptive thermogenesis (12, 13), with BAT thermogenesis being a key contributor of the latter.

Although thyroid thermogenesis is known for more than a century (14), new innovative concepts are emerging; these regard the involvement of the hypothalamus in TH induced-thermogenesis, the ability of TH to induce the browning of WAT, and the identification of 3,5-diiodo-L-thyronine (3,5-T2) as an active TH derivative able to enhance BAT thermogenesis.

Here, we provide an overview on the effects induced by TH and 3,5-T2 on BAT thermogenesis, pointing the attention on aspects suggesting the involvement of central adrenergic stimulation.

BAT-MEDIATED THERMOGENESIS

The single heat-producing unit of BAT is the brown adipocyte, it contains triglycerides within multiple small vacuoles and numerous mitochondria. Each cell interacts with noradrenergic fibers of sympathetic nervous system (SNS) and is surrounded by capillaries. When an increased rate of heat production is needed, a signal is transmitted *via* the SNS to each brown adipocyte. The released norepinephrine (NE) primarily binds to the brown adipocytes' β3 adrenergic receptors and activates intracellular signaling that leads to the hydrolysis of triglycerides and the release of free fatty acids (FFAs) (1). At the mitochondrial level, FFAs are then oxidized, thus furnishing reduced substrates for the respiratory chain that actively pumps protons from the matrix to the inner membrane space and generates proton motive force. In BAT, uncoupling protein-1 (UCP1) is directly activated by FFAs and mediates the re-entry of protons into the matrix, not associated to ATP synthesis, leading to (i) energy dissipation contained in the proton motive force as heat and (ii) substrate oxidation, uncoupled by the synthesis of ATP. Thus, in BAT, UCP1 "converts fat to heat" (**Figure 1**).

Prolonged exposure to cold and β-adrenergic stimuli triggers a trophic response through activation of mitochondriogenesis, contributes to the increase of the thermogenic capacity

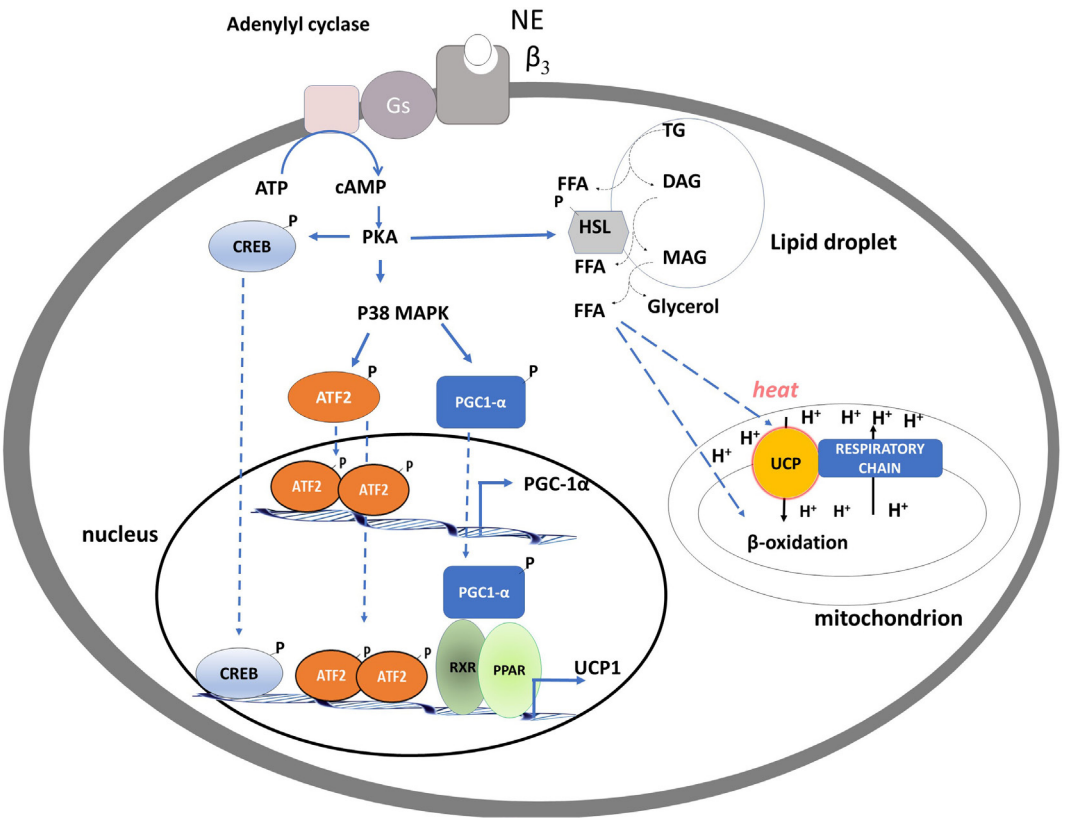

FIGURE 1 | Schematic representation of processes activated by noradrenaline in brown adipocytes leading to brown adipose tissue thermogenesis, mitochondrial biogenesis, and fatty acid oxidation. PKA activation of CREB and p38 MAP kinase leads to an enhancement of the transcription of uncoupling protein-1 (UCP1) and PGC-1α genes. PKA, by phosphorylating hormone sensible lipase leads to the hydrolysis of triglycerides. Free fatty acids released are used as fuel substrate at the mitochondrial levels and as UCP1 activator.

of brown adipocytes, and promotes the browning processes of WAT (15).

The molecular events involved in the NE-induced BAT thermogenesis include activation of the cAMP/PKA signaling pathway, downstream stimulation of the p38α MAPK, and recruitment and p38-dependent activation of PGC-1α [peroxisome proliferator-activated receptor γ coactivator-1] (16) (**Figure 1**).

In BAT, PGC-1α coordinates the expression of genes that stimulate mitochondrial biogenesis and a thermogenic program (17). Indeed, mice lacking PGC-1α are extremely cold sensitive (18, 19), because of a defective thermogenesis, likely due to an impaired mitochondrial program for fatty-acid β-oxidation and electron transport, accompanied by reduced induction of UCP1 and type 2-deiodinase (D2) (see below). In nuclei, PGC-1α coactivates nuclear respiratory factors-1 and -2, which regulate expression of a nuclear-encoded transcription factor essential for replication, maintenance, and transcription of mitochondrial DNA: mitochondrial transcription factor A (mt-TFA). Intra-mitochondrial PGC-1α associates with nucleoids and forms a multiprotein complex with mt-TFA at the mitochondrial DNA transcription start site, thus having a putative role as a transcriptional coactivator of mtTFA (20).

TH AND BAT THERMOGENESIS

Thyroid hormones are essential for the full thermogenic response of BAT, and normal systemic thyroid status is essential for cold-induced adaptive thermogenesis. The thermogenic response of BAT to TH is the result of the synergistic interactions of the hormones with the SNS (21, 22).

Brown adipocytes express both TH receptors alpha (TRα) and beta (TRβ) that control distinct and fundamental pathways for adequate BAT thermogenesis. TRα mediates synergism between TH signaling and the SNS, whereas TRβ is involved in T3 mediated regulation of UCP1 transcription (23, 24). Indeed, the disruption of TRβ-mediated signaling leads to defective adaptive thermogenesis and reduced UCP1 expression (24, 25), while the TRβ agonist GC-1, when applied in association with NE to isolated brown adipocytes, increases UCP1 expression but NE responses result blunted (24).

This TRβ-dependent mechanism is also crucial to induce UCP1 expression in WAT, thus suggesting a role for TH-signaling in the "browning" phenotype of WAT (26).

The intracellular action of TH is regulated by the amount of cellular T3 available for receptor binding, with deiodinase 2 (D2) playing a crucial role. Brown adipocytes express D2, a TH activating enzyme that catalyzes the deiodination of T4 to T3. Within a few hours of cold exposure, because of D2 activation, intracellular T3 levels increase threefold, resulting in higher T3 receptor occupancy nearly reaching saturation (27–29).

D2 is also crucial for the synergism between TH and NE signaling (22), as supported by the evidence that transgenic D2 null mice show cold intolerance, despite normal plasma T3 concentrations (30). NE leads to an enhancement of D2 levels by promoting its de-ubiquitination and by enhancing its gene transcription (31). As a result, tissue levels of T3 increase, thus amplifying the SNS-induced effects, such as lipolysis and stimulation of the UCP1 gene transcription.

Hyperthyroidism stimulates both basal and facultative thermogenesis. Specifically, alongside enhanced thermogenesis hyperthyroid mice displayed increased BAT mass (32, 33), mitochondrial content, oxidative capacity, and UCP1 protein levels (33). T3 also increases nuclear and mitochondrial PGC-1α levels, pointing to a coordinative effect of this iodothyronine in these two organelles, thus activating mitochondrial biogenesis and BAT thermogenesis. Hyperthyroidism also induces "browning" of WAT (32). In addition to TH, thyrotropin receptor signaling is also involved in BAT formation in the hyperthyroid state, probably *via* upregulation of browning factors such as PRDM6, PGC-1α, and UCP1 (34).

In hyperthyroid rodents, compensatory mechanisms are triggered to limit the response of BAT to both NE and TH. Specifically, an excess of thyroxine (i) promotes ubiquitination and degradation of D2, thus protecting BAT from the elevated serum levels of TH and (ii) reduces β3 receptor density, thus toning down sympathetic stimulation (35, 36). Conversely, hypothyroidism reduces obligatory thermogenesis, accompanied by a compensatory increase in BAT stimulation (21). In fact, hypothyroid BAT shows signs of adrenergic stimulation, such as enhancements of NE levels (32) and sympathetic innervations (33). Despite of this, the lack of T3 limits the thermogenic response of the tissue to the NE stimulation (37). Indeed, cAMP generation is greatly reduced in isolated brown adipocytes obtained from hypothyroid animals as a result of modifications of the receptor, its interaction with Gi proteins, and resulting adenylyl cyclase levels (35, 36, 38). In line with this, hypothyroid animals present impaired BAT thermogenesis, therefore, developing severe hypothermia resulting in death after a few days of exposure to cold (39). Lower BAT activity/thermogenesis despite enhanced sympathetic tones in hypothyroidism has been recently confirmed by a technique termed "*in vivo* small animal 18F-FDG PET/MR" (32). Moreover, histological analysis revealed that BAT from hypothyroid animals shows more lipid-depleted adipocytes, typified by an increased number of unilocular adipocytes, similar to what is observed in white adipocytes (32, 33).

Interestingly, in hypothyroid mice, a compensatory "browning" of WAT seems to occur as a response to the decreased heat production due to BAT inactivity. Markers for this event are increased expression levels of brown fat specific genes such as UCP1 and Cidea, and the multilocular UCP1-positive phenotype of some adipocytes in both iWAT and gWAT (32, 40).

CENTRAL EFFECT OF TH IN ACTIVATION OF BAT

Recent studies revealed that T3 is a central inducer of BAT by directly stimulating the hypothalamic pathway (41). In definite hypothalamic centers, and specifically in Sf1 neurons of ventromedial nucleus (VHM) (42, 43), T3 selectively increases *de novo* lipogenesis, leading to the activation of the SNS and the induction of BAT (41). T3-induced lipogenesis is mediated by AMP-activated protein kinase (AMPK), a key kinase regulating lipid metabolism that, when activated by phosphorylation, switches-on fatty acid oxidation rate and switches-off lipogenesis (44).

Following intracerebroventricular administration, T3 causes a rapid dephosphorylation of AMPK. The crucial role played by AMPK in the T3-induced BAT thermogenesis through the SNS is shown in experiments of VMH-selective genetic ablations of AMPK or TH receptors: AMPK ablation increases the sympathetic BAT tone and subsequent BAT activation, which is blunted by inhibition of β3 adrenergic receptor activity in BAT (41). Ablation of TH receptors in hyperthyroid rats significantly inhibits BAT thermogenesis (41–43).

The mechanism linking T3-induced AMPK de-phosphorylation in VHM to the activation of SNS seems related to the ability of AMPK to influence ceramide levels as well as to endoplasmatic reticulum (ER) stress (42, 43). Indeed, at the hypothalamic level, ceramide-induced lipotoxicity triggers ER stress and leads to a decreased sympathetic tone in BAT, thus impairing BAT thermogenesis (45). Thus, T3-induced hypothalamic AMPK dephosphorylation leads to a decrease in ceramide synthase activity, and consequently ceramides levels, resulting in a reduction of ER stress (see **Figure 2**).

In BAT, following ICV administration of T3, the expression of thermogenic markers (UCP1, PGC1α, D2, hormone sensitive lipase, lipoprotein lipase) occurs in association with a decreased lipid droplet (LD) content as well as an enhancement of the mitochondrial size (41–43). Furthermore, an increase in the BAT-mediated uptake of fatty acids and their subsequent utilization as fuel substrates at the mitochondrial level is observed, plausibly mediated by increased activity of the AMPK signaling pathways (42, 43).

Interestingly, the effects of ICV administration of T3 on energy expenditure, thermogenesis, and body weight are abolished in UCP1-deficient mice (46), suggesting the importance of BAT in TH metabolic regulation.

Another significant effect to consider is the ability of TH to regulate WAT browning through a central mechanism, since ICV infusion of T3 increases the browning of iWAT (46).

3,5-T2: A THYROID HORMONE DERIVATIVE THAT ENHANCES BAT THERMOGENESIS

The research field concerning the control of energy metabolism by the thyroid is no longer restricted to T3 since growing evidence indicates that some of its derivatives could have biological effects. Among these, 3,5-T2 plays a significant role [for review, see Ref. (47–49)].

3,5-T2 influences the activity of metabolically active tissues such as skeletal muscle, liver, and BAT. Some of the effects induced by this iodothyronine are manifested in a short-term within 1 h of its administration, and experimental evidence indicates that

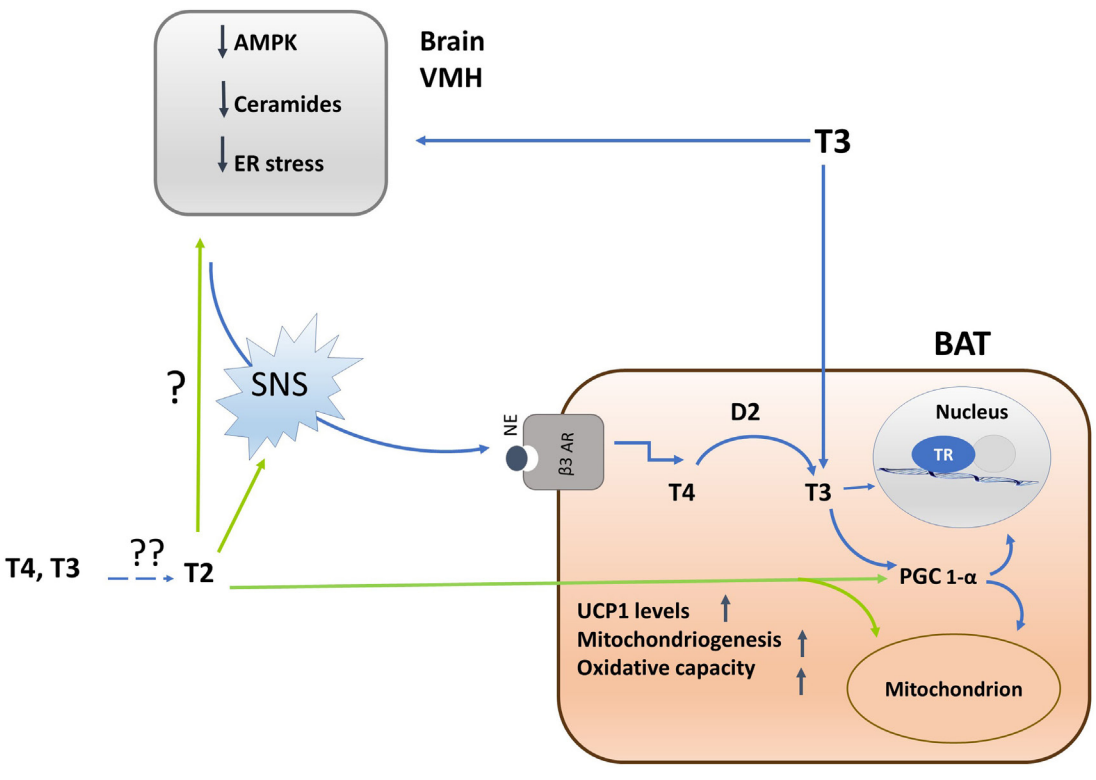

FIGURE 2 | Schematic representation of the mechanisms by which iodothyronines (T3 and 3,5-T2) activates brown adipose tissue (BAT) thermogenesis and of the interrelations between central and direct effects of iodothyronines on the tissue. Single question point indicates that, despite of the fact that 3,5-T2 enhances BAT adrenergic tone, now, there are no experiments indicating whether this effect is brain-mediated or not. Double question points indicate that there are unresolved questions such as: where is 3,5-T2 coming from? Is it coming from the circulation or is it locally formed by deiodination? Is D2 involved? Experimental evidences are needed.

mitochondria are a direct target of 3,5-T2 (47, 48, 50). The ability of 3,5-T2 to exert a calorigenic effect when injected into rats (33, 39, 51–54) suggests that 3,5-T2 could be involved in the regulation of energy metabolism in physiological situations requiring a surplus of energy expenditure, such as cold exposure. Such a possibility is substantiated by the evidence that hypothyroid rats do not survive in the cold (4°C), but their survival is improved by 3,5-T2 administration. Indeed, enhanced oxidative capacity of metabolically active tissues, among these BAT, underlies the ability of 3,5-T2 to improve cold tolerance of hypothyroid rats (55).

When administered intraperitoneally to hypothyroid rats housed at thermoneutrality, 3,5-T2 reverses the "white-like" appearance of brown adipocytes characteristic of such animals (see above), enhancing the percentage of multilocular versus unilocular cells. 3,5-T2 also decreases the diameter of LDs and increases the tissue's mitochondrial content, diagnostic for BAT activation (33).

Chronic intraperitoneal 3,5-T2 administration to hypothyroid rats improves maximal tissue oxidative capacity by increasing the activity of cytochrome c oxidase (COX) (33, 39, 51). Within the mitochondrial respiratory chain, apart from transferring electrons to oxygen molecules, COX actively pumps protons from the mitochondrial matrix to the intermembrane space, thus contributing to generation of the proton-motive force, which in BAT, due to the presence of UCP1, is dissipated as heat at the expense of ATP synthesis. The ability of 3,5-T2 to enhance COX activity in BAT of hypothyroid rats seems to be the result of a dual mechanism: (1) an increase in tissue content of mitochondria, (2) a direct effect of the iodothyronine on the enzyme (33). In fact, the stimulatory effect of 3,5-T2 occurs following both intraperitoneal administration and addition to BAT homogenates. The *in vitro* effect of 3,5-T2 is plausibly the result of a direct interaction of the iodothyronine with subunit-Va of the COX complex (56), an interaction that was previously reported to prevent the allosteric inhibition exerted by ATP on the enzyme (57). Chronic administration of 3,5-T2 to hypothyroid rats enhanced the expression of UCP1; moreover, in isolated mitochondria, both the inhibition of UCP1-mediated respiration/thermogenesis by GDP and its reactivation by fatty acids, blunted in hypothyroid condition, were enhanced by 3,5-T2 (33). Taken together, these data imply that the 3,5-T2-mediated activation of COX in BAT triggers mitochondrial thermogenesis through the action of UCP1.

3,5-T2 enhances mitochondrial BAT content, and plausibly, PGC-1α is the putative molecular determinant for this effect. Administration of 3,5-T2, in fact, rapidly increases nuclear and mitochondrial PGC-1α levels, indicating a tight coordination between these organelles, hence programming toward mitochondrial biogenesis and thermogenesis (33) (see **Figure 2**).

3,5-T2 increases the cellular number of nervous fibers that are immune-reactive to tyrosine hydroxylase, a catecholamine-synthesizing enzyme whose expression is related to noradrenergic tone indicating that this iodothyronine increases the sympathetic tone, thus suggesting that part of the thermogenic effect induced by 3,5-T2 in BAT is due to SNS activation. Since adrenergic stimulation of brown adipocytes induces the expression of VEGF [mediated by β-adrenoreceptor/cAMP/PKA signaling pathway (58)], it is plausible that the sympathetic activation promotes the induction of angiogenesis responsible for the higher BAT vascularization observed in 3,5-T2-treated animals (33). The improved BAT vascularization as part of the action of 3,5-T2 on the activation of BAT allows increased blood supply that is crucial to support the higher demand for oxygen and substrates.

CONCLUSION

Recent progress in the field concerning the activation of BAT thermogenesis by TH indicates that their metabolic effect both involves their direct action in the target tissue as well as centrally mediated actions through stimulation of specific regions of the brain.

In addition, TH metabolites have emerged as biological active molecules; among these, 3,5-T2 has a calorigenic effect, mimicking the effect induced by T3 on BAT, and emerging data indicate that 3,5-T2 enhances sympathetic tone of BAT.

In relation to this, several questions arise: (i) are endogenous levels of 3,5-T2 relevant to energy balance in normal human physiology, or is 3,5-T2 the basis of a potential pharmacological approach to contrast obesity? (ii) are the effects exerted by T3 on BAT thermogenesis due to T3 itself, or part of these effects are due to its conversion to 3,5-T2?, and (iii) are the thermogenic effects of 3,5-T2 in part mediated centrally?

Unfortunately, studies performed so far do not allow to unambiguously answer the questions above.

Indeed, key experiments concerning the detection of 3,5-T2 serum and tissues levels, in different physiological conditions or following T3 administration are few or lacking. Moreover, despite an extrathyroidal production of 3,5-T2 from T4 have been suggested in humans (59) and in rats an increase in 3,5-T2 serum levels following T3 *in vivo* administration (52) was observed, the identification of the enzyme involved in 3,5-T2 formation from TH has not yet been achieved. It would be a crucial step in the understanding of the basal physiological mechanisms regulating 3,5-T2 availability, and whether some T3 effects are mediated by its conversion into 3,5-T2. New information in this field may reveal future perspectives to allow a deeper understanding of whether 3,5-T2 is a direct stimulator of metabolism with the potential to be an extra level for the regulation of energy metabolism by the thyroid or if it could be used as pharmacological approach to contrast dysmetabolic diseases.

AUTHOR CONTRIBUTIONS

FC, AG, ES, and AL contributed with research and writing. FG and AL participated in the conceptual aspect of the mini review and reviewed the article. AL oversaw, assembled, and reviewed the article. FC and AG contributed equally to the study.

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3,5-Diiodo-L-Thyronine Increases Glucose Consumption in Cardiomyoblasts Without Affecting the Contractile Performance in Rat Heart

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3,5-diiodo-L-thyronine (T2) is an endogenous derivative of thyroid hormone that has been suggested to regulate energy expenditure, resting metabolic rate and oxygen consumption with a mechanism that involves the activation of mitochondrial function. In this study, we focused on the cardiac effects of T2, which have been poorly investigated so far, by using both *in vitro* and *ex vivo* models. As a comparison, the response to T3 and T4 was also determined. Rat cardiomyoblasts (H9c2 cells) were used to determine T2, T3, and T4 uptake by high-performance liquid chromatography–tandem mass spectrometry. In the same experimental model, MTT test, crystal violet staining, and glucose consumption were investigated, using T2 concentrations ranging from 0.1 to 10 μM. To assess cardiac functional effects, isolated working rat hearts were perfused with T2, T3, or T4 in Krebs-Ringer buffer, and the hemodynamic variables were recorded. T2 was taken up by cardiomyoblasts, and in cell lysate T2 levels increased slowly over time, reaching higher concentrations than in the incubation medium. T2 significantly decreased MTT staining at 0.5–10 μM concentration ($P < 0.05$). Crystal violet staining confirmed a reduction of cell viability only upon treatment with 10 μM T2, while equimolar T3 and T4 did not share this effect. Glucose consumption was also significantly affected as indicated by glucose uptake being increased by 24 or 35% in cells exposed to 0.1 or 1.0 μM T2 ($P < 0.05$ in both cases). On the contrary, T3 did not affect glucose consumption which, in turn, was significantly reduced by 1 and 10 μM T4 (−24 and −41% vs control, respectively, $P < 0.05$ and $P < 0.01$). In the isolated perfused rat heart, 10 μM T2 produced a slight and transient reduction in cardiac output, while T3 and T4 did not produce any hemodynamic effect. Our findings indicate that T2 is taken up by cardiomyoblasts, and at 0.1–1.0 μM concentration it can modulate cardiac energy metabolism by increasing glucose consumption. Some evidence of toxicity and a transient impairment of contractile performance are observed only at 10 μM concentration. These effects appear to be specific for T2, since they are not reproduced by T3 or T4.

Keywords: T2, heart perfusion, cardiomyoblasts, glucose consumption, T2 uptake

INTRODUCTION

3,5-Diiodo-L-thyronine (T2), an endogenous metabolite of thyroid hormones (TH), has been described as a peripheral mediator of several TH metabolic effects (1). Although conversion of 3,5,3′-triiodothyronine (T3) to T2 has not yet been demonstrated *in vitro*, indirect evidence indicates that T2 is, indeed, formed from T3 *in vivo*, through deiodination (2). Both T2 and T3 increase resting metabolic rate, but the response to T2 shows a more rapid onset (3). These findings have induced to hypothesize that T2 might mediate some of the short-term effects of TH and that it might be involved, like T3, in physiological processes leading to increased energy expenditure (3). While the response to T3 was principally mediated by nuclear receptors, the effects of T2 were independent of protein synthesis (4) suggesting that they were probably due to a direct interaction with mitochondria.

Several reports have shown that, in rats, acute or chronic T2 administration causes significant changes in mitochondrial activities, stimulating fatty acid oxidation, and decreasing hepatic lipid accumulation (5–9). At the dosage 0.25 μg/g body weight for 4 weeks i.p. (10), T2 prevented body weight gain in rats fed with a high-fat diet, without inducing T3-related undesirable side effects, namely tachycardia, cardiac hypertrophy, and decreased TSH levels. In this models, T2 stimulated mitochondrial uncoupling, decreased ATP synthesis, and increased hepatic fatty acid oxidation rate, thus counteracting obesity. Additionally, T2 induced biochemical and structural shifts toward glycolytic myofibers and prevented an increase in serum triglycerides and cholesterol (10, 11). On the whole, it has been clearly established that T2 exerts hepatic antilipidemic effects, but its physiological relevance in skeletal and cardiac muscle is still unclear. In skeletal muscle, Moreno et al. (12) reported increased insulin sensitivity upon T2 administration, particularly increased protein kinase B phosphorylation, and sarcolemmal GLUT4 accumulation. In another investigation, T2 induced proton leak in skeletal muscle mitochondria obtained from hypothyroid rats (13).

Chronic administration of a low T2 dosage to two healthy volunteers increased resting metabolic rate and decreased body weight, by inducing a reduction in steatosis and in the total serum cholesterol levels, without affecting cardiac function (14). Conversely, a recent study reported that, at a higher dosage (2.5 μg/g), T2 administration exerted thyromimetic effects, since it induced cardiac hypertrophy and an overall genomic effect similar to that produced by the TH (T3) (15). However, the cardiac effects of T2 have not been extensively investigated so far. Therefore, in this study we explored the functional, metabolic, and toxic effects of T2 using both *in vitro* and *ex vivo* models of cardiac preparations.

MATERIALS AND METHODS

Chemicals

H9c2 (2–1) rat cardiomyoblast cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). [^{3}H]-ryanodine was obtained from New England Nuclear (Milan, Italy). Ryanodine was purchased from Abcam, UK. Unless otherwise specified, all reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). Solvents for HPLC–MS/MS measurements were HPLC-grade, and the other chemicals were reagent-grade.

Cell Culture and Treatment

Rat cardiomyoblasts (cell line H9c2) were cultured in DMEM supplemented with 10% (vol/vol) FBS, 1 mM pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 and subcultured before confluence. To assess glucose uptake, H9c2 were seeded in six-well plate (5×10^5 cells/well), grown to 80% of confluence with standard medium and washed twice with PBS before treatment. Then, cells were exposed for 4 h to exogenous T2 (0.1–10 μM) in 1 ml of the same DMEM base (phenol free) supplemented with 0.7 mg/ml glucose. Control cells were incubated with DMEM containing the same volume of vehicle. Cell culture medium was then collected and glucose concentration was evaluated in medium with a spectrophotometric assay kit (Sigma-Aldrich). Hexokinase activity was assessed in cell lysate with a colorimetric assay kit (BioVision, Milpitas, CA, USA). Enzyme activity was evaluated following kit protocol. The absorbance was read at 450 nm. Metabolite concentrations were referred to the total protein content of whole-cell lysates (16).

Uptake of T2 and HPLC–MS/MS Assay Technique

The experiments aimed at evaluating hormone uptake were performed as previously described, with minor modifications (17). Briefly, cells (H9c2) were seeded into 24-well plate (8.5×10^4 cells/well) and grown to 80% confluence. At the start of each experiment, the culture medium was removed, and after washing with PBS, fresh medium containing 100 nM T2, 50 nM T3 or T4 was added. The plate was returned to a humidified atmosphere of 5% CO_2 at 37°C, and the medium was removed from each well at specific time points and frozen at −80°C until extraction. At the end of the experiment, cell plates were washed with PBS and frozen. Cell lysis was carried out using 100 μl of 0.1 M NaOH and samples were neutralized by adding 10 μl of 1 M HCl. After pH neutralization, 390 μl MeOH were added and samples collected and centrifuged for 10 min at 14,000 × *g*, and the supernatant was evaporated under N_2 at 40°C. Dry samples were then reconstituted using 50 μl of water/acetonitrile (70:30 by volume) containing 0.1% formic acid, and analyzed using HPLC coupled tandem mass spectroscopy, as elsewhere described (17). Cell culture medium was extracted using a liquid–liquid method: 1 ml of methyl tert-butyl ether was added and then the mixture was vigorously shaken for 30 s. Then the mixture was briefly spinned at 14,000 × *g* to completely separate the organic and aqueous phases. The organic phase was then collected and the extraction process was repeated twice. At the end of the procedure, the collected organic phases were evaporated under N_2 at 40°C. Dry samples were then reconstituted using 50 μl of water/acetonitrile (70:30 by volume) containing 0.1% formic acid and analyzed using HPLC coupled tandem mass spectroscopy assay, as detailed below. The positive ion mode method included: ionspray

voltage, 5.00 kV; gas source 1, 70; gas source 2, 55; turbo temperature, 650°C; entrance potential, 10 V; collision-activated dissociation gas pressure, 12 mPa. HPLC runs were based on the following mobile-phase gradient: solvent A, methanol/acetonitrile 1:4, containing 0.1% formic acid; solvent B, water containing 0.1% formic acid. The gradient started at 5% A increasing to 65% at 8.5 min, then to 100% at 9 min, maintained until 11 min, with subsequent re-equilibration at 5% for further 2.5 min. The flow rate was 0.4 ml/min.

The selected reaction monitoring (SRM) method transitions and the related parameters for T2 were set as follows.Transitions are: 525.9→352.9, 525.9→381.8, 525.9→479.9 KDa; declustering potential 87.0 kV; collision energy 40.8, 27.6, and 26.0 kV, respectively; collision exit potential 10.3,11.2, and 14.2 kV, respectively. The SRM method transitions for the detection of T3 and T4 were set as described previously (17). Medium peak area at time zero was taken as reference area for the other time points.

Cell Viability Test

Cell viability was assessed by using two different assays: the 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) test (18) and crystal violet staining (19). Cells were seeded in 96-well microtiter plate at a density of 5,000–10,000. Twenty four hours after, T2, T3, or T4 were added at different concentrations and cell viability was determined 24 h after incubation. MTT (0.5 mg/ml) was added to the medium, and after an additional 4 h incubation, SDS–HCl (0.05 mg/ml) was added to solubilize formazan salt. After 18 h, the absorbance of the solution was read at 570 nm in a microplate reader (BioRad Laboratories, Italy). Since T2 is known to affect mitochondrial activity and MTT test is based on mitochondrial function, crystal violet staining was performed according to Feoktistova et al. (19) with minor modifications. Upon iodothyronine treatment, cells were washed gently with warmed PBS, then stained 10 min at room temperature with crystal violet solution (0.2% crystal violet in 2% ethanol). The plate was washed twice with deionized water, and then a 1% SDS solution was added to each well, and the plate was agitated until complete solubilization of the staining that was read at 570 nm.

Isolated Heart Perfusion and Ryanodine Binding

Experimental procedures were approved by the ethical committee of the University of Pisa (protocol no. 51814/2016). The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996).

Male Wistar rats (275–300 g body weight), fed a standard diet, were anesthetized with a mixture of ether and air. The heart was then quickly excised and perfused according to the working heart technique, as described previously (17). The perfusion buffer included (mM): NaCl, 118; $NaHCO_3$, 25; KCl, 4.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 1.5; glucose, 11. Perfusions were carried out using 200 ml of recirculating buffer, which was equilibrated with a mixture of O_2 (95%) and CO_2 (5%). Temperature was kept between 36.8 and 37°C, and the pH was 7.4. Powerlab/200 (ADInstruments, Castle Hill, Australia) was used for hemodynamic variable acquisition. The height of the atrial chamber was set at 20 cm, corresponding to a filling pressure of 15 mmHg. During the experiment, after an equilibration period of 10 min, T2, T3, or T4 (0.1–10 μM) was added to the perfusion buffer and hearts were perfused for another 50 min. In the control group, hearts were perfused only with standard buffer and vehicle. At the end of the perfusion, hearts treated with or without 10 μM T2 were homogenized in five volumes of 300 mM sucrose and 10 mM imidazole (pH 7.0 at 4°C) and high affinity ryanodine binding was determined on the crude homogenate (20). Briefly, vesicles were incubated at 37°C in a buffer containing 25 mM imidazole (pH 7.4 at 37°C), 1 M KCl, 0.4–40 nM [3H]ryanodine (6 Ci/mmol), 0.950 mM EGTA, and 1.013 mM $CaCl_2$ (free Ca^{2+} concentration was 20 μM). After 60 min, the binding reaction was stopped by filtration through cellulose nitrate filters which were washed twice with 25 mM imidazole and 1 M KCl (washing buffer). Radioactivity was counted at 60% efficiency in TRI-CARB 2800 TR Liquid Scintillation Analyzer (Perkin Elmer, Italy). Incubations were performed in duplicate and nonspecific binding was measured in the presence of 10 μM unlabeled ryanodine. Saturation experiments were analyzed by nonlinear fitting of a single binding site model.

Statistical Analysis

Results are expressed as the mean ± SEM. Differences between groups were analyzed by one-way or two-way ANOVA as detailed for each figure. In the experiments aimed at determining differences vs a single control group, Dunnett's *post hoc* test was applied. When the experimental setting included only two groups, statistical differences were determined by unpaired *t*-test. The threshold of statistical significance was set at $P < 0.05$. GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA, USA) was used for data processing and statistical analysis.

RESULTS

Cellular Uptake of Iodothyronines

The results of the T2 uptake experiments in H9c2 cells are shown in **Figure 1**. Uptake was carried out with medium (0.5 ml) containing 100 nM T2 (50 pmol/well). In lysate, T2 concentration increased over time reaching a value of about 14 nM (7 pmol/well) after 24 h, while in medium steady state concentration was reached after 6 h, and it averaged about 110 nM (55 pmol/well). So the overall recovery of T2, after 24 h infusion, was slightly higher than 100%. Since lysate volume was in the order of 0.02–0.03 ml, the actual cellular concentration at the end of the treatment can be estimated to be about 250–350 nM. From preliminary experiments, similar results were obtained in the presence of 50 nM T3 or T4, whose cellular uptake after 24 h was on the order of 10 and 2% of the medium concentration, respectively (data not shown).

Cell Viability

After 24 h incubation with T2, MTT assay was carried out in H9c2 cell lines. As shown in **Figure 2A**, incubation with T2 at concentrations ≥500 nM caused 15–16% decrease in MTT

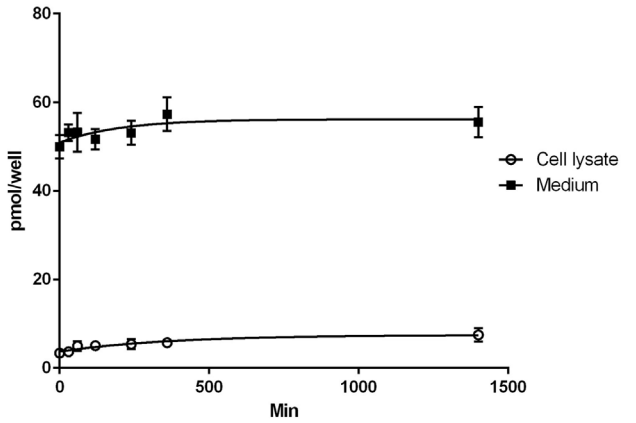

FIGURE 1 | Uptake of T2. Results of T2 uptake in H9c2 cell during 24 h of incubation with 100 nM T2 in medium. Assays were performed at different times in the incubation medium and in the cell lysate. Values are mean ± SEM of results derived from three uptake experiments.

staining, compared to vehicle ($P < 0.05$ by one-way ANOVA test and Dunnett's *post hoc*). This may imply decreased cell viability (and/or reduced oxidative metabolism). To get a better insight, we also performed crystal violet staining. Crystal violet binds to DNA and proteins of cells that did not lose their adherence, and is considered as an alternative index of viability. As shown in **Figure 2C**, crystal violet staining was reduced ($P < 0.05$) only at the highest T2 concentration (10 μM), whereas it was not affected by equimolar T3 or T4 and it was actually increased in the presence of 0.1 μM T3 ($P < 0.05$ vs control). Similar results were obtained in MTT staining by T3 ($P < 0.05$ 0.1 μM vs control), while 10 μM T4 significantly reduced cell viability by 33% ($P < 0.001$ vs control, **Figure 2B**).

Glucose Consumption and Hexokinase Activity

To assess glucose consumption, H9c2 cells were incubated for 4 h in phenol red-free DMEM containing 0.7 g/l glucose, as described in Section "Materials and Methods." At the end of treatment, glucose concentration was assayed in the medium and the results were expressed as the difference between the initial and final concentrations, and normalized to the total protein content of cell lysates. As shown in **Figure 3A**, T2 caused a 23–30% increase in glucose consumption if administered at the concentrations of 0.1 or 1 μM ($P < 0.05$ vs control by one-way ANOVA test and Dunnett's test), by a mechanism which does not involve hexokinase, as revealed by enzymatic activity being unaffected by T2 (**Figure 3D**). At 10 μM T2 concentration, glucose consumption was also enhanced, but the difference vs control did not reach statistical significance (0.066 ± 0.003 vs 0.055 ± 0.003 mg glucose/mg protein, P = NS). By comparison, glucose consumption was not affected by 0.1–10 μM T3 (**Figure 3B**), while it was significantly reduced in cell exposed to 1–10 μM T4, by 24 and 41%, respectively ($P < 0.05$ and $P < 0.001$, respectively; see **Figure 3C**).

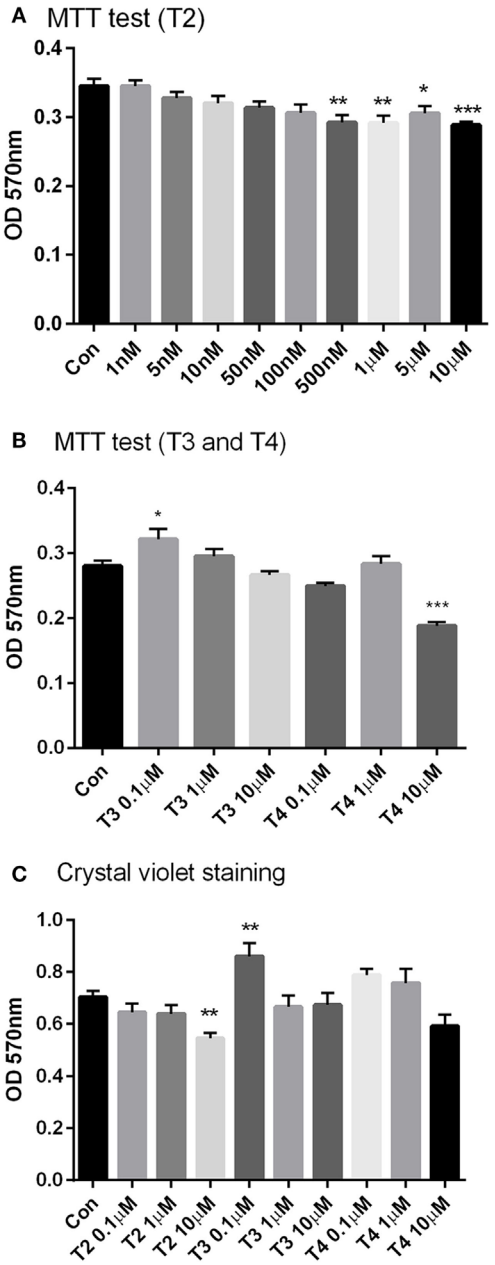

FIGURE 2 | Cell viability. H9c2 cells were incubated for 24 h with a wide range of T2 (1 nM–10 μM), T3, and T4 (0.1–10 μM) concentrations in medium incubation and then the cell viability tests, MTT test **(A,B)**, and crystal violet assay **(C)** were performed. All treatments received the same amount of vehicle. Control group was incubated with medium containing the same volume of vehicle. Data are plotted as means of 4–6 replicas ± SEM [one-way ANOVA, $P < 0.0001$, Dunnett's *post hoc* test for multiple comparison, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control (con), n = 4–6 per group].

Heart Perfusion and Ryanodine Binding

The results of the perfusion experiments are shown in **Figure 4**. Baseline values of the hemodynamic variables averaged as follows: aortic flow (AF) 37.3 ± 0.6 ml/min, coronary flow (CF) 19.3 ± 0.6 ml/min, cardiac output (CO) 56.7 ± 0.7 ml/min,

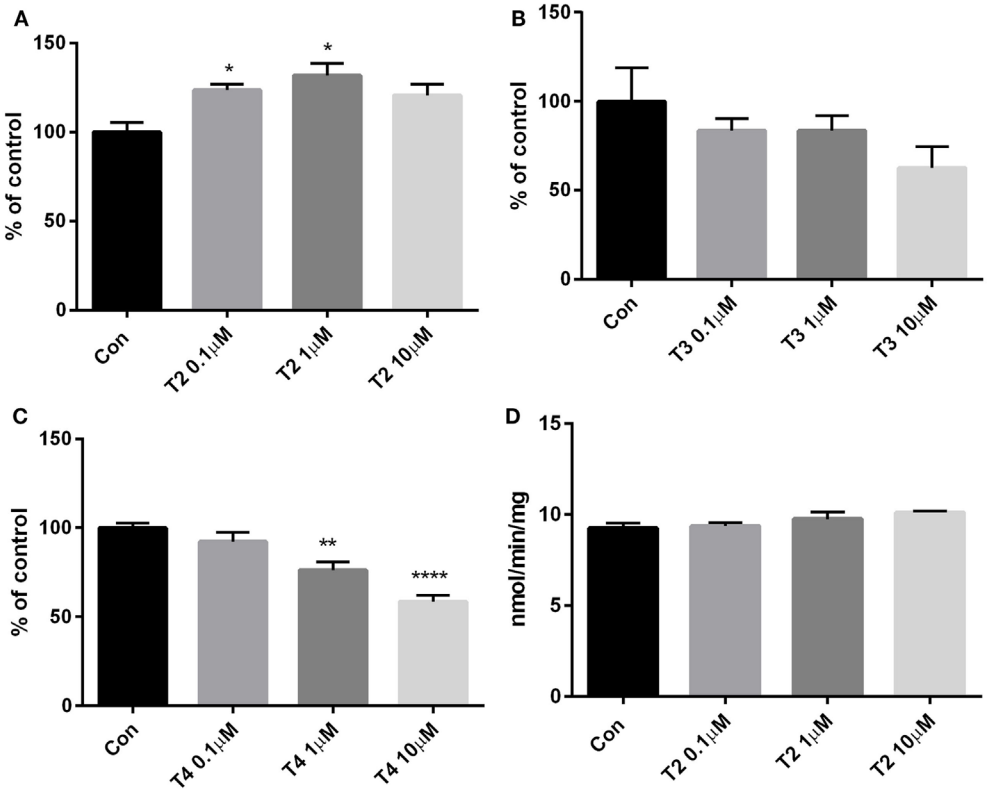

FIGURE 3 | Glucose consumption and hexokinase activity. Glucose concentration was assayed in cell medium after 4 h treatment with **(A)** T2, **(B)** T3, or **(C)** T4 (0.1–10 μM). Results are the difference between the initial glucose concentration in medium (0.707 mg/ml, assessed with the glucose assay kit) and the final concentration, normalized to the total content of protein in lysate. Control cells were incubated with medium containing the same volume of vehicle. **(D)** Hexokinase activity assay was performed in cell lysates upon treatment with T2. Values are mean ± SEM of 3–4 replicates and are expressed as percent of control in the glucose consumption or as NADH nmol/min/mg in the enzyme activity. [one-way ANOVA, $P < 0.05$, Dunnett's *post hoc* test for multiple comparison, $^{*}P < 0.05$, $^{**}P < 0.01$ vs control (con), $n = 3$–4 per groups].

heart rate (HR) 215.6 ± 9.8 beats/min, and peak systolic aortic pressure (PAP) 182.8 ± 6.8 mmHg. T2 (0.1-10 μM) was added 10 min after the beginning of the perfusion, and hearts were perfused for another 50 min with T2, while in the control group the same amount of vehicle was added. After 20 min of perfusion with 10 μM T2, a slight but significant reduction was observed in CO and in AF (AF: −10–12% at 20–40 min $P < 0.05$; CO: 9–10% at 20–30 min $P < 0.05$; see **Figures 4A,C**). The other hemodynamic variables, namely the CF, HR, and PAP were not affected by T2 perfusion (**Figures 4B,D,E**). As a comparison, perfusions with 0.1, 1, and 10 μM T3 or T4 did not produce any significant changes in contractile performance (**Figures 4F–H**, AF, CF, and CO).

In ryanodine binding experiments, crude homogenates of hearts perfused with 10 μM T2 were analyzed. We explored only the concentration that affected, albeit only transiently, the CO. As shown in **Figure 5**, T2 did not modify ryanodine binding: either the number of binding sites (**Figure 5A**) or the affinity for ryanodine (**Figure 5B**) were unchanged by T2 perfusion (Bmax averaged 107.0 ± 7.9 vs 119.7 ± 7.3 fmol/mg; Kd averaged 0.45 ± 0.14 vs 0.54 ± 0.16 nM, P = NS in all cases).

DISCUSSION

T2 is a putative derivative of the TH whose effects on lipid metabolism are well established. On the contrary, the cardiac actions of T2 are less known and still controversial. In the present work, we analyzed the effects of T2 on cardiac tissue in perfused rat hearts and H9c2 cell cultures, using equimolar dosages of T3 and T4 as a control.

We observed that T2 can be taken up and accumulated in cardiomyoblasts. T2 was rapidly absorbed, since its presence was detected in cell lysate after a few minutes of incubation, with a complete recovery after 24 h, suggesting that no T2 catabolites were produced under these experimental conditions, although additional investigations are needed to verify this hypothesis. At the end of the treatment, cell lysate concentration was estimated to average about 250–350 nM, exceeding the medium concentration by over 2–3-fold. Either MCT8 or MCT10 might be responsible for T2 uptake, since these transporters are relatively nonspecific for iodothyronines (21). However the molecular identity of T2 transporters is still unknown.

The absence of significant T2 catabolism is consistent with the fact that rat cardiomyocytes, and in general rodent

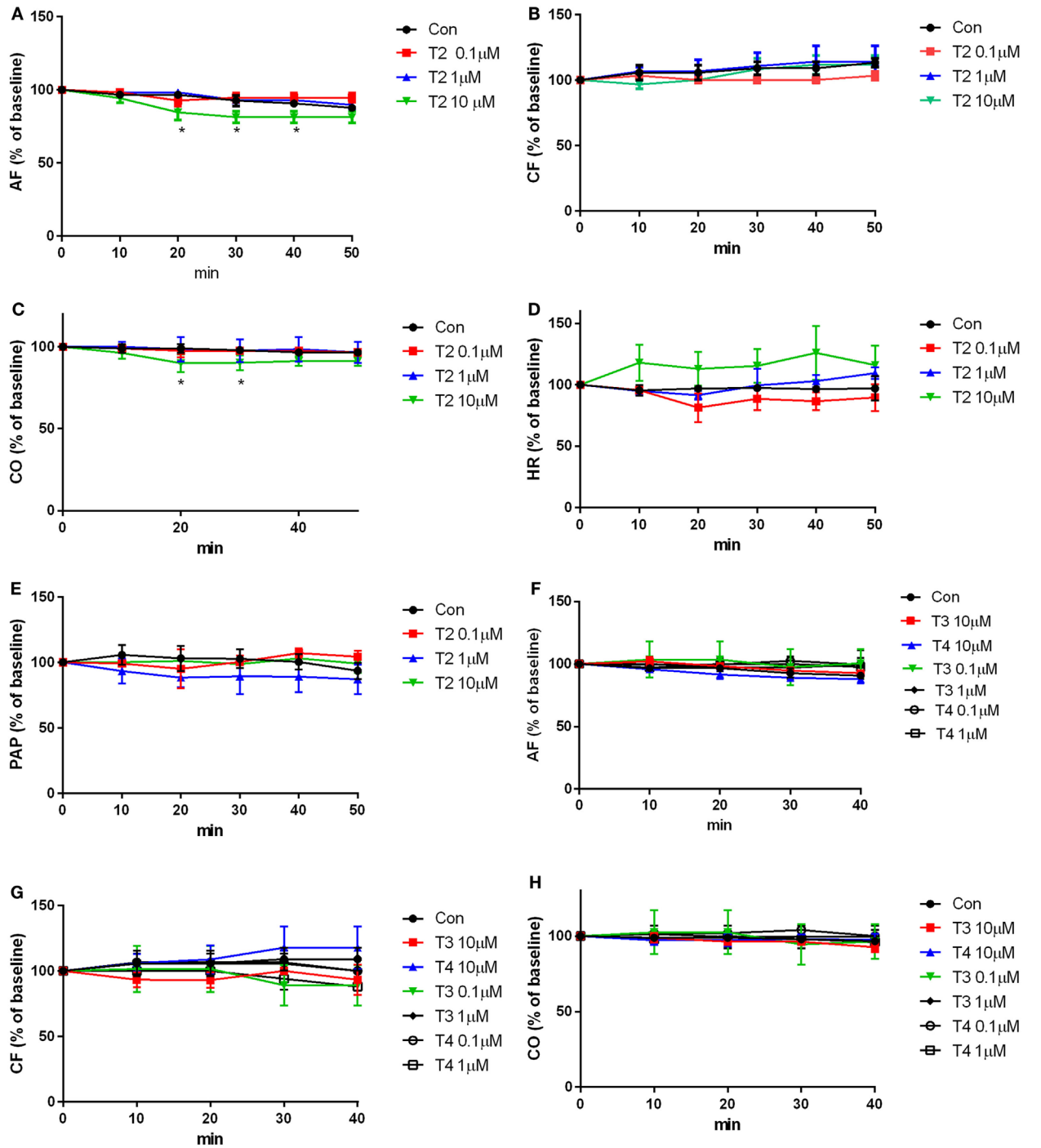

FIGURE 4 | Contractile performance. Heart was perfused for 50 min with buffer containing the vehicle or iodothyronines (0.1–10 μM). Hemodynamic variables were measured every 10 min of perfusion and are expressed as percentage of the basal values, that were measured after 10 min of perfusion, i.e., before any addition to the perfusion buffer supplemented with **(A–E)** T2, **(F–H)** T3, **(F–H)** T4, or vehicle. Results represent mean ± SEM of three hearts per group. [*$P < 0.05$ for the effect of treatment, by two-way ANOVA, Dunnett's *post hoc* test for multiple comparison, *$P < 0.05$ vs control (con), $n = 3$ per groups]. Abbreviations: AF, aortic flow; CF, coronary flow; CO, cardiac output; HR, heart rate; PAP, peak systolic aortic pressure.

hearts, express low concentration of type II deiodinase, which has higher affinity for T3 and T4 than type I deiodinase (22). Although type III deiodinases might be more abundant in cadiomyoblasts, considering their fetal origin and capacity of proliferation (type III deiodinase expression is correlated to proliferative activity and pluripotency) (22), their effect appeared to be negligible. We cannot exclude that T2 catabolism might be balanced by local production, since H9c2 cell line

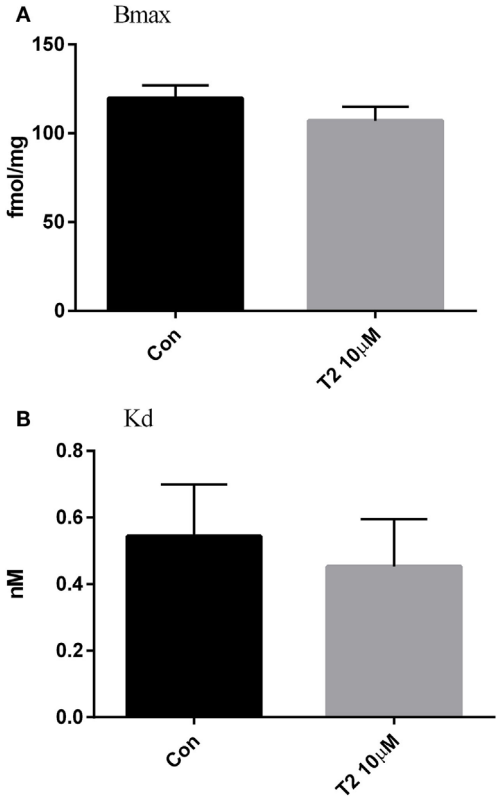

FIGURE 5 | Ryanodine binding. Effect of T2 on the density of ryanodine binding sites [Bmax, fmol/mg, **(A)**] and affinity for ryanodine [Kd, nM, **(B)**] in crude cardiac homogenates. Ryanodine binding was determined in ventricle homogenate after 50 min of perfusion. Histograms represent mean ± SEM derived from three hearts per group. Unpaired *t*-test yielded *P* = ns for the difference between groups.

have been reported to produce low levels of TH in an appropriate environment (23).

The glucose consumption results showed that T2 increased cardiomyocyte glucose uptake, as already demonstrated in skeletal muscle by Moreno et al. (12), who have described an increase in myofiber glycolytic activity. Consistently, T2 has been reported to be able to increase GLUT4 concentrations on plasma membrane, by increasing AKT phosphorylation by insulin (12). This might suggest that the higher glucose uptake was a consequence of an increase in glycolytic flow, even though the hexokinase activity was not influenced by T2, as revealed by enzymatic assay. The latter appears to be a primary metabolic effect, since the hemodynamic variables were not modified, and the energy need for contraction was, therefore, putatively unchanged, as confirmed also by the absence of changes in sarcoplasmic reticulum calcium channels.

As to the underlying molecular mechanism, the relatively short time course of our experiments (1–4 h) does not suggest the occurrence of genomic effects. This is consistent with several previous studies [reviewed by Moreno et al. (24)], and with the observation that T2 has a low affinity for nuclear TH receptors (25), and in any case it is relatively selective for TR beta, while TR alpha is the predominant cardiac isoform (26, 27). On the whole, it is likely that T2 activates short-term mechanisms, acting directly on specific protein targets, possibly located in mitochondria (28).

In vitro viability tests indicated that T2 produced only minor toxic effects upon a chronic (24 h) treatment. MTT staining was slightly but significantly reduced at concentrations exceeding 500 nM. Since the MTT test depends on mitochondrial function, we also used the crystal violet assay, finding out some evidence of T2 toxicity only at the highest concentration (10 μM). As reported (19), crystal violet is a stain for viable adherent cells, able to bind to proteins and DNA. Cells undergoing cell death lose their adherence and are subsequently lost from the population of cells, reducing the amount of staining in cell culture. So, the decreased MTT staining observed at lower concentrations may be related to a reduced oxidative metabolism due to changes in mitochondrial function. On the other hand, the toxic effect was not reproduced by equimolar T3 and T4, and interestingly 10 μM T2, but not equimolar T3 or T4, produced a slight and temporary reduction of the CO in the isolated working rat heart model.

Our experimental findings are consistent with the clinical results reported by Antonelli et al. (14) upon a 4-week treatment (300 μg/day) in healthy volunteers: T2 decreased body weight, and enhanced resting metabolic rate without affecting cardiac contractility. On the contrary, Jonas et al. (15) observed that T2 induced cardiac hypertrophy in mice after 2 weeks of treatment at 2.5 μg/g.

Differences in T2 concentration are a crucial issue, potentially able to account for these discrepancies. The T2 dosages used in previous studies (28–31) were widely different, namely 0.1–100 nM (29), 10 nM (28), 0.01–10 μM (31), or 1–10 μM (30). Different serum levels have also been measured: depending on the assay method applied to quantify T2, its circulating concentration ranged from 20–50 to 400–500 pM (32–38). Higher serum concentrations (50–400 nM) were measured by Jonas et al. (15) in mice treated for 14 days with 0.25–2.5 μg/g T2. Interestingly10 μM T2, the highest dosage reported in *in vitro* studies (30, 31) was the only one for which a slightly cytotoxicity was observed in our cell line.

In conclusion, our findings show that T2 can increase cardiac glucose consumption even though the contractile performance was unchanged. In our model, only minimal toxic effects were observed, at concentrations substantially higher than those associated with metabolic effects. So far, T2 serum concentration has not been accurately assessed, and further investigations will be needed to develop, standardize, and validate appropriate analytical methods. Once endogenous T2 levels are determined, it will be possible to investigate its potential physiological, pathophysiological, or pharmacological relevance for the heart and other tissues.

DATA AVAILABILITY STATEMENT

Datasets are available on request. The raw data supporting the conclusions of the manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

Experimental procedures were approved by the ethical committee of the University of Pisa (protocol no. 51814/2016, for organ

explants). The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996).

AUTHOR CONTRIBUTIONS

GS, MN, and LL designed and carried out cell culture experiments. SF performed the *ex vivo* experiments on heart. AS carried out mass spectrometry measurements. RZ revised the manuscript and performed statistical analysis. SG designed and supervised the experimental work and wrote the manuscript.

FUNDING

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Central Effects of 3-Iodothyronamine Reveal a Novel Role for Mitochondrial Monoamine Oxidases

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3-Iodothyronamine (T1AM) is the last iodinated thyronamine generated from thyroid hormone alternative metabolism found circulating in rodents and in humans. So far, the physiopathological meaning of T1AM tissue levels is unknown. Much is instead known on T1AM pharmacological effects in rodents. Such evidence indicates that T1AM acutely modifies, with high potency and effectiveness, rodents' metabolism and behavior, often showing inverted U-shaped dose–response curves. Although several possible targets for T1AM were identified, the mechanism underlying T1AM behavioral effects remains still elusive. T1AM pharmacokinetic features clearly indicate the central nervous system is not a preferential site for T1AM distribution but it is a site where T1AM levels are critically regulated, as it occurs for neuromodulators or neurotransmitters. We here summarize and discuss evidence supporting the hypothesis that central effects of T1AM derive from activation of intracellular and possibly extracellular pathways. In this respect, consisting evidence indicates the intracellular pathway is mediated by the product of T1AM phase-I non-microsomal oxidation, the 3-iodothryoacetic acid, while other data indicate a role for the trace amine-associated receptor, isoform 1, as membrane target of T1AM (extracellular pathway). Overall, these evidence might sustain the non-linear dose–effect curves typically observed when increasing T1AM doses are administered and reveal an interesting and yet unexplored link between thyroid, monoamine oxidases activity and histamine.

Keywords: 3-iodothyronamine, 3-iodothyroacetic acid, histamine, trace amine-associated receptors, monoamine oxidases

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BACKGROUND

3-Iodothyronamine (T1AM) is the last iodinated thyronamine produced by thyroid hormone (TH) metabolism and supposed to mediate TH non-genomic effects. From its identification in rodent tissues (1), T1AM was included within the family of the trace amines (TAs) and identified as an endogenous ligand for trace amine-associated receptors (TAARs), a family of constitutively active G protein-coupled receptors with species and tissue-specific expression cross-talking with the dopaminergic and the serotoninergic transmission (2, 3). This latter feature raised the interest around TAAR agonists, and then on T1AM, as potential treatments for controlling craving, pain, and neuropsychiatric illnesses. Consistently, the determination of endogenous T1AM brain levels or T1AM pharmacological administration would play diagnostic or therapeutic roles,

respectively, in clinical manifestations of diseases involving dopamine release. However, after more than 10 years from T1AM discovery, whether central effects described following pharmacological administration of T1AM can be ascribed to TAAR activation remains an open issue, and we have no indications of T1AM levels in psychiatric or in drug-abusing patients. An important contribution to this uncertainness derives from the lack of pharmacological antagonists of TAARs with favorable pharmacokinetic features for *in vivo* administration. Instead, based on the current knowledge, T1AM presents a puzzling pharmacological profile suggesting a role as a cell messenger, behaving as a hormone and/or a neuromodulator, a function likely mediated also by 3-iodothryoacetic acid (TA1), the product of T1AM non-microsomal phase-I metabolism. T1AM may also interact with high-affinity targets including G protein-coupled receptors as TAAR1, or ion channels (4–6). We here summarize and discuss evidence indicating that behavioral effects of T1AM deserve to receive the attention of the investigators since, in the central nervous system, the pharmacokinetic features of this amine include a novel role for mitochondrial monoamine oxidases (MAO).

T1AM PHARMACOKINETIC

Few Notes on Tissue Distribution

Consisting evidence indicates that the tandem mass spectrometry represents the most reliable method to determine T1AM tissue levels, whereas some difficulties remain for measuring T1AM plasma levels (7) because T1AM administered to rodents circulated almost completely bound reversibly and with high affinity to Apo-B100, the main protein fraction of LDL and VLDL lipoproteins (8). This evidence indicated that T1AM has a specific plasma protein carrier, a condition usually reserved to hormones and vitamins and controlling amine pharmacokinetic.

LDL and VLDL are physiologically implicated in regulating the homeostasis of triglycerides and cholesterol, including their intracellular transport, by activating respective receptors (VLD-R and LDL-R), which are expressed almost ubiquitously but concentrated in the liver. Apo-B100 is the determinant for the recognition of lipoprotein receptors, their endocytosis, and recycling from plasma membrane. According to Roy et al. (8), the interaction of VLDL or LDL with their respective hepatic receptors represents a highly effective mechanism for the intracellular transport of T1AM. Once inside cells, T1AM, as well as cholesterol and triglycerides, may activate their own intracellular targets. In particular, since triglycerides may activate PPAR alpha while oxysterols the liver X receptor, genomic effects of T1AM may also represent epiphenomena derived from the activation of these targets (9, 10). Chiellini et al. (11) produced indications about T1AM distribution in mouse tissues. Following i.p. administration of [125I]-T1AM, the radioactivity recovered in tissues confirmed the liver and the gallbladder were preferential sites of T1AM distribution, reinforcing the link between T1AM, lipoproteins, and their receptor signaling, while the adipose tissue and skeletal muscle were described as sites where T1AM accumulated. Likewise, a small percentage of the radioactivity administered was recovered in the brain where such percentage could be even overestimated due to the accumulation of radioactive metabolites of T1AM. If we consider that circulating T1AM is almost completely bound to ApoB-100, it becomes relevant to understand how this amine can cross the blood–brain barrier (BBB) and if this passage represents a limit for T1AM degradation (**Figure 1**).

The BBB consists of a specialized network of fenestrated capillaries in tight relation with astrocytes projecting to neurons, working as a physical barrier to the passage of humoral factors, including cholesterol and other lipids. Cholesterol flux is directed by lipoproteins but only the high-density lipoproteins can traverse the BBB. However, apolipoproteins, including Apo-E, are present in brain areas and in the cerebrospinal fluid (12). In addition, since endothelial cells express Apo-B100 (13), it can be argued apolipoproteins can be synthetized in brain areas. In this respect, astrocytes, which also express high levels of VLDL-R and LDL-R (14), have been indicated as the most likely candidate cell for apolipoprotein synthesis. Overall, the presence of apolipoproteins and of their receptors suggests that apolipoproteins may have functions in the brain. Consistently, evidence indicated lipoproteins can stimulate the cognitive function in the hippocampus and regulate energy balance in the hypothalamus (14).

3-Iodothyronamine is physiologically present in mouse thyroid, and it distributes in the gland following pharmacological administration. In the thyroid, the half-life of T1AM was the highest among the tissues analyzed (11).

Manni et al. (15) demonstrated that, following i.c.v. injection of T1AM, this amine was recovered in the systemic circulation, thus indicating it can pass but also exit the BBB. Interestingly, T1AM systemic bioavailability significantly increased when its oxidative metabolism was inhibited, i.e., mice pretreated with clorgyline, a mitochondrial MAO inhibitor. Consistently, Laurino et al. (16) demonstrated T1AM disappearance from the medium of cultured organotypic hippocampal slices associated with the contemporaneous accumulation of TA1 and that that, in mouse brain, T1AM pharmacologically administered is metabolized to TA1.

T1AM Metabolism

For its chemical structure, T1AM has the requisites to be a substrate for amine oxidases including MAO (17), ubiquitous enzyme activities, and semicarbazide-sensitive amine oxidases (SSAOs), a heterogeneous class of enzymes localized on cell plasma membranes. Two isoforms of MAO have been cloned, i.e., MAO-A and MAO-B (18) with MAO-A mainly present in catecholaminergic and MAO-B in serotonergic and histaminergic neurons and astrocytes (19, 20).

Both MAO and SSAO are included within the pattern of non-microsomal phase-I metabolizing enzymes. Due to the absence of SSAOs in the brain, T1AM oxidative metabolism at this site can be carried on only by MAO. Lehmphul et al. (17) first demonstrated T1AM is a substrate for MAO-B opening to a new function for MAO-B (21). Furthermore, the involvement of MAO catalysis in T1AM degradation implies physiopathological and pharmacological consequences. In fact, the extent of T1AM degradation would depend on MAO-B tissue expression levels and on pathological conditions altering MAO-B promoter

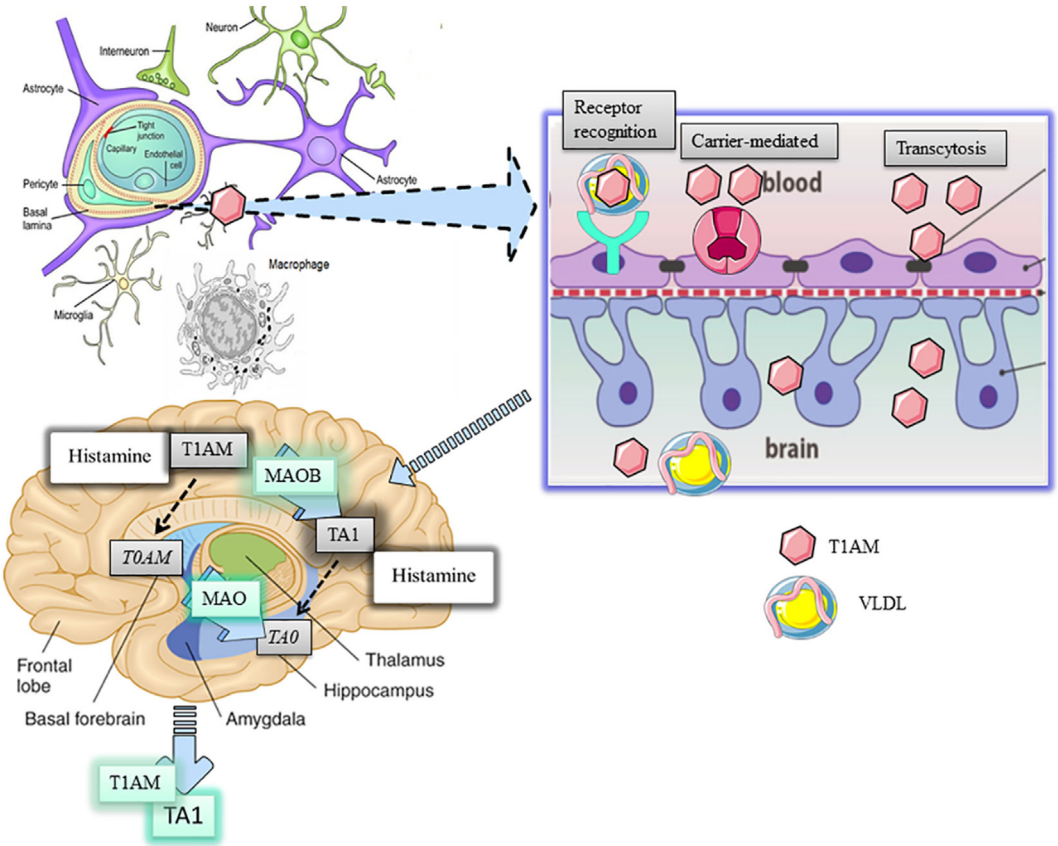

FIGURE 1 | Distribution and metabolism of 3-iodothyronamine (T1AM) in the brain. T1AM is physiologically present in the brain of mice and its levels increse after pharmaoclogical administrtaion thus indicating T1AM can pass the blood brain barrier (BBB). The mechanism of T1AM passage through the BBB remains elusive yet. Three possible hypotheses are presented, including endocytosis of lipoprotein receptor, carrier-mediated transport, or transcytosis of free T1AM. One inside the brain, T1AM is manly metabolized to 3-iodothyroacetic acid (TA1) by the activity of mitochondrial monoamine oxidases (MAO).

activity, including diabetes, inflammation, thyroid diseases, or aging. Secondarily, T1AM oxidation is expected to be modified by treatments including inhibitors of MAO-B as those used in the control of the early symptoms of Parkinson's disease (18).

In addition, since MAO catalysis produces hydrogen peroxide, reactive aldehydes, and ammonia, the degradation of T1AM by MAO-B potentially alters the cell redox state (19). The impact of T1AM degradation on neuron redox state might represent a novel and interesting issue to explore.

Furthermore, the fact that in the thyroid a very high mitochondrial MAO-A, and low MAO-B, is present (22) might account for the long half-life of the amine in the gland.

The Effects of T1AM Suggests the Amine Behaves as a Hormone or as a Neuromodulator

Experimental data indicate T1AM is a potent and effective modifier of the behavior and of the metabolism of rodents. Such effects onset within 15–30 min from amine administration and often show inverted U-shaped dose–effect curves. This characteristic suggests increasing T1AM doses (i) rapidly induce desensitization of a target(s) or (ii) recruit different targets controlling opposite effects. Whatever the mechanism, the inverted dose–effect curve indicates it is possible to predict the dose of T1AM that needs to be administered to observe a desired effect (**Figure 2**) and stresses the importance to study T1AM at its lowest effective dose.

Furthermore, the acute central effects of T1AM are followed by long-term effects based on the activation of gene transcription. Whether these effects are driven directly by T1AM, TA1, or indirectly by T1AM or TA1-induced release of neurotransmitters is unknown yet. Considering the current knowledge, histamine has been identified as one of the neurotransmitters involved in T1AM effects.

Histamine in the brain accumulates in mast cells, astrocytes (23), and in the few histaminergic neurons that are concentrated in the hypothalamus from which they project to most of the brain areas including the hippocampus and cortex (24). Neuronal histamine is implicated in memory, control of the sleep/wake cycle, feeding, thermoregulation, motility, and it is considered an endogenous neuroprotective mediator against excitotoxicity (25, 26). Histamine in the brain is mainly degraded by *N*-methyl transferase producing *N*-methylhistamine (27) which is, in turn, a good substrate for MAO-B. Consequently, histamine and T1AM may compete for MAO-B catalysis.

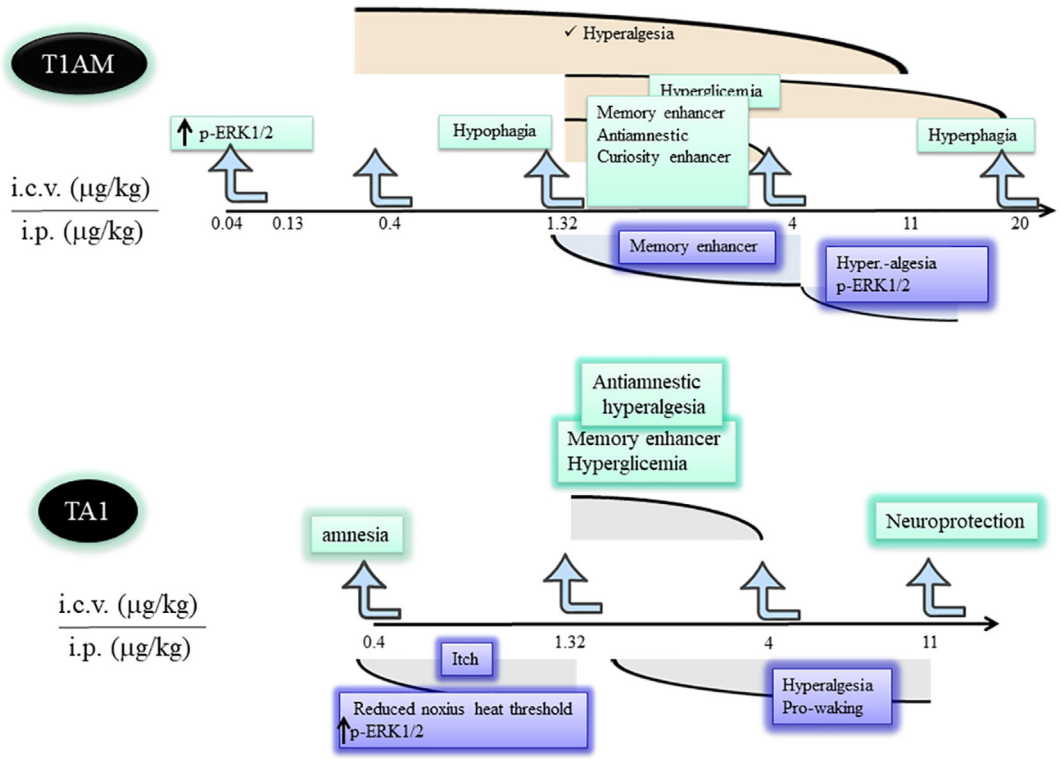

FIGURE 2 | Differences and similarities between T1AM and TA1 pharmacological effects. T1AM and TA1 share pharmacological effects that are described by inverted dose–effect curves. The cartoon indicates similarities and differences among central effects of equimolar doses of T1AM and TA1. Abbreviations: T1AM, 3-iodothyronamine; TA1, 3-iodothyroacetic acid; T0AM, thyronamine; MAO, monoamine oxidases.

Hypothalamic Effects of Pharmacologically Administered T1AM

Scanlan et al. (1) first described that high T1AM doses (50 mg/kg) induced a deep reduction of mice body temperature. Later, Doyle et al. (28) demonstrated T1AM rescued neurons from excitotoxicity by inducing hypothermia in an experimental model of cerebral ischemia. Some years later, James et al. (29) clarified that the hypothermia was secondary to increased heat dispersion caused by tail vasodilation (29) and indicated T1AM as a hypothalamic regulator of the temperature set point of warm–sensitive neurons. This mechanism might also explain the potent reduction of pain threshold to hot stimuli described after T1AM administration in mice (15).

Dhillo et al. (30) reported that T1AM reduced feeding of *ad libitum*-fed mice, and Ju et al. (31) described the amine shifted energy substrate utilization from glucose to lipids. Overall, this evidence contributed to delineate T1AM as a compound inducing a hypometabolic status. However, Manni et al. (15) unmasked the hyperphagic effect of the amine. In fact, the authors found that, at condition of fasting, i.c.v. injected low T1AM doses (1.3 μg/kg) still induced hypophagia and rapidly increased plasma glucose. Instead, injection of higher T1AM doses induced hyperphagia without raising plasma glycemia. Because of this, T1AM can be considered as a potent hypophagic compound able to counteract the fasting-induced tendency of mice to overfeeding. This effect is likely sustained by the increase of plasma glycemia. Consistently, at doses producing hyperphagia, plasma glycemia was not modified. In any case, the raise of plasma glycemia was a hypothalamic-mediated effect and it went in the opposite direction compared with the anti-hyperglycemic effect described for other TAs (32).

In addition, Manni et al. (15) demonstrated, for the first time, that T1AM oxidative metabolism was essential for T1AM hyperphagia. In fact, in mice pretreated with clorgyline, an inhibitor of MAO activity, hyperphagia disappeared while the hypophagic effect remained. Then, hypophagia could derived from the recognition of a high-affinity target (extracellular pathway), while hyperphagia depended on some products of T1AM oxidative deamination, including TA1 (intracellular pathway).

James et al. (29) reported that T1AM stimulated mice wakefulness when injected in the preoptic region, confirming the activity on hypothalamus. Furthermore, T1AM injected i.c.v. or systemically administered induced hyperalgesia to hot stimuli (33). The stimulation of peripheral sensory neurons translated in pERK1/2 of the interneurons of dorsal root ganglia (DRG), thus involving the spinothalamic pathway in the transmission of pain transmission (34).

T1AM Activity at Hippocampus

Among the main interesting effects elicited by T1AM is its activity on memory circuits possibly indicating some effectiveness of the amine in diseases characterized by memory impairments (35).

3-Iodothyronamine given i.c.v. or systemically, increased pERK1/2 and pAKT levels in different brain areas, including the hippocampus and hypothalamus (33), it acutely produced an increase in mice exploratory activity of learning and reverted drug-induced amnesia (36). Furthermore, T1AM not only stimulated learning but also consolidated memory in the 24-h training session (33, 34) an effect consistent with pERK1/2, a master regulator of *de novo* synthesis of proteins necessary for memory consolidation. Interestingly, stimulation of learning was not observed in mice pretreated with clorgyline and with anti-histaminergic treatments. This evidence confirmed the role of MAO-B catalysis in the generation of a pro-learning compound and indicated the involvement of the histaminergic system (5, 34).

Interestingly, recent *in vitro* evidence obtained in isolated striatal slices gave indication on a receptor-mediated mechanism of T1AM. In particular, Zhang et al. (37) demonstrated T1AM, by activating TAAR1, promoted phosphorylation of thyroxine hydroxylase, thus prompting the synthesis of dopamine and its release. Interestingly enough, in the striatum, the main MAO isoform expressed is type B, which is the enzyme involved in T1AM (but also dopamine) degradation (20). Furthermore, other recent evidence also indicates that the release of dopamine in the striatum may be controlled by histamine released from the tuberomammillary nucleus, *via* H1 and H2 receptors (38). Overall, these results suggest T1AM might sustain dopamine synthesis and release acting at high-affinity targets (TAARs) and, indirectly, releasing histamine from the hypothalamic neurons. Both mechanisms might sustain an effectiveness of T1AM in basal ganglia diseases.

Furthermore, T1AM was also indicated as an activator of the serotoninergic type 1 subtype b receptor in virtue of heterodimerization of this receptor with TAAR1 (39), a finding that suggests possible antidepressant effectiveness of the amine.

However, the effects of T1AM on dopamine release and on the activation of the serotonin receptor need to be conclusively demonstrated in *in vivo* settings.

SIMILARITIES AND DIFFERENCES IN BEHAVIORAL EFFECTS INDUCED BY PHARMACOLOGICAL ADMINISTRATION OF T1AM AND TA1

If TA1 is T1AM active principle, then the pharmacological administration of TA1 should reproduce at least some of the effects described for T1AM. This assumption does not consider that TA1 pharmacologically administered might not have the same pharmacokinetic features of the acid produced *in situ* by T1AM deamination. Among these differences, TA1 pharmacokinetic does not include the activity of MAO.

Musilli et al. (40) studied the effect of TA1 i.c.v. injected on memory, pain, and plasma glycemia. This study indicated TA1 effect on memory was more composite than that of T1AM. In fact, at a low dose (0.4 μg/kg) the acid produced amnesia while at 1.32 and 4 μg/kg it stimulated learning without inducing memory consolidation. Interestingly, only the pro-learning effect was prevented by anti-histaminergic treatments. In particular, in the presence of zolantidine, TA1 turned out to be amnestic at all the doses tested. These differences in respect of T1AM effects on memory might relay on the pharmacokinetics of the acid but definitively prove that activation of the histaminergic system is triggered by TA1. However, TA1 present in the synaptic cleft may interact also with its own targets involved, for instance, in the amnestic effect. At the same pro-learning doses, TA1 induced hyperalgesia and hyperglycemia showing inverted U-shaped dose–effect curves (41) (**Figure 2**). Hyperalgesia and hyperglycemia were again modulated by anti-histaminergic drugs.

Hoefig et al. (42) reported that TA1 i.p. administered to mice at doses much higher (5 mg/kg) than those used by Manni et al. (15), did not induce hypophagia, hyperglycemia, and hypothermia thus confuting the hypothesis TA1 was the mediator of T1AM hypothalamic effects. Moreover, before concluding on this, it is noteworthy to recall that T1AM was not able to induce hyperglycemia when administered at high doses and the hypophagic effect of T1AM was found independent of MAO inhibition (15). Collectively, these data instead reinforced the hypothesis that T1AM and TA1, pharmacologically administered, have the possibility to activate their own target but that they share a common intracellular mechanism.

Laurino et al. (41) indicated that TA1, systemically administered, deeply affected mouse sensitivity to noxious and painful stimuli. As for T1AM, the reduction of pain threshold resulted to be dependent on histamine release and associated with ERK1/2 activation in DRG neurons.

3-Iodothryoacetic acid, as T1AM, systemically administered, increased mouse vigilance. In particular, TA1 stimulated the wakefulness of mice induced to sleep by a high ethanol dose (43), without interfering with the GABaergic transmission.

TA1 Mechanism of Action: Possible Hypotheses

How TA1 activates the histaminergic system remains to be elucidated. In this respect, it is unlikely that TA1 works as a histamine type 3 receptor antagonist since it lacks the necessary chemical requisites to recognize the receptor. From our study, we can also exclude TA1 may interact at muscarinic and GABA-A receptors (43).

The study from Bellusci et al. (34) indicated the chemical requisite for activating the histaminergic system. In particular, synthetic analogs of T1AM, lacking the iodide atom and the diphenoxyl moiety, were reported to stimulate, as T1AM, memory acquisition and retention depending on MAO activity and on H1 receptor activation. Such results indicated that (i) the presence of the iodide and of the diphenoxyl moiety are not essential for activating the histaminergic system and (ii) the activation of the histaminergic system is secondary to intracellular accumulation of an acid compound that stimulates histamine release.

CONCLUSION

Pharmacological evidence indicates T1AM activates signaling pathways in different brain areas including the hypothalamus and the hippocampus, reproducing in rodents' behavioral effects typically mediated by these brain areas. Such behaviors are dependent

on T1AM metabolism carried on by phase-I non-microsomal enzymes, producing TA1, and on the activation of the histaminergic system-mediated accumulation of TA1.

Furthermore, T1AM may also interact with high-affinity targets on plasma membrane, including TAAR1. Of these two mechanisms, one may prevail over the other depending on T1AM doses or on the effect studied.

The study of T1AM pharmacological profile allowed to reveal a novel and yet unexplored link between T1AM, MAO (MAO-B), and the histaminergic system.

AUTHOR CONTRIBUTIONS

EL and AL participated in performing experiments discussed in this review. In addition, they participated with LR in paper construction.

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Thyroid Hormones, Thyromimetics and Their Metabolites in the Treatment of Liver Disease

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The signaling pathways activated by thyroid hormone receptors (THR) are of fundamental importance for organogenesis, growth and differentiation, and significantly influence energy metabolism, lipid utilization and glucose homeostasis. Pharmacological control of these pathways would likely impact the treatment of several human diseases characterized by altered metabolism, growth or differentiation. Not surprisingly, biomedical research has been trying for the past decades to pharmacologically target the 3,5,3′-triiodothyronine (T3)/THR axis. *In vitro* and *in vivo* studies have provided evidence of the potential utility of the activation of the T3-dependent pathways in metabolic syndrome, non-alcoholic fatty liver disease (NAFLD), and in the treatment of hepatocellular carcinoma (HCC). Unfortunately, supra-physiological doses of the THR agonist T3 cause severe thyrotoxicosis thus hampering its therapeutic use. However, the observation that most of the desired beneficial effects of T3 are mediated by the activation of the beta isoform of THR (THRβ) in metabolically active organs has led to the synthesis of a number of THRβ-selective thyromimetics. Among these drugs, GC-1, GC-24, KB141, KB2115, and MB07344 displayed a promising therapeutic strategy for liver diseases. However, although these drugs exhibited encouraging results when tested in the treatment of experimentally-induced obesity, dyslipidemia, and HCC, significant adverse effects limited their use in clinical trials. More recently, evidence has been provided that some metabolites of thyroid hormones (TH), mono and diiodothyronines, could also play a role in the treatment of liver disease. These molecules, for a long time considered inactive byproducts of the metabolism of thyroid hormones, have now been proposed to be able to modulate and control lipid and cell energy metabolism. In this review, we will summarize the current knowledge regarding T3, its metabolites and analogs with reference to their possible clinical application in the treatment of liver disease. In particular, we will focus our attention on NAFLD, non-alcoholic steatohepatitis (NASH) and HCC. In addition, the possible therapeutic use of mono- and diiodothyronines in metabolic and/or neoplastic liver disease will be discussed.

Keywords: T3, T2, thyromimetics, thyroid hormone receptor, NASH, hepatocellular carcinoma, regenerative medicine, liver cell proliferation

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THYROID HORMONE T3 AND LIVER DISEASE

Thyroid hormones (THs), in particular 3,5,3'-triiodo-L-thyronine (T3), have long been recognized to regulate multiple physiological processes, including fetal development, cell growth, homeostasis, as well as carbohydrate, lipid, protein, and mineral metabolism (1). Through the years, numerous effects of the thyroid hormone excess (a result of administration of exogenous TH or excessive activity of the thyroid gland) on lipid metabolism have been reported, such as increased metabolic rate, weight loss, lipolysis, lowering of serum cholesterol levels; in addition, the improvement in myocardial contractility has been described (2). Although the physiological actions of TH affect almost every organ, liver is one of the most important targets of TH. In fact, alteration of cellular TH signaling has been reported to cause liver-associated diseases, such as non-alcoholic fatty liver disease (NAFLD) and hepatocellular carcinoma (HCC), major health problems worldwide (3). NAFLD, which includes two pathologically distinct conditions associated to different prognoses—non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH)—is the most common liver disorder in Western countries, affecting 17–46% of adults (4–6). In particular, the acronym NASH, coined by Ludwig et al. (7), describes a liver disease that histologically mimics alcoholic hepatitis but occurs in individuals whose alcohol consumption is nil or negligible. The diagnosis of NASH provides important prognostic information and indicates an increased risk of fibrosis progression, cirrhosis and HCC (6). Despite its high prevalence and potentially serious consequences, so far no effective drug treatment for NASH has been provided. In this regard, exogenous T3 administration showed encouraging results in lowering hepatic fat content in various experimental models of NAFLD. A nutritional model often utilized in NAFLD studies is the choline-devoid methionine-deficient (CMD) diet that results in massive hepatic accumulation of triglycerides and liver injury with close pathological and biochemical similarities to human NASH (8, 9). In this model, T3 completely prevented the development of hepatic steatosis by increasing fatty acid mitochondrial and peroxisomal β-oxidation and decreasing the expression of liver-type fatty acid-binding protein (L-FABP) (10). Most important, T3 also promoted regression of pre-existing fatty change. The disappearance of hepatic triglycerides was associated with a strong decrease of lipid peroxidation, cyclooxygenase-2 (COX-2) expression, phospho-signal transducer and activator of transcription 3 (phospho-STAT3) and phospho-stress-activated protein kinase/c-Jun NH2-terminal kinase (phospho-SAPK/JNK) levels (10), usually activated in inflammatory processes (11). The following analysis of the effects of T3 on lipid homeostasis *in vivo*, its effects on plasma free fatty acid (FFA) levels, hepatic gene expression, and mitochondrial respiration rates, evaluated in Sprague-Dawley rats, demonstrated that, in the liver, T3 reduced the mRNA levels of sterol regulatory element binding protein-1c (SREBP-1c) and apolipoprotein C3 (ApoC3) and increased apolipoprotein A1 (ApoA1), peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) levels (12). Moreover, mRNA levels of carnitine palmitoyltransferase-1 (CPT-1), which catalyzes an essential step for the mitochondrial uptake of long-chain fatty acids and their subsequent β-oxidation in the mitochondria (13), tended to be increased following treatment with T3. Further, a recent report revealed that T3 decreases hepatic fat content in the livers of rats fed high fat diet (HFD); this effect was associated with a concomitant increase of CPT-1 protein levels (14). On the other hand, T3 did not lower triacylglycerol levels in ob/ob mice; this was likely due to the relatively low dose used in this study as chronic T3 administration at higher doses was poorly tolerated in these animals. Moreover, a pronounced increase in heart weight was found in T3-treated animals (12). In the attempt to clarify the mechanisms underlying the anti-steatotic capacity of T3, some studies on T3-regulated cellular pathways that lead to the generation of free fatty acids from stored lipid droplets in the liver, suggested that TH stimulation of fatty acid β-oxidation is also coupled with induction of hepatic autophagy to deliver fatty acids to mitochondria (15). With regard to human data, a recent report, presenting an example of a patient with NASH complicated by Graves' disease, indicated that hyperthyroidism may improve the pathological condition of NASH (16). Besides obesity, NAFLD is also associated with another manifestation of the metabolic syndrome, the insulin resistance (17). Although the cellular mechanism underlying this association is still not fully elucidated, it is believed that by products of the *de novo* lipogenesis, that is up-regulated in NAFLD, favor the hepatic insulin resistance (18). Several investigations with animal models of NAFLD clearly indicate that reduction in fatty liver results in an amelioration of insulin resistance. These experimental evidences suggest that TH, or their analogs, may elicit an insulin sensitizing and antidiabetic effect by reducing hepatic fat accumulation.

Although experimental evidences indicate that T3 actions on hepatic metabolism are mediated exclusively by peripheral actions, either through membrane or nuclear receptors, a growing interest for the study of possible central mechanisms has recently emerged. In particular, Alvarez-Crespo and colleagues have demonstrated that chronic central infusion of T3 significantly affects thermogenesis and adipose tissue activity (19). Although the effects on hepatic metabolism was not investigated, it is possible that similar central effects of T3 on liver also exist.

As previously mentioned, the diagnosis of NASH is associated with an increased risk of HCC (6). It follows that reversal of fat accumulation/liver damage may reduce HCC development. In view of the positive results of T3 on NASH, the effects of T3 administration have been also investigated in experimental models of HCC. Significantly, this tumor type represents the second cause of global cancer-related deaths (20, 21). Since the efficacy of traditional pharmacological therapies and

Abbreviations: HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PH, partial hepatectomy; R-H, Resistant-Hepatocyte; T3, 3,5,3′-triiodo-L-thyronine; THR, Thyroid hormone receptor.

of new developed drugs, such as the multikinase inhibitors sorafenib and regorafenib, and their ability to produce a significant survival benefit remains still questionable (22), there is an urgent need to develop novel therapies for HCC. Several studies collectively indicate that disruption of TH signaling is involved in the development of HCC (23–25). Interestingly, *in vivo* studies showed that a short treatment with T3 accelerated the regression of chemically induced hepatic pre-neoplastic lesions in rats subjected to the Resistant-Hepatocyte (R-H) model of hepatocarcinogenesis (26). This experimental model consists of hepatocyte initiation through a single dose of the carcinogen diethylnitrosamine (DENA) and promotion of initiated hepatocytes through a short-term exposure to 2-acetylaminofluorene (2-AAF) combined with 2/3 partial hepatectomy (PH) (27). Using this protocol, the authors observed that only 50% of rats exposed to repeated cycles of T3 developed HCC, with no sign of lung metastasis (26). An anti-preneoplastic effect of T3 was also observed in the CMD nutritional model (28). T3 has been also recognized as a strong inducer of liver cell proliferation in rats and mice (29–31), which performs its hepatomitogenic effect in the absence of activation of transcription factors, such as AP-1, NF-κB or STAT3, associated with an increased expression of *c- fos, c-jun,* or *c-myc* proto-oncogenes and an increase in the mRNA and protein levels of cyclin D1 (31). The potent hepatomitogenic effect of T3 was demonstrated not only for intact liver, but also during the regenerative response in rodents after 70% partial or 90% subtotal hepatectomy (32–35). These results strongly indicate the possible therapeutic use of T3 in conditions characterized by an impaired regenerative ability (such as aged livers) or when a rapid growth stimulation of the liver is required.

DELETERIOUS EFFECTS OF T3

The above mentioned beneficial effects of T3 treatment on liver disease are unfortunately counterbalanced by harmful effects on the heart, muscle and bone, as increased T3 levels are known to induce thyrotoxicosis marked by tachycardia, arrhythmia, muscle wasting, and also reduced bone mineralization and alteration of central nervous system development (36, 37). Since all these actions hamper the potential therapeutic use of T3, successive attempts were aimed at identifying THs derivatives, with the purpose of reaping the benefits from T3 treatment by separating the therapeutic actions of T3 from its deleterious effects. The demonstration that the antitumoral effect of T3 is associated with an activation of the THR-mediated pathway in hepatocytes clearly indicates that the effect is mediated by a peripheral action of T3.

THYROMIMETICS, THRβ LIGANDS, AND LIVER-TARGETED TH AGONISTS

TH exert their physiological effects by binding to specific nuclear receptors (38–41), the thyroid hormone receptors (THR) α and β, that exhibit conspicuous differences in expression pattern. The β isoform is the major THR expressed in the liver, whereas the α isoform is particularly abundant in the heart (38, 39). The observation that most of the desired therapeutic effects of T3 in the liver are mediated by the activation of the isoform β of THRs and that THR activation in extrahepatic tissues leads to altered cardiovascular function and thyroid hormone axis (THA) suppression, has led to the synthesis of a number of THRβ- and organ-selective (liver-selective) thyromimetics. Although isoform selective agonists of the THRs have been developed primarily for the treatment of hypercholesterolemia, as an alternative strategy to 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase) inhibitors (known as statins) (42, 43), their use has been also proposed for the treatment of several metabolic disorders, including NAFLD and even HCC. In the last 20 years, an improved understanding of THR structure and function has led to the development of several potent thyromimetics, including GC-1, GC-24, KB141, KB2115, and MB07344, the active form of the prodrug MB07811(43–45).

GC-1 (3,5-dimethyl-4 (4′-hydroxy-3′- isopropylbenzyl)-phenoxy) acetic acid, also known as Sobetirome), the first THRβ agonist to be generated and a scaffold compound for the development of other TH derivatives, has been synthetized in 1998. The structural changes presented by GC-1, with respect to the natural hormone T3, allowed GC-1 to demonstrate comparable affinity for THRβ1 to that of T3 and a 10-fold lower affinity to THRα1 compared with T3 (46). Successively, GC-1 has been described to combine both, organ- and THRβ1-selectivity, which enhanced hepatic targeting (47). Importantly, in the same study, GC-1 did not elicit harmful effects on heart weight, heart rate and mRNAs coding for proteins related to cardiac contraction, such as myosin heavy chain α (MHCα), MHCβ and sarcoplasmic reticulum calcium adenosine triphosphatase (Serca2) (48). With regard to the liver disease, Perra *et al.* (10), employing the CMD diet, demonstrated that, the therapeutic effects of T3 on NAFLD were shared by GC-1, which prevented the development of hepatic steatosis and promoted regression to pre-existing fat accumulation in the same model. The reduction in hepatic triglycerides was accompanied by a concomitant decrease of lipoperoxidation (10). GC-1 ameliorated hepatic steatosis also in other animal experimental models, such as ob/ob mice and Western diet-fed LDLR$^{-/-}$ mice (49). Furthermore, GC-1 treatment prevented the development of hepatic steatosis in rats placed on high fat diet; however, at the same time, it caused hyperglycemia and insulin resistance (50). With regard to tumorigenesis, a 14-day treatment with GC-1 caused an almost complete disappearance of hepatic pre-neoplastic lesions in rats subjected to the R-H model of hepatocarcinogenesis (28). Similar to what observed with T3, following GC-1 treatment, the induction of a preneoplastic hepatocyte differentiation program preceded the loss of the preneoplastic lesions, as confirmed by a progressive loss of fetal markers such as the placental form of glutathione S-transferase (GST-P) and gamma glutamyl transpeptidase (GGT) and reacquisition of glucose 6-phosphatase (G6Pase) and adenosine triphosphatase (ATPase), two proteins expressed in normal differentiated liver. An anti-preneoplastic effect of GC-1 was also reported in DENA and CMD diet fed rats (28). These results, together with the finding that the isoform β of THRs is profoundly down-regulated

in rat preneoplastic lesions and in rat and human HCC (51), strongly suggest that reactivation of the TH-THR axis may have a strong impact on HCC progression. Another evidence highlightening GC-1 as an attractive candidate in HCC therapy stems also from a recent study (52). By exploiting a model of HCC driven by the co-expression of S45Y-β-catenin and hMet using SBTT and HTVI (53), the authors found that GC-1 exerted a notable anti-tumoral effect by decreasing tumor burden without affecting β-catenin and its downstream targets, therefore, demonstrating its safety for use in chronic liver diseases (52).

Columbano and co-workers demonstrated that GC-1 mimics also the effect of T3 as a powerful inducer of hepatocyte proliferation in rats and mice (54, 55). Furthermore, pre-treatment of mice with GC-1, prior to partial hepatectomy, caused a significant increase in hepatocyte proliferation, suggesting that GC-1 administration confers a regenerative advantage following 2/3 PH (56). In the light of these results, GC-1 might result beneficial also in the area of hepatic regenerative medicine.

The nineties witnessed also the development of GC-1 derivative, GC-24 (57). Although an important improvement in key metabolic parameters, such as plasma triglycerides, body fat, glucose tolerance, and insulin sensitivity were achieved with GC-24 treatment without affecting cardiac weight in rats fed a high-fat diet, GC-24 treatment did not manage to restore hypercholesterolemia, increased hepatic cholesterol content, elevated non-esterified free fatty acids (NEFA) and IL6 levels (58).

Although another THRβ1 agonist, KB141 [3,5-dichloro-4-(4-hydroxy-3-isopropylphenoxy) phenylacetic acid], was successively identified yielding promising results in preclinical animal models, such as significant cholesterol, lipoprotein (a), and body-weight reduction with minimal cardiac side effects, it has not been pursued for human use (45, 59). Moreover, in contrast to GC-1, it was observed that it did not accumulate preferentially in the liver (60, 61).

More recently, novel THR-selective analogs have been reported in the literature, namely KB2115 (Eprotirome, 3-((3,5-dibromo-4-(4-hydroxy-3-(1-methylethyl)-phenoxy)-phenyl)-amino-3-oxopropanoic acid) and MB07811 (2*R*,4*S*)-4-(3-chlorophenyl)-2-[(3,5-dimethyl-4-(4′-hydroxy-3′-isopropylbenzyl)phenoxy)methyl]-2-oxido-[1,3,2]-dioxaphosphonane). KB2115, a compound containing two bromides, was shown to have minimal uptake in non-hepatic tissues, to be more liver-specific than GC-1 and do not provoke deleterious effects on the heart (62, 63). In order to determine whether KB2115 would be a more effective therapy for NAFLD and hepatic insulin resistance compared with GC-1, the effects of KB2115 treatment were evaluated in high-fat diet-fed male Sprague-Dawley rats. Similar to what observed with GC-1, KB2115 treatment effectively prevented the development of hepatic steatosis in this animal model. Unlike GC-1 administration, KB2115 treatment did not lead to fasting hyperglycemia, although it still resulted in fasting hyperinsulinemia. Moreover, in contrast to GC-1, KB2115 treatment did not cause a significant change in glycerol turnover, or in plasma non-esterified fatty acid concentration (50). Similar to GC-1, KB2115 was able to reduce the burden of hepatic steatosis also in ob/ob mice, markedly reducing hepatic triglyceride levels (49). Furthermore, KB2115 shares the hepatomitogenic activity of T3 and GC-1 in the absence of significant signs of liver toxicity, indicating its utility for regenerative therapies in liver transplantation or other surgical settings (64). At present, no data regarding the effect of KB2115 on HCC development/progression are available.

Relevant data confirming the possible importance of THR agonists in the treatment of liver disease have been also obtained with the prodrug MB07811, which undergoes first-pass hepatic extraction and its cleavage generates the negatively charged THR agonist (3,5-dimethyl-4-(4′-hydroxy-3′-isopropylbenzyl)phenoxy)methylphosphonic acid (known as MB07344) (65). Comparison of the effects of MB07811 with T3 and a non-liver-targeted TR agonist, KB-141, on the expression of THR agonist-responsive genes in the liver and extrahepatic tissues, demonstrated enhanced liver targeting for MB07811 with increased cardiac sparing. Importantly, limiting THR activation to the liver resulted in the reduction not only in cholesterol but also in both hepatic and plasma triglyceride levels in Diet-Induced Obese (DIO) mice (65). Successively, using several experimental models of NAFLD, such as Zucker diabetic fatty (ZDF) rats, *ob/ob* mice, diet-induced obese (DIO) mice, Cable and colleagues (12) demonstrated that MB07811 markedly reduced hepatic steatosis, plasma FFA and triglycerides. Importantly, no sign of liver fibrosis or other histological liver damage, transaminase increase were observed after treatment with MB07811. No increase in heart weight was also reported. This antisteatotic activity of MB07811 was likely attributable to an increased metabolic rate in liver and an increased rate of mitochondrial β-oxidation, in particular of CPT-1 levels.

Concerning human clinical trials, GC-1, KB2115 and MB07811 were reported to reduce significantly low-density lipoprotein (LDL)-cholesterol and triglycerides levels (45, 66, 67). However, despite these encouraging results, no compounds that reached human clinical trials developed into therapeutics so far. KB2115 was discontinued after long-term dosing in dogs, demonstrating adverse effects on cartilage (68). Similarly, no phase II trials for GC-1 and MB07344 have been performed (43).

RECENT ADVANCES IN THRβ LIGANDS

More recently, a new, liver-directed with low extra-hepatic permeability, THR-β agonist has been developed, the 2-[3,5-Dichloro-4-(5-isopropyl-6-oxo-1,6-dihydropyridazin-3-yloxy)phenyl]-3,5-dioxo-2,3,4,5-tetrahydro[1,2,4]triazine-6-carbonitrile, also known as MGL-3196 (69). MGL-3196 resulted to be 28-fold selective for THRβ over THRα in an *in vitro* functional coactivator recruitment assay. Notably, compared with other compounds, no impact on the central thyroid axis and no cardiac effects in a rat heart model were also reported for MGL-3196. In mice, this drug was able to induce a reduction in cholesterol and in liver size, which is secondary to reduction

of liver triglycerides. Apparently, no effect on bone mineral density (BMD) or heart or kidney size was detected in MGL-3196-treated animals (69). By contrast, while T3 treated animals demonstrated cholesterol lowering, there was no effect on liver size. In humans, 60% reduction in triglycerides was observed for doses ranging from 50 to 200 mg, in the absence of drug-related adverse events (70). Using HFD-fed mice, it was shown that at human equivalent doses, MGL-3196 reversed and prevented progression of lipid, inflammatory and fibrotic markers of NASH in this model without the adverse effects of T3. Importantly, beneficial effects of MGL-3196 have been also observed in NASH patients, as this THRβ agonist significantly decreased hepatic fat in patients with NASH compared with placebo group. At the moment, MGL-3196 is in Phase 2 for treatment of NASH. As discussed for T3, the modulation of cell metabolism by synthetic THR agonists could be mediated by both central and peripheral actions, with a prevalence of the latter. On the other hand, the proliferative and antineoplastic actions of some agonists appear to be exclusively mediated by their binding to the target cell, as also demonstrated by their *in vitro* activity.

TH METABOLITES: 3,5-DIIODO-L-THYRONINE (T2)

Although TH actions were thought to be classically mediated by T3 and 3,5,3′,5′-tetraiodo-L-thyronine (thyroxine or T4), over the last decades, another TH-related compound, 3,5-diiodo-L-thyronine (T2), received marked attention as it was demonstrated to be a bioactive compound. T2, similar to T4, T3 and rT3 (reverse T3), is one of four natural iodothyronines with significant, biological activities. Although experimental *in vitro* evidences for the conversion of T3 to T2 are still lacking, indirect evidence indicates that T2 is the product of the deiodination of the outer ring of T3 and it is reported to have 50–1,000 times lower affinity for THR than T3 (71, 72). TH deiodination can be mediated by three selenoenzymes: type 1 deiodinase (D1), type 2 D2, type 3 D3 (73); among these enzymes, D2 (71) results as the most likely candidate involved in T2 formation (72). Literature data published in the past years proposed that both THR- and non-genomic actions may be elicited by T2; in addition, T2 has been suggested to mimic some of the effects of T3 on liver metabolism (74–76). The results and mechanisms regarding the T2 actions have been explored both *in vitro* and *in vivo*. With regard to the *in vitro* models, the effects of T2 administration were evaluated using primary cultures of rat hepatocytes exposed to an oleate/palmitate mixture in order to obtain "fatty hepatocytes," a condition mimicking fat accumulation induced *in vivo* by high fat diet (HFD) (77). Similar to T3, the addition of T2 to "fatty hepatocytes" resulted in a significant reduction of lipid content and lipid droplet diameter, as well as in the activities of acyl-CoA oxidase (AOX), a rate-limiting enzyme of peroxisomal β-oxidation, and antioxidant enzymes (78). The beneficial anti-steatotic effects of T2 *in vivo* have been described in several models of NASH, with particular attention to animals fed HFD, a model which has the advantage to mimic most features of human fat overload and overnutrition and allows to study obesity and liver related disorders (79). Notably, T2 was able not only to prevent hepatic steatosis (80) but also to reverse pre-existing hepatic fat accumulation (81), similarly to what observed with T3 (10). In detail, the simultaneous co-treatment of T2 to rats receiving a HFD caused a complete disappearance of hepatic steatosis and a significant reduction in the serum triglyceride and cholesterol levels when compared with rats receiving HFD alone. These effects were the consequence of stimulation of hepatic fatty acid oxidation and CPT activity. No change in heart rate during or at the end of the treatment was observed (80). T2 was also able to markedly reverse HFD-induced hepatic steatosis. Regression of fat accumulation was associated to enhanced fatty acid oxidation rate, CPT activity and improved mitochondrial oxidative stress (81). Successive proteomic studies (82) provided an integrated view of the metabolic adaptations occurring after HFD+T2 treatment. In particular, pathways involved in fatty acid and ketone-bodies metabolism, amino acid and nitrogen metabolism, urea cycle, respiratory chain activity and reactive oxygen species (ROS) production were identified (82). Further studies demonstrated that T2 administration prevented HFD-induced insulin resistance (83). They also suggested that T2 does not act via THRβ, but directly *activates* the hepatic nuclear sirtuin 1 (SIRT1) which was identified as a main factor in mediating the effects of T2. This nuclear deacetylase, that modulates lipid metabolism and enhances mitochondrial activity (84), has been already recognized to contribute to the regulation of hepatic gene expression by T3 through a direct interaction with THRβ (85–87). In addition to the induction of oxidative pathways, T2 was reported to up-regulate the levels of apo-lipoprotein B, the major protein component of very low-density lipoprotein (VLDL) (88), indicating that T2 stimulates lipoprotein secretion to reduce the hepatic fat excess (89). Moreover, in the same study, T2 treatment was shown to affect the levels of adipose triglyceride lipase (ATGL), which selectively performs the first and rate-limiting step of triglycerides hydrolysis to generate diacylglycerol and fatty acids, triggering the lipolytic cascade (90). Very recently, using a targeted metabolomics approach, Iannucci and colleagues (14) deepened and compared the effects of T2 and T3 on the early metabolic adaptation in the livers of rats fed HFD. Both T2 and T3 diminished hepatic triglyceride levels, strongly induced autophagy and intra-hepatic acylcarnitine flux, while preventing the generation of sphingolipid-ceramides, which are known to contribute to the oxidative stress in NASH (91). The effects of T3 and T2 on insulin/growth factor signaling effectors such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) were also investigated. Evaluation of phosphorylation of extracellular signal-regulated kinase (ERK) and Protein kinase B (AKT), demonstrated that only T2 was able to rescue the impairment in AKT and MAPK/ERK pathways caused by HFD (14). As recently demonstrated, although both T2 and T3 have the capacity to regulate lipid accumulation in the liver of rats fed a HFD, they act by different molecular mechanisms to achieve inhibition of hepatic lipid accumulation. While the effects exerted by T2 are accompanied by a decreased lipogenesis and an increased fatty acid oxidation, those exerted by T3 are primarily dependent

on increased fatty acid oxidation (92). Although T2 reduced hepatic triglyceride content also in diet-induced obese mice, increased heart weights indicated potential cardiac side effects going beyond hepatic thyromimetic actions (93). However, it should be underlined that the dose of T2 chosen for this study was 10-fold higher than those reported in previous studies, in which prevention of hepatic lipid accumulation without undesirable side effects was observed.

No deleterious side effects on the thyroid axis or at the cardiac level, together with an increased basal metabolic rate and decreased body weight, were observed in two euthyroid subjects who underwent T2 administration. Serum levels of FT3, FT4 and TSH resulted unchanged in these patients (94).Recently, a novel functional analog of T2, 3-[4-(7-hydroxy-6-methyl-indan- 4-ylmethyl)-3,5-dimethyl-pyrazol-1-yl]-propionic acid (TRC150094, TRC) has been tested in rats receiving a HFD. A significant increase in mitochondrial fatty acid import and oxidation led to the reduction of the hepatic triglyceride content in these animals (95). TRC induced also a reduction in cholesterol levels. Importantly, no undesirable effects were observed on heart rate or heart weight in HFD+TRC-treated rats. Next, oral administration of TRC150094 to obese Zucker spontaneously hypertensive fatty rats (obese ZSF1) decreased hepatic steatosis by inducing a significant increase in mitochondrial respiration as well as by increasing fatty acids oxidation (96). Since most of the metabolic effects on hepatocytes elicited by T2 are mediated by a direct action on mitochondria and are reproducible *in vitro*, it is likely that they are mediated by peripheral actions. Nonetheless, the possibility that T2 affects hepatic metabolism by a central action cannot be excluded and requires more experimental evidences. No data regarding the potential therapeutic use of T2 for HCC have been reported so far.

It should be underlined that D3 action generates 3,3′,5′-triiodothyronine (reverse T3), from T4 and another form of T2, 3,3′-diiodo-L-thyronine (3,3′-T2) from T3 (72, 97). However, the comparison of the effects of T3, 3,5-T2 and 3,3′-T2 on metabolic parameters and glucose levels in HFD-fed mice revealed that 3,3′-T2 lacks beneficial metabolic effects and even worsens metabolic parameters in this experimental model. These differences cannot be attributed to differences in the affinities for THRs as 3,3′-T2 acts as a more effective and potent THR agonist that binds THRs with higher affinity (98).

TH METABOLITES: 3-IODOTHYRONAMINE (T1AM)

The biogenic amine 3-iodothyronamine (T1AM) is another noteworthy endogenous TH metabolite that has recently been demonstrated to have profound effects on glucose and lipid metabolism at physiological concentrations. Interestingly, these effects were, at least in part, mediated by its actions on hepatocytes.

Tandem mass spectrometry coupled with HPLC allowed to detect T1AM in the blood and in several tissues from both rodents and humans. Tissue concentration of this amine was found to range from 0.2 to 0.3 nM in the blood, to 1–90 nM in other tissues. Although literature is not concordant on the absolute concentration of T1AM in blood and tissue, it is widely accepted that this amine concentrates in some organs, such as liver, brain, and muscle (99, 100). Differently from T3 and its synthetic analogs, T1AM does not bind to the THR, consequently does not share with TH the classical cardiovascular side effects. Most of the effects of this amine is thought to be mediated by the trace amine-associated receptor 1 (TAAR1), a G protein-coupled membrane receptor (46, 101). T1AM can also interact, albeit at lower affinity, with α2A adrenergic receptors and more additional targets, such as apolipoprotein B100, mitochondrial ATP synthase, and membrane monoamine transporters. The physiologic importance of these low affinity interactions is still a matter of investigation. Despite the growing interest for T1AM, the biosynthetic pathway responsible for its production remains quite unclear. The similarity of this amine with T3 allows to speculate that T1AM may be synthesized from this hormone through decarboxylation and deiodination (102–104). Nonetheless, it is unclear where these reactions really occur. In healthy subjects there is a significant correlation between T3 and T1AM serum concentrations (105), suggesting a possible role of the thyroid gland in T1AM synthesis; however, the evidence that thyroidectomized patients treated with thyroxine have normal serum T1AM values, supports the alternative hypothesis that T1AM may be the result of an extrathyroidal metabolism of thyroid hormone (106). Rat peripheral tissues display intracellular T1AM concentration ranging from 5.6 ± 1.5 pmol/g in lung to 92.9 ± 28.5 pmol/g in liver: These concentrations are much higher than those measured in serum (up to 20-fold that measured in the serum (0.3 ± 0.03 pmol/ml) (99). Unfortunately, these results do not allow to clarify if the high tissue concentrations of T1AM resulted from (i) the ability of cell to concentrate it, (ii) intracellular biotransformation of TH, or, (iii) local biogenesis. Experiments performed in H9c2 cardiomyocytes and isolated and perfused rat heart, indicate that intracellular T1AM originate from an active Na^+ dependent cellular uptake of the amine, and, to a lower extent, from intracellular biotransformation of T3 (99). *In vivo* experiments, using hypothyroid mice treated with an isotope-labeled T4 [heavy-T4 (H-T4)] that can be distinguished from endogenous T4 by mass spectrometry, indicate that T1AM is not an extrathyroidal metabolite of T4, but it is produced by an independent biosynthetic process. Despite these results, the site of T1AM synthesis remains elusive. Nonetheless, the wide organ distribution and relative high concentrations of T1AM, clearly points to a physiological role of this amine. The administration of supra physiological concentrations of T1AM, in rodents, lead to hypothermia, decreased cardiac contractility, behavioral alterations, weight loss, activation of energetic metabolism from lipids, increased gluconeogenesis and impaired insulin secretion.

T1AM IN NAFLD

The effects of T1AM on body weight and energetic metabolism prompted the interest for this amine in pathological conditions characterized by obesity and triglyceride accumulation within

the liver. These conditions are often associated to patients with NAFLD, a condition for which there is no effective pharmacological treatment (107). Early findings indicated that central nervous system could be the principal responsible for the acute alterations in glucoregulatory hormones and glucose metabolism seen after T1AM administration to adult male rats (108). On the other hand, the finding that T1AM reaches the higher concentrations in the liver supports the hypothesis that its metabolic effects might be partly mediated by hepatocytes. T1AM uptake, as well its effects on hepatic glucose and ketone body production, has been investigated in perfused liver from healthy rats and in HepG2 r cancer cells (109). When HepG2 cells were exposed to a T1AM (up to 1.0 μM), they concentrated the amine by 6- to 8-fold. Ex vivo experiments with perfused liver confirmed a significant T1AM uptake. In both conditions, T1AM administration produced a significant increase in glucose production, whereas a significant amount of ketone bodies was seen in the liver but not in HepG2 cells known for their limited ketogenic capability (110). This effect may be dependent on pyruvate and amino acid catabolism, so that pyruvate shift toward gluconeogenesis would cause less acetyl-CoA to be available for ketogenesis.

These findings are consistent with a shift of pyruvate toward gluconeogenesis and direct stimulation of fatty acid catabolism occurring in normal hepatocytes (109). Recent investigations confirmed that chronic T1AM administration induces transcriptional changes in hepatocytes and adipocytes consistent with a more lipolytic metabolic pattern (111). Since these effects were confirmed by *in vivo* experiments and were associated to a significant weight loss without changes in food consumption (112), T1AM, or synthetic analogs should be seriously considered as novel therapeutic agents for obese patients with associated NAFLD.

T1AM AND LIVER CANCER

Differently from metabolism-related diseases, much less is known about the effects of T1AM on liver carcinogenesis and neoplastic hepatocytes. Only few papers have investigated the effect of T1AM on cancer cells (113, 114). In a recent paper it was found

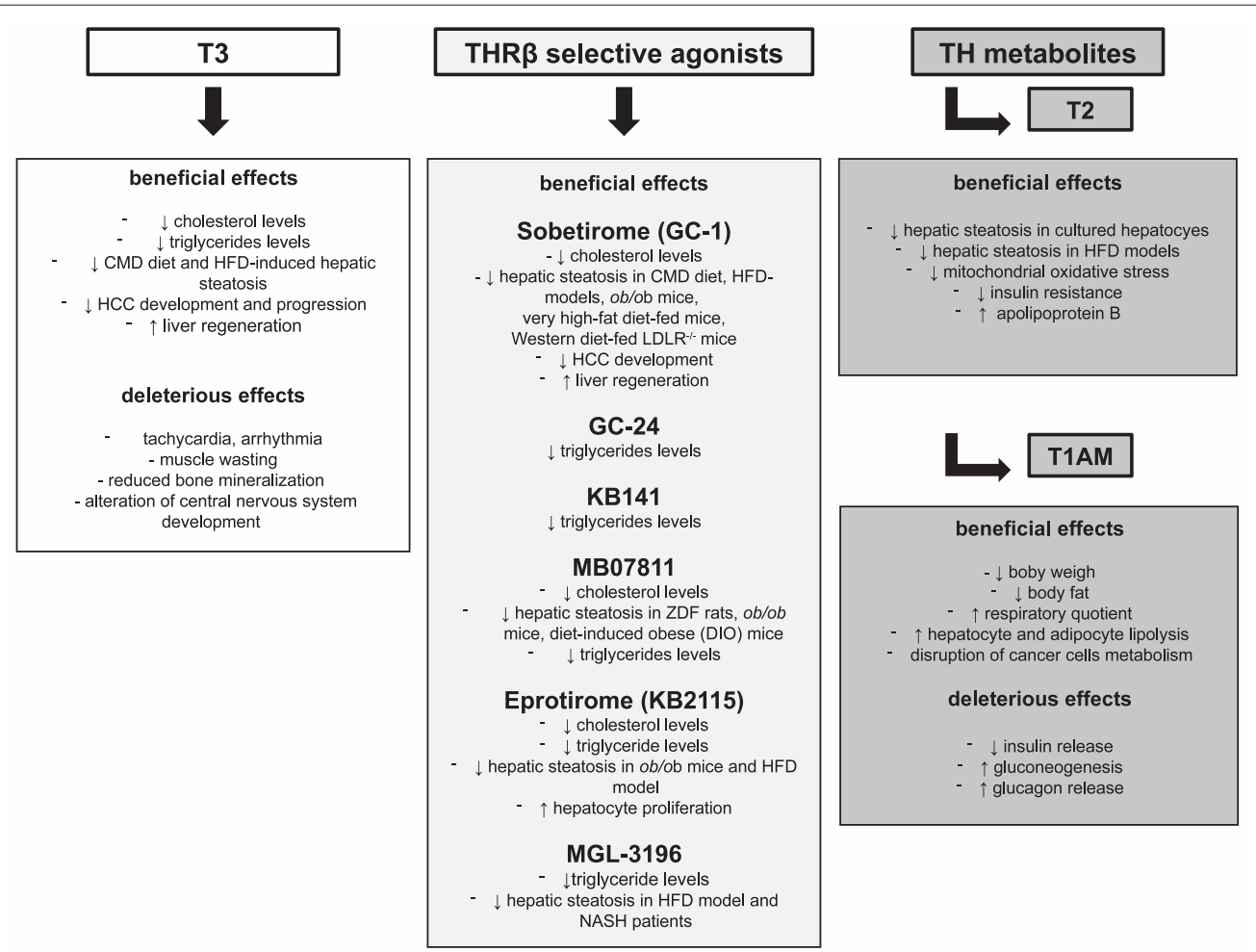

FIGURE 1 | Beneficial and deleterious effects exerted by T3, THRβ selective agonists, and TH metabolites.

that micromolar doses of T1AM and its structural analog SG-2, are able to reduce viability and growth rate of the liver cancer cell line HepG2 and the breast cell line MCF7 (114) The exact mechanism by which T1AM altered cell viability and growth rate of these cancer cells is not entirely clear. However, the mitochondrial localization of T1AM suggest a role in metabolic disruption of cancer cell function. The gene expression study suggests that the mechanism of action of T1AM possibly involves its ability to induce upregulation of the expression of the mitochondrial genes *SIRT 4 and SIRT 5.* The upregulation of other genes, such as *G6PD, p53, HIF1a,* and *SIRT 1*, could be indirectly mediated by the metabolic effects of T1AM.

T1AM AND TREATMENT OF LIVER DISEASE

Similar to T3, also T1AM modulates lipid and glucose metabolism through central and peripheral actions, as demonstrated by its ability to modulate insulin resistance and hypothalamus-pancreas-thyroid axes in mice after intracranial administration (115). More experimental data are needed to better dissect these two mechanisms of action. Available data are still insufficient to establish a direct role of T1AM and its possible use in the treatment of liver cancer, as further investigations in *in vivo* models are needed. Nonetheless, the possibility that T1AM treatment may result in a reduced risk of liver cirrhosis and HCC development, as a consequence of its potential effect on NAFLD progression should be intensely pursued. A short summary of both beneficial and deleterious effects exerted by T3, THRb selective agonists, and TH metabolites has been reported in **Figure 1**.

CONCLUSION

Liver disease represents an increasing health problem in many countries. From a clinical point of view, a treatment resulting in induction of fatty acid oxidation, decrease in serum triglyceride and cholesterol levels, reduced hepatic steatosis and body weight, without deleterious effects on the heart, might represent a very attractive therapeutic option for many patients. TH, their analogs and metabolites share several desired effects on glucose and lipid metabolism; thus a therapeutic strategy based on their use, as single agents or in association, might be effective for the treatment of liver diseases, such as NAFLD and HCC, that are currently devoid of any effective drug.

AUTHOR CONTRIBUTIONS

MAK: literature revision and drafting of the article. AP: drafting of the article and figure preparation. AC: coordination of all the work, critical revision of the manuscript and final approval.

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REVIEW
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Similarities and Differences in the Peripheral Actions of Thyroid Hormones and Their Metabolites

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Thyroxine (T4) and 3,5,3'-triiodothyronine (T3) are secreted by the thyroid gland, while T3 is also generated from the peripheral metabolism of T4 by iodothyronine deiodinases types I and II. Several conditions like stress, diseases, and physical exercise can promote changes in local TH metabolism, leading to different target tissue effects that depend on the presence of tissue-specific enzymatic activities. The newly discovered physiological and pharmacological actions of T4 and T3 metabolites, such as 3,5-diiodothyronine (3,5-T2), and 3-iodothyronamine (T1AM) are of great interest. A classical thyroid hormone effect is the ability of T3 to increase oxygen consumption in almost all cell types studied. Approximately 30 years ago, a seminal report has shown that 3,5-T2 increased oxygen consumption more rapidly than T3 in hepatocytes. Other studies demonstrated that exogenous 3,5-T2 administration was able to increase whole body energy expenditure in rodents and humans. In fact, 3,5-T2 treatment prevents diabetic nephropathy, hepatic steatosis induced by high fat diet, insulin resistance, and weight gain during aging in Wistar male rats. The regulation of mitochondria is likely one of the most important actions of T3 and its metabolite 3,5-T2, which was able to restore the thermogenic program of brown adipose tissue (BAT) in hypothyroid rats, just as T3 does, while T1AM administration induced rapid hypothermia. T3 increases heart rate and cardiac contractility, which are hallmark effects of hyperthyroidism involved in cardiac arrhythmia. These deleterious cardiac effects were not observed with the use of 3,5-T2 pharmacological doses, and in contrast T1AM was shown to promote a negative inotropic and chronotropic action at micromolar concentrations in isolated hearts. Furthermore, T1AM has a cardioprotective effect in a model of ischemic/reperfusion injury in isolated hearts, such as occurs with T3 administration. Despite the encouraging possible therapeutic use of TH metabolites, further studies are needed to better understand their peripheral effects, when compared to T3 itself, in order to establish their risk and benefit. On this basis, the main peripheral effects of thyroid hormones and their metabolites in tissues, such as heart, liver, skeletal muscle, and BAT are discussed herein.

Keywords: 3, 5-T2, T1AM, thyroid hormone, deiodinase, thyroid hormone analogs

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INTRODUCTION

Thyroid hormones affect development, growth, and metabolic control, therefore being indispensable to normal development and body energy expenditure (1).

Thyroid dysfunction such as hypothyroidism is implicated in growth and developmental impairment, changes of lipid, and cholesterol metabolism, cardiovascular diseases, and a decreased metabolic rate. In contrast, hyperthyroidism is a catabolic syndrome that is related to increased metabolic rate, tachycardia, and loss of lean body mass (2).

The thyroid gland produces both thyroxine (T4) and 3,5,3'-triiodothyronine (T3) (3) and every tissue expresses the enzymes that are able to metabolize these hormones, the so-called iodothyronine deiodinases that remove iodine atoms from iodothyronines. Type 1 deiodinase (D1) is found in the thyroid, liver, and kidneys, while type 2 deiodinase (D2) is expressed in the central nervous system, human thyroid, skeletal muscle, and brown adipose tissue (BAT), and both of them convert T4 into T3 through the outer ring deiodination reaction. Type 3 deiodinase (D3) inactivate T4 by its conversion into reverse T3 (rT3) through the inner ring deiodination that can also be catalyzed by D1. Under physiological conditions, D3 is expressed in the brain, placenta, and pancreas, but can be induced in other tissues under pathophysiological circumstances.

The activity of deiodinases impact both serum and tissue levels of T4 and T3, as described when animals are exposed to cold temperatures (4). Also, the regulation of decidua and placenta deiodinase activities correspond to the best example of space temporal regulation of thyroid hormones metabolism that impact on the fetus physiology (5–8). Recently, several thyroid hormone metabolites have been detected in human placenta, and future studies are necessary to address their possible effects during pregnancy (9).

It has been proposed that D1 contributes to serum T3 concentrations due to its catalytic site that is believed to face the extracellular space, while D2 is an endoplasmic reticulum resident protein that is important for intracellular T3 availability, and T3 receptor saturation in tissues. Besides the deiodinase reactions, TH undergo tissue-specific metabolism that includes sulfation, glucuronidation, deamination, and decarboxylation (1).

Since both T3 and rT3 are substrates for deiodinases, diiodothyronines are also produced, namely 3,5-diiodothyronine (3,5-T2), 3,3'-diiodothyronine (3,3'-T2), and 3',5'-diiodothyronine (3',5'-T2). Iodothyronines can also be decarboxylated giving rise to a phenethylamine derivative called a thyronamine (TAM), such 3-iodothyronamine (T1AM) (10). 3,5-T2, and T1AM are the most studied metabolites to date and they play significant physiological roles. The endogenous presence of these metabolites was confirmed by the development of specific immunoassays to detect 3,5-T2 (11) and T1AM (12).

Thyroid hormones actions can be separated into two major groups: (1) the central effects that consist of a direct signaling on the central nervous system, and (2) the peripheral effects that correspond to direct effects in responsive tissues. T3 controls energy expenditure via central and peripheral pathways. For example, T3 stimulates specific neurons of the ventromedial nucleus, which activate the sympathetic nervous system that in turn innervates the BAT and leads to adaptive thermogenesis (13); concomitantly, T3 acts directly in the BAT and activates the thermogenic program by the control of lipid metabolism and uncoupling protein 1 (UCP1) activation (14). The aim of this review is to discuss the peripherical effects of thyroid hormones and their metabolites isolating the similarities and differences in their actions and the promising use of 3,5-T2 and T1AM as therapeutic agents.

BIOSYNTHETIC ROUTES OF TH METABOLITES 3,5-T2, AND T1AM

In terms of structure, thyronamines differ from thyroid hormones and their deiodinated derivatives due to the absence of the carboxylate group in the beta-alanine side chain (15). Therefore, it has been suggested that T1AM is produced from TH precursors by both deiodination and decarboxylation reactions. However, the sequence of reactions for thyronamines biosynthesis and the organs where they occur are still poorly defined.

3-iodothyronamine (3-T1AM) production was believed to occur in the thyroid, to be dependent on the presence of the sodium-iodide symporter and thyroperoxidase, and did not seem to involve extrathyroidal metabolism of T4 (16). However, FRTL-5 cells incubated with T4 were unable to produce T1AM (17). *In vitro* experiments also showed significant 3-T1AM production in H9c2 rat cardiomyoblasts exposed to T3, which questions the importance of thyrocytes for thyronamine production (18).

T1AM synthesis from TH requires the decarboxylation of the L-amino acid moiety. Initially, it was proposed that the aromatic L-amino acid decarboxylase (AADC) (19) mediated T1AM biosynthesis, but it has recently been demonstrated that patients lacking functional AADC activity exhibited normal serum 3-T1AM levels (20). The generation of extrathyroidal TH metabolites was confirmed due to the detection of 3,5-T2 in thyroidectomized individuals (11) and the finding that serum T1AM was even higher in thyroidectomized and radioiodine-treated patients than in healthy individuals (12). The studies in T4-substituted thyroid cancer patients lacking functional thyroid tissue suggest extrathyroidal 3-T1AM production, whereas studies using labeled T4 in mice indicate intrathyroidal formation. The contradictory results might be due to the different administrations routes, once thyroidectomized individuals received T4 orally, whereas the mice received it intraperitoneally (21). Elegantly, it was shown that the intestine can produce 3-T1AM from T4 and 3,5-T2 (21) due to the expression of the molecular machinery required for 3-T1AM biosynthesis: ornithine decarboxylase (ODC) and all the three deiodinase isoforms (D1, D2, and

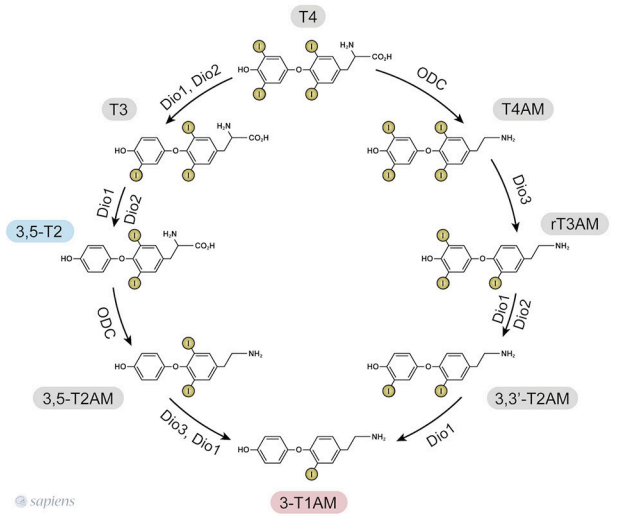

FIGURE 1 | 3,5-T2, and 3-T1AM biosynthetic pathways from thyroid hormones T4 and T3, as identified in murine intestinal tissue (15, 21). ODC—ornithine decarboxylase. Dio—iodothyronine deiodinase.

D3). Consequently, ODC was identified as the first enzyme able to decarboxylate thyroid hormones (21). Purified human ODC can, in fact, mediate decarboxylation of T4 and 3,5-T2 to T4AM, and 3,5-T2AM, respectively, as shown in **Figure 1** (21).

Based on the ability of ODC to decarboxylate only T4 and 3,5-T2 (21), the only way to produce other thyronamines is from T4AM and T2AM. However, cardiac cells exposed to T3 were able to yield T1AM (18). Since purified ODC does not decarboxylate T3 to yield T3AM (21), one can speculate that T3 might be deiodinated into 3,5-T2 prior to the decarboxylation step.

Since the one step iodothyronine decarboxylation reaction to yield thyronamine was identified, the following question was posed: could thyronamines be metabolized by iodothyronine deiodinases? D1 exhibits both phenolic and tyrosyl ring deiodination activities (**Figure 1**), while D2 and D3 are specific for to the position of deiodination. D2 only catalyses the phenolic or outer ring deiodination, e.g., the conversion of T4 into T3, whereas D3 only catalyses the deiodination of the tyrosyl or inner ring, e.g., the conversion of T4 into rT3.

Interestingly, rT3AM can be readily deiodinated by all the three deiodinase isozymes, such as occurs with rT3 (15). However, apparently ODC does not convert rT3 into rT3AM (21), suggesting that the source of rT3AM is T4AM. Therefore, a synchronized order of events involving decarboxylation and deiodination takes place for T1AM formation. In contrast, T4AM is not a substrate for neither D1 nor D2 but is instead a substrate of D3, leading to rT3AM formation. Deiodination of rT3AM by D1 and D2 then produce T1AM, thus providing a specific biosynthetic pathway for endogenous T1AM production from T4AM, which would result from the decarboxylation of T4 followed by D3 deiodination (15, 22). The 3,5-T2, and 3-T1AM biosynthetic routes from T4 are shown in **Figure 1**.

ENDOGENOUS LEVELS OF TH METABOLITES 3,5-T2, AND T1AM

Serum levels of 3,5-T2 have been reported to be around 0.24 nM in euthyroid humans (23), and they seem not to differ in thyroid dysfunction states (11). However, a study reported reduced free T3 levels together with elevated 3,5-T2 levels in patients with postoperative atrial fibrillation (24). 3,5-T2 might also be present in tissues, but so far it has not been possible to detect endogenous 3,5-T2 in heart tissue (10), but 1.5 fmol of 3,5-T2/100 g tissue could be detected in rat liver (25).

T1AM was detected in brain within different ranges such as: 0.4 (26), <1 (27) and 49 pmol/g of tissue (28). T1AM (pmol/g of tissue) is found at different levels in different rat brain regions, such as: 60.4 in cerebral cortex, 20.9 in hemisphere white matter and 23.2 in cerebellum (18). In human serum, T1AM was detected in the range of 0.15–0.20 pmol/ml, while 0.3 pmol/ml were detected in rat serum (18). T1AM (pmol/g of tissue) was also identified in concentrations higher than the serum levels in many other rodent tissues, such as: heart (6.6), liver (92.9), kidney (36.08), skeletal muscle (25.02), stomach (15.46), and lung (5.6) (18). Other studies found 7 pmol T1AM/g (29) and 68 pmol T1AM/g (30) of rat liver, showing a variable value depending of the species and the methods of analyses used.

Recently, a study has compared the endogenous T1AM levels in different tissues and its tissue levels after 7 days of administration of two different T1AM doses (1 mg and 5mg/100 g b.w.) (31).

Endogenous T1AM and 3,5-T2 are generated from decarboxylation and deiodination reactions catalyzed by enzymes that expressed in different tissues (18, 21, 32–34). Thus, it is possible that the regulation of the local synthesis of these metabolites might also be important for their physiological effects.

REGULATION OF OXYGEN CONSUMPTION BY THYROID HORMONE METABOLITES

T3 is able to increase oxygen consumption and decrease body fat, however the undesirable side effects of T3 administration limit its therapeutic use for the control of metabolic disorders, which favored the development of TH analogs designed to exert no deleterious effects on the heart and lean body mass loss (35).

Almost 30 years ago, the TH metabolite 3,5-T2 was shown to increase oxygen consumption in isolated hepatocytes (36), what was afterwards corroborated using blood mononuclear cells (37) and also *in vivo* in rats (38, 39), mice (40), and humans (41). Since the metabolic effect of 3,5-T2 was faster than T4 or T3 treatment, and without the need of new protein synthesis, the authors postulated that 3,5-T2 could act trough a post-translational mechanism, independently of genomic action [For recent review see (42)].

Both T3 and 3,5-T2 were able to increase the basal metabolic rate to the same extent in hypothyroid rats induced by

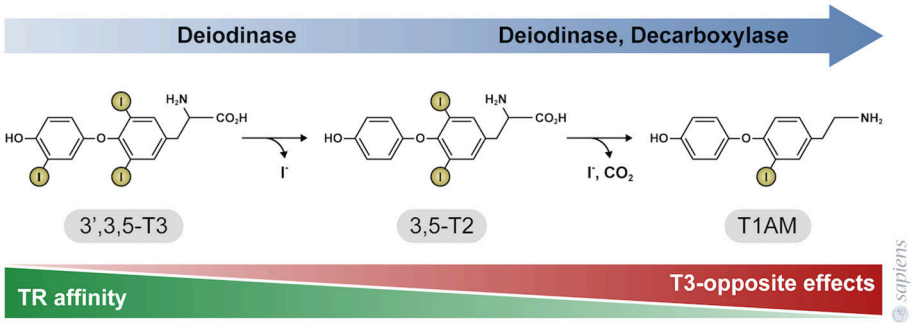

FIGURE 2 | Thyroid hormone metabolites 3,5-T2, and 3-T1AM. 3,5-T2 is generated by the outer ring deiodination of T3, and T1AM is generated by enzymatic deiodination and decarboxylation of T4 or 3,5-T2, as shown in **Figure 1**. The affinity of thyroid hormone nuclear receptors (TR) for these metabolites is significantly lower, when compared to T3. TR affinity is approximately 500-fold lower for 3,5-T2, whereas TR do not bind to T1AM that in turn exerts effects that are opposite to T3 and 3,5-T2 actions.

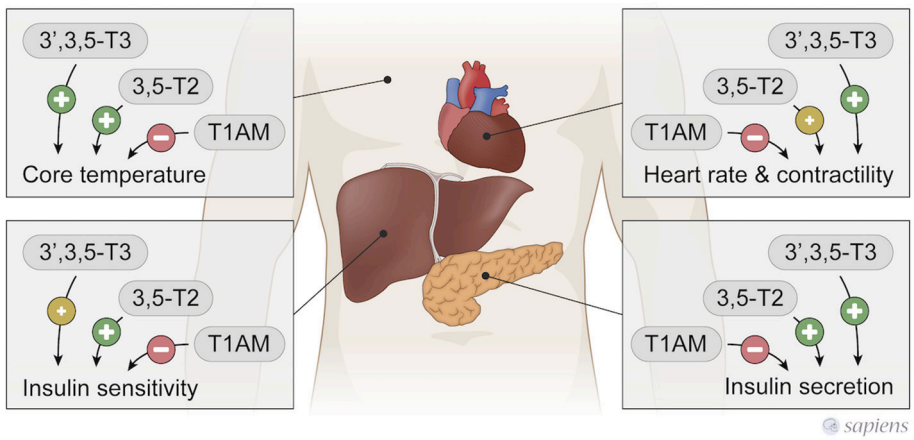

FIGURE 3 | T3, 3,5-T2, and T1AM actions in peripherical tissues. 3,5-T2, and T3 have similar actions, while T1AM exerts T3 and 3,5-T2 opposite effects.

propylthiouracil (PTU) and iopanoic acid administration (43). However, a single dose of 3,5-T2 was not sufficient to increase the metabolic rate in euthyroid rats differently from T3, but the chronic treatment with 3,5-T2 increased metabolic rate in aging euthyroid rats (39) and in high fat diet fed euthyroid rats (38). In contrast, a single dose of T1AM rapidly induced a hypometabolic state in rodents and hamster, with a significant decrease in the core temperature during the first hours after administration (27, 44) and persisting for 8 days of administration in mice (45). This opposite action show that thyroid hormones metabolites might also counteract the classical actions mediated by T3. However, even though T1AM has opposite effects from those of 3,5-T2 on oxygen consumption, both stimulate lipolysis, as produced by T1AM administration to spontaneously overweight mice (45) and by 3,5-T2 treatment of rats fed a high fat diet (HFD) (38).

The metabolic pathways implicated in these effects described above will be discussed in the next section separately, isolating each peripherical tissue that can contribute to the higher oxygen consumption rate, such as skeletal muscle, BAT, and liver.

T3 positively regulates mitochondria function and induces mitochondrial biogenesis. The peroxisome proliferator activated receptor gamma (PPAR gamma) co-activator 1α (PGC-1α) is a transcriptional co-activator regulated by T3 that mediates biological programs related to energy metabolism that coordinates mitochondrial biogenesis (46). PGC-1α cDNA was first cloned from BAT, and the protein was described to be involved in the induction of UCP1 (47). To date, it has been accepted that PGC-1α plays indispensable roles in glucose and fatty acid metabolism, mitochondrial biogenesis and adaptive thermogenesis (48). Notably, the PGC-1α gene contains a TH responsive element (49) and both T3 (50) and 3,5-T2 (51) rapidly induce PGC-1α expression.

GENOMIC AND NON-GENOMIC ACTIONS OF THYROID HORMONES

T3 primarily exerts its effects by binding to thyroid hormone nuclear receptors (TR) that affect gene transcription through

thyroid hormone responsive elements (TRE) in the promoter region of target genes. TR alpha and beta isoforms are encoded by two genes that are differentially expressed in various tissues. The distribution of these receptors is heterogeneous among the different tissues, and as a result some physiological effects of T3 are TR isoform specific. For example: TR beta plays an essential role in the negative regulation of thyroid stimulating hormone (TSH) secretion (52), whereas TR alpha mediates the positive chronotropic effects of thyroid hormones in the heart (53). Moreover, some physiological effects might require the activation of both TR alpha and TR beta in the same tissue, such as occurs in BAT. Although previous studies claim that TH metabolite 3,5-T2 might only act through non-genomic mechanisms, recent studies have demonstrated that both 3,3-T2, and 3,5-T2 can weakly interact with both TR alpha and TR beta, when compared with T3 (**Figure 2)** (54, 55). Despite the lower affinity of 3,5-T2 for TR beta (55), its ability to exert negative feedback at the hypothalamus-pituitary axis (39) indicate that 3,5-T2 pharmacological actions might also be dependent on the *in vivo* TR beta transactivation.

3,5-T2, and T3 interact with long and short TR beta1 isoforms in teleost, respectively (55, 56). These findings strongly suggest the existence of different signaling pathways for hormone and its metabolite, indicating that the specific response can be, at least in part, due to the differential expression of these receptors. T3 upregulates TR beta, whereas 3,5-T2 downregulates the long TR beta1 isoform (57).

Thyroid hormones can also act through non-genomic mechanisms by binding to sites in the plasma membrane, such as alpha V beta 3 integrin (58), and the activation of cytoplasmic proteins such as AMPK, PI3K/Akt (59) and MAPK (60). Similarly, 3,5-T2 is as a potent stimulator of the same signaling pathways involving AMPK (38) and PI3K/Akt (59), showing a similar partner of non-genomic actions when compared to T3.

Another cellular target of 3,5-T2 is the mitochondria. 3,5-T2 may stimulate cellular respiration via a direct action involving mitochondria by the interaction with cytochrome C Oxidase (COX) to impair the allosteric ATP inhibition of COX, which decreases the respiratory efficiency (61). 3,5-T2 also increases Sirtuin 1 activity, a nuclear deacetylase (62). Interestingly, T3 is not able to stimulate COX (63) and Sirtuin 1 (62) to the same extent as 3,5-T2, showing important differences concerning the non-genomic actions of these hormones.

T1AM has no affinity for TR beta or TR alpha [**Figure 2**; (30)]; T1AM can act through different mechanisms depending of the cellular type (64, 65), such as via the trace amine-associated receptor that activate the adenylyl cyclase and protein kinase A signaling pathway (66), the inverse agonist action on the alpha 2A -adrenergic receptor (67), the stimulation of the transient melastatin 8 channel receptors, increasing intracellular calcium and MAPK ERK1/2 pathway (68, 69) and the Sirtuin 6 and 4 function (31, 70). Additionally, T1AM also inhibits mitochondria F0F1 ATPase activity (71).

THYROID HORMONES, THEIR METABOLITES AND THE HYPOTHALAMUS-PITUITARY-THYROID AXIS

Thyroid gland is under the positive control of TSH, and in turn T4 and T3 negatively control the hypothalamus-pituitary axis, through a classical negative feedback loop. T3 downregulates the secretion of Thyrotropin Releasing Hormone (TRH) in the medio basal hypothalamus and of TSH in the pituitary. Locally produced T3 originates from T4 deiodination by D2 (72), and the metabolite 3,5-T2 is also able to downregulate hypothalamic TRH mRNA expression and serum TSH levels in rats (39), leading to central hypothyroidism, what was also confirmed in mice (40, 54). However, the only study in 2 human volunteers demonstrated that 3,5-T2 for 3 weeks did not change serum thyroid hormone levels despite the stimulating effect on metabolic rate (41).

The intracerebroventricular administration of T1AM decreased plasma free T3 in mice (73), and more recently, 3-T1AM was shown to act directly on the thyroid gland (74), since the administration of 3-T1AM to mice for seven days decreased the thyroid mRNA contents of NIS, thyroglobulin, and pendrin but did not interfere with the hypothalamus-pituitary axis (74).

THYROID HORMONE METABOLITES AND THE HEART

Thyroid hormones are required to maintain heart rate, myocardial contractility and vascular function (75). The genomic effects of TH on the heart that control chronotropic and inotropic features are mainly mediated by the TH-specific receptor TR alpha1 (53). High T3 levels are related to increased myocardial contractility and electrical conduction, and arrhythmias events, while low T3 promotes opposite effects, such as bradycardia (10). As a result, both hyper-and hypothyroidism are important risk factors for cardiovascular diseases.

The heart is able to locally adjust the metabolism of TH through the modulation of D3 activity, as described in myocardial infarction (76) and dilated cardiomyopathy (77). Thus, ischemic insults promote local hypothyroidism, which results in the lower expression of T3 responsive genes that are involved in contractile apparatus and energy metabolism, what is interpreted as a counter regulatory mechanism to reduce oxygen consumption after an ischemic event (78). Animals deficient of D3 show a worst infarct progression (77), showing the importance of cardiac D3 induction to attenuate the classical T3 effects and/or produce other metabolites that could play a key role in disease prognosis.

Critically ill patients usually present decreased circulating T3 levels, what is called the "low T3 syndrome" or the "euthyroid sick syndrome" (79). The low serum T3 is at least in part a consequence of the higher D3 activity that is induced in some tissues (76, 78). The relevance of thyroid hormones to cardiac function motivated several authors to test thyroid hormones

replacement after myocardial infarct (MI). Many strategies were adopted including treatment some hours after MI (at 72h after MI) (80) or 13 weeks after MI (81). In summary, T3 effects on cardiac remodeling is listed: (1) increased mitochondrial content due higher PGC-1α expression after T3 treatment (82), (2) decreased fibrosis development due to increased metalloproteinases expression (83),)3) decreased apoptosis signaling from mitochondrial (82, 84), and (4) activation of ERK to promote angiogenesis in myocardium (85, 86). Most of these effects were considered to be secondary to a positive effect on mitochondria, which is a classical target of T3 (80, 87). To our knowledge, the 3,5-T2 treatment after MI has not been assessed so far. Some encouraging results showed that 3,5-T2 treatment was potentially able to modulate the same pathways as T3 in skeletal muscle (59), in BAT (51), in liver (62) and in kidney (88), indicating that 3,5-T2 might also be a very promising agent in cardiac diseases.

Chronic 3,5-T2 treatment did not significantly change neither heart weight (38, 39) nor heart rate (39, 89). However, higher doses of 3,5-T2 caused cardiac hypertrophy in rats and mice, just as T3 does (39, 40, 54). In humans, the only clinical study using low doses of 3,5-T2 in two volunteers showed a significant increase in resting metabolic rate, without changes in cardiac function evaluated by echocardiography (41).

It has been demonstrated that the prejudicial cardiac remodeling is mediated in part by overactivation of sympathetic nervous system from the paraventricular nucleus PVN (90). To isolate the central from the direct peripherical cardiac effects of thyroid hormones, some studies have tested the ischemic and reperfusion model using isolated hearts. (91) demonstrated a protection against reperfusion injury mediated by TR alpha, when acute T3 administration preceded the ischemic insult (91), however the possible cardioprotective effects of 3,5-T2 have not been reported yet. Recently, 3,5-T2 increased glucose consumption in isolated rat hearts, contrary to what was observed for T3 and T4 in the same conditions. This direct 3,5-T2 effect on cardiomyocytes was not associated with alterations in contractile performance (92).

In contrast, T1AM treatment in mice (27) or administered to isolated rat hearts (30, 93) induced bradycardia, which is opposite to the classical T3 effect (**Figure 3**). Furthermore, T1AM protected hearts against ischemia without significant hemodynamic actions (94). The balance between D2 and D3 activities could favor the formation of different metabolites in the cardiomyocyte, but further studies are necessary for the comprehension of how the thyroid hormones are metabolized in the heart under different pathophysiological conditions, and whether these different metabolites may play a key role in the regenerative process and cardiac remodeling.

THYROID HORMONES METABOLITES AND THE BROWN ADIPOSE TISSUE

The overall cellular effects of T3 that impact on energy expenditure are based on its ability to decrease the efficiency of ATP synthesis and increase its turnover. T3 stimulates thermogenesis and thus allows the maintenance of body internal temperature under cold exposure (2, 95). Facultative thermogenesis takes place mainly in the BAT and it involves several mechanisms, such as the disruption of the proton gradient across the inner mitochondrial membrane, which mediated by the mitochondrial UCP1 (96). In mitochondria, the energy derived from the electrochemical proton gradient across the inner mitochondrial membrane is used by ATP synthase to produce ATP. The passive flux of protons (proton leak) through the inner membrane through UCP1, dissociates oxygen consumption in the electron transport chain from ATP synthesis, and thus the energy is somehow dissipated and energy expenditure increases (95–97).

Facultative thermogenesis is T3 dependent, as illustrated by the fact that hypothyroid rats do not survive after few hours of cold exposure (98). During cold exposure, an intense activity of sympathetic flux and norepinephrine (NE)/cAMP/protein kinase A signaling activates D2 activity in BAT (99) to increase the local T3 production and consequently thyroid hormone receptor saturation (4). Local T3 controls the expression of many proteins implicated in the turnover of lipid metabolism (14), and lipogenesis is very important for the maintenance of lipid stores that will be used to provide free fatty acids to activate UCP1. Like hypothyroid mice, the D2 deficient mice are unable to survive in low temperature for a long period of time. Apart from the mitochondria effects, T3 also increases the cell membrane permeability to Na^+ and Ca^+ ions and activate the Na^+/K^+ ATPase pump, increasing heat dissipation due to ATP hydrolysis. Among all the cell adaptations discussed before, mitochondria biogenesis is a key event for thermogenesis process (47).

3,5-T2 administration restores the thermogenic program of BAT from hypothyroid rats mainly through mitochondria activation, what is similar to T3 effects (51, 100). To investigate the possible central effect of 3,5-T2 in the cerebral nuclei that control neuronal sympathetic activation, the authors demonstrated that the turnover of NE in BAT was not increased in hypothyroid rats treated with 3,5-T2. In turn, hypothyroid rats treated with either T3 or 3,5-T2 showed the same induction levels of PGC-1α, showing a direct peripheral effect of 3,5-T2. On the other hand, T3 is also able to increase the sympathetic flow to BAT through hypothalamic AMPK inhibition (13), while 3,5-T2 does not show this central effect. The mechanism of action of 3,5-T2 in BAT is not completely defined, although both TR alpha and TR beta seem to mediate the regulatory effects of T3 on facultative thermogenesis (101). It has been proposed that TR alpha induces lipolysis in synergism with the adrenergic system, while the induction of UCP1 expression is mediated by TR beta (101).

In contrast to 3,5-T2 (51), the TR beta agonist (CG-1) did not restore the normal thermogenic function in hypothyroid mice. BAT UCP1 levels are normal in hypothyroid mice treated with GC-1, although they remain cold intolerant due to inadequate BAT cellular response to norepinephrine (101).

In contrast to 3,5-T2 and T3 thermogenic effects, a single T1AM administration rapidly induces a hypometabolic state in rodents, acutely decreasing body temperature in a

dose dependent manner (27, 44). In fact, T1AM induces profound hypothermia, however the mechanisms involved are not completely known (**Figure 3**). T1AM administration induce tail vasodilatation, which could lead to heat loss; however, this effect seems to occur through central mechanisms, since the intracerebroventricular administration of T1AM also produces vasodilatation secondary to hypothalamic activation (102).

THYROID HORMONES METABOLITES AND THE SKELETAL MUSCLE

The skeletal muscle represents approximately 40% of body mass and significantly contributes to energy expenditure and oxygen consumption. Besides that, skeletal muscles contribute to the maintenance of blood glucose levels. Hence, skeletal muscles are important targets for the treatment of obesity (103).

Thyroid hormones influence both the type and distribution of the skeletal muscle fibers, their metabolic program and contractility apparatus (104). Muscular disorders occur in both hypo- and hyperthyroid patients (105). In skeletal muscle, T3 increases the transcription of MyoD (106), stimulates myosin heavy chain IIa (MHC IIa) (107) and the sarcoendoplasmic reticulum adenosine triphosphatase 1 isoform (SERCA1) expressions (108) through genomic actions. Under the effect of T3, the skeletal muscle energy metabolism is switched into an oxidative mitochondrial metabolism mediated by PGC-1α (50) and after exercise muscle D2 activity increases, which might be involved in the local T3 production necessary for these metabolic changes (109). T3 genomic actions significantly differ in muscle fibers due to the pattern of TR expression that are more abundant in slow-twitch than in fast-twitch muscles (110). As well as T3, 3,5-T2 also caused a slow to fast-twitch muscle transition and induced a shift toward a glycolytic phenotype, increasing key enzymes of glycolysis, such as phosphofructokinase (PFK) (89).

Apart from the well-known genomic actions of T3 in skeletal muscle, T3 administration increases the phosphorylation of AMPK and P38, preferentially in slow-twitch when compared to fast-twitch muscles (60). Both genomic and non-genomic pathways have been described to control mitochondrial biogenesis, a crucial step by which thyroid hormones can increase oxygen consumption.

When hypothyroid rats receive a single dose of 3,5-T2, AMPK phosphorylation increases in gastrocnemius muscle, a type of muscle that presents both slow- and fast-twitch fibers, while a single injection of T3 also induced the phosphorylation of both AMPK and acetyl CoA carboxylase (ACC) and a persistent phosphorylation of Akt. These changes lead to increased carnitine palmitoyl transferase (CPT) activity, fatty acid oxidation and increased GLUT4 translocation to the membrane (59). In rats fed a HFD, 3,5-T2 long-term administration enhanced Akt phosphorylation in skeletal muscle, increased lipid oxidation and ameliorated insulin resistance (38).

AMPK activation is a potential candidate to mediate the increased fatty acid oxidation detected in skeletal muscle after TH administration. AMPK activity was augmented by T3 in euthyroid (60) and hypothyroid rats (59) and by 3,5-T2 in euthyroid HFD fed (62) and hypothyroid rats (111). AMPK phosphorylation increased in the gastrocnemius of hypothyroid rats 1 hour after 3,5-T2 administration, which might explain the increased fatty acid oxidation (111).

T3 stimulates the electron transfer from cytosolic NADH through the mitochondrial alpha-glycerophosphate dehydrogenase (α-GPD), promoting a consequent loss of chemical energy as heat, due to the synthesis of only two ATP molecules from NADH instead of three (112). Differently, the acute treatment of hypothyroid rats with 3,5-T2 was sufficient to increase oxygen consumption related to FAD metabolism and did not affect the mitochondrial respiration when it was stimulated with malate, suggesting that the 3,5-T2 effect is independent of NADH metabolism and supporting the idea that lipid beta oxidation is responsible for the increased oxygen consumption (111).

The alteration in skeletal muscle mitochondrial proton conductance could also affect energy expenditure. A single dose of 3,5-T2 on mitochondrial parameters of hypothyroid rats was able to induce mitochondrial uncoupling due to increased substrate oxidation and the proton leak, probably through non-genomic actions (111). Both T3 and 3,5-T2 treatment for 4 weeks increased the UCP3 mRNA expression levels in skeletal muscle in diet induced obesity in mice (54).

Lastly, another effect of T3 is the ability to increase glucose uptake independent of insulin in skeletal muscle cells. T3 rapidly increases GLUT4 expression in skeletal muscle and its trafficking to the plasma membrane (113). In L6 muscle cells an increased glucose uptake independent of GLUT was reported in the first 30 min of T3 action (114). 3,5-T2 was also able to up-regulate sarcolemma membrane-associated GLUT4 protein content followed by increased insulin sensitivity in HFD fed rats (89). In contrast, another study did not show any effect of 3,5-T2 in GLUT4 expression in skeletal muscle (54).

Apparently, T1AM has an opposite effect when compared with 3,5-T2 and T3 actions in skeletal muscle. T1AM administration increases lipolysis (44, 45) and decreases carbohydrate utilization, as demonstrated after respiratory quotient (RQ) analysis (44). Protein breakdown also increases in mice after administration of T1AM for 8 days, as observed by Nuclear Magnetic Resonance spectroscopy (45). Recently, T1AM decreased oxygen consumption and cell diameter in cultured C2C12 myotubes. Suppression of AKT phosphorylation and mTOR activation together with increased catabolic pathways, such as ubiquitin E3 ligase, were demonstrated after T1AM treatment (115).

THYROID HORMONES METABOLITES AND THE LIVER

Liver insulin resistance secondary to lipid accumulation is one of the initiating steps related to obesity induced by high fat diet

(116). The mechanisms involved in the development of non-alcoholic fatty liver disease (NAFLD) are: (1) increased hepatic lipogenesis, and/or (2) higher lipolysis in adipocytes.

T3 regulates lipid metabolism in the liver and induces both fatty acid oxidation and lipogenesis. Apart from its ability to induce lipolysis, T3 also increases the expression of genes involved in hepatic lipogenesis, such as SPOT14, ACC, and fatty acid synthase (FAS) (117), and is responsible for increased plasma triglycerides and decreased serum cholesterol in humans (118).

T3 administration was not efficient to treat NAFLD, contrary to what was observed when rats received the selective TR beta agonist MB07811 that significantly increased hepatic fatty acid oxidation, and decreased liver steatosis (119). In relation to cholesterol, TR beta ligands act through increased expression of scavenger receptor B type I (SR-BI) that promotes hepatic uptake of cholesterol, lowering serum cholesterol levels (120). TR beta is probably the isoform that mediates most of the liver T3 effects on lipid metabolism (35). However, using a luciferase expression vector containing the human uncoupling protein 3 (UCP3) promoter, a known target of T3 through TR beta activation suggested that TR beta is not implicated in liver 3,5-T2 responses (62).

The regulation of the D1 gene by T3 is mediated by TR beta, and T3 stimulates both hepatic and kidney D1 activities (1) through genomic actions. Rats treated with 3,5-T2 have low serum T3 and increased hepatic and kidney D1 activities (39), suggesting a T3-like genomic effect of 3,5-T2 mediated by TR beta in the liver D1 regulation (rather than a non-genomic action). However, a recent study using rats fed with HFD showed that the expression levels of D1 mRNA increased with T3 but not with 3,5-T2 (116). However, the differences may be attributed to the different doses of 3,5-T2 used, the period of time of treatment and the animal model.

3,5-T2 was able to increase oxygen consumption of hepatocytes faster than T4 or T3 (36). Interestingly, it was demonstrated that T4 and T3 actions were dependent on deiodination since PTU pre-treatment attenuated their effects, suggesting that 3,5-T2 was the metabolite responsible for the effects of thyroid hormones on liver oxygen consumption (25). Due to its remarkable ability to increase hepatocytes oxygen consumption and the subsequent findings that it had minor board of genomic effects, 3,5-T2 was tested as a therapeutic agent to treat NAFLD in animal models [for review see (121, 122)]. 3,5-T2 treatment was shown to prevent or treat hepatic steatosis and obesity induced by HFD, increasing insulin sensitivity in rats (38). Senese et al. (116) compared T3 and 3,5-T2 administration in a HFD induced NAFLD model, and the decrease in liver lipid accumulation mediated by 3,5-T2 was accompanied by a down regulation in the expression of lipogenic enzymes like SPOT14, ACC, and FAS, different from T3 effects (116). In contrast, in rat liver mitochondria T1AM decreases oxygen consumption and increases reactive oxygen species release through the inhibition of complex 3 activity (123). Also, T1AM increase the content of glutathione in liver cells and thus their antioxidant ability (70).

There are several significant differences between T3 and its metabolite 3,5-T2, and T1AM, concerning their molecular mechanisms of action (31, 121). T3 stimulates both hepatic lipogenesis and fatty acid oxidation, while 3,5-T2, and T1AM induce fatty acid oxidation and inhibit lipogenesis.

Daily 3,5-T2 administration for 4 months increased AMPK activity and could explain the increased lipid oxidation by hepatocytes (38), decreased liver lipid accumulation, and increased insulin sensitivity, which could prevent the accumulation of lipid in liver and also in skeletal muscle (62). Part of these beneficial effects of 3,5-T2 were not altered by the inhibition of AMPK by compound C. Thus, sirtuin might also participate in the pathway activated by 3,5-T2 (62). It is well-known that sirtuin activation normalizes the expression of gluconeogenic and lipogenic enzymes in liver, and *in vitro* experiments demonstrated that 3,5-T2, and stimulates while T3 decreases sirtuin activity (62). Recently, T1AM treatment of overweight mice led to increased net weight loss and decreased cholesterol levels. Part of these T1AM beneficial effects seem to be a consequence of increased Sirtuin 6 and decreased Sirtuin 4 expressions (31). Additionally, It has been previously shown that 3,5-T2 induces SIRT1-mediated deacetylation of the promoter of SREBP- 1c in liver, reducing its expression (62).

In liver, insulin-dependent Akt activation suppresses gluconeogenesis through the regulation of key enzymes, such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6 phosphatase (G6Pase) (124). Recently, Da Silva Teixeira et al. (54) have shown that neither T3 nor 3,5-T2 trigger hepatic Akt phosphorylation, suggesting that these ligands do not alter hepatic insulin sensitivity through Akt activation (54). However, the gluconeogenesis was stimulated by T1AM in perfused rat liver and HepG2 cells, concomitant to the stimulation of fatty acid oxidation (29). Most studies with 3,5-T2 demonstrated amelioration in glucose tolerance, insulin sensitivity and liver steatosis (38, 39, 62). Therefore, both 3,5-T2, and T1AM could be tested as therapeutic agents against NAFLD.

T1AM ameliorates lipid profile in a model of polycystic ovary syndrome induced by glucocorticoids administration (70). On the other hand, T1AM might also induce insulin resistance due to decreased carbohydrate utilization by the cells (44, 45) and to decreased insulin secretion by beta-cells of the pancreas islets (125, 126). T1AM intraperitoneally resulted in decreased insulin secretion probably through the activation of alpha2A adrenergic receptors (Adra2a) in the beta cells of the pancreas, since the effect is abrogated in Adra2a knockout mice (126). Recently, it was shown that T1AM reduces the ATP turnover, which was implicated in the decrease of insulin secretion upon glucose stimulation (125). However, this acute *in vivo* effect of T1AM was not observed when a lower dose of T1AM was used and for a longer period of time (67). In contrast, 3,5-T2 stimulates insulin secretion upon glucose stimulation (127).

In short-term fasted male mice, the intracerebroventricular (icv) injection of T1AM (26) caused improved memory, hypophagia, and also peripheral effects such as reduced peripheral insulin sensitivity and higher plasma glucose levels (**Figure 3**), again highlighting the opposite effects of T1AM on glucose handling in relation to T3 and 3,5-T2.

TABLE 1 | Overview of 3,5-T2, and T1AM effects on Core temperature, Heart, Insulin sensibility: Liver-adipocyte-muscle and Pancreas.

Core temperature	**Dose /100g b.w.**	**Experiment**	**Effect(s)**
3,5-T2			
(51)	25 μg	HypoT rats	Maintenance of temperature in cold environement
(40)	25-250 μg	HFD, mice	Increased core temperature (with 250 μg/100g b.w.)
(54)	125-1250 μg	HFD, mice	Increased core temperature (with 1250 μg/100g b.w.)
T1AM			
(27)	5 mg	Mice	Transitory hypothermia
(44)	5 mg	Hamster and Mice	Transitory hypothermia
Heart	**Dose /100g b.w.**	**Experiment**	**Effect(s)**
3,5-T2			
(38)	25 μg	Euthyroid rats	Heart weight and HR unchanged
(39)	25-50-75 μg	Euthyroid rats	Increased H/b.w. (only 75 μg/100g b.w.). HR unchanged
(40)	25-250 μg	HFD, mice	Increased H/b.w. (only 250 ug/100g b.w.)
(54)	125-1250 μg	HFD, mice	Increased H/b.w. (with both doses used)
(92)	0,1-10 μM	Isolated rat heart	Increased glucose consumption (0,1-1 μM)
			Reduced contractile performance (10 μM)
T1AM			
(27)	5 mg /ED50=29 μM	Mice/Isolated rat heart	Bradycardia/reduced cardiac output *in vitro*
(30)	18-38 μM	Isolated rat heart	Reduced HR and contractility performance
(94)	0.125-12.5 μM	Isolated rat heart	Cardioprotection after ischemia insult
Liver-Adipocyte-muscle	**Dose /100g b.w.**	**Experiment**	**Effect(s)**
3,5-T2			
(36)	1 pM	Liver from HypoT rats	Increased oxygen consumption
(38)	25 μg	Euthyroid rats HFD	Increased FAO, hepatic steatosis atteunuated
(62)	25 μg	Euthyroid rats HFD	Increased FAO, increased insulin sensitivity
(39)	25-50-75 μg.	Euthyroid rats	Increased insulin sensitivity
(54)	125-1250 μg	HFD, mice	Reduced hepatic glucose output
(116)	25 μg	HFD, euthyroid rats	Increased lypolysis and decreased lipogenesis genes
T1AM			
(123)	10^{-7}-10^{5} M	Liver from HypoT rats	Reduced oxygen consumption, increased H_2O_2 release
(45)	1 mg	Mice (overweight)	Increased FAO
(70)	2.5 mg	Mice (PCOS)	Antilipogenic and enhanced protection to oxidative stress
(31)	1-2.5 mg	Mice (overweight)	Shift of metabolism from carbohydrates to lipids
Pancreas	**Dose /100g b.w.**	**Experiment**	**Effect(s)**
3,5-T2			
(62)	25 μg	HFD, euthyroid rats	Blood Insulin levels unchanged
(39)	25-50-75 ug	Euthyroid rats	Blood Insulin levels unchanged
(127)	0.1 nM/l - 0.1 μM/L	Human islet and cells	Increased insulin secretion.
(54)	125-1250 μg	HFD, mice	Reduced insulin levels (with 1250 μg/100g b.w.)
T1AM			
(126)	50 mg/10 uM(*in vitro*)	Mice/isolated islets	Increased blood glucose/Reduced insulin secretion *in vitro*
(73)	13 μg	Mice (icv)	Hyperglicemia
(67)	0.5 mg	Mice	Basal fasting glucose and glucose tolerance unchanged
(125)	100 nM	Murine Beta-Cells	Reduced insulin secretion

HypoT, Hypothyroid; HFD, High fat diet; H/b.w, Heart weight/body weight; HR, heart rate; Icv, intracerebroventricular; FAO, Fatty acid oxidation; PCOS, polycystic ovary syndrome.

THYROID HORMONES METABOLITES AND THE KIDNEY

Thyroid hormones affect kidneys size, weight, and structure. Hypothyroidism decreases, whereas hyperthyroidism increases kidney weight (128). Thyroid hormones affect the kidneys through direct as well as indirect mechanisms since T3 influences systemic hemodynamic parameters and exerts important cardiovascular effects.

As described for patients with myocardial infarction, low serum T3 levels are associated with poor prognosis of patients with chronic kidney disease (129). Notably, renal function

was improved by T3 treatment in patients with renal failure and severe hypothyroidism (130). Daily T3 administration for 4 weeks decreased urinary albumin excretion and attenuated the collagen accumulation and renal fibrosis in a model of diabetic nephropathy in mice. T3 decreased TGFβ expression and increased PI3K activity in kidney; the treatment with PI3K inhibitor abolished the beneficial effect of T3 in all parameters of renal function (131).

In an experimental model of diabetic nephropathy, 3,5-T2 treatment for 12 weeks also reduced fibrosis markers such as Fibronectin, Collagen IV and TGFβ expression ameliorating the renal function (88). Also, the restauration of sirtuin expression and activity was observed in diabetic rats treated with 3,5-T2, and the presence of sirtinol, an inhibitor of deacetylases, abolished the positive effect of 3,5-T2. It has been suggested that persistent JNK1 activation leads SIRT1 inhibition through increased protein degradation (88) and 3,5-T2 treatment attenuated JNK phosphorylation, what could be the mechanism of protection induced by 3,5-T2 that is apparently different from the pathways activated by T3 that are dependent on PI3K.

We cannot rule out that the anti-hyperglycaemic effect of both T3 and 3,5-T2 might also contribute to their protective effects on diabetic nephropathy models.

FINAL REMARKS

Substantial data described in the last 30 years show a consistent action of metabolites of thyroid hormones in several cell types and pathophysiological conditions, opening new chapters about the broad spectrum of thyroid hormones action, and the synthesis and action of their metabolites (**Table 1**).

The pathway for 3,5-T2 formation is believed to be the outer ring deiodination of T3 probably mediated by D2 that catalyses the removal of the outer-ring iodine from thyroxine (T4). Conversion of T3 into 3,5-T2 requires outer-ring deiodination, which can be catalyzed by either D1 or D2.

Nuclear receptors have high affinity for T3. T3 to T2 conversion promotes specific binding of 3,5-T2 to different isoforms of TRs, but this metabolite also exerts potent non-genomic actions that control many cellular reactions.

3,5-T2 conversion to T1AM (Deamination and deiodination reactions) produces a hormone metabolite that does not seem to bind to TR and apparently exert opposite effects in relation to its precursors biological actions.

rT3 is the product of D3 deiodination that was described as an inactivating enzyme, however rT3 can also be a precursor of T1AM, a metabolite that counteracts many classical T3 actions, such as tachycardia and body temperature regulation.

In the future, a better understanding about the differences in the cellular pathways regulated by T3 and its metabolites is of great interest in order to possibly unravel novel therapeutic targets for the control of prevalent diseases.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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3,5-Diiodothyronine: A Novel Thyroid Hormone Metabolite and Potent Modulator of Energy Metabolism

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Over 30 years of research has demonstrated that 3,5-diiodo-L-thyronine (3,5-T2), an endogenous metabolite of thyroid hormones, exhibits interesting metabolic activities. In rodent models, exogenously administered 3,5-T2 rapidly increases resting metabolic rate and elicits short-term beneficial hypolipidemic effects; however, very few studies have evaluated the effects of endogenous and exogenous T2 in humans. Further analyses on larger cohorts are needed to determine whether 3,5-T2 is a potent additional modulator of energy metabolism. In addition, while several lines of evidence suggest that 3,5-T2 mainly acts through Thyroid hormone receptors (THRs)- independent ways, with mitochondria as a likely cellular target, THRs-mediated actions have also been described. The detailed cellular and molecular mechanisms through which 3,5-T2 elicits a multiplicity of actions remains unknown. Here, we provide an overview of the most recent literature on 3,5-T2 bioactivity with a particular focus on short-term and long-term effects, describing data obtained through *in vivo* and *in vitro* approaches in both mammalian and non-mammalian species.

Keywords: thyroid hormones, energy balance, mitochondria, insulin resistance (IR), obesity, fatty acids oxidation

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INTRODUCTION

Thyroid hormones [3,5,3′,5′-tetraiodo-L-thyronine (T4) and 3,5,3′-triiodothyronine (T3), THs] play critical roles in differentiation, growth, and metabolism (1, 2). THs act via the nuclear thyroid hormone receptors (THRs), through different modes of action which, accordingly with Flamant (3), can be classified as: THR-dependent signaling of TH with direct binding to DNA; THR-dependent signaling of TH with indirect binding to DNA and THR-dependent signaling of TH without DNA binding; however, also THR-independent TH signaling is involved in TH mode of action (4). The different modes of action may be coupled, and several reports have recently shown that several TH metabolites act accordingly (5–8).

3, 5-diiodo-L-thyronine (3,5-T2) has emerged as a biologically active iodothyronine (9–11). Mitochondria and bioenergetic mechanisms seem to be major targets of 3,5-T2. Here, we review the most recent findings on the peripheral actions of 3,5-T2 and discuss the possible role of 3,5-T2 in the modulation of thyroid-related effects in organisms ranging from non-mammals to humans.

THE RAPID EFFECTS OF 3,5-T2 ON ENERGY METABOLISM

At 1 pM concentration, 3,5-T2 stimulates oxygen consumption more rapidly than T3 in perfused hypothyroid rat liver (12). Acute administration of T3 and 3,5-T2 to rat enhances mitochondrial activities (13, 14), with 3,5-T2 producing more rapid events (within 1 h) than T3 (after 24 h) and cycloheximide-independent (15).

The rapid increase in mitochondrial oxygen consumption by 3,5-T2 is reflected at the whole animal level (16). Injecting a single dose of 3,5-T2 (25 μg/100 g BW) to rats simultaneously administered with propylthiouracil (P) and iopanoic acid (I) (referred to as P+I, which results in severe hypothyroidism and inhibition of all the deiodinase activities) results in an increased resting metabolic rate (RMR) which is more rapid (within 6 h) than that induced by T3 (15 μg/100 g BW, effect seen within 25 h) (17). The effects following T3 injection in this study were like those reported by Tata (18), who injected the same dose. Simultaneous injection of Actinomycin D blocked the effects of T3 but not of 3,5-T2 (17), thus excluding the involvement of transcription in the effects of 3,5-T2. Indeed, it has been shown that the affinity of 3,5-T2 for human THRβ is 60-fold lower compared to T3 (19). Moreover, when injected into euthyroid rats, the effect of T3 on RMR is evident 25 h earlier than in P+I animals and is independent of Actinomycin D, suggesting that the effect of T3 injection could be due, at least in part, to the *in vivo* formation of 3,5-T2 from T3 as supported by its inhibition by P+I treatment and by the increased 3,5-T2 serum and liver levels following T3 injection into euthyroid rats (20).

The addition of nanomolar concentrations of 3,5-T2 significantly increases cytochrome oxidase (COX) activity (21) as demonstrated by specific binding of radioactive 3,5-T2 to subunit Va, and by complete reversal of its effect on respiration by a monoclonal antibody to this subunit (22, 23). The addition of 3,5-T2 to a liposome-reconstituted COX complex results in partial uncoupling which could explain its *in vivo* thermogenic effect (24). Binding sites detected by photoaffinity labeling in the rat liver cytosol (25) and by radioligand binding and displacement experiments in rat liver mitochondria (22) and cell membrane [shown by the *in vitro* activation of the Na^+/H^+ exchanger (26)] support the involvement of these organelles in the rapid action of 3,5-T2 (10, 26–29). The 3,5-T2 mitochondrial binding was maximal at pH 7.0 and the values for the apparent association constant and the binding capacity were $0.5 \pm 0.04 \times 10^8$ M^{-1} and 0.4 ± 0.04 pmol/mg mitochondrial protein respectively (21–23, 30). A top-down elasticity analysis shows that 3,5-T2 (within 1 h from injection into euthyroid rats) stimulates hepatic activity of both cytochrome c-oxidizing and -reducing components of the respiratory chain (31). 3,5-T2 also rapidly stimulates skeletal muscle mitochondrial activity and uncoupling (32, 33). 3,5-T2 rapidly increases mitochondrial Ca2+ uptake through which the iodothyronine could increase mitochondrial activity and respiration (34). More recently, the rapid effects of 3,5-T2 on intracellular Ca2+ and NO through plasma membrane and mitochondrial pathways in pituitary GH3 cells (35) further support mitochondria as a principal target of 3,5-T2 effects.

Moreover, 3,5-T2 has direct and rapid effects (within 1 h) on mitochondrial F(o)F (1)-ATP synthase activity in the liver of hypothyroid rats (36), increases mitochondrial respiration rates, increases mitochondrial uncoupling and reduces H_2O_2 production (37).

THE EFFECTS OF LONG-TERM ADMINISTRATION OF 3,5-T2 ON ENERGY METABOLISM

Chronic administration of 3,5-T2 into P+I cold-exposed rats increases the energy capacity of the heart, skeletal muscle, liver, and brown adipose tissue (BAT), improving their survival in the cold (38).

Chronic administration of 3,5-T2 into P+I rats induces significant stimulation of lipid β-oxidation (39), and upregulates rat-liver mitochondrial F(o)F(1)-ATP synthase by GA-binding protein/nuclear respiratory factor-2, thus providing new insights into the 3,5-T2 role on bioenergetic mechanisms (40).

When injected into P+I rat, 3,5-T2 increases skeletal muscle lipid handling through FAT/CD36 and mitochondrial oxidation (41), activates thermogenesis, with UCP1 likely acting as the molecular determinant of this effect, and increases the sympathetic innervation and vascularization of BAT (42).

THE HYPOLIPIDEMIC EFFECTS OF 3,5-T2

The effects of 3,5-T2 on energy metabolism has prompted research *in vitro* and *in vivo* on whether and how 3,5-T2 administration could improve adiposity and associated disorders.

IN VITRO STUDIES

Primary rat hepatocytes exposed to the classical oleate/palmitate (2:1 ratio) mixture have been employed as *in vitro* model of "fatty hepatocytes" to assess the effects of 3,5-T2 and T3 (doses of 10^{-7} or 10^{-5}M for 24 h) on lipid metabolism (43). 3,5-T2 and T3 reduce the number and average sizes of lipid droplets, thus making stored triglycerides (TGs) more accessible to enzymes acting on the catabolism/secretion of free fatty acids. More recently, 3,5-T2 has been shown to reduce lipid excess in fatty hepatocytes by recruiting triglyceride lipase on the lipid droplet surface (44). 3,5-T2 also reduces lipid content and triggers phosphorylation of Akt in an insulin receptor-independent manner when incubated with NAFLD-like rat primary hepatocytes (45). Furthermore, 3,5-T2 enhances glucose-induced insulin secretion in both rat β-cells and human islets (46).

When exposed to an oleate/palmitate (2:1 ratio) mixture and treated with 3,5-T2 or T3 (doses of 10^{-7} or 10^{-5}M for 24 h), FAO rat hepatoma cell lines, defective for functional THRs, show reduced TGs content, reduced number and size of lipid droplets and stimulated mitochondrial uncoupling (47), supporting a THR-independent TH signaling mechanisms which involve both

3,5-T2 and T3 through stimulation of mitochondrial uncoupled respiratory activity (47).

In HepG2 cells, 3,5-T2 blocks the proteolytic cleavage of SREBP-1 without affecting its expression, thus reducing fatty acid synthase expression in a way dependent on the concurrent activation of MAPK, ERK, and p38 and Akt and PKC-δ pathways (48).

IN VIVO STUDIES

Hypolipidemic effects have been studied *in vivo* by using several animal models (49). Simultaneous 3,5-T2 (25 μg/100 g BW) administration for 4 weeks to rats feeding a high-fat diet (HFD) prevents fatty liver and increases in body weight by increasing fatty acid oxidation rate and mitochondrial uncoupling to burn fat (50). Reductions in serum TGs and cholesterol levels (50), as well as improved insulin sensitivity (51), are also associated with 3,5-T2 administration. 3,5-T2 elicits the deacetylation of hepatic peroxisome proliferator-activated receptor gamma coactivator 1-alpha and sterol regulatory element binding protein-1c (SREBP-1c) through direct induction of silent mating type information regulation 2 homolog 1 (SIRT1) activity, resulting in increased fatty acid oxidation and decreased lipogenesis, respectively (51). Though both 3,5-T2 and T3 decrease the expression of hepatic SREBP-1c, 3,5-T2 (administered at a daily dose of 25 μg/100 g BW to high-fat diet- fed rats for 1 week), in contrast to T3 (administered at a 10-fold lower dose), does not directly induce the expression of the TRE-containing SREBP-1c lipogenic target genes [acetyl-CoA carboxylase and fatty acid synthase (52). This, at least in part, explains the effectiveness of 3,5-T2 in preventing hepatic fat accumulation and insulin resistance. Iannucci (53) showed that both 3,5-T2 and T3 exert lipolytic effects in the liver mediated by autophagy and increased fatty acid oxidation although the metabolic profiles suggested that there may be some differences in the mechanism(s) and magnitude of their metabolic effects. 3,5-T2 ameliorates muscle glucose uptake by increasing the response to insulin of Akt/PKB phosphorylation and induces structural and biochemical shifts toward glycolytic myofibers (54), thus enhancing muscle glycolytic capacity producing metabolic benefits (55–57), reminiscent of those induced by resistance exercise (58). Mitochondria adapt to the glycolytic phenotype of gastrocnemius muscle both in terms of metabolism and of dynamic with 3,5-T2 being able in reverting the HDF-associated expression pattern of proinflammatory factors (59). At the doses of 25 μg 3,5-T2/100 g BW for 4 weeks no signs of suppression of the hypothalamus-pituitary-thyroid (HPT) axis and cardiac hypertrophy are detected.

In streptozotocin-treated rats, 3,5-T2 (at the dose of 25 μg/100 g BW for 12 weeks) protects against renal damage in diabetic nephropathy through SIRT1-dependent deacetylation and inactivation of subunit p65 of NF-kB, thus inhibiting the inflammatory process related to this disease (60). Ball in rats (61), reported that 3,5-T2 is more effective in inducing hepatic malic enzyme gene expression than suppressing circulating TSH, indicating that tissue- and gene-selective effects of 3,5-T2 are not only related to differences in binding of this thyromimetic ligand to various TR isoforms but also to distinct local cellular ligand availability.

3,5-T2 administration to HFD-obese Wistar rats was also shown to reduce pre-existing hepatic fat accumulation through increased mitochondrial fatty acid oxidation coupled with less efficient utilization of substrates and reduced oxidative stress (62). A proteomic study showed that 3,5-T2 counteracts several HFD-induced changes in the protein profile, mostly in the mitochondria (63). Moreover, blue native-PAGE (BN-PAGE)/in-gel activity analysis revealed that 3,5-T2 treatment results in stimulation (vs. HFD) of respiratory complexes, thus explaining, at least in part, the anti-steatosis effect of 3,5-T2. Administration of 3,5-T2 [subcutaneously injected at doses of 25, 50, or 75 μg/100 g BW for 90 days] to chow-fed rats aged 3–6 months significantly reduces body mass and improves glucose tolerance, while heart rate and mass remain unchanged, TSH levels remain normal in rats receiving 25 μg of 3,5-T2 /100 g BW but are slightly lowered in rats that received 50 and 75 μg of 3,5-T2 /100 g BW (64). In apparent contrast, 3,5-T2 administration to Sprague Dawley rats fed a safflower-oil based HFD fails to improve NAFLD or insulin sensitivity (65). One reason for this discrepancy may be that an unsaturated fat-predominant plant oil-based diet is used (65) that could mask the hypolipidemic effects of 3,5-T2 with saturated fat-predominant animal fat-based diets (50, 51, 54, 62). Furthermore, Sprague Dawley and Wistar rats display differences in both lipoprotein metabolism and endocrine function (66).

In diet-induced obese mice, daily administration of 3,5-T2 (250 μg/100g BW for 14 or 28 days i.p.) shows beneficial effects on adiposity, serum leptin, and energy expenditure (67). The lower dose of 3,5-T2 suppress βTSH transcripts, thus suggesting a risk of interference of 3,5-T2 on the HPT axis as well as on the heart (67). Lean and diet-induced obese male mice treated for 4 weeks with a 3,5-T2 dose of 2.5 μg/g BW, show an altered expression of genes encoding hepatic xenobiotic-metabolizing enzymes involved in catabolism and inactivation of xenobiotics and TH as well as in hepatic steroid and lipid metabolism (68). Hence, the administration of this high dose of 3,5-T2 might exert adverse hepatic effects.

3,5-T2 (1.25 mg/100 g BW via daily gavage) reduces circulating total and LDL cholesterol as well as the liver level of apoB and circulating levels of both apoB48 and apoB100, but, at the same time, reduces plasma T4 levels in Western type diet fed low-density lipoprotein receptor knockout mice (69). Both 3,5-T2 and T3 administration significantly reduce nuclear HNF4α protein content, while 3,5-T2, but not T3, decreases the expression levels of the HNF4α transcriptional coactivator PGC-1α. Lower PPARα levels are found only following T3 treatment while both T3 and 3,5-T2 lower liver X receptor α nuclear content (70). 3,5-T2 (1.25 mg/100 g BW) decreases body weight and blood glucose levels through reductions in GLUT2 levels and changes in hepatic glucose output in obese mice showing to produce signs of thyrotoxicosis (71). Taken together, these studies suggest the possibility that the "thyrotoxic effects" of 3,5-T2 may be dependent upon possible differences between experiments in rats vs. mice, normal weight vs. obese, or euthyroid vs.

hypothyroid animals, age, diet and temperature of exposure. It is important to note, however, that 3,5-T2 did not suppress TSH as strongly as T3 and that the cardiac readouts may represent an adaptation to increased metabolic rate, perhaps implying potential for separation of desirable effects from thyrotoxic effects.

At the current stage, no validated technique is available to accurately measure intracellular levels of 3,5-T2. Resolving this issue will bring to light to what extent 3,5-T2 is taken up in the tissues and how this relates to the effects of the exogenous administrations described above.

THE PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL ROLES OF 3,5-T2 IN HUMANS

A case report (72) involving two participants revealed that administration of 3,5-T2 to humans (1–5 μg/kg BW) rapidly (after 4–6 h) increased RMR. Chronic 3,5-T2 administration (28 days, approximately 5 μg/kg BW) increases RMR by approximately 15% and decreases body weight by approximately 4 kg in both participants. Principal clinical parameters show no significant changes and no side effects (i.e., cardiac abnormalities) are observed.

As mentioned above, reliable quantification methods to measure endogenous levels of 3,5-T2 have been lacking (73–75) and the data reported so far need independent analytical confirmation.

Recently, a mouse monoclonal antibody based on a new competitive chemiluminescence immunoassay was developed (76) to investigate the origin and action of 3,5-T2 in humans under several conditions. Data by Pietzner (77) in euthyroid human serum point toward a physiological link between 3,5-T2 (with a concentration of 0.22–0.33 nM) and glucose metabolism as well as TH homeostasis. Pietzner (78) screened the urine metabolome for associations with serum 3,5-T2 concentrations in healthy individuals, resulting in a median serum concentration of 0.24 nM. The detected metabolites are related to glucose and lipid metabolism, as well as the response to oxidative stress or drug metabolism, and are in concordance with previously published rat liver proteome analyses (63). Dietrich (79) reported elevated concentrations of 3,5-T2 (0.59 ± 0.07 nM vs. 0.39 ± 0.04) in cardiac Nonthyroidal Illness Syndrome (NTIS) suggesting that 3,5-T2 elevations in NTIS could explain why patients with low-T3 syndrome substituted with T4/T3 do not benefit from exogenous TH administration. Langouche (80), in critically ill patients reported a 30% higher serum 3,5-T2 concentration than healthy volunteers which are not independently correlated with TH.

Although recent studies in human gave some indications on the physiological and pathophysiological roles of 3,5-T2 in humans, further analyses on larger samples of euthyroid individuals are needed to obtain a more comprehensive picture of the role of 3,5-T2 in humans.

THE EFFECTS OF 3,5-T2 IN NON-MAMMALIAN SPECIES

The effects of 3,5-T2 on metabolic efficiency is conserved across species. 3,5-T2 rapidly stimulates pyruvate-fuelled mitochondrial respiration of liver and muscle from goldfish *Carassius auratus* (81). After 12 or 24 h, 3,5-T2 rapidly decreases type 2 deiodinase (D2) activity in the liver of killifish, whilst not affecting type 1 deiodinase (D1) activity; moreover, after a 24 h exposure, 3,5-T2 (like T4 and T3) inhibits both D1 and D2 transcription (82). 3,5-T2 also regulates thermal acclimation in *Danio rerio* (83) and growth in tilapia (84). 3,5-T2 binds to and activates a specific long TRβ1 isoform that contains a nine-aminoacid insert at the beginning of the ligand-binding domain, whereas T3 can interact also with a different TRβ1 isoform that lacks this insert (19). Hernández-Puga reported that 3,5-T2 represses THRβ expression and impairs its up-regulation by cortisol possibly through a transrepression mechanism (85). Very recently, Olvera (86) reported that in tilapia cerebellum 3,5-T2 specifically regulates gene sets involved in cell signaling and transcriptional pathways, while T3 regulated pathways related to cell signaling, immune system, and lipid metabolism.

CONCLUSIONS

Thirty years of research using mammalian and non-mammalian *in vivo* and *in vitro* models has generated substantial data on the biological effects of 3,5-T2. However, a debate is open concerning the side-effects of 3,5-T2, an issue that needs to be investigated by performing more comprehensive studies in humans and animal models to fully evaluate any potential risks.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Global Disruption of α2A Adrenoceptor Barely Affects Bone Tissue but Minimizes the Detrimental Effects of Thyrotoxicosis on Cortical Bone

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Evidence shows that sympathetic nervous system (SNS) activation inhibits bone formation and activates bone resorption leading to bone loss. Because thyroid hormone (TH) interacts with the SNS to control several physiological processes, we raised the hypothesis that this interaction also controls bone remodeling. We have previously shown that mice with double-gene inactivation of α2A- and -adrenoceptors (α2A/2C-AR$^{-/-}$) present high bone mass (HBM) phenotype and resistance to thyrotoxicosis-induced osteopenia, which supports a TH-SNS interaction to control bone mass and suggests that it involves α2-AR signaling. Accordingly, we detected expression of α2A-AR, α2B-AR and α2C-AR in the skeleton, and that triiodothyronine (T3) modulates α2C-AR mRNA expression in the bone. Later, we found that mice with single-gene inactivation of α2C-AR (α2C-AR$^{-/-}$) present low bone mass in the femur and HBM in the vertebra, but that both skeletal sites are resistant to TH-induce osteopenia, showing that the SNS actions occur in a skeletal site-dependent manner, and that thyrotoxicosis depends on α2C-AR signaling to promote bone loss. To further dissect the specific roles of α2-AR subtypes, in this study, we evaluated the skeletal phenotype of mice with single-gene inactivation of α2A-AR (α2A-AR$^{-/-}$), and the effect of daily treatment with a supraphysiological dose of T3, for 4 or 12 weeks, on bone microarchitecture and bone resistance to fracture. Micro-computed tomographic (μCT) analysis revealed normal trabecular and cortical bone structure in the femur and vertebra of euthyroid α_{2A}-AR$^{-/-}$ mice. Thyrotoxicosis was more detrimental to femoral trabecular bone in α2A-AR$^{-/-}$ than in WT mice, whereas this bone compartment had been previously shown to present resistance to thyrotoxicosis in α2C-AR$^{-/-}$ mice. Altogether these findings reveal that TH excess depends on α2C-AR signaling to negatively affect femoral trabecular bone. In contrast, thyrotoxicosis was more deleterious to femoral and vertebral cortical bone in WT than in α2A-AR$^{-/-}$ mice, suggesting that α2A-AR signaling contributes to TH actions on

cortical bone. These findings further support a TH-SNS interaction to control bone physiology, and suggest that α2A-AR and α2C-AR signaling pathways have key roles in the mechanisms through which thyrotoxicosis promotes its detrimental effects on bone remodeling, structure and resistance to fracture.

Keywords: thyroid hormone, thyrotoxicosis, sympathetic nervous system, α2A-adrenoceptor, cortical bone, trabecular bone, bone remodeling

INTRODUCTION

Thyroid hormone (TH) is recognized as an important regulator of bone remodeling, having a key role in the maintenance of bone mass and bone integrity (1). TH affects the skeleton indirectly, modulating the expression or action of other hormones and factors, such as GH and IGF-1 (2, 3), but also acts directly in the skeleton, controlling the proliferation, differentiation and/or activity of the main skeletal cells (4–7). Thyroid hormone receptors (TR) were detected in osteoblasts (8–11), osteocytes (9), osteoclasts (9, 12) and chondrocytes (13). In conditions of TH deficiency, both bone formation and resorption are decreased, leading to a state of low bone turnover. In this condition bone mass may be slightly increased or unchanged (14–16). In contrast, TH excess increases both osteoblastic and osteoclastic activities, but the latter is favored, leading to negative balance of calcium and bone loss (17–20). Therefore, thyrotoxicosis is an established cause of secondary osteoporosis, due to high bone turnover, with accelerated bone loss (16, 21, 22).

Over the last two decades, data has uncovered the sympathetic nervous system (SNS) as another potent regulator of bone remodeling (23). Evidence shows that SNS activation increases bone resorption and decreases bone formation leading to bone loss (24, 25), in a process that involves β2 adrenoceptors (β2-AR), expressed in osteoblasts (26, 27). However, studies by our group showed that female mice with global double inactivation of α2A and α2C adrenoceptors (α2A/C-AR$^{-/-}$ mice) present a striking high bone mass (HBM) phenotype along with improved resistance to fracture, increased bone formation rate and decreased bone resorption, regardless of presenting increased sympathetic outflow and, therefore, a chronic increase in serum levels of catecholamines (28). The α2-adrenoceptor (α2-AR) family comprises α2A-, α2B- and α2C-AR subtypes (29–31), which are expressed in presynaptic membranes of adrenergic neurons, where they act as autoreceptors (receptors stimulated by the neurotransmitter released by the neuron where they are located) inhibiting the release of catecholamines (32, 33), especially norepinephrine (NE). Therefore, α2A/C-AR$^{-/-}$ mice represent a mouse model of chronic sympathetic hyperactivity (34). Thus, the HBM phenotype in these animals revealed that β2-AR is not the only adrenoceptor involved in the control of bone turnover and raised the hypothesis that α2-AR subtypes also mediate SNS signaling in the skeleton (28). Accordingly, we and others found expression of α2A-, α2B- and α2C-AR in osteoblasts (28, 35, 36), osteoclasts, osteocytes and chondrocytes (28). In addition, *in vitro* findings reveal direct α2-AR signaling-mediated actions of the SNS in the skeleton (28, 37).

An important characteristic of TH is its interaction with the SNS to regulate several physiological processes (38). It is well known that TH-SNS interactions are necessary for maximum thermogenesis, lipolysis and lipogenesis (39). Interestingly, several clinical manifestations of thyrotoxicosis are indicative of increased adrenergic activity, such as tachycardia, increased cardiac output, increased glycogen and lipid mobilization, enhanced thermogenesis, tremor, hyperkinetic behavior, and sweating (40). In contrast, responses to adrenergic stimulation are low or blunted during hypothyroidism, leading, for example, to cold intolerance and limited metabolic responses to exercise (38, 41). Considering that both SNS hyperactivity and thyrotoxicosis have osteopenic effects, we raised the hypothesis that a TH-SNS interaction also occurs to regulate bone remodeling. The fact that treatment of hyperthyroid patients with propranolol, a β adrenoceptor antagonist, corrects the thyrotoxicosis-induced hypercalcemia (42) and decreases the urinary excretion of hydroxyproline, a biochemical marker of bone resorption (43), support a possible TH-SNS interaction to control bone remodeling, in addition to suggest that this interaction depends on the β-AR signaling pathway.

Recent studies by our group further support the hypothesis of a TH-SNS interaction to control bone remodeling, and reveals that α2-AR signaling has an important role in this interaction (28, 37, 44). We found that young adult female α2A/2C-AR$^{-/-}$ mice are resistant to the deleterious effects of thyrotoxicosis on bone mass, on cortical and trabecular bone microarchitecture, and on bone resistance to fracture (37). In addition, *in vitro* studies bring evidence that TH-SNS interactions are likely to occur locally in the skeleton, via α2A-AR and/or α2C-AR signaling. Later, with the aim of discriminating the roles of the different α2-AR isoforms, we evaluated the bone phenotype of mice with the single gene inactivation of α2C-AR subtype (α2C-AR$^{-/-}$) (44), which mRNA expression had been previously shown to be downregulated by TH in the femur (37). While α2A/2C-AR$^{-/-}$ mice present a generalized phenotype of HBM (28), we found that α2C-AR$^{-/-}$ animals present higher trabecular bone mass in the vertebra and lower trabecular bone mass in the femur (when compared with WT mice), which was accompanied by decreased bone strength in the femur and tibia (44). This heterogeneous bone phenotype of α2CAR$^{-/-}$ mice reinforces the hypothesis that the SNS regulates bone remodeling and structure, via α2C-AR signaling, and suggests that this regulation is not the same across the skeleton, but rather may vary depending on the skeletal site. In spite of this heterogeneous bone phenotype, α2C-AR$^{-/-}$ mice present resistance to the thyrotoxicosis-induced bone deterioration in both skeletal sites (femur and vertebra),

likewise α2A/2C-AR$^{-/-}$ mice (37, 44). These findings strongly suggest that the mechanism of action of TH to promote bone loss depends on α2C-AR subtype signaling. To confirm this hypothesis, in the present study, we characterized the bone phenotype of mice with global gene inactivation of only α2A-AR subtype, and the skeletal responses of these single KO animals (α2A-AR$^{-/-}$ mice) to a 4- or 12-week-long-chronic condition of thyrotoxicosis.

MATERIALS AND METHODS

Animals and Treatment

All the experimental procedures were carried out in accordance with the ethical principles and guidelines for animal research set by the Brazilian Society of Animal Experimentation, and were approved by the Ethics Committee on Animal Use (ECAU) of the Institute of Biomedical Sciences, University of São Paulo (protocol number 35/page 85/book 02). A cohort of female congenic C57BL/6J (B6) mice with gene inactivation of α2A-adrenoceptors (α2A-AR$^{-/-}$) (34) and their wild-type (WT) controls (B6) were studied. All animals were 30 days old at baseline. The animals were kept under light- and temperature-controlled conditions (alternating cycles of light/dark for 12 h at a temperature of approximately 25°C), with *ad libitum* access to food and water. Thyrotoxicosis was induced by daily and i.p. administration of T3 (Sigma, St Louis MO, USA), at a dose of 7 μg T3/100g•body weight (BW)/day, which is equivalent to 20 times the physiological dose of T3 per day. T3-untreated (-) animals received daily i.p. injections of saline. Animals were treated with T3 or saline for 4 or 12 weeks, at the same time of the day each day. Animals were weighed once a week to follow changes in BW over the experimental period, in order to indirectly monitor a thyrotoxic state and with the purpose of adjusting the amount of T3 to be administered, to maintain the supraphysiological dose (20xT3), during the whole treatment period. Female mice were grouped (7 animals per group) as follows: WT and α2A-AR$^{-/-}$ (T3-untreated animals, receiving saline), WT+T3 and α2A-AR$^{-/-}$ + T3. At the end of the treatment period, animals were euthanized by exposure to CO2.

Serum T3 and T4 Assay

In order to confirm a thyrotoxic state, serum levels of T3 and thyroxine (T4) were measured at the end of the experimental period. Just after euthanasia, blood was collected by cardiac puncture. Serum was isolated by centrifugation and serum levels of total T4 and T3 were measured using radioimmunoassay commercial kits (RIA-gnost T4 and RIA-gnost T3; CIS Bio International, Gif-sur-Yvette, France). For the T4 and T3 assays, standard curves were built in our laboratory with a pool of charcoal-stripped mouse serum. Blood samples were always collected 2 h after the last T3 administration.

Fat, Skeletal Muscles and Heart Mass

Considering the characteristic effects of thyrotoxicosis on fat mass, skeletal muscle mass and heart mass (45, 46), we measured these parameters at the end of the treatment period to also confirm a thyrotoxic state. Immediately after euthanasia, the axillar and retroperitoneal fat pads; the extensor digitorum longus (EDL), gastrocnemius and rectus femoris muscles; and the heart were dissected out and weighed for wet-mass determination. The skeletal muscles and heart samples were dehydrated at 60°C for 48 h and weighed again for dry-mass determination. Fat and heart masses were expressed in milligrams per gram of BW, whereas muscle masses were expressed inn milligrams per tibial or femoral length.

Micro-Computed (μCT) Analysis of the Femur and L5

Bone structural parameters of the right femur and the fifth lumbar vertebra (L5) were obtained using the μCT unit Skyscan 1174 (Bruker MicroCT, Kontich, Belgium), and the CtAn Software, version 1.5 (Bruker MicroCT). The X-ray settings were standardized to 100 kV for the baseline specimens, with an exposure time of 590 ms. A 0.05-mm-thick aluminum filter and a beam-hardening algorithm were used to minimize beam-hardening artifacts. The vertebral body of L5 and the distal metaphysis of the femur were the selected regions of interest (ROI). The trabecular bone parameters analyzed are trabecular volume (BV/TV); trabecular number (Tb.N); trabecular thickness (Tb.Th); trabecular separation (Tb.Sp); structure model index (SMI), which indicates the prevalence of rod-like (cylinder) and plate-like trabeculae (an ideal plate and cylinder have SMI values of 0 and 3, respectively) (47); trabecular pattern factor (Tb.Pf), a trabecular connectivity index (lower indexes indicate better connectivity of the trabecular structure) (48); trabecular porosity (Tb. Po); and trabecular bone mineral density (BMD). The cortical bone parameters analyzed are the total tissue area (T.Ar), which is the total area delimited by the periosteum, including the medullary and cortical area; cortical bone area (Ct.Ar); medullary area (Ma.Ar), cortical bone thickness (Ct.Th); periosteal perimeter (Ps.Pm); endocortical or endosteal perimeter (Ec.Pm); cortical porosity (Ct.Po), and cortical BMD.

Three-Point Bending Test of the Femur

The right femurs were dissected and submitted to the three-point bending test, using the Instron testing system Model 3344 (Instron Corporation, MA, USA). During the test, the anterior cortex of the femur was placed in compression and the posterior cortex in tension. The distal and proximal extremities of the femur were supported on two anvils, spaced by a distance that equals the half of the femoral length (femoral length/2). A force was applied perpendicularly to the longitudinal axis of the bone, at the midpoint between the two supports, in the anteroposterior direction of the femur, by a crosshead, at a constant velocity of 5 mm/min, until the bone completely ruptured. To stabilize the specimen, a small preload (5% of the average maximum load) was applied before real testing. The applied force and the displacement of the crosshead were monitored and registered, at a sampling rate of 80 Hz, by the Bluehill software (version 3, Instron Corporation). The following biomechanical parameters were obtained and evaluated: Maximum load (N), which corresponds to the highest force applied during the test (maximum force at which the bone

is able to withstand); tenacity (mJ), which is the bone's ability to withstand a fracture; and stiffness (N/mm), a measure of the rigidity of the bone tissue.

Real-Time PCR

Expression of α2A-AR, α2B-AR, α2C-AR, β1-AR, and β2-AR were determined by real-time PCR in the whole femur. The femurs were dissected and then crushed in a steel mortar and pestle set (Fisher Scientific International, Inc., Hampton, NH) precooled in dry ice. The crushed bones were transferred to microfuge tubes precooled in ice and total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. Total RNA was reverse transcribed using RevertAid-H-Minus M-MuLV Reverse Transcriptase (Fermentas, Hanover, MD) to synthesize the first-strand cDNA, which was used as a template. SYBR Green Super Mix (Applied Biosystems, Warrington, UK) was used for the real-time PCR using the ABI Prism 7500 sequence detector (Applied Biosystems, Foster City, CA). All primers used in this study [α2A-AR, forward (F): CGC AGG CCA TCG AGT ACA A and reverse (R): GAT GAC CCA CAC GGT GAC AA (NM_007417.3); α2B-AR, forward (F): TCC CTC TGG GAG GCA AGT G and reverse (R): GGC CAG GAT TCC AGA CCA TT (NM_ 009633.3); α2C-AR, F: CAT GGG CGT GTT CGT ACT GT and R: CAG GCC TCA CGG CAG ATG (NM_007418.3); β1-AR, forward (F): TCG TCC GTC GTC TCC TTC TAC and reverse (R): ACA CCC GCA GGT ACA CGA A (NM_007419); β2-AR, forward (F): GCC ACG ACA TCA CTC AGG AAC and reverse (R): CGA TAA CCG CAC TGA GGA TGG (NM_007420); 18S, F: GTA ACC CGT TGA ACC CCA TT and R: CCA TCC AAT CGG TAG TAG CG (NM_11188)] were designed using the Primer Express software (Applied Biosystems^{TM}) and were synthesized (Integrated DNA Technologies, Coralville, IA) specifically for real-time PCR. All CT values were normalized using 18S as the internal control, which was validated for this study, showing to be stable (its expression did not vary due to mice lineage or T3 treatment). Relative gene expression quantification was assessed by the CT method, as described previously by Livak and Schmittgen (49). The final values for samples are reported as fold induction relative to the expression of the control, with the mean control value being arbitrarily set to 1.

Statistical Analysis

The statistical significance was determined by the Two-way analysis of variance (ANOVA), followed by the Tukey's test. Results were expressed as mean ± standard error of the mean (SEM). For all tests, $p < 0.05$ was considered statistically significant. For the statistical tests, we used the GraphPad Instat Software (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

Effect of T3 Treatment on Serum Levels of T3 and T4

Serum levels of T3 were significantly higher (7- to 11-fold) in WT and α2A-AR$^{-/-}$ animals treated with T3 for 4 or 12 weeks, when compared with their respective saline-treated controls (**Figure 1A**). On the other hand, T4 concentrations (**Figure 1B**) were significantly lower (44–64%) in WT and α2A-AR$^{-/-}$ animals treated with T3 (vs. saline-treated animals), which reflects the suppression of the hypothalamic-pituitary-thyroid (HPT) axis promoted by T3 excess (50), and confirms a thyrotoxic state in both WT and KO animals. Serum levels of T3 and T4 were not different between WT and α2A-AR$^{-/-}$ animals.

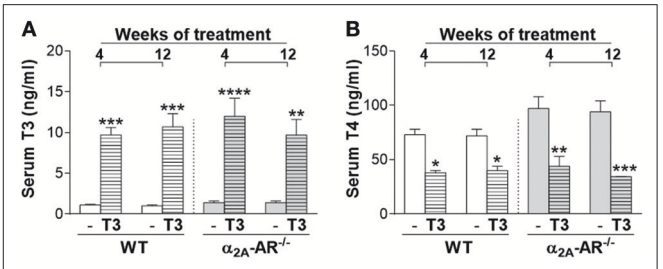

FIGURE 1 | Serum levels of T3 and T4 in α_{2A}-AR$^{-/-}$ and WT mice. Animals were treated with a supraphysiological dose of T3 (7 μg/100 g BW/day) or saline (-) for 4 or 12 weeks, by daily i.p. injections. Values are expressed as mean ± SEM ($n = 7$ per group). *$P < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ vs. the respective saline-treated mice, by Two-way ANOVA followed by Tukey's test.

Effect of T3 Treatment on Body Composition and Heart Mass

To indirectly confirm a thyrotoxic state, we evaluated the effect of T3 treatment on body composition and heart mass. **Figure 2A** shows that α2A-AR$^{-/-}$ mice presented 8–13% lower BW than WT mice from weeks 4 to 12 of this study, when animals were in the age range of 58–114 days. T3 treatment lead to lower BW in WT animals (**Figure 2B**), on weeks 5, 10, 11, and 12 of treatment (7–8%). In α2A-AR$^{-/-}$ animals (**Figure 2C**), BW decreased 11 and 9% after 1 and 2 weeks of T3 treatment, respectively; but then tended to be higher in T3 treated KO animals as compared with saline-treated KO mice. In fact, on week 11, BW was 9% higher in KO mice treated with T3. In WT animals, T3 treatment significantly decreased the axillar fat mass, after 12 weeks of treatment (33%); and the retroperitoneal fat mass, after 4 and 12 weeks of treatment (66 and 62%, respectively), which was not observed in KO animals (**Figures 3A,B**). The retroperitoneal fat mass showed to be 42% lower in euthyroid KO animals vs. euthyroid WT animals by the end of the 4th week of this study, when animals were 58 days old (**Figure 3B**), but later these differences were not present anymore. Four weeks of treatment with T3 did not significantly affect the mass of EDL and rectos femoris muscles both in WT and KO animals (not shown), but significantly decreased by 19% the gastrocnemius mass in both WT and KO animals, whereas 12 weeks of treatment with T3 only decreased the gastrocnemius mass in WT animals (19%), but not in KO animals (**Figure 3C**). Treatment with the supraphysiological dose of T3 (20xT3) promoted cardiac hypertrophy, evidenced by the increased dry mass of the heart (between 17 and 27%), in both WT and KO animals after 4 and 12 weeks of treatment (**Figure 3D**).

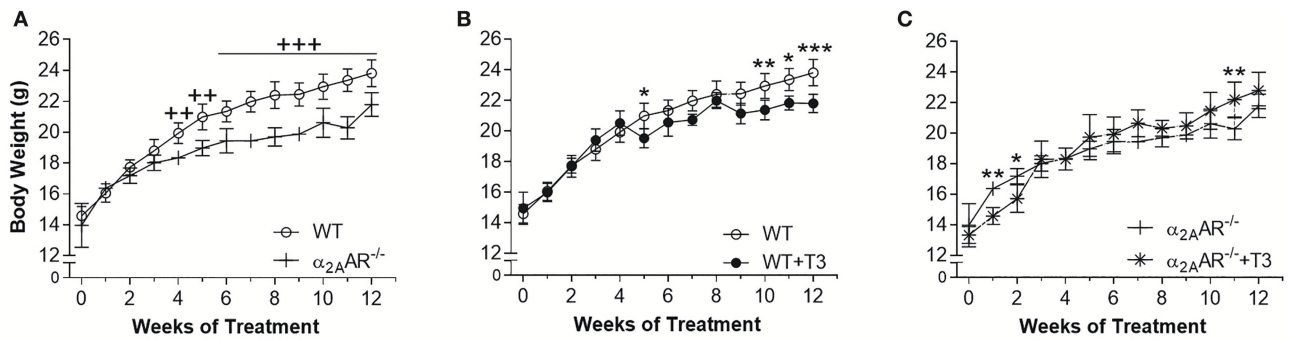

FIGURE 2 | Effect of thyrotoxicosis on body weight of α_{2A}-AR$^{-/-}$ and WT mice. Animals were treated with a supraphysiological dose of T3 (7 μg/100 g BW/day) or saline for 12 weeks, by daily i.p. injections. Body weight was measured every week. Values are expressed as mean ± SEM ($n = 7$ per group). $^{++}P < 0.01$, and $^{+++}p < 0.001$ vs. WT; and $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. the respective saline-treated mice, by Two-way ANOVA followed by Tukey's test.

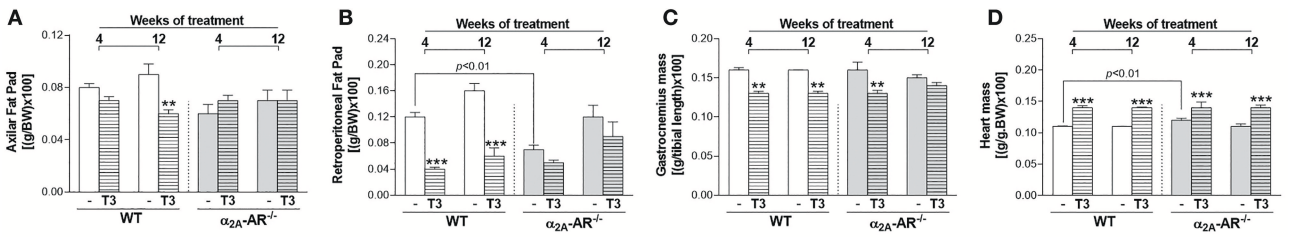

FIGURE 3 | Effect of thyrotoxicosis on fat mass, muscle mass and heart mass of α_{2A}-AR$^{-/-}$ and WT mice. Animals were treated with a supraphysiological dose of T3 (7 μg/100 g body mass/day) or saline (-) for 4 or 12 weeks, by daily i.p. injections. Values are expressed as mean ± SEM ($n = 7$ per group). $^{**}P < 0.01$ and $^{***}p < 0.001$ vs. the respective saline-treated mice, by Two-way ANOVA followed by Tukey's test. *P*-values above the bars indicate differences between WT and KO mice.

Effect of T3 Treatment on Trabecular and Cortical Bone of the Femur

The μCT analysis of the femur showed that trabecular and cortical bone parameters were not different between WT and α2A-AR$^{-/-}$ mice treated with saline (**Figure 4**). As expected, T3 treatment caused deleterious effects on both trabecular and cortical compartments of the femur, but trabecular bone of KO animals showed to be more sensitive to T3 effects than that of WT animals (**Figures 4A–H**). The 4-week treatment with T3 significantly decreased BV/TV (54%; **Figure 4A**), Tb.N (55%; **Figure 4B**), and trabecular BMD (74%; **Figure 4H**); and increased Tb.Sp (54%; **Figure 4D**) only in KO, but not in WT animals. The 12-week treatment with T3 significantly decreased BV/TV (**Figure 4A**) and Tb.N (**Figure 4B**), and increased Tb.Sp (**Figure 4D**) and Tb.Po (4G) in both WT and KO animals (78 and 72%, 76 and 73%, 55 and 59%, and 8 and 6%, respectively). However, this longer treatment increased SMI (27%; **Figure 4E**), which indicates an increase in rod-like trabeculae (vs. plate-like trabeculae); enhanced Tb.Pf (2.1-fold; **Figure 4F**), which indicates a lower trabecular connectivity; and decreased trabecular BMD (74%; **Figure 4H**) only in KO animals, and not in WT animals. Interestingly, some cortical bone parameters of the femur were more affected by thyrotoxicosis in WT animals, while others were more affected by T3 in α2A-AR$^{-/-}$ animals (**Figures 4I–Q**). T3 treatment for 4 weeks practically had no effect on cortical bone of both WT and KO animals, except for Ct.Po, which was increased 2.5 times in WT mice, but not in KO animals. The longer T3 treatment (12 weeks) decreased Tt.Ar (8%), Ct.Ar (7%) and cortical BMD (10%) only in KO mice. On the other hand, the 12-week T3-treatment decreased Ct.Th (15%) and increased Ec.Pm (68%) and Ma.Ar (58%) only in the femur of WT animals, which suggests that TH-induced endocortical bone resorption depends on α2A-AR signaling.

Effect of T3 Treatment on Trabecular and Cortical Bone of the Vertebra (L5)

Trabecular and cortical bone parameters of the vertebral body of L5 were not different between euthyroid WT and α2A-AR$^{-/-}$ mice as well (**Figure 5**), except for trabecular BMD that was 2.5-fold higher in KO mice on the fourth week of this study, when animals were 58-day old, but this difference was no longer observed by the end of the study, when animals were 114 days old (**Figure 5H**). Trabecular bone of L5 also showed to be more sensitive to thyrotoxicosis in α2A-AR$^{-/-}$ mice than in WT animals, but this difference was only observed after the shorter period of T3 treatment (4 weeks). T3 treatment for 4 weeks practically had no effect on the trabecular bone of L5 in WT mice, it only decreased Tb.Th (7%) in these animals

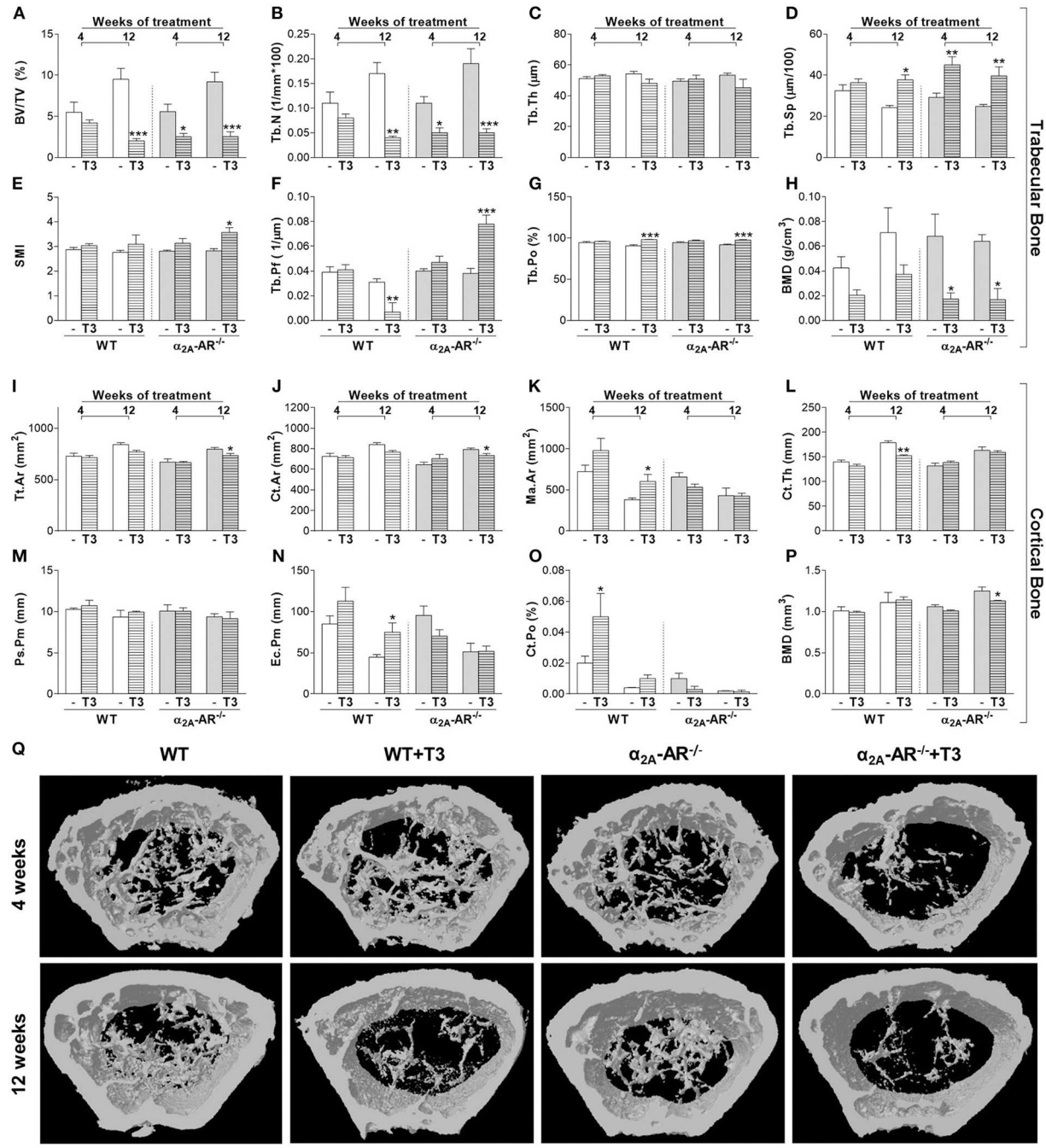

FIGURE 4 | Effect of thyrotoxicosis on µCT parameters of trabecular and cortical bone of the femur in α_{2A}-AR$^{-/-}$ and WT mice. Animals were treated with a supraphysiological dose of T3 (7 µg/100 g body mass/day) or saline (-) for 4 or 12 weeks, by daily i.p. injections. Values are expressed as mean ± SEM ($n = 7$ per group). *$P < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. the respective saline-treated mice, by Two-way ANOVA followed by Tukey's test. BV/TV, trabecular bone volume; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; SMI, structure model index; Tb.Pf, trabecular pattern function; Tb.Po, trabecular porosity; BMD, bone mineral density; Tt.Ar, total tissue area; Ct.Ar, cortical area; Ma.Ar, medullary area; Ct.Th, cortical thickness; Ps.Pm, periosteal perimeter; Ec.Pm, endocortical perimeter; and Ct.Po, cortical porosity. **(A–H)** Trabecular bone microarchitecture. **(I–P)** Cortical bone microarchitecture. **(Q)** µCT images of the distal methaphysis of the femur.

(**Figure 5C**). In contrast, in α2A-AR$^{-/-}$ mice, 4 weeks of T3 treatment decreased BV/TV (27%; **Figure 5A**) and Tb.N (22%; **Figure 5B**), and increased Tb.Sp (13%; **Figure 5D**) and Tb.Po (3%; **Figure 5G**). Twelve-weeks of T3 treatment was slightly more detrimental to WT mice. T3 increased Tb.Sp and decreased Tb.N and trabecular BMD in both WT and KO animals (13 and

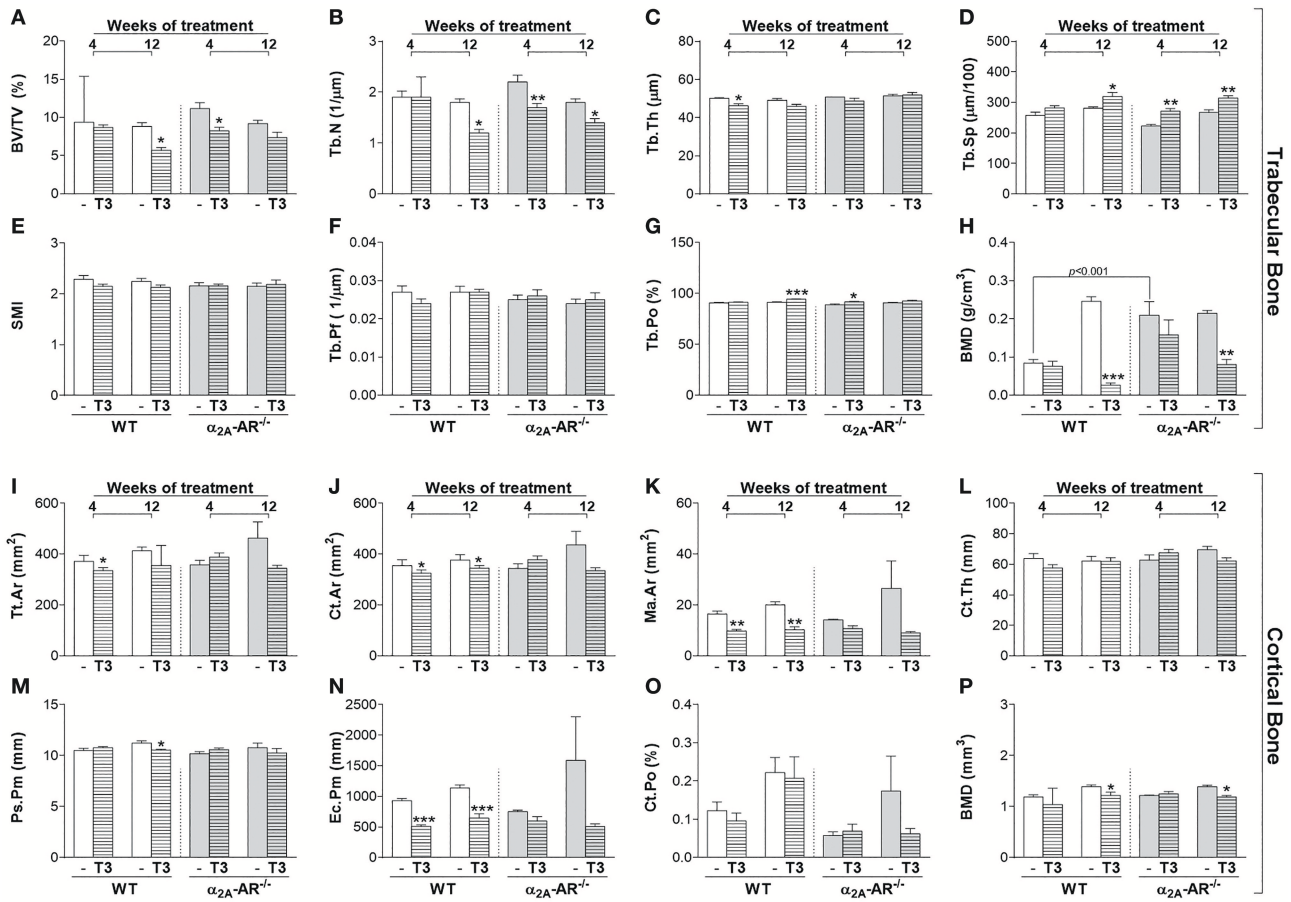

FIGURE 5 | Effect of thyrotoxicosis on μCT parameters of trabecular and cortical bone of the vertebral body of L5 in α_{2A}-AR$^{-/-}$ and WT mice. Animals were treated with a supraphysiological dose of T3 (7 μg/100 g body mass/day) or saline (-) for 4 or 12 weeks, by daily i.p. injections. Values are expressed as mean ± SEM ($n = 7$ per group). *$P < 0.05$, **$p <$ 0.01, and ***$p < 0.001$ vs. the respective saline-treated mice, by Two-way ANOVA followed by Tukey's test. BV/TV, trabecular bone volume; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; SMI, structure model index; Tb.Pf, trabecular pattern function; Tb.Po, trabecular porosity; BMD, bone mineral density; Tt.Ar, total tissue area; Ct.Ar, cortical area; Ma.Ar, medullary area; Ct.Th, cortical thickness; Ps.Pm, periosteal perimeter; Ec.Pm, endocortical perimeter; and Ct.Po, cortical porosity. **(A–H)** Trabecular bone microarchitecture. **(I–P)** Cortical bone microarchitecture.

17%, 33 and 23%, and 89 and 62%, respectively); but decreased BV/TV (35%) and increased Tb.Po (3%) only in WT animals (**Figures 5A,G**). In contrast to trabecular bone, cortical bone of L5 showed to be more sensitive to thyrotoxicosis in WT than in α2A-AR$^{-/-}$ mice. T3 treatment decreased Tt.Ar (10% after 4 weeks), Ct.Ar (8% after 4 and 12 weeks), Ma.Ar (41 and 49% after 4 and 12 weeks, respectively), Ps.Pm (6% after 12 weeks) and Ec.Pm (45 and 43% after 4 and 12 weeks, respectively) only in WT animals (**Figures 5I–K,N**, respectively), but not in KO animals. BMD was decreased after 12 weeks of T3 treatment in both WT and KO animals in about 12% (**Figure 5P**). These data suggest that TH interacts with α2A-AR signaling to promote its detrimental effects on cortical bone of the vertebra.

Effect of T3 Treatment on Biomechanical Parameters of the Femur

Through the three-point bending test, we analyzed the effect of thyrotoxicosis on the femoral resistance to fracture. According to the μCT data, all the biomechanical parameters analyzed were not different between WT and α2A-AR$^{-/-}$ animals. T3 treatment for 4 weeks had no effect on any parameter. On the other hand, when T3 treatment was extended to 12 weeks, it promoted a significant decrease in maximum load (17%) and tenacity (54%), only in WT animals and not in α2A-AR$^{-/-}$ animals (**Figures 6A,B**); but significantly decreased stiffness (**Figure 6C**) in both WT and KO animals (24 and 21%, respectively). These findings suggested that α2A-AR signaling mediates the detrimental effects of TH on the femoral ability to resist fracture.

Effect of T3 on mRNA Expression of α2 and β Adrenoceptors

T3 treatment had no effect on the mRNA expression of α2A-AR and α2B-AR (**Figure 7A**) in the whole femur of WT and/or KO mice (**Figures 7A,B**). On the other hand, T3 significantly decreased α2C-AR and β1-AR mRNA expression (44 and 50%,

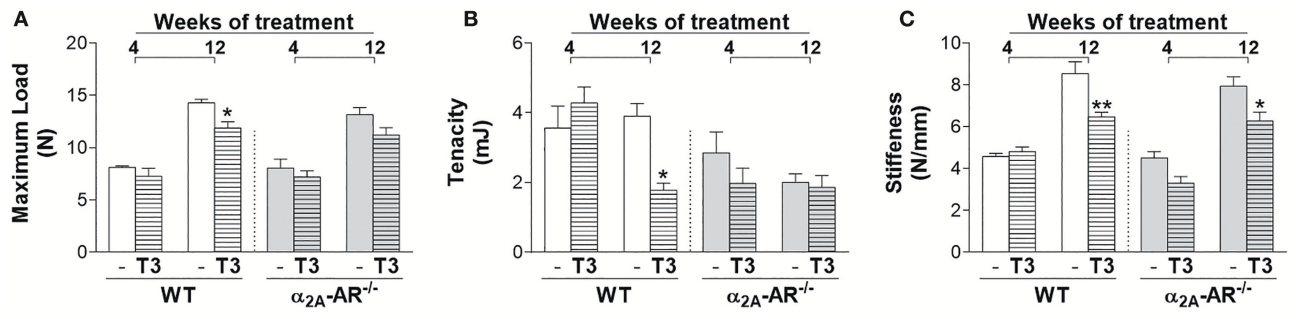

FIGURE 6 | Effect of thyrotoxicosis on biomechanical parameters of the femur of α_{2A}-AR$^{-/-}$ and WT mice. Animals were treated with a supraphysiological dose of T3 (7 μg/100 g body mass/day) or saline (-) for 4 or 12 weeks, by daily i.p. injections. Values are expressed as mean ± SEM ($n = 7$ per group). *$P < 0.05$ and **$p < 0.01$ vs. the respective saline-treated mice, by Two-way ANOVA followed by Tukey's test.

respectively) in the femur of WT mice, but not of α2A-AR$^{-/-}$ mice (**Figures 7C,D**). On the other hand, β2-AR mRNA expression (**Figure 7E**) was decreased by T3 treatment in both WT and KO femurs (53 and 52%, respectively). **Figure 7** also shows that the femoral mRNA expression of α2B-AR, α2C-AR, β1-AR and β2-AR is not different between WT and α2A-AR$^{-/-}$ mice.

DISCUSSION

In the present study, we first characterized the skeletal phenotype of mice with single-gene inactivation of α2A-AR (α2A-AR$^{-/-}$ mice), in an attempt to dissect the specific roles of α2-AR subtypes in bone physiology. Surprisingly, the μCT analysis of the trabecular and cortical bone parameters of the femur and vertebra (vertebral body of L5) showed no differences between α2A-AR$^{-/-}$ mice and their WT controls in euthyroidism. These observations contrast with mice with global double-gene inactivation of α2A- and α2C-AR (α2A/C-AR$^{-/-}$ mice), which exhibit a generalized HBM phenotype (28). These double KO mice present increased trabecular and cortical bone in the femur and vertebra, but mainly in the vertebra (vs. femur) and in the trabecular bone (vs. cortical bone) (**Table 1**), which initially suggested an osteopenic role of α2A- and/or α2C-AR signaling in the bone tissue. Accordingly, histomorphometric analysis showed that these animals present increased bone formation and decreased bone resorption, with higher BMD mainly in the vertebra (28). We later studied mice with global single-gene KO of α2C-AR (α2C-AR$^{-/-}$ mice) (44). We found that α2C-AR$^{-/-}$ mice present a HBM phenotype in the vertebra, with increased trabecular bone, but normal cortical bone (**Table 1**). The collective analysis of the vertebral phenotype of α2A/C-AR$^{-/-}$ mice (increased trabecular and cortical bone) and α2C-AR$^{-/-}$ mice (increased trabecular bone) indicated that α2C-AR signaling mediates osteopenic actions of the SNS on vertebral trabecular bone and suggested a negative action of α2A-AR signaling on vertebral cortical bone (28, 44). The present finding that euthyroid α2A-AR$^{-/-}$ mice present a normal skeletal phenotype strengthens the role of α2C-AR signaling in mediating detrimental actions of the SNS on trabecular bone of the vertebra; on the other hand, it does not sustain, but does not exclude, a detrimental action of α2A-AR signaling on vertebral cortical bone.

In contrast to the HBM phenotype in the vertebra, α2C-AR$^{-/-}$ mice showed a low bone mass (LBM) phenotype in the femur, with lower trabecular bone and nearly normal cortical bone (**Table 1**) (44). The lower trabecular content in the femur of α2C-AR$^{-/-}$ mice raised two hypotheses: (i) α2C-AR signaling has anabolic actions on trabecular bone of the femur; and/or (ii) α2A-AR signaling has a predominant role in mediating osteopenic actions of the SNS on trabecular bone of the femur. Considering this latter hypothesis, one could expect increased trabecular bone in the femur of α2A-AR$^{-/-}$ mice. However, the current study showed normal trabecular bone phenotype in the femur of euthyroid α2A-AR$^{-/-}$ mice, which does not sustain a predominant catabolic role of α2A-AR signaling in euthyroid conditions, but supports anabolic actions of α2C-AR signaling on the trabecular compartment of the femur. On the other hand, negative actions of α2A-AR signaling on femoral trabecular bone cannot be excluded, as it will be discussed next.

It is important to consider that all these mouse models present increased SNS outflow, since α2A-AR and α2C-AR are autoreceptors that inhibit the secretion of catecholamines (32, 33). The first one is the major presynaptic regulator of sympathetic NE release (32, 33), whereas α2C-AR is the main feedback receptor of adrenaline secretion from the chromaffin cells in the adrenal medulla (51), in addition to contribute to inhibition of NE release from sympathetic nerves (34). Therefore, it is important to consider that other adrenoceptors may be activated in these α2-AR KO mouse models. Evidence suggests that β2-AR is the main adrenoceptor to directly mediated SNS actions in the skeleton. Pharmacological blockade and activation of β2-AR signaling, respectively, increases and decreases bone mass (52–54), whereas global or osteoblast-specific genetic ablation of β2-AR in mice results in a HBM phenotype by 6 months of age (27, 55). Activation of β2-AR signaling inhibits osteoblast proliferation and activity and induces the expression of RANKL (receptor activator of nuclear factor kappa-B ligand), which binds to its receptor, RANK, in osteoclast precursor

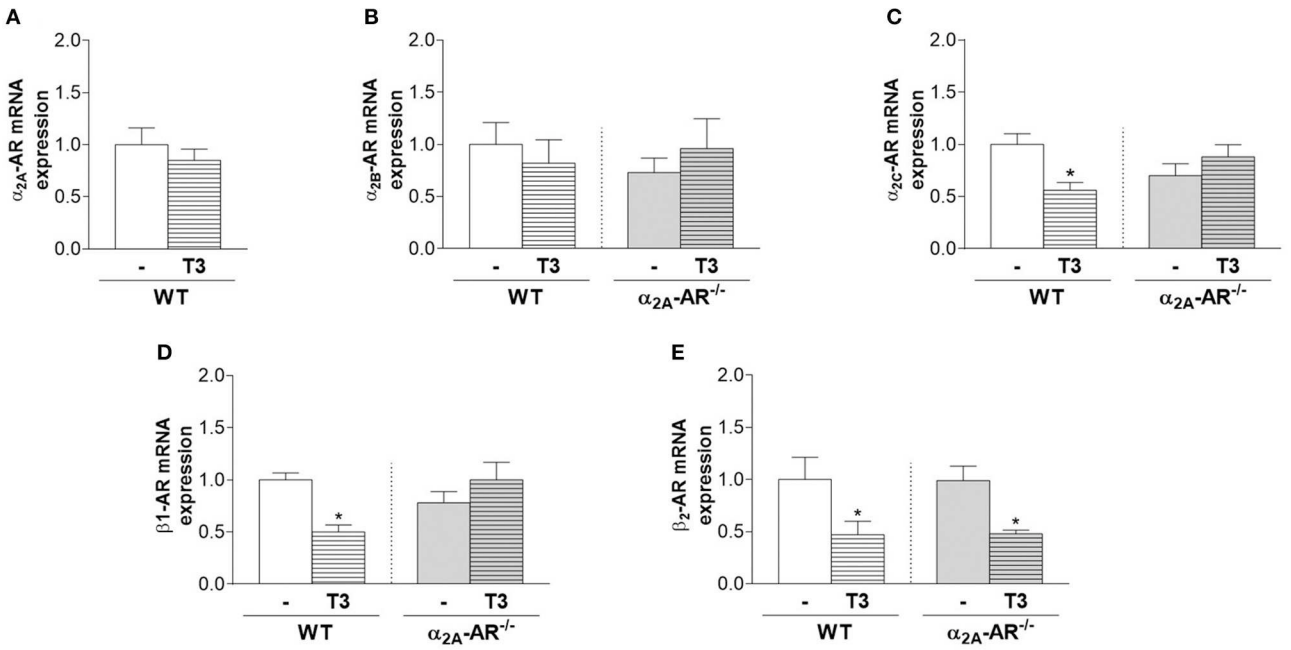

FIGURE 7 | Effect of thyrotoxicosis on the relative mRNA expression of α_{2A}-, α_{2B}-, α_{2C}-, β1- and β2-adrenoceptors in the femur. Animals were treated with a supraphysiological dose of T3 (7 μg/100 g body mass/day) or saline (-) for 12 weeks, by daily i.p. injections. mRNA expression in the whole femur was determined by real-time PCR analysis. Values are expressed as mean ± SEM ($n = 7$ per group). *$P < 0.05$ vs. the respective saline-treated mice, by unpaired Student *t*-test or by Two-way ANOVA followed by Tukey's test.

cells or in mature osteoclasts to activate osteoclastogenesis and to increase osteoclastic activity, respectively (27). On the other hand, β1-AR signaling seems to exert anabolic actions in the skeleton and, therefore, opposite effects in relation to β2-AR signaling. Nevertheless, euthyroid β1-AR$^{-/-}$ mice show decreased trabecular bone in the vertebra but normal trabecular bone in the femur (56). There is also evidence that α1 adrenoceptors have a role in regulating osteoblast function (57, 58). Recently, α1B-AR signaling was shown to be required for bone formation and α1B-AR$^{-/-}$ mice was shown to display reduced trabecular bone in the femur (59). Thus, the trabecular phenotypes in the femur of α2A/2C-AR$^{-/-}$, α2C-AR$^{-/-}$, and α2A-AR$^{-/-}$ mice are expected to rely on a balance/unbalance among the actions of α2A-AR, α2C-AR and the other adrenoceptors, which adds great complexity to the actions of the SNS in the skeleton. Considering the well characterized actions of β2-AR signaling in the bone tissue, the increased trabecular bone in the femur of α2A/2C-AR$^{-/-}$ mice suggests that lack of α2A-AR results in anabolic effects that could overcome the negative effects of β2-AR activation and the lack of α2C-AR-mediated anabolic actions (**Figure 8A**). On the other hand, in α2C-AR$^{-/-}$ mice, the osteopenic actions of α2A-AR and β2-AR and the lack of α2C-AR-mediated anabolism result in lower trabecular bone content (**Figure 8B**). Finally, in α2A-AR$^{-/-}$ mice (**Figure 8C**), a balance between α2C-AR-mediated anabolism and β2-AR-mediated catabolism could result in normal trabecular bone mass (as in WT controls). It is noteworthy that in the present study, the mRNA expression of α2B-, α2C-, β1 and β2-adrenoceptors was not different between WT and KO animals, but, as discussed above, it is expected that the activation of these receptors differs between WT and KO mice. Further studies are necessary to investigate the actions and interactions among all these adrenoceptors to control bone remodeling.

Regarding the femoral cortical bone, α2A-AR$^{-/-}$ mice present no alterations; α2A/2C-AR$^{-/-}$ mice present an increase only in volumetric BMD (28), suggesting negative actions of α2-AR signaling in this parameter; whereas α2C-AR$^{-/-}$ mice present an increase in Ma.AR (44), which is suggestive of increased endosteal resorption. This finding suggests anabolic actions of α2C-AR signaling also in the cortical bone of the femur. Accordingly, α2A-AR$^{-/-}$ mice show normal (same as WT mice) resistance to fracture whereas α2C-AR$^{-/-}$ mice display reduced resistance to fracture (**Table 1**), determined by the three-point bending test (decreased maximum load and resilience). It is noteworthy that this test measures mostly the resistance of the femoral diaphysis to fracture, which is a skeletal site mainly composed by cortical bone. These findings, therefore, support a positive role of α2C-AR signaling in the cortical bone and in its ability to resist fracture.

To add insights to our understanding about the interaction of TH with the SNS to control bone physiology, and to clarify the specific roles of α2-AR subtypes in this process, we evaluated the bone responses of α2A-AR$^{-/-}$ mice to 4 and 12 weeks of daily treatment with 20xT3. This daily treatment increased serum levels of T3 and decreased serum levels of T4 in both WT and

TABLE 1 | Trabecular and cortical bone microarchitecture and femoral resistance to fracture in α2-AR KO mouse models.

	α2A/2C-AR$^{-/-}$ (28)	α2C-AR$^{-/-}$ (44)	α2A-AR$^{-/-}$
VERTEBRA			
Trabecular	↑BV/TV, ↑Tb.N ↓Tb.Pf and ↓SMI	↑BV/TV, ↑Tb.N, ↑Tb.Th ↓Tb.Sp	n.d.
Cortical	↑Ct.Th,↑BMD, ↑Ct.BV ↓Ma.Ar, ↓Ec.Pm	n.d.	n.d.
FEMUR			
Trabecular	↑BV/TV ↓Tb.Pf and ↓SMI	↓BV/TV, ↓Tb.N ↑Tb.Sp	n.d.
Cortical	↑BMD	↑Ma.Ar	n.d.
Fracture resistance	↑Maximum load	↓Maximum load	n.d.

Bone microarchitecture was determined by μCT analysis and bone resistance to fracture by the three-point bending test. Arrows indicate if each parameter decreased (↓) or increased (↑) in KO mice vs. WT mice. n.d. indicates no difference vs. WT. Animals were 114 day-old when the tests were performed. μCT parameters: BV/TV, trabecular bone volume; Tb.N; trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; Tb.Pf, trabecular pattern factor; SMI, structure model index; BMD, bone mineral density; Ct.Th, cortical thickness; Ct.BV, cortical bone volume; Ma.Ar, medullary area; Ec.Pm, endocortical perimeter. Biomechanical parameter: maximum load and resilience.

KO mice, which reflects the suppression of the HPT axis by TH excess (50), and confirms a thyrotoxic state in these animals. To further confirm a thyrotoxic state, we investigated the effect of T3 treatment on body composition. TH excess for 4 and 12 weeks promoted the characteristic effects of thyrotoxicosis in WT mice, including some decrease in BW, fat mass (axillar and retroperitoneal fat pads) and muscle mass (gastrocnemius mass). Besides, this T3 treatment increased heart mass, which reflects cardiac hypertrophy, a known consequence of toxic levels of TH (60, 61). Responses of α2A-AR$^{-/-}$ mice to thyrotoxicosis were different from those of WT mice in several parameters, including bone parameters, as it will be discussed below. KO animals showed an 8–13% lower BW than WT mice from 58 days of age until the end of the study (8 weeks later), which is probably explained by increased lipolysis. There is evidence that α2-AR signaling has antilipolytic actions (62, 63). Thus, the increased NE release and the impaired α2-AR-dependent antilipolysis are likely to facilitate the lipolytic action of β-AR (64). Accordingly, α2A-AR$^{-/-}$ animals presented lower retroperitoneal fat pad than WT mice. Surprisingly, TH failed to promote reductions in the retroperitoneal and axillar fat pads in KO animals, suggesting a complex role of α2A-AR in lipolysis, which remains to be investigated. Heart mass was increased in 58-day-old saline-treated α2A-AR$^{-/-}$ mice (vs. WT mice), which reflects cardiac hypertrophy because of the enhanced sympathetic activity (34). Nevertheless, TH promoted cardiac hypertrophy in α2A-AR$^{-/-}$ mice as much as in WT mice, further confirming a thyrotoxic state in both mouse lineages.

Regardless of the normal bone phenotype in euthyroid α2A-AR$^{-/-}$ mice, in general, trabecular bone showed to be more sensitive to thyrotoxicosis, whereas cortical bone showed to be resistant to the osteopenic effects of thyrotoxicosis in these KO animals, when compared with their WT controls. In order to gain insights into the role of each α2-AR subtypes on the TH-SNS interaction to control bone physiology, we compared these new findings with the already published data of α2A/C-AR$^{-/-}$ and α2C-AR$^{-/-}$ mice (28, 37, 44). The higher sensitivity of trabecular bone to TH in α2A-AR$^{-/-}$ mice contrasts the lower sensitivity of trabecular bone to TH in α2A/C-AR$^{-/-}$ and α2C-AR$^{-/-}$ mice (37, 44), which suggests that α2C-AR signaling has a key role in the mechanism by which TH excess promotes its detrimental effects on trabecular bone. On the other hand, cortical bone of all these KO models showed some degree of resistance to thyrotoxicosis (37, 44), suggesting that α2A-AR and α2C-AR signaling contributes to TH actions on cortical bone.

The current study showed that TH excess for 4 weeks decreased BV/TV, Tb.N and increased Tb.Sp and Tb.Po only in the vertebra of α2A-AR$^{-/-}$ mice, whereas WT mice showed only a reduction in Tb.Th. However, after 12 weeks of TH treatment, vertebral trabecular bone of WT mice showed to be slightly more sensitive to the detrimental effects of thyrotoxicosis than α2A-AR$^{-/-}$ mice, since 20xT3 decreased BV/TV and increased Tb.Po only in WT animals. Conciliating these new findings with the observation that vertebral trabecular bone of α2C-AR$^{-/-}$ mice shows resistance to thyrotoxicosis after 4 and 12 weeks of T3 treatment (44), we could suppose that α2C-AR signaling is the main α2-AR subtype to mediate TH actions on trabecular bone of the vertebra until 4 weeks of thyrotoxicosis, but that, in longer situations of thyrotoxicosis (12 weeks), α2A-AR signaling could also contribute to the detrimental effects of TH excess. Interestingly, cortical bone of the vertebra showed to be more sensitive to thyrotoxicosis in WT than in α2A-AR$^{-/-}$ mice. Thyrotoxicosis negatively affected Ct.Ar, Ma.Ar, Ps.Pm, and Ec.Pm in WT mice and not in α2A-AR$^{-/-}$ mice. Similar effects of toxic levels of T3 were also observed in α2A/2C-AR$^{-/-}$ mice (37), which were resistant to TH-induced decreases in Ct.Th, T.Ar, Ct.Ar, Ct.BV, and Ma.Ar, and to TH-induced increases in Ct.Po. and in α2C-AR$^{-/-}$ mice (44), which were resistant to TH-induced decreases in T.Ar and increases in Ec.Pm.' Altogether, these findings suggest that both α2A-AR and α2C-AR signaling pathways mediate TH actions on the cortical compartment of the vertebra.

The mechanisms by which TH interacts with the SNS to control bone morphophysiology in the femur seems to be different than that in the vertebra. As discussed before, α2A/2C-AR$^{-/-}$ mice present increased trabecular (mainly) and cortical bone in the femur (37); α2C-AR$^{-/-}$ mice present lower trabecular bone and nearly normal femoral cortical bone (44); whereas α2A-AR$^{-/-}$ mice present normal trabecular and cortical bone in the femur (**Table 1**). Considering these findings, anabolic roles of α2C-AR signaling in the trabecular compartment of the femur have emerged. The study of α2A/2C-AR$^{-/-}$ and α2C-AR$^{-/-}$ mice showed that trabecular and cortical compartments of the femur are resistant to detrimental effects of thyrtotoxicosis (37, 44). In contrast, the present study shows that thyrotoxicosis was clearly more deleterious to the trabecular bone of the femur in α2A-AR$^{-/-}$ mice than in WT mice, whereas cortical bone of the femur was less sensitive to thyrotoxicosis in α2A-AR$^{-/-}$ mice. Altogether, these studies suggest that α2C-AR signaling, but not α2A-AR, is necessary for TH to promote its detrimental

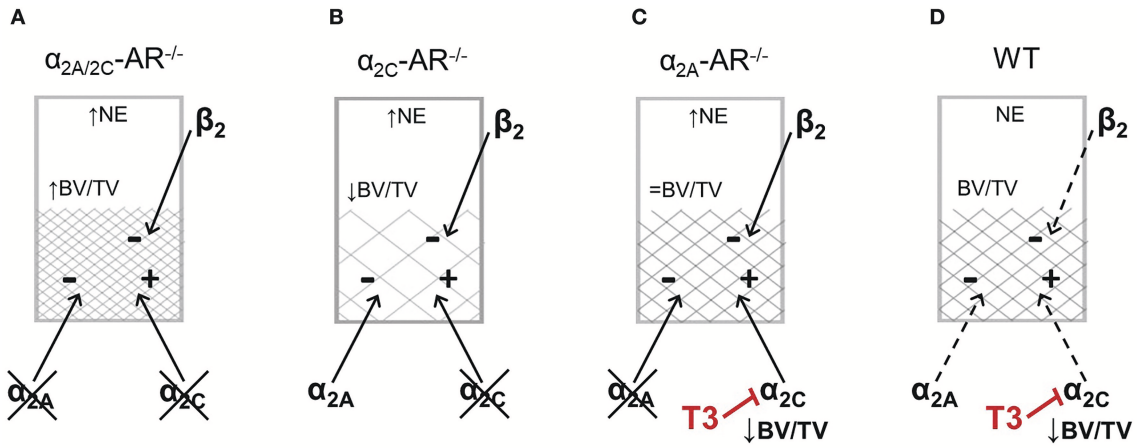

FIGURE 8 | Schematic representation of a possible balance between α_2-AR and β2-AR signaling to control femoral trabecular bone in the distal metaphysis of the femur in $\alpha_{2A/2C}$-AR$^{-/-}$, α_{2C}-AR$^{-/-}$ and α_{2A}-AR$^{-/-}$ and WT mice. All KO mouse models present increased norepinephrine (NE) release and, therefore, increased sympathetic activation. **(A)** In $\alpha_{2A/2C}$-AR$^{-/-}$ mice, the lack of α2A-AR predominantly results in anabolic actions that overcomes the catabolic effects of β2-AR activation and the lack of α_{2C}-AR anabolic actions, resulting in increased trabecular bone volume (BV/TV). **(B)** In α2C-AR$^{-/-}$ mice, the osteopenic actions of α2A-AR and β2-AR and the lack of α2C-AR-mediated anabolism results in lower BV/TV. **(C)** In α2A-AR$^{-/-}$ mice, a balance between α2C-AR-mediated anabolism and β2-AR-mediated catabolism could result in normal BV/TV. **(D)** In WT mice, since NE release is normal, α2-AR and β2-AR are functioning at basal levels, and in equilibrium, to maintain bone mass. **(C,D)** T3 inhibits α2C-AR signaling, which suppresses the α2C-AR-mediated trabecular anabolism, leading to decreased BV/TV. ↑, ↓ and = vs. WT. This is a simplified scheme that does not includes other adrenoceptors that may mediate some of the effects observed in this study.

effects in the trabecular bone of the femur, whereas both α2A-AR and α2C-AR mediate TH effects in the femoral cortical bone.

The three-point bending test showed that thyrotoxicosis decreased femoral resistance to fracture (lower maximum load and tenacity) in WT mice, but not in α2A-AR$^{-/-}$ animals. This finding is consistent with the more deleterious effects of thyrotoxicosis in the cortical bone of WT mice. Similar results were observed in α2A/2C-AR$^{-/-}$ and α2C-AR$^{-/-}$, which also showed resistance to the detrimental effects of TH on bone ability to resist fracture. These findings, therefore, support a role of both α2A-AR and α2C-AR signaling pathways in mediating detrimental actions of TH on cortical bone of the femur.

The fact that α2C-AR signaling arises as having anabolic actions on trabecular and cortical femoral bone while seems to be necessary for TH to promote its deleterious effects in the femur is intriguing. We, therefore, suggest that one mechanism by which TH promote its deleterious effects in the femur is decreasing α2C-AR signaling (**Figures 8C,D**), which would decrease bone anabolism and favor catabolism. Accordingly, we found that T3 decreases α2C-AR mRNA expression in the femur of WT mice. This effect, however, was not observed in α2A-AR$^{-/-}$ mice. It is important to consider that TH actions on the expression of adrenoceptors are normally modest or nonexistent, and usually cannot explain the T3-SNS interactions (38). Evidence shows that TH usually modulates more distal cellular effectors in the adrenoceptors signaling pathways (38). Further studies will be necessary to confirm if T3 really suppresses α2C-AR signaling in bone cells and in which level it occurs. We also found that T3 had no effect on α2A- and α2B-AR mRNA expression. On the other hand, T3 decreased femoral mRNA expression of β1-AR in WT but not in α2A-AR$^{-/-}$ mice, whereas decreased β2-AR mRNA expression in both WT and α2A-AR$^{-/-}$ mice. The relevance of these modulations cannot be extrapolated at this moment, but the effects of T3 on the expression of these adrenoceptors suggest that the modulation of adrenoceptor signaling may be a point of interaction between TH and the SNS to regulate bone mass.

It is important to consider that besides their function as autoreceptors, α2-AR subtypes are also expressed in non-adrenergic neurons, where they can operate as heteroreceptors to regulate the release of several neurotransmitters in the central and peripheral nervous system, including serotonin, GABA and dopamine, among other neurotransmitters (65). In addition to neuronal locations, α2-AR subtypes are also present in several non-neuronal cells/tissues (vascular vessels, pancreatic islets, etc.), which actions have emerged as relevant to the body (65). The widespread expression of α-AR subtypes in the central and peripheral nervous system, and in non-neuronal cells adds further complexity to the understanding of the roles of α2-AR signaling in several physiological processes, including bone metabolism. An important limitation of the present study is that it is based on mouse models of global gene inactivation of α2-AR subtypes. Thus, the interference of factors from the central and peripheral nervous system, as well as interferences from systemic and local factors may have occurred in the bone effects of TH observed in the present study. In addition, these global mouse models do not allow the discrimination of the contribution of central and local TH-SNS interactions. Nevertheless, we have previously shown that clonidine (CLO), an α2-AR agonist, increases osteoclastogenesis, *in vitro*, in mouse marrow cells derived from WT animals, but not from α2A/C-AR$^{-/-}$ mice. In contrast, phentolamine, a nonspecific α-AR antagonist, decreases osteoclast formation in cultures of marrow cells derived from WT but not in the same cells derived from α2A/C-AR$^{-/-}$ animals

(28). In addition, further *in vitro* studies showed that CLO or T3 alone decreased proliferation of calvaria-derived osteoblasts isolated from WT mice, which was further decreased when cells were treated with CLO combined with T3 (CLO+T3). These effects, however, were completely blocked or reversed in α2A/2C-AR$^{-/-}$ mice-derived cells (37). These *in vitro* studies and the expression of all α2-AR isotypes in the main skeletal cells (28) support direct actions of the SNS on the skeleton and show that a TH-SNS interaction is likely to occur locally in the skeleton, via α2A-AR and/or α2C-AR signaling. Thus, these studies suggest that one mechanism by which TH regulates bone physiology directly in the skeleton involves a local interaction with α2-AR signaling.

In summary, euthyroid α2A-AR$^{-/-}$ mice present normal trabecular and cortical bone in the femur and vertebra, in addition to present normal resistance to fracture. The combined analysis of the bone phenotypes of α2A/C-AR$^{-/-}$, αC-AR$^{-/-}$, and α2A-AR$^{-/-}$ mice suggests that: (i) α2-AR signaling mediates SNS-actions mainly in trabecular bone; (ii) α2C-AR signaling predominantly mediates osteopenic actions of the SNS on trabecular bone of the vertebra; (iii) α2C-AR signaling has anabolic actions mainly on trabecular bone but also on cortical bone of the femur. Regardless of the normal bone phenotype in euthryoidism, α2A-AR$^{-/-}$ mice responds differently than WT mice to thyrotoxicosis. The higher sensitivity of trabecular bone to TH in α2A-AR$^{-/-}$ mice and the lower sensitivity of trabecular bone to TH in α2A/C-AR$^{-/-}$ and α2C-AR$^{-/-}$ mice (37, 44) points out α2C-AR signaling as a key factor to mediate the detrimental actions of TH on trabecular bone, particularly in the femur. On the other hand, the lower sensitivity of cortical bone to thyrotoxicosis in α2A/C-AR$^{-/-}$, α2C-AR$^{-/-}$, and α2A-AR$^{-/-}$ mice (37, 44) suggests that both α2A-AR and α2C-AR signaling pathways contribute to detrimental actions of TH on cortical bone of both femur and vertebra. Altogether, these novel findings further sustain a TH-SNS interaction, involving α2-AR signaling, to regulate bone remodeling, and, therefore, bone mass, bone integrity and, ultimately, bone resistance to fracture.

AUTHOR CONTRIBUTIONS

GM: Conceived and performed experiments, carried out data collection and analysis, and wrote the manuscript; MT: performed experiments, carried out data collection and analysis, and reviewed the manuscript; MS, BN-P, and MM-R: performed experiments, carried out data collection, and reviewed the manuscript; PB: Conceived the study and experiments, carried out data analysis and reviewed the manuscript; CG: Conceived the study and experiments, carried out data analysis and wrote the manuscript.

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REVIEW
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A Renewed Focus on the Association Between Thyroid Hormones and Lipid Metabolism

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Thyroid dysfunction, manifesting as either overt or subclinical hypothyroidism, negatively affects lipid metabolism: this leads to hypercholesterolemia which progressively increases the risk for cardiovascular disease and, potentially, mortality. Hypercholesterolemia in hypothyroidism is mainly due to a reduction in low-density lipoprotein (LDL) receptor activity, this accompanied by concomitant diminishing control by triiodothyronine (T3) of sterol regulatory element-binding protein 2 (SREBP-2), which modulates cholesterol biosynthesis by regulating rate-limit degrading enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA) activity. Recently, 3,5-diiodothyronine (T2), a natural thyroid hormone derivative, was found to repress the transcription factor carbohydrate-response element-binding protein (ChREBP) and also to be involved in lipid catabolism and lipogenesis, though via a different pathway than that of T3. While thyroid hormone could therapeutically reverse the dyslipidemic profile commonly occurring in hypothyroidism, it should be borne in mind that the potency of the effects may be age-and sex-dependent. Thyroid hormone administration possibly also sustains and enhances the efficacy of hypolipidemic drugs, such as statins, ezetimibe and proprotein convertase subtilisin/kexin type 9 (PCSK9), in patients with dyslipidemia and hypothyroidism.

Keywords: cholesterol, LDL, TSH, hypothyroidism, triglyceride, thyroxine

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INTRODUCTION

The link between thyroid diseases and serum cholesterol was firmly established following the classic article by Mason et al. on Christmas Day of 1930, shedding light on the significance of thyroid function in cholesterol metabolism (1). In 1951, Scow reported increased fat accumulation in the tissues of hypothyroid mouse models, as compared to controls, thereby corroborating the observations of von Noorden, in Vienna in 1900, that the thyroid gland plays a pivotal role in the development of "fatty disease" (2, 3). Since then, hypothyroidism has been associated with obesity and hypercholesterolemia, the extent of the latter usually being greater in primary than in secondary hypothyroidism due to the more severe condition of the primary form (4, 5).

The prevalence of hypothyroidism is 1.4–13% in patients with hyperlipidemia, indicating that thyroid failure is common and may often go undetected in these patients (6, 7). Lipid levels increase in a graded fashion as thyroid function declines, while patients with TSH values between 5.1 and 10 mIU/L have significantly higher mean total cholesterol (TC) and low-density lipoprotein-cholesterol (LDL-C) levels as compared to euthyroid subjects. In a retrospective cohort study aiming to determine the prevalence of thyroid dysfunction in 8,795 patients of various

races/ethnicities, TSH was found to be high in 5.2% of the 49.5% of patients who were diagnosed with hyperlipidemia (8). Specifically, 3.5% had a TSH level of 5 to 10 mIU/L and 1.7% had a TSH level >10 mIU/L, suggesting that even subclinical hypothyroidism (SCH) might be a secondary cause of hyperlipidemia and thus be linked to coronary heart disease (CHD).

In hypothyroidism, the dyslipidemia is mainly caused by a shift to increased synthesis over degradation rate, with the elevated levels of TC, chiefly LDL-C, providing the substrate for lipid peroxidation by reactive oxygen species (ROS), this resulting in oxidative stress (9). Moreover, the synthesis and rate of catabolism of fatty acids in hypothyroidism is decreased and the lipolytic sensitivity of white fat cells is blunted (10). Crucially, the coexistence of dyslipidemia and hypothyroidism is closely linked to the development of CHD, which is the leading cause of death in most parts of the world (11). The fact that the degree of thyroid failure influences the metabolism of cholesterol has often given rise to discussions during interdisciplinary meetings as to whether the thyroid should first be treated and what is the cut-off of thyrotropin (TSH) to start treatment (12).

The development of powerful hypolipidemic drugs, such as proprotein convertase subtilisin/kexin type 9 (PCSK9), which may be optimally combined with L-thyroxine (L-T4) to treat severe forms of familial hypercholesterolemia and hypothyroidism has opened up a new field of research investigating the underlying mechanisms connecting thyroid function to lipids (13).

The aim of this review is to examine the knowledge acquired mainly over the past decade concerning the intriguing connections between thyroid hormone and lipid metabolism at the molecular level and to present a clinical assessment.

THE VARIOUS MECHANISMS CONNECTING THYROID HORMONE TO LIPIDS

Cholesterol is generated in the liver by the enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) and is transported through the circulation by lipoproteins, these being classified according to their size and density. Among the various lipoprotein subfractions, LDL-C has attracted much attention (14) due to its atherogenicity, susceptibility to oxidation and potential to predict risk for CHD. Meanwhile, also of interest is high-density lipoprotein cholesterol (HDL-C), due to its mediation of cholesterol reverse transport from the circulation to the liver and the cardiovascular protective effects that it exerts. Thyroid hormone is the main regulator of lipid metabolism by stimulating the mobilization and degradation of lipids as well as *de novo* fatty acid synthesis in the liver (15). T3 actions are mediated via modulation of gene expression and cell signaling pathways, while cholesterol synthesis is mediated by the sensing of intracellular cholesterol in the endoplasmic reticulum via sterol regulatory element binding proteins (SREBP)−1 and−2, the transcription factor that positively regulates the expression of LDL receptor (LDLR) and cholesterol synthesis (15, 16). After cleavage by specific proteases, SREBP migrates to the nucleus and acts as a transcription factor binding to the sterol regulatory element (SRE) which stimulates the transcription of the LDLR and HMG-CoA reductase genes.

Moreover, T3 is known to regulate thermogenesis and reduce body weight by stimulating brown adipose tissue (BAT) activity and increasing mitochondrial uncoupling protein 1 (Ucp1) gene transcription (17). Recently, the carbohydrate-responsive element-binding protein (ChREBP) was identified as a T3 target gene in BAT, since stimulation of ChREBP by T3 results in a 5.2-fold upregulation of Ucp1, this indicating that thyroid hormone fine-tunes hepatic lipogenesis via modulation of both SREBP-1 and ChREBP gene expression (18). Interestingly, this process is likely to be mediated through activation of thyroid hormone receptor beta (TRβ), which has been found in the liver and adipocytes (19). It is highly interesting that the metabolite of triiodothyronine (T3), 3,5-diiodo-l-thyronine (T2), although mimicking the actions of T3 on hepatic lipids, exerts an inhibitory effect on *de novo* lipogenesis that renders this compound a potential selective drug for certain metabolic conditions (15, 20). Transfection studies have shown that T2 does not act via TRβ but through an increase of hepatic nuclear sirtuin 1 (SIRT1) activity that targets peroxisome proliferator-activated receptor (PPAR)-γ coactivator (PGC-1α) and SREBP-1c; these deacetylation processes result in mitochondrial biogenesis and downregulation of lipogenic genes (21). T3 exerts its effects through both mechanisms: genomic, consisting in linking T3 to nuclear receptors that bind responsive elements in the promoter of target genes, and non-genomic, by αvb3 receptor-mediated MAPK/ERK and PI3K/Akt/mTOR-C1 activation (22). T2, unlike T3, as was shown in HepG2-cells, determines the block of proteolytic cleavage of SREBP-1 without affecting its expression at the transcriptional or translational level (23). T2 concurrently activates MAPKs ERK and p38, of the Akt and PKC-δ pathways, resulting in apoptosis of HepG2 cells. In animals fed a high-fat diet (HFD), T2 prevented both adiposity and body weight gain by increasing lipid mobilization and hepatic beta-oxidation. On the other hand, by increasing lipid catabolism, T3 enhances hepatic lipid accumulation while also simultaneously elevating lipogenesis. The above observations highlight the different molecular mechanisms of T3 and T2 activity in their inhibition of hepatic lipid accumulation (23).

Cholesterol is converted to cholesteryl esters by lecithin-cholesterol acyltransferase (LCAT) and is transferred from HDL to apolipoprotein B (apoB)-containing lipoproteins by cholesterol ester transfer protein (CETP) (24). CETP has a pivotal role in the reverse cholesterol transport mechanism, a bilateral key process protecting vessel walls against atherosclerosis. The enzyme hepatic lipase (HL) regulates the hydrolysis of HDL2 to HDL3, whereas the lipoprotein lipase (LPL) catabolizes serum triglycerides and transports free cholesterol to HDL (5, 24). The activity of CETP, HL and LPL is regulated by thyroid hormone, this strongly pointing to its crucial influence in cholesterol metabolism. Moreover, thyroid hormone increases the flow of bile acids (BA) causing the depletion of intrahepatic cholesterol and the enhancement of cholesterol synthesis in the liver and of hepatic uptake of cholesterol from the circulation, thus

maintaining the balance of hepatic cholesterol (25). By contrast, in hypothyroidism, a decline of TH levels results in slowing of BA flow, marked diminution in the rate of cholesterol secretion into the bile, increase of intrahepatic cholesterol despite the decrease in cholesterol biosynthesis and decrease of hepatic uptake of cholesterol from the circulation (26) (**Figure 1**). In parallel with lowering of cholesterol excretion there is a rise in LDL-C by a factor of ∼3, the latter being due to decreased LDL-receptor (LDLR) activity accompanied by suppression of the uptake of LDL by the LDLR receptor in the liver. Indeed, LDLR mRNA levels decrease by nearly 50%, indicating that LDLR is regulated at the mRNA level (27). The result appears to be reduced catabolism and turnover, which would account for the presence of dyslipidemia in hypothyroidism.

The effects of thyroid hormone on the various fractions of lipids are presented in **Table 1.**

Recently, a cross-sectional investigation conducted in 250 patients with Graves' orbitopathy (GO) revealed that those with disease lasting <44 months had higher serum TC and LDL-C levels, an association confirmed by the results of the Clinical Activity Score (CAS) in untreated patients (28). The findings suggest that cholesterol levels above 191 mg/dl could represent a novel independent risk factor for GO. These results have more recently been corroborated in 86 consecutive patients with new-onset GD (29). Serum levels of TC and LDL-C were significantly higher in patients with GO (211.6 ± 44.0 and 135.3 ± 41.3 mg/dL, respectively) than in those without GO (176.0 ± 27.2 and 106.6 ± 23.9 mg/dL, respectively). No relationship between GO severity and activity and cholesterol was found. The mechanisms are not clear. The fact that the associations between TC and LDL-C were observed in patients with GO of recent onset points to the potential involvement of autoimmune process *per se* and to the possibility that TC is involved in the induction of oxidative stress which plays an important role in the pathogenesis of GO. Meanwhile, the recent finding that TSH-β mRNA was positively associated with serum TC and LDL-C and, moreover, that it was detected in adipose tissue (30), might partially substantiate these results and hypotheses.

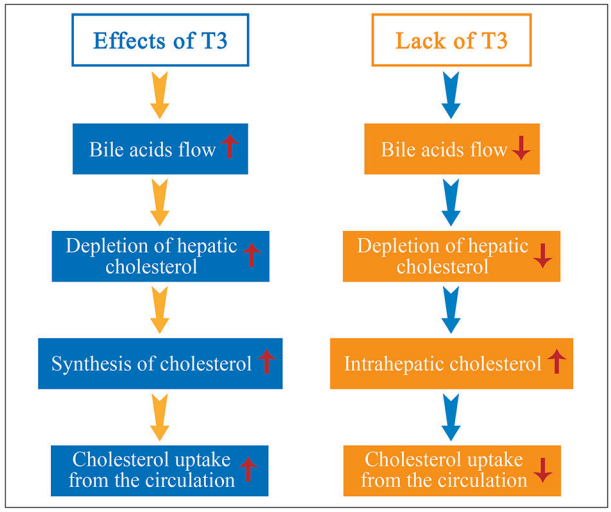

FIGURE 1 | Thyroid hormone (TH) increases the synthesis of cholesterol and bile acids (BA) flow, resulting in depletion of hepatic cholesterol and enhancement of cholesterol uptake from the circulation to the liver. By contrast, in hypothyroidism, diminution of TH results in slowing of BA flow, marked diminution in the rate of cholesterol secretion into the bile, increase of intrahepatic cholesterol despite the decrease in cholesterol biosynthesis, and decrease of hepatic uptake of cholesterol from the circulation.

TRIGLYCERIDE REMNANTS, RESIDUAL CVD RISK, AND THYROID HORMONE

In spite of the fact that LDL–C is the primary lipid target for cardiovascular disease (CVD) prevention, other lipid measures should be undertaken to assess individuals with well controlled LDL-C levels who are still exposed to high residual risk of CVD which is the term used to define the CVD risk that remains despite intensive statin treatment (31). Residual CVD risk is mainly determined by hypertriglyceridemia, elevated small dense LDL particles, reduced HDL-C and HDL particle numbers, increased triglyceride (TG)–rich lipoproteins or remnant lipoproteins (RLPs) and postprandial hyperlipidemia, also known collectively as the atherogenic dyslipidemia complex (32). Markers of residual risk include a number of biochemical parameters, such as non-HDL-C, that reflect the cholesterol content of RLPs (33), as previously shown in diabetic patients (34). RLPs increase intimal cholesterol deposition and activate several proinflammatory, proapoptotic and procoagulant pathways (35). Medium-sized TG-rich RLPs, present in mild to moderate hypertriglyceridemia, can enter the arterial wall and cause atherosclerosis (36). Indeed, clinical conditions related to high CVD risk, such as obesity, metabolic syndrome (MetS) and type 2 diabetes mellitus (T2DM), are associated with RLPs accumulation and elevated inflammatory markers which remain despite statin treatment (37).

In this regard, although overt hypothyroidism (OH) has been traditionally associated with increased levels of cholesterol, an elevation of TG and RLPs levels both in overt and SCH patients have also been reported. Furthermore, hypertriglyceridemia, one of the main components of the MetS, was found to be associated with higher TSH values in several populations that included patients with MetS. In fact, in the MJ Health Screening database with 94,434 participants, patients with MetS were at a 21% excess risk of developing SCH, while after 4.2 years, when individual components were analyzed, an increased risk of SCH was associated with high serum triglycerides (38).

It is of note that subtle changes in TH have a significant opposite effect on plasma VLDL-TG concentration, which was found to be higher in SCH, this due to greater secretion of large TG-rich VLDL particles from the liver, and lower in subclinical hyperthyroidism than in euthyroid subjects (39). The action of thyroid hormones on triglyceride metabolism includes both *de novo* lipogenesis via the transcription of several key lipogenic genes (40) and increased hydrolysis: the latter is regulated by its actions on LPL (41) and HL activity, the enzyme that converts intermediate-density

TABLE 1 | The effects of thyroid hormone on the various fractions of lipids.

	TC	LDL-C	HDL-C	Triglycerides	ApoB	Lp(α)
LT4	↓	↓	*n* (↓)	↓	↓	*n* (↓)*

TC, Total Cholesterol; LDL-C, Low Density Lipoprotein Cholesterol;
HDL-C, High Density Lipoprotein Cholesterol;
*ApoB, Apolipoprotein B; LP(α), Lipoprotein (α). *Depending on lipoprotein (α) molecular weight.*

lipoprotein (IDL) to LDL (42, 43). With regard to LPL, it has been reported that T3 can stimulate its activity both by upregulating apolipoprotein AV (ApoAV) (44) and by decreasing its inhibitor angiopoietin-like proteins 3 (ANGPTL3) (45).

Thus, one explanation for the increase in plasma TG levels in hypothyroidism is the decreased clearance of TG-rich lipoproteins through lower enzymatic activity. In this context, a decline of HL activity in SCH was reported in association with impaired chemical composition of isolated LDL particles due to TG enrichment in middle-aged women with SCH (46). HL activity was subsequently found to be lower in the presence of VLDL remnants/IDL, which are identified via an electrophoretic qualitative method in the fasting plasma of patients with SCH (47). By use of an immunoaffinity-chromatography method for evaluating the cholesterol concentration of RLPs known as remnant-like particle cholesterol (RLP-C), increased RLP-C levels in SCH women were conclusively confirmed (48). Moreover, among the latter group of patients, RLP-C levels decreased upon 6 months of L-T4 treatment together with an increase in HL activity (48). As immunoaffinity-chromatography is not routinely performed, the presence of RLPs can also be estimated by assessing non-HDL-C or non-fasting remnant cholesterol (non-fasting TC minus HDL-C minus LDL-C) (49). It is of note that there have been previous reports of a reduction of TG-rich remnants in the serum of patients with thyroid hormone deficiency following L-T4 treatment (50) and of the presence of postprandial hyperlipidemia in hypothyroidism (51).

Notwithstanding that hypertriglyceridemia in the setting of hypothyroidism develops as a result of impaired removal of endogenous TG (52), and increased hepatic production of triglycerides (53). Gjedde et al. (54) have described an overproduction by the liver of TG-rich particles in OH patients in the presence of unaltered rates of lipolysis. The normal lipolysis and high TG concentrations indicates increased TG synthesis. Similarly, plasma kinetics of an artificial TG-rich emulsion labeled with radioactive TG and cholesteryl esters was unaffected in SCH by comparison with euthyroid individuals, suggesting that TG-rich lipoprotein metabolism is unimpaired in SCH and increased levels of these atherogenic lipoproteins are mainly due to overproduction by the liver (55). In this study, TG and phospholipid transfer to HDL was indeed seen to be significantly lower in SCH subjects than in controls. In a further study, VLDL-TG and VLDL-apolipoprotein B-100 (apoB-100) kinetics were assessed in SCH patients (55). A larger secretion of large TG-rich very low-density lipoprotein (VLDL) particles from the liver with consequent higher plasma VLDL-TG concentration was detected in SCH subjects.

Another possible way to explore TG metabolism is by examining postprandial hyperlipidemia, a predictor of atherogenesis (56). By means of an oral lipid tolerance test, Tanaci et al. (52) found postprandial hyperlipidemia (an increase of TG levels by 80% or more) equally more prevalent in OH and SCH than in euthyroid individuals. Moreover, patients with a TSH level higher than 5 mIU/L had a 7-fold increased risk of postprandial hyperlipidemia, indicating that postprandial TG metabolism is affected in hypothyroidism. Arikan et al. (57) also compared euthyroid, OH and SCH patients and detected higher TG levels 8 h postprandially in hypothyroid patients than in controls. In a more recent study, apolipoprotein B48 (Apo B48), a marker of intestinally derived lipoprotein, was found to be higher in overt and SCH compared to euthyroid patients (58), though the effect of SCH was milder than that of OH. On the other hand, hypothyroidism was not observed to be associated with chylomicronemia (non-fasting TG above 177 mg/dL) in 108,711 individuals from the Copenhagen General Population Study in whom obesity and T2DM were the main risk factors (59). These results are at odds with an epidemiological study in which subjects with hypertriglyceridemia had a higher risk for SCH (60). When TG levels were higher than 150 mg/dL, this risk increased ∼2.0-fold in men and 1.4-fold in women.

In summary, although the atherogenic role of LDL-C is well known and hypercholesterolemia is the most frequent lipoprotein alteration in hypothyroidism, it should be noted that higher TG-enriched lipoprotein levels are also present in the serum of hypothyroid patients. These RLPs in hypothyroidism arise from decreased lipolysis of lipoproteins due to lower enzymatic activity and possibly also through increased production by the intestine and the liver. Since RLPs have been recognized as highly atherogenic, the relevance of their presence in the serum of hypothyroid patients without replacement therapy is a fertile subject for future research.

AGE, LIPID METABOLISM, AND THE THYROID

While age is an established risk factor for dyslipidemia and thus for CVD, the increased proportion of cardiac events in later life may also be attributed to the presence of other comorbidities (61). Nevertheless, despite the lack of pertinent data and the risk of adverse effects from lipid-modifying therapy, the latter approach is generally deemed worthwhile in elderly patients (62). In these subjects, it is particularly important to rule out secondary dyslipidemia, such as hypothyroidism, to avoid unnecessary statin treatment in polymedicated individuals.

Notably, it has been reported that hypothyroidism frequently occurs in dyslipidemic patients (63, 64) and several guidelines (6, 65, 66) recommend case-finding with TSH determination in such a setting. However, given that slightly higher TSH values are considered physiological as age advances, care has to be taken to distinguish between the two conditions, i.e., physiologic or pathologic TSH elevation, especially when replacement with L-T4 is being considered (67). To make this decision, it is helpful to check for the presence of dyslipidemia, while also taking into account the levels of TSH elevation together with positive thyroid peroxidase antibody (TPOAB) titers.

Several studies have demonstrated that lower thyroid function can be a risk factor for a worse lipid profile in elderly patients. In a previous cross-sectional study of 2,799 individuals aged 70–79 years, TSH > 5.5 mIU/L was associated with a 9 mg/dL higher cholesterol level (68). Furthermore, in another cross-sectional study including 30,656 participants, lipid values even within the normal TSH range were directly associated with TSH values in men above 50 years of age (69). For women, associations were statistically significant in all age groups except for HDL cholesterol in women below 50 years of age. However, due to the nature of this cross-sectional study, it is not clear whether the association between higher lipid values and TSH, within the euthyroid range, is due to high TSH values *per se* or to mildly decreased thyroid function.

Elderly patients appear to be at greater risk of developing MetS and the resultant hypothyroidism could contribute to the increased prevalence. Studies were thus conducted to examine whether the prevalence and incidence of MetS are heightened due to augmented TSH levels. Once again, both, SCH and MetS were observed to be linked to increased risk of CHD. Meanwhile, the association between thyroid function and the prevalence and incidence of MetS in older patients were determined by analyzing the data from the Health, Aging and Body Composition Study, a prospective cohort of 3,075 community-dwelling US adults (70). It was shown that SCH with a TSH > 10 mIU/l was significantly associated with increased prevalence but not incidence of MetS (70). In the Longitudinal Aging Study Amsterdam, recruiting 1,187 participants (590 men and 597 women) between the ages of 65 and 88 years, it was observed that subjects with a serum TSH level above 2.28 mIU/L [odds ratio (OR) = 1.68; 95% confidence interval (CI) 1.19–2.37] had a significantly increased prevalence of MetS compared with subjects with serum TSH below 1.04 mIU/L (71). It is therefore evident that increasing TSH is likely to be associated with increasing prevalence of MetS.

The contribution of hypothyroidism to age-related dyslipidemia has been addressed by Tognini et al. who stratified 2,308 patients according to age and TSH values (72). In this study, those patients with higher TSH levels showed a continuous worsening of lipid profile with age, which was noted even in subjects just over the age of 65. More specifically, the above study reported that in patients older than 65 years, both serum LDL-C and LDL/HDL-C values significantly increased further in those with TSH between 3.6 and 10 mIU/L, while in patients with TSH > 10 mIU/L, TC levels increased as well. The main finding of this study is that hypothyroidism, both SCH and OH, could aggravate the rise of lipids with age; thus, age proved to be the most influential factor as regards serum TC levels, TSH following in importance.

In the same line of research, a subsequent study assessed the role of SCH in lipid profiles in elderly subjects and compared it with younger individuals. The analyzed population consisted of 17,046 middle-aged and elderly patients derived from the REACTION study conducted across China from 2011 to 2012 (73). After adjustment for multiple factors, it was found that each 1 mU/L increase in TSH was related to an increase of TC and LDL-C as age increased. Thus, the effect of TSH proved to be stronger on cholesterol levels in moderately old subjects (60–69 years) than in younger subjects (40–49 years). Furthermore, SCH, categorized into mild and severe (TSH <10 or >10 mIU/L) in moderately old subjects, increased the prevalence of hypercholesterolemia (≥240 mg/dL) ∼1.50- and 2.27-fold, respectively, when compared with younger individuals (73). Conversely, in disagreement with previous observations (72), the study showed that the plateau in lipid levels commonly observed with advanced age was still present despite TSH elevation.

More questions arise when one looks at lipoprotein alterations associated with SCH in the oldest old segment of the population, albeit very limited data regarding lipid metabolism and thyroid function are available for this age group. The concept of reverse physiopathology in which higher TSH values are deemed desirable for increased survival seems to apply in this scenario (74).

In brief, it has been postulated that higher TSH values in the elderly are a physiological adaptation of the aging process. Hence, the association between mild thyroid failure and lipid profile in the elderly is still to be elucidated. The fact remains, nevertheless, that TSH levels above the reference range among older patients, and particularly those with levels over 10 mIU/L, have proven to be associated with higher cholesterol levels than what is observed in younger individuals.

TREATMENT WITH L-T4: WHY, WHO, AND HOW

Dyslipidemia, including secondary dyslipidemia that commonly arises in hypothyroidism, is one of the main risk factors for the development of atherosclerosis, the latter arising through changes in lipid profile, arterial hypertension, inflammation and/or oxidative stress which contribute to endothelial dysfunction (75) (**Figure 2**). Lipid status worsens, along with TSH levels. Therefore, TSH values can be considered a good predictor of cardiovascular disease, notably when its levels are above 10 mIU/L (75). In particular, a TSH above 2.5 mIU/L in women of childbearing age may induce oxidative damage to membrane lipids and unfavorably alter the lipid profile, suggesting that TSH levels in this population should preferably be maintained below 2.5 mIU/L (76).

The influence of SCH on lipids is proportional to the degree of TSH elevation and becomes increasingly evident as SCH progresses to OH, this process additionally precipitating any predisposition the patient may have to atherosclerosis (9). Of note, given that progression of the disease is accompanied by

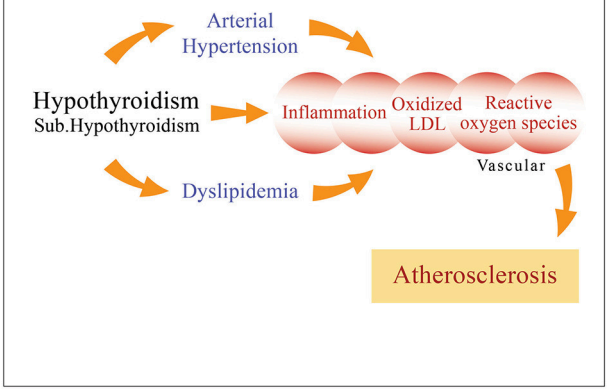

FIGURE 2 | Long-term overt hypothyroidism, as well as subclinical hypothyroidism to a lesser degree, when left untreated cause dyslipidemia and arterial hypertension (diastolic), as well as inflammation characterized by oxidative stress and generation of reactive oxygen species, which may induce endothelial dysfunction, thereby promoting atherosclerosis.

significantly decreased nitrite and nitrate levels, nitric oxide (NO) levels could be a reliable biomarker for cardiovascular risk in SCH (77). Recently, a retrospective analysis of 82 thyroidectomized patients with differentiated thyroid cancer showed that following stimulation by recombinant human TSH (rhTSH), serum T3 concentrations decreased (from 1.91 to 1.81 nmol/L; $p < 0.001$), while T4, fT4, and rT3 remained unchanged (78). Thus, these results may suggest that TSH has a direct effect on peripheral TH metabolism by decreasing T3 in patients receiving L-T4 and that the decline in T3 may cause the dyslipidemic profile. Moreover, after rhTSH stimulation, both apoB-100 and TG statistically significantly increased from 0.90 to 0.92 g/L ($p = 0.03$) and from 1.98 to 2.50 mmol/L ($P < 0.001$), respectively, while HDL-C decreased from 0.98 to 0.81 mmol/L ($p < 0.001$). It is of note that the decreasing effect of L-T4 was more pronounced in short-term (<6 months) rather than in long-term (>6 months) studies.

The efficacy of L-T4 replacement to normalize the lipid profile of hypothyroid patients is related to the degree of disease, being more evident in OH. Though there is no consensus regarding the lipid-lowering effect of replacement treatment with L-T4 in patients with SCH, a recent search in PubMed, the Cochrane Library, ClinicalTrials.gov and EMBASE, in order to compare substitution to placebo treatment or observation, revealed clear benefits of L-T4 administration for reducing TC and LDL-C in SCH patients with TSH < 10 mIU/L (79).

In a meta-analysis of 13 studies with 247 patients aiming to estimate the expected decline in serum lipid concentrations following treatment with LT4, the mean diminishment in serum TC concentration wa-0.20 mmol/L (−7.9 mg/ dL), with a 95% confidence interval of −0.09 to −0.34 while the decrease in serum LDL-C concentration was −0.26 mmol/L (−10 mg/dL), with a 95% confidence interval of −0.12 to −0.41 (80). The results show that LT4 therapy in SCH patients lowers TC and LDL-C, the high levels of reduction possibly being attributable to higher pretreatment TC levels and suboptimal LT4 doses.

Currently, the guidelines recommend against treatment in the elderly in those cases where serum TSH concentrations are between the upper normal limit of the reference range and 10 mIU/L (81). These recommendations are supported by a recent case control study evaluating the association between LT4 therapy and mortality in individuals 65 years or older with SCH and TSH values <10 mIU/L (82). On a multivariate analysis treatment with LT4 was associated with significantly increased mortality (HR = 1.19 CI 1.03–1.38) in patients 65 years or older with SCH and TSH < 10.

However, it is suggested that L-T4 treatment may be considered in patients between 45 and 65 years, and particularly in subjects with comorbidities like dyslipidemia, arterial hypertension and/or insulin resistance.

Moreover, a study enlisting 100 patients, mean age 53.8 yr and mean TSH 6.6 mIU/L, who were treated for 12 weeks with L-T4 100 mcg daily, demonstrated that L-T4 therapy for SCH appreciably lowers cardiovascular risk factors while concurrently often alleviating symptoms of tiredness (83). This same treatment succeeded in lowering TC from 231 to 220 mg/dl and LDL-C from 143 to 131 mg/dl. Of particular note is the fact that the FT4 increase was the most significant variable predicting a decrease in TC.

In this connection, serum paraoxonase-1 (PON-1) activity (arylesterase activity), which may attenuate oxidative stress and thus protect against CVD, was estimated in 2,206 euthyroid individuals, to assess a possible relationship between serum PON-1 activity and TSH and TH (84). Since serum PON-1 activity was inversely associated with serum FT4 levels, other mechanisms could explain the previously reported lipid oxidation reported in hypothyroid patients (84).

It is noteworthy that in an LDLR knock-out mice study, TH reduced circulating TC and VLDL-C levels by about 70% (85). Circulating values of both apoB, apolipoprotein B (apo)B48 and apoB100 were significantly decreased, indicating that TH may reduce apoB lipoproteins via a non-LDLR pathway.

The effects of overt hypothyroidism and SCH on lipids concentrations are presented in **Table 2.**

THYROMIMETICS AND LIPIDS

The different tissue specificity of TH receptors (TRs) causes variable TH effects. Thus, TRα-1 is present in skeletal and cardiac muscle, TRβ-1 is predominant in liver, kidney, and brain, TRα-2 is most prevalent in brain and testis, whereas TRβ-2 is present in the anterior pituitary gland, hypothalamus, and cochlea. TH has been shown to be effective in dyslipidemia, via its lipid-lowering action in the liver, and also in obesity. This differential pattern of TRs distribution stimulated the search for compounds with tissue-specific action on TR, leading to the development of compounds like GC1 and KB141 which selectively act on the β1 isoform of TR (86).

TABLE 2 | The impact of overt (OH) and subclinical hypothyroidism (SCH) on serum lipids concentration.

	TC	LDL-C	HDL-C	Triglycerides	ApoB
OH	↑	↑	↓	*n* (↑)	↑
SCH TSH<10 mU/L	*n* (↑)	*n* (↑)*	*n* (↓)	*n*	*n*
SCH TSH>10 mU/L	↑	↑	*n* (↓)	*n*	↑

TC, Total Cholesterol;
LDL-C, Low Density Lipoproteins Cholesterol; HDL-C, High Density Lipoproteins Cholesterol; ApoB, Apolipoprotein B;
*LP(α), Lipoprotein (α). *Depending on age (<60 y or >60 y) and gender as women are more affected.*
Total and LDL-C and triglycerides increased with increasing TSH, while HDL-C decreased across the entire reference range, with no indication of a threshold effect and presumably due to gradually increased activity of CETP.

The selective TRβ modulator GC-1 enhances several steps in reverse cholesterol transport and reduces serum cholesterol independently of LDL-R. In a study aiming to determine whether GC-1 reduces atherosclerosis and the mechanisms that are involved, apoE-deficient mice were fed an atherogenic diet ± GC-1 (87). GC-1 reduced cholesteryl esters in the aorta after 20 weeks. More recently, eprotirome, a liver-selective thyroid hormone receptor agonist, was developed and was shown to be effective in treating dyslipidemia, this due to the lipid-lowering action of TH in the liver, as well as in the treatment of obesity.

In another recent study, 236 patients were enrolled: 80 were randomly allocated to receive placebo, 79 to receive 50 μg eprotirome, and 77 to receive 100 μg eprotirome (88). There was an increase of 9% (95% CI-2 to 20) in mean LDL cholesterol concentrations in the placebo group, whereas in the 50 μg eprotirome group the concentrations dropped by 12% (−28 to 4%; $p = 0.0677$ vs. placebo) and in the 100 μg eprotirome group they fell by 22% (−32 to −13%; $p = 0.0045$ vs. placebo).

CO-TREATMENT WITH STATINS, EZETIMIBE, AND PROPROTEIN CONVERTASE SUBTILISIN/KEXIN TYPE 9

Though statins are first-line treatment in the majority of patients with dyslipidemia, it must be acknowledged that they often cannot reverse the CVD risk and, moreover, that adverse effects frequently occur, compromising patient compliance (89). The side effects ranging from clinically benign myalgia to rare but life-threatening rhabdomyolysis, are likely to be dose dependent. Hence, clinicians should take into consideration a series of factors such as DM, vitamin D deficiency that potentially increase this risk. Also potentially dangerous is the coexistence of statin therapy with undiagnosed hypothyroidism, since this may elevate the risk of myopathy and even rhabdomyolysis (90). It was recently shown in a multivariate model analyzing 59,597 statin-users and non-users, that both OH and SCH were associated with an increased risk for T2DM (DM) (RR 1.53 [95% CI 1.31–1.79] and 1.75 [1.40–2.18], respectively) (91). However, OH elevated the risk for T2DM independently of use or non-use of statins, while SCH -associated risk for T2DM was prominent only upon statin use. More generally speaking, the above data emphasize the importance of identifying and treating hypothyroid patients as a means of lowering the risk for T2DM and CVD.

Another argument in favor of considering the possible presence of hypothyroidism during statin treatment is that untreated hypothyroid patients on statins rarely reach their lipid goals, the effects of atorvastatin having been demonstrated to be considerably reduced in hypothyroid patients as compared with euthyroid individuals (92). Moreover, in an animal study where LDLR expression was abolished, L-T4 still reduced lipid values. It was shown that liver production of apoB was markedly reduced by thyroid hormones and thyromimetics, thus opening up parallel avenues for statin-resistant patients (93).

Furthermore, statin therapy, as shown in a meta-analysis, produces a significant increase in plasma proprotein convertase subtilisin/kexin type 9 (PCSK9) concentrations (94). PCSK9, a serine protease, is significantly involved in the regulation of LDLR expression and apoB cholesterol metabolism. Hepatic PCSK9 protein expression, activity and secretion have been shown to affect cholesterol homeostasis and to increase LDLR degradation (95). Both currently available PCSK9 inhibitors, alirocumab and evolocumab, bind to PCSK9 preventing its binding to the LDLR: this results in continuous LDLR recycling at the hepatic cell surface and clearing of LDL-C particles, causing a decrease in plasma LDL-C concentration (95). It has been proposed that TH, by stimulating LDLR activity and clearance of LDL-C via PCSK9, may support this mechanism (13). Also of interest is the fact that the correlation of circulating PCSK9 levels with thyroid function, even in the normal range, may be blunted by obesity (96).

As a final note, treatment with ezetimibe, a selective inhibitor of Niemann-Pick C1-Like 1 (NPC1L1), an apical membrane cholesterol transporter of enterocytes, reduces intestinal cholesterol absorption (97, 98). Identification and characterization of NPC1L1 has established this protein as a critical intestinal sterol transporter that regulates whole body cholesterol metabolism (99). Moreover, ezetimibe potently inhibits the uptake and absorption of biliary and dietary

cholesterol from the small intestine without affecting the absorption of fat-soluble vitamins, triglycerides, or bile acids.

Ezetimibe treatment also increases extrahepatic reverse cholesterol transport. Notably, in patients with sitosterolemia, ezetimibe, while reducing TC intestinal absorption, simultaneously decreases circulating 5α-stanol levels. Therefore, by thus lowering circulating 5α-stanols while increasing FT3/FT4, presumably by enhancing conversion of T4 to T3, it has the potential to improve thyroid hormone status (100). It is accordingly suggested that ezetimibe, together with statins, be combined with thyroxine, thereby both ameliorating thyroid function and reducing TC and LDL-C by actively inhibiting the synthesis and intestinal absorption of TC (13).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Metabolic Effects of the Intracellular Regulation of Thyroid Hormone: Old Players, New Concepts

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Thyroid hormones (THs) are key determinants of cellular metabolism and regulate a variety of pathways that are involved in the metabolism of carbohydrates, lipids and proteins in several target tissues. Notably, hyperthyroidism induces a hyper-metabolic state characterized by increased resting energy expenditure, reduced cholesterol levels, increased lipolysis and gluconeogenesis followed by weight loss, whereas hypothyroidism induces a hypo-metabolic state characterized by reduced energy expenditure, increased cholesterol levels, reduced lipolysis and gluconeogenesis followed by weight gain. Thyroid hormone is also a key regulator of mitochondria respiration and biogenesis. Besides mirroring systemic TH concentrations, the intracellular availability of TH is potently regulated in target cells by a mechanism of activation/inactivation catalyzed by three seleno-proteins: type 1 and type 2 iodothyronine deiodinase (D1 and D2) that convert the biologically inactive precursor thyroxine T4 into T3, and type 3 iodothyronine deiodinase (D3) that inactivates TH action. Thus, the pleiotropic effects of TH can fluctuate among tissues and strictly depend on the cell-autonomous action of the deiodinases. Here we review the mechanisms of TH action that mediate metabolic regulation. This review traces the critical impact of peripheral regulation of TH by the deiodinases on the pathways that regulate energy metabolism and the balance among energy intake, expenditure and storage in specific target tissues.

Keywords: Thyroid hormone, Deiodinases, energy metabolism, Thyroid hormone receptors, local regulation of thyroid function

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INTRODUCTION

Thyroid hormones (TH) have long been known to regulate energy metabolism (1). Patients with TH dysfunction often have symptoms of metabolic dysregulation, including fatigue and weight changes (2). Indeed, pathological excess of THs in humans raises the basal metabolic rate (BMR) while TH deficiency is accompanied by a decreased BMR (2). TSH and TRH levels are also critical determinants of whole body energy metabolism. In fact, they exert thyroidal and non-thyroidal effects and thus integrate signals from nutritional status and the adrenergic nervous system with a fine regulation of THs production (3). The wide spectrum of THs effects on body metabolism is exerted mainly by stimulating catabolic and anabolic reactions and by regulating turnover of fats, carbohydrates and proteins (1). A peculiar feature of TH-dependent metabolic regulation is the acceleration of the rates of anabolic and catabolic reactions (4). For instance, TH increases fat mobilization thereby leading to increased concentrations of fatty acids in plasma as well as to enhanced oxidation of fatty acids. THs stimulate insulin-dependent glucose uptake, and both gluconeogenesis and glycogenolysis. Therefore, the action of THs culminates

in promoting futile cycles that contribute significantly to the increase oxygen consumption seen in thyrotoxicosis ("hyperthyroidism"). Thyroid hormones also stimulate ion cycling by altering membrane permeability, the expression of ion pumps and the characteristics of these pumps (5–8).

The classic endocrine view of TH biology is that THs are produced and secreted by the thyroid gland for transport to target tissues. Accordingly, TH concentrations determine the extent of hormonal regulation and generate downstream effects in peripheral cells. Classical regulation of the thyroid gland involves the hypothalamic–pituitary–thyroid axis, whereas low TH concentrations trigger a negative feedback that results in the release of both thyroid releasing hormone (TRH) from the hypothalamus and thyroid stimulating hormone (TSH) from the pituitary gland (9, 10). However, besides the capacity of the thyroid gland to produce the correct amount of THs, the periphery can modify the TH signal in time and space. Indeed, while plasma concentrations of TH are relatively stable, tissues can coordinate TH levels through the cell-autonomous regulation of TH transporters, deiodinases and TH receptors (11). The iodothyronine deiodinase family of selenoproteins is constituted by three enzymes, D1, D2, and D3. These enzymes are present in specific tissues, and regulate TH activation and inactivation (12). The differential expression of deiodinases enables close control of T3 and its prohormone, T4, by removing iodine moieties ("deiodination") at different sites of the phenolic or tyrosylic ring of the TH hormones (13). T4 has a long half-life and is converted to the active form, T3, within cells by the activating deiodinases (D1 and D2) that catalyze outer ring deiodination. The third deiodinase, D3, terminates TH action by inactivating T3 and T4 by removing the iodine at the inner ring (13). The local regulation of TH at intracellular level enables wide fluctuations of TH in local tissues and is a powerful tool with which to modulate TH action without perturbing systemic TH levels.

The correlation of a Thr92Ala polymorphism in the DIO2 gene, encoding protein D2, with altered glycemic control, obesity and type 2 diabetes mellitus (T2DM) (14–16), as well as the association of genetic variants of the DIO1 gene, encoding protein D1, with insulin resistance (17), reinforces the clinical relevance of the peripheral T4-to-T3 conversion in metabolic control.

In this review we summarize the role of the local control of TH by the deiodinases in the metabolic program of cells in the context of the tissue-specific impact of deiodination on energy metabolism, and discuss the effect of local alteration of TH on the metabolic functions of the body.

METABOLIC ROLE OF TH AND THE DEIODINASES

Although each cell of the body is virtually a TH target, the TH signal is differentially integrated in each tissue depending on the cell-autonomous machinery. Therefore, the action of TH on whole body metabolism is best evaluated by examining the specific contribution of TH and its modulating enzymes to energy metabolism in the context of each target tissue. The relative roles of most components of the TH signaling pathways have been assessed in mouse models of inducible, tissue-specific activation or inactivation of deiodinases, receptors and transporters (1). These studies revealed how different TH-induced processes contribute to regulating metabolic homeostasis in humans (**Figure 1**).

LIVER

There is an intricate relationship between TH metabolism and liver (1, 18, 19). Thyroid hormones regulate hepatic function by modulating the basal metabolic rate of hepatocytes; the liver in turn metabolizes the THs and regulates their systemic endocrine effects (20). In the liver, TH regulates lipid metabolism mainly through the T3-TRβ (TH receptor beta) (1), and the downstream regulation of cholesterol homeostasis (synthesis and efflux), bile acid synthesis and fatty acid metabolism (1). The local control of TH metabolism in liver is mediated by the expression of all three deiodinases. D1 is highly expressed in liver, where it contributes to plasmatic T3 homeostasis and mediates the clearance of rT3 from the circulation (21). D1 expression is highly sensitive to T3 levels to such an extent that it is an indicator of the thyroid state of the liver (22). Despite the high D1 levels in the liver, the intracellular T3 level in hepatocytes is not mediated by D1, but by the other TH-activating deiodinase, D2 (23). The liver is the paradigm of spatio-temporal regulated expression of D2 that is transiently turned on in the neonatal mouse liver between the first and the fifth post-natal day. During this short time, a peak of hepatic D2 expression occurs and rapidly declines to background levels (24). This brief peak of D2 produces an excess of T3 that modifies the methylation and the expression pattern of thousands of hepatic genes thereby increasing future susceptibility to diet-induced obesity and liver steatosis (24). Mouse models of hepatocyte-specific D2 inactivation (Alb-D2KO) do not undergo this physiological increase of liver T3 at birth, with a consequent delay in neonatal expression of lipid-related genes and a phenotype of resistance to obesity and liver steatosis (24). These fundamental changes during perinatal life indicate that the thyroid state of specific tissue impacts on whole body metabolism thereby affecting the phenotype in adult life (25). Finally, D3 is almost undetectable in the liver of healthy individuals, but robust re-activation of D3 has been found in regenerating liver tissue, in certain hepatic tumors and in sera and liver samples from critically ill humans, thus influencing the systemic thyroid status (26). These results suggest that D3 plays a role in the tissue response to injury and in the imbalance of TH homeostasis commonly observed during critical illness.

PANCREAS

Thyroid hormone plays a critical role in the development, maturation, and function of pancreatic cells, where T3 is required for the physiological maturation of pancreatic β-cells to glucose-stimulated insulin-secreting cells (27). Pancreatic cells express both TRα and TRβ isoforms and the activated T3-TR

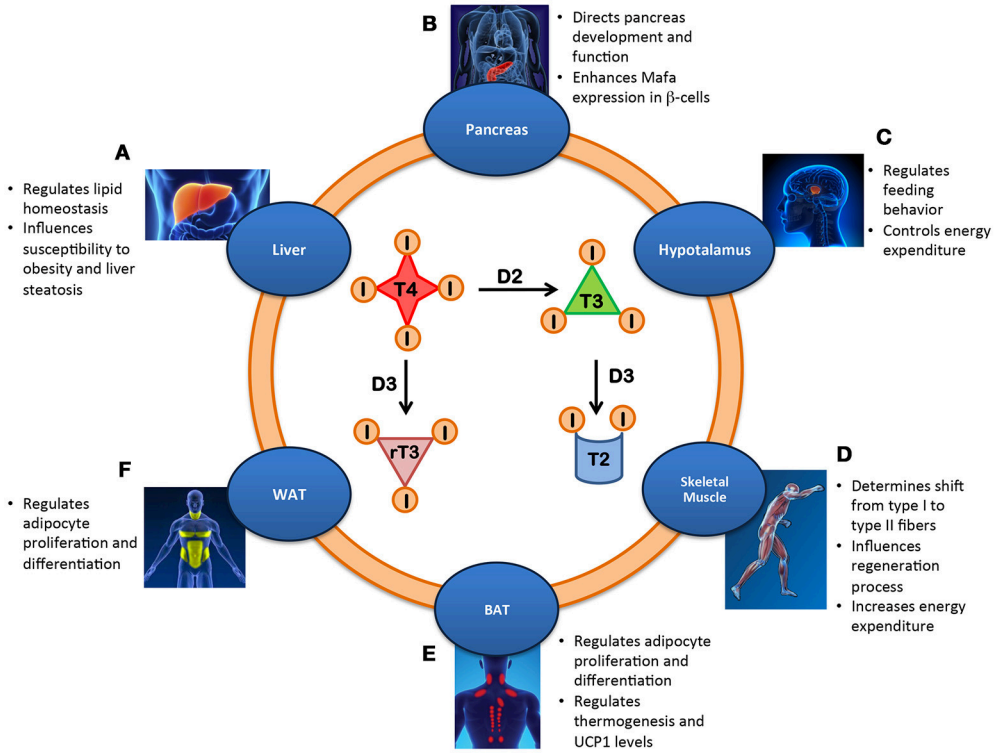

FIGURE 1 | Metabolic effects of the intracellular regulation of thyroid hormone in different tissues. **(A)** Liver: Lipid homeostasis is regulated by local T3 level thereby influencing susceptibility to obesity and liver steatosis. **(B)** Pancreas: The balance between the deiodinases controls the development and function of β-cells by enhancing Mafa transcription factor and inducing insulin secretion. **(C)** Hypothalamus: Local availability of TH regulates feeding behavior and controls energy expenditure. **(D)** Skeletal Muscle: Increased T3 levels in skeletal muscle promote the shift from type I to type II fibers, influence regeneration and increase energy expenditure. **(E)** BAT: D2-mediated TH activation regulates UCP1 expression and thermogenesis, adipocyte proliferation and differentiation and body weight. **(F)** WAT: Local T3 metabolism regulates adipocyte proliferation/differentiation.

complex directly bind to the promoter of islet transcription factor Mafa thereby resulting in its activation (27, 28). However, the exact physiological role of TH in glucose homeostasis remains controversial (29, 30). Although numerous *in vitro* and *ex vivo* studies have demonstrated that T3 mediates positive effects on β-cell function, exposure to high doses of TH results in a phenotype of glucose intolerance. Indeed, hyperthyroidism is associated with glucose intolerance consequent to decreased insulin secretion (31, 32) and to stimulation of hepatic gluconeogenesis (33). Probably, in hyperthyroid conditions, impaired insulin secretion is not sufficient to suppress high hepatic glucose production. Accordingly, the prevalence of diabetes mellitus in hyperthyroid patients is approximately double that of non-affected subjects (34). In contrast, systemic hypothyroidism is associated with reduced hepatic gluconeogenesis and enhanced insulin sensitivity, as demonstrated by the onset of a hypoglycemic state after an insulin injection (35). While during vertebrate development, reduced TH levels are important for normal function and for glucose homeostasis of pancreatic β-cells, exposure to high TH doses induces apoptosis of pancreatic β-cells (36). In this context, the TH hormone-inactivating deiodinase D3 plays a fundamental role in lineage fate decisions and endocrine cell specification (34). Indeed, studies in D3KO mice demonstrated that the reduction D3-mediated of TH action is critical for normal maturation and function of pancreatic β-cells (34). D3KO mice exhibited a glucose intolerant phenotype due to impaired glucose-stimulated insulin secretion, reduced size, and absolute mass of pancreatic islet and β-cells, decreased insulin content, and reduced expression of key genes involved in glucose sensing, insulin synthesis, and exocytosis (34). The pancreatic phenotype of the D3KO mice is proof that attenuation of TH-signaling via D3 activation is essential for normal development.

HYPOTHALAMUS

Peripheral TH signals are integrated within the hypothalamus and processed into coordinated responses to regulate energy balance. The center for regulation of food intake and of body weight is the melanocortin system, constituted by three neuronal populations: the pro-opiomelanocortin (POMC)-expressing neurons, the neuropeptide Y (NPY) and agouti-related peptide (AgRP)-co-expressing neurons and the melanocortin 4 receptor (MC4R)-expressing neurons (37, 38). The POMC neurons exert an anorexigenic function by activating MC4R neurons,

which induce a reduction of food intake and increased energy expenditure. On contrary, NPY/AgRP neurons are the orexigenic neurons: by antagonizing the action exerted by POMC on MC4R, they increase food intake and decrease energy expenditure. All these neurons are sensitive to the TH signal that can either activate or inhibit melanocortin neurons, and thus, it is not surprising that local TH metabolism plays a critical role in appetite and feeding regulation. Changes in central T3 levels occur in various metabolic conditions (39), for example elevated T3 levels have been found in the hypothalamus during fasting (40). Fasting induces alterations in the thyroid state, namely, a reduction in pituitary D2 levels and liver D1 levels correlated with low peripheral T3 levels in the presence of increased hypothalamic D2 activity. The high D2 activity in the hypothalamus causes an increase of local T3 concentrations, which in turn activate orexigenic NPY/AgRP neurons and inhibit anorexigenic POMC neurons, thereby inducing hyperfagia (1). The molecular mechanism underlying TH-mediated NPY/AgRP activation resembles that in brown adipose tissue (BAT) in which T3 increases uncoupling protein 1 (UCP1) activity. In fact, high T3 levels in the hypothalamus during fasting, consequent to D2 activation, promote UCP2 expression and stimulate mitochondrial proliferation in orexigenic NPY/AgRP neurons, so promoting their activity and stimulating rebound feeding upon food deprivation. The increase of T3 in the hypothalamus also causes TRH mRNA suppression (40, 41). Therefore, under food deprivation, despite a reduction in peripheral TH levels, there is a localized increase in T3 within the hypothalamus, which in turn increases orexigenic signals and decreases TRH production. The hypothalamus probably maintains low TH levels to preserve energy stores, which would be dissipated in hyperthyroid condition.

The fundamental role of deiodinases in the regulation of energy balance in brain has been demonstrated in mouse models of deiodinases depletion (42). Despite the low TH circulating levels in adult *Dio3*$^{-/-}$ mice, their central nervous system is in a hyperthyroid state (42). The enhanced TH levels alter the functioning of the hypothalamic circuitries, including the leptin-melanocortin system, thereby regulating energy balance and adiposity. In detail, *Dio3*$^{-/-}$ mice have decreased adiposity, but an abnormally functioning leptin-melanocortin system associated with leptin resistance (43). The hypothalamic D2-mediated T4 to T3 conversion is important for the photoperiodic response of the gonads (44) in which fine-tuned D2 and D3 expression tightly regulates LH stimulation (45).

SKELETAL MUSCLE

Skeletal muscle represents 40–50% of the total body mass in humans and is crucial for metabolism, heat generation and maintenance of posture. TH influences skeletal muscle contraction, regeneration and metabolism (46). All components of the TH signaling process, from TR to TH transporters (MCT8 and MCT10), and D2 and D3, are expressed in the skeletal muscle of rodents and humans (47). During skeletal muscle development, D2 is up-regulated, particularly during the first postnatal days, and decreases at day 30, although its activity returns to high levels during differentiation of muscle stem cells (12, 48, 49). In particular, during post-injury regeneration processes, D2 mRNA is up-regulated to enable correct myoblast differentiation (50). D2 is a target of FOXO3, which is a protein involved in myocyte fusion and metabolism as well as in atrophy and autophagy (12). Loss of D2 impairs stem cell differentiation and prevents up-regulation of myogenic transcription factor MyoD thereby increasing the proliferative potential of muscle stem cells. D2-mediated TH in skeletal muscle influences also muscle fibers. High TH levels induce a shift from type I fibers (slow) to type II fibers (fast), which results in up-regulation of sarcoendoplasmic reticulum $Ca2^{+}$ATPase, of glucose transporter 4 (GLUT4) and of uncoupling protein 3 (UCP3) thereby producing heat and increasing energy expenditure (51). D2-dependent T3 activation influences insulin response in skeletal muscle (52). Indeed, D2KO mice are insulin-resistant, which demonstrates the relevance of D2 in glucose homeostasis. In humans, a common polymorphism of the *Dio2* gene, the Thr92Ala substitution in protein D2, which partially impairs enzymatic activity, has been correlated with insulin resistance and diabetes (53, 54). Furthermore, muscle fibers respond to cold through TH-related mechanisms, namely increased glucose uptake, activation of oxidative pathways and increased mitochondria biogenesis (55, 56). Interestingly, D2 is up-regulated in muscle after 4 h of cold exposure (57). Moreover, D2 is up-regulated in response to such metabolic signals as bile acids and insulin (1, 58) and during exercise under β-adrenergic stimulus in order to amplify TH signaling and regulate PGC-1α expression (59, 60). Coordinated D2-D3 expression is required to fine-tune intracellular TH availability during muscle stem cell differentiation, and *in vivo*, during muscle regeneration (47). While D2 is essential for a correct T3 surge and the subsequent differentiation of muscle stem cells, D3 fosters muscle stem cell proliferation by lowering TH availability during the early phases of the myogenic program (47). This dual regulation is so critical that D3-depletion *in vivo* causes massive apoptosis of proliferating satellite cells and drastically impairs a full regeneration process. These studies highlight the pivotal role of the intracellular TH coordination by the deiodinases in muscle physiology.

BROWN ADIPOSE TISSUE

Brown adipose tissue is characterized by multilocular lipid droplets and numerous mitochondria, and governs heat production (61). In fact, BAT is activated in response to a high fat diet or cold exposure in order to protect the organism from weight gain and hypothermia. Thyroid hormone critically influences BAT activity (62). The most obvious metabolic role of D2 is the regulation of energy expenditure in the BAT of small mammals, including human newborns. During cold exposure, the sympathetic nervous system induces D2 expression in brown adipocytes, thereby promoting local T4-to-T3 conversion, and activation of the transcription of target genes involved in the thermogenic program (63). Loss of function of D2 reduces the level of UCP-1, which is normally up-regulated at RNA

level by TH. D2 is thus considered a marker of BAT activity (1, 57). Interestingly, global D2KO mice are resistant to diet-induced obesity, highly tolerant to glucose, and have a deficit in respiratory quotient at 22°C, while at 30°C they become more susceptible to obesity and develop intolerance to glucose (64, 65). T3 regulates the expression of several genes during adipogenic differentiation, among which GPD, ME, PEPCK, S14, FAS, and GLUT4 (66, 67). While D2 activity is important during differentiation, D3 is considered a mitogenic marker in brown pre-adipocytes. In fact, D3 mRNA and activity are induced by bFGF and aFGF in proliferating brown pre-adipocytes (68). In BAT, T3 also accelerates fatty acid oxidation and lipogenesis through the action of the ACC and ME lipogenic enzymes. Consequently, D2KO mice have reduced fatty acid oxidation and lipogenesis (4).

WHITE ADIPOSE TISSUE

The primary function of white adipose tissue (WAT) is to store energy in the form of single large lipid droplets, although it also secretes the leptin and adiponectin adipokines. White adipocytes differ anatomically and physiologically from brown adipocytes. However, the latter may appear at sites corresponding to WAT, in a so-called process of WAT "browning" caused by a thermogenic stimulus, such as prolonged cold exposure (69), or treatment with β_3-adrenergic receptor activators (70). The brown adipocytes in WAT are often called "inducible," "beige," or "brite." D1 and D2 are barely expressed in epidermal WAT, the adipose tissue that, contrary to inguinal WAT, is never converted in BAT. All TR isoforms and the TH transporter MCT8 are expressed in human subcutaneous adipose tissue (71). D1 expression in epidermal WAT is only 1% of the D1 found in the liver. Similarly, the D2 mRNA level is 7% of the D2 in BAT (72). Interestingly, D1 expression and activity are increased in the subcutaneous and visceral WAT of obese subjects (71). On the other hand, D2 is up-regulated in beige/brite adipocytes and its expression is correlated to increased energy expenditure (73). A high-fat diet stimulates D1 and leptin expression, while caloric restriction decreases D1 activity as well as leptin levels, and increases levels of the leptin mediator SCD-1. Leptin overexpression increases D1 activity and down-regulates SCD-1 expression (74). Similar to brown adipocytes, in white adipocytes, D2 plays an important role in lipogenesis and in the regulation of the expression of genes related to adipocyte differentiation, while D3 sustains the proliferation of white adipocytes (61). Interestingly, thyroidectomized mice have an increased level of both D1 and D2 (72). Moreover, D2 is expressed also in human pre-adipocytes although its role is unclear (75).

FUTURE DIRECTIONS AND CONCLUSIONS

Monodeiodination is quantitatively the most important pathway of TH activation. Within peripheral tissue, multiple pathways modulate TH availability. These pathways govern the action and regulation of deiodinase expression, the action of TH transporters, and the expression and crosstalk of TH receptors with multiple partners. This intricate network of TH modifiers increases the sensitivity and the speed of responses to changes induced in the internal and external environment by the thyroid signal. The price to be paid for this is an intricate regulation of each component in time and space. Given the vast spectrum of metabolic body functions regulated by the TH signal, the deiodinases represent a powerful tool with which to modulate cellular metabolism in specific tissues without perturbing systemic levels of THs. Consequently, the development of drugs that target deiodinase action is the next challenge in this field. Extensive work is still required to delineate the kinetics and regulation of the deiodinase enzymes in specific tissues to understand the full spectrum of their biological roles. Thus, pharmacological research is poised to develop deiodinase modulators aimed at driving specific metabolic outcomes. Targeting tissue-specific TH actions may result in novel and safe therapeutic options for metabolic dysfunctions.

AUTHOR CONTRIBUTIONS

AC and DD wrote the manuscript. MD wrote and supervised the manuscript.

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Assay of Endogenous 3,5-diiodo-L-thyronine (3,5-T_2) and 3,3′-diiodo-L-thyronine (3,3′-T_2) in Human Serum: A Feasibility Study

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3,5-diiodo-L-thyronine (3,5-T2) is an endogenous derivative of thyroid hormone with potential metabolic effects. It has been detected in human blood by immunological methods, but a reliable assay based on mass spectrometry (MS), which is now regarded as the gold standard in clinical chemistry, is not available yet. Therefore, we aimed at developing a novel *ad-hoc* optimized method to quantitate 3,5-T2 and its isomers by MS in human serum. Serum samples were obtained from 28 healthy subjects. Two ml of serum were deproteinized with acetonitrile and then subjected to an optimized solid phase extraction-based procedure. To lower background noise, the samples were furtherly cleaned by hexane washing and acetonitrile precipitation of residual proteins. 3,5-T2 and its isomers 3,3′-T2 and 3′,5′-T2 were then analyzed by HPLC coupled to tandem MS. Accuracy and precision for T2 assay were 88–104% and 95–97%, respectively. Recovery and matrix effect averaged 78% and +8%, respectively. 3,5-T2 was detected in all samples and its concentration averaged (mean ± SEM) 41 ± 5 pg/ml, i.e., 78 ± 9 pmol/l. In the same samples the concentration of 3,3′-T2 averaged 133±15 pg/ml, i.e., 253±29 pmol/l, while 3′,5′-T2 was not detected. 3,5-T2 concentration was significantly related to 3,3′-T2 concentration ($r = 0.540$, $P < 0.01$), while no significant correlation was observed with either T3 or T4 in a subset of patients in which these hormones were assayed. In conclusion, our method is able to quantify 3,5-T2 and 3,3′-T2 in human serum. Their concentrations lie in the subnanomolar range, and a significant correlation was detected between these two metabolites in healthy individuals.

Keywords: thyroid hormones, thyroid hormones metabolites, 3, 5-diiodo-L-thyronine, mass spectrometry, T_2

INTRODUCTION

The term thyroid hormones, classically referred to both 3,5,3′-triiodothyronine (T_3) and thyroxine (T_4), seems nowadays to be simplistic; indeed, it has been shown that some T_3 and T_4 metabolites, particularly 3,5-diiodothyronine (3,5-T_2) and 3-iodothyronamine, are independent chemical messengers, with specific metabolic effects (1).

Two deiodinase enzymes, namely D1- and D3-deiodinase, can potentially catalyse the synthesis of distinct diiodothyronines: 3,5-diiodothyronine (3,5-T_2), 3,3′- diiodothyronine (3,3′-T_2) and 3′,5′- diiodothyronine (3′,5′-T_2) (2). Until recent years, all T_2 isomers were regarded as inactive metabolites of T_3 and T_4, because of their very low affinity for nuclear thyroid hormone receptors. However, this view has been challenged by the observation that 3,5-T_2 can also interact with mitochondrial targets and that administration of exogenous 3,5-T_2 to experimental animals produces significant functional effects on lipid metabolism and mitochondrial function (3).

A major pitfall in the investigations about the alleged physiological and pathophysiological relevance of 3,5-T_2 is the difficulty in assaying its low serum or tissue concentration, either in animal models or in human.

Polyclonal antibody-based radio-immunoassays were developed in the 1970's. The first radio-immunoassay from Meinhold et al. allowed detection of serum 3,5-T_2 in 10 normal subject (100 pmol/L) and in 5 hyperthyroid patients (380 nmol/L) (4). Subsequently, in 1982 Faber et al. reported a serum 3,5-T_2 concentration close to 100 pmol/L with a RIA method based on gel separation and antibody extraction (GSAE) (5). Further RIA methods have been subsequently employed, but high variability was reported for serum 3,5-T_2 concentration, i.e., between 10 and 190 pmol/L (6). In 2014, Lehmphul et al. developed a new CLIA method for 3,5-T_2 evaluation with a lower detection limit of 200 pmol/L. The average concentration was 430 pmol/L in 31 hypothyroid patients and 310 pmol/L in 24 hyperthyroid patients, vs. 290 pmol/L in the control healthy group, although this difference was not statistically significant (7).

Mass spectrometry-based methods should provide a more specific and sensitive quantification technology for the assay of thyroid hormone and their metabolites, including 3,5-T_2. In recent years, several groups have published method for 3,5-T_2 detection based on Liquid Chromatography-tandem Mass Spectrometry (LC-MS-MS) (8), but so far, the application of these techniques to human serum samples usually produced negative results.

Due to these methodological challenges, the aim of this study was to develop an offline pre-analytical sample preparation (sample extraction) procedure and an LC-MS-MS method for quantification of T_2 isomers in human serum.

MATERIALS AND METHODS

Sample Collection

Serum samples were obtained from 28 patients, including 8 healthy volunteers and 20 women undergoing endocrinological screening and found to be euthyroid. Serum was obtained by using the remaining part of samples obtained for independent clinical indications. The study had the approval of the local Ethical Committee. All subjects gave a written informed consent and since no additional blood drawings were performed, ethical committee approval was not required.

Reagents and Materials

3,3′,5,5-tetraiodo-L-thyronine (T_4), 3,3′,5-triiodo-L-thyronine (T_3), 3,3′,5′-triiodo-L-thyronine (rT_3), 3,5-diiodo-L-thyronine (3,5-T_2), 3,3′-diiodo-L-thyronine (3,3′-T_2), 3,3′,5,5-tetraiodo-L-thyronine-$^{13}C_6$ ($^{13}C_6$-T_4), and 3,3′,5-triiodo-L-thyronine-$^{13}C_6$ ($^{13}C_6$-T_3), were provided by Sigma-Aldrich (Saint Louis, MO, USA). Acetonitrile (LC-MS grade), methanol (LC-MS grade), ultra-pure water (LC-MS grade), hexane (HPLC grade), glacial acetic acid (ACS grade), hydrochloric acid (ACS grade), and ammonium hydroxide (ACS grade), were also from Sigma - Aldrich. 3,3′-diiodo-L-tyronine-$^{13}C_6$ ($^{13}C_6$-T_2) was purchased from Isosciences (Ambler, PA, USA), while 3,5-diiodo-L-tyronine-$^{13}C_9$-^{15}N ($^{13}C_9^{15}N$-T_2) was kindly provided by Prof. Thomas S. Scanlan (Portland, OR, USA).

Sample Preparation

The optimization of the sample preparation procedure, as well as the instrumental method development, took advantage of the expertise gained in the assay of T_4, T_3, rT_3, and some of their metabolites in human and animal biological matrices (9–11). The general strategy of the pre-analytical procedure consisted in using a relatively large amount of starting material (human serum) that was concentrated up to 40-fold. Since this approach tends to generate a high matrix effect, additional steps were introduced to ensure extensive cleaning of the sample, both before and after the extraction step. Briefly, 2 mL of serum from each sample were placed in a 2 mL microcentrifuge tube (Eppendorf, Hamburg, Germany) and spiked with appropriate internal standards amount in order to achieve the final concentration of 1 ng/mL, 5 ng/mL, 50 ng/mL, and 200 ng/mL for $^{13}C_6$-T_2, $^{13}C_9^{15}N$-T_2, $^{13}C_6$-T_3, and $^{13}C_6$-T_4, respectively. After a 2 min shaking at room temperature, the sample was split into 4 aliquots of 500 μL each, and all of them was added with 500 μL of a mixture containing ice-cold acetonitrile (79% as v/v), water (20%), and formic acid (1%) for deproteinization. After vortexing and sonication (15 min), 1 mL of ice-cold acetonitrile was added to each aliquot, which was then shacked for 2 min, sonicated for 15 min, and centrifuged for 15 min at 14,000 × g. The resulting supernatants were warmed up to 40°C and evaporated up to ∼0.5 mL under a gentle stream of nitrogen. The liquid residues from the different aliquots of the same sample were pooled and loaded onto an Agilent (Santa Clara, CA, USA) Bond-Elut Certify 130 mg SPE cartridge, previously conditioned by sequential wetting with 2 mL of methanol and 3 mL of water. After washing with 2 mL of water, 2 mL of 0.1 M HCl, and 5 mL of methanol, the column was dried, and the sample was eluted with 2 mL of methanol/ammonium hydroxide (95:5 by volume). The eluate was dried under a gentle stream of nitrogen and reconstituted with 200 μL of water/acetonitrile (70:30) containing 0.1% formic acid, and then shaken for 6 min at room temperature. The reconstituted solution was washed with 600 μL of hexane to extract lipid residues, which were then removed, while the aqueous solution containing the analytes was added with 500 μL acetonitrile, shaken for 2 min, and centrifuged for 15 min. After removing the pellet, the supernatant was dried under nitrogen and stored at −20°C until processing. Immediately before analysis, it was

reconstituted with 50 μL of water/acetonitrile (70:30) containing 0.1% formic acid, shaken for 15 min, centrifuged for 15 min and injected (20 μL of the supernatant) into the HPLC-MS-MS system.

Instrumental Layout and Operative Conditions

Samples were processed by using an instrument layout consisting of an AB Sciex API 4000 triple quadrupole mass spectrometer (Concord, ON, Canada), equipped with an electrospray (ESI) Turbo V ion source, coupled to an Agilent 1290 Infinity UHPLC system (Santa Clara, CA, USA), including autosampler outfitted with peltier tray, binary pump, and column oven, used for the chromatographic separation. A ten port divert valve (Valco Instruments Co. Inc., Huston, TX, USA) allowed the discarding of both head and tail of the HPLC runs, while a quaternary HPLC pump (Series 200, PerkinElmer, Boston, MA, USA) supplied the mass spectrometer with solvent when the flow from the analytical system was diverted to waste, to prevent the occurrence of high voltage discharge in the ion source. Chromatographic separation was performed by a Phenomenex (Torrance, CA, USA) Gemini C18 110 Å, 3 μm, 50 x 2 mm HPLC column protected by a C18 4 x 2.0 mm ID security guard cartridge. Data acquisition and system control were carried out by an AB Sciex Analyst® version 1.6.3 software.

Twenty micro liters of each sample was injected into the Agilent UPLC system, where it was submitted to chromatographic separation by a mixture methanol/acetonitrile (20/80) added with 0.1% formic acid, as solvent A, and water also containing 0.1% of formic acid, as solvent B, under the gradient conditions shown in **Table 1**. The chromatographic column was kept at 20°C.

Mass spectrometry method was based on selected reaction monitoring (SRM) in positive ion mode and made use of three transitions for each compound, one of which was used as a quantifier (Q) and the others as qualifier (q). All of them were monitored using optimized declustering potential (DPs), collision energies (CEs), and collision exit potentials (CXPs), which are reported in **Table 2**. Further operative parameters were: nebulizer current (NC), 5.0 KV; gas source 1 (GS1) zero air, 70; gas source 2 (GS2) zero air, 55; source temperature (TEM), 650°C; entrance potential (EP), 10 V; IQ1 lens potential, −9.8 V; stubby rod Q1 (ST),−15.5 V; collision gas (CAD) nitrogen, operative pressure with CAD gas on, 5.7 mPa.

Method Performances

Quality control was performed according to Matuszewski et al. (12). Accuracy is the ratio of measured concentration to spiked concentration after adding known amounts of analytes at two different concentration levels: precision is the coefficient of variation (standard deviation/mean) of repeated measurements within the same assay under the same conditions as described above; recovery is defined as the ratio of internal standard spiked before extraction to internal standard spiked after extraction; matrix effect is defined as the ratio of internal standard

TABLE 2 | Mass spectrometry operative parameters.

Analyte	SRM transition	Operative Parameters		
		DP	CE	CXP
3,5-T_2; 3,3′-T_2	525.9 → 352.9 (q)	87	40.8	10.3
	525.9 → 381.8 (q)		27.6	11.2
	525.9 → 479.9 (Q)		26.0	14.2
T_3; rT_3	651.8 → 478.9 (q)	76	47.7	13.7
	651.8 → 508.0 (q)		31.2	14.8
	651.8 → 605.9 (Q)		31.3	17.9
T_4	777.8 → 604.8 (q)	82	52.8	17.0
	777.8 → 633.9 (q)		36.0	18.6
	777.8 → 731.9 (Q)		34.0	22.0
$^{13}C_6$-T_2	531.9 → 358.9 (q)	87	40.8	10.3
	531.9 → 387.9 (q)		27.6	11.2
	531.9 → 485.9 (Q)		26.0	14.2
$^{13}C_9^{15}$N-T_2	535.9 → 361.9 (q)	87	40.8	10.3
	535.9 → 390.8 (q)		27.6	11.2
	535.9 → 488.9 (Q)		26.0	14.2
$^{13}C_6$-T_3	657.8 → 484.9 (q)	76	47.7	13.7
	657.8 → 514.0 (q)		31.2	14.8
	657.8 → 611.9 (Q)		31.3	17.9
$^{13}C_6$-T_4	783.8 → 610.8 (q)	82	52.8	17.0
	783.8 → 639.9 (q)		36.0	18.6
	783.8 → 737.9 (Q)		34.0	22.0

TABLE 1 | Program of HPLC pumps.

	HPLC Quaternary Pump				HPLC Binary Pump			
Step	Total time (min)	Flow rate (μl/min)	Methanol (%)	Water (%)	Total time (min)	Flow rate (μl/min)	Solvent A (%)	Solvent B (%)
0	0.1	50	50	50	0.1	400	5	95
1	3.0	200	50	50	3.0	400	5	95
2	9.5	50	50	50	8.5	400	65	35
3	13.5	50	50	50	9.0	400	100	0
4					11.0	400	100	0
5					11.5	400	5	95
6					13.5	400	5	95

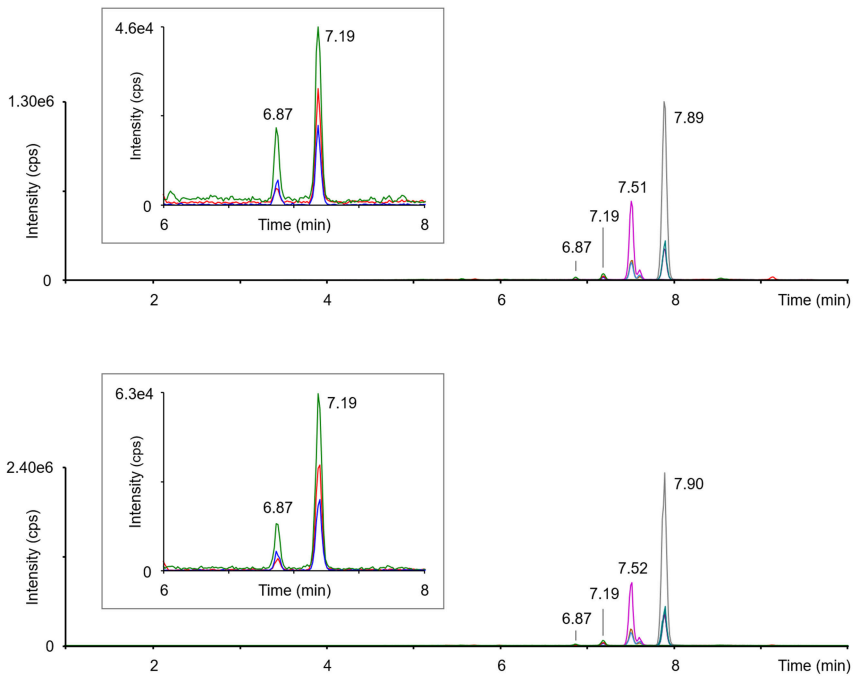

FIGURE 1 | Representative chromatograms obtained in two different subjects. The green, red, and blue tracings, reported also as an expanded view in the framed panels, refer to the three transitions monitored for 3, 5-T_2 (6.87 min.) and 3,3′-T_2 (7.19 min.), namely *m/z* 529.9 → 352.9, 529.9 → 381.8, and 525.9 → 479.9. Three more peaks are attributable to T_3 (7.51–7.52 min.), rT_3 (small peak next to T_3, at 7.60–7.61 min), and T4 (7.89–7.90 min.). Peak identity was confirmed by the comparison to appropriate standards, as detailed in the methods section.

spiked after extraction to internal standard dissolved in the reconstitution solvent.

Statistical analysis was performed with GraphPad Prism version 7.0 for Windows (GraphPad Software, San Diego, CA). Data are reported as mean ± SEM. Correlation analysis was performed through linear regression, taking $P = 0.05$ as the conventional limit of statistical significance.

RESULTS

Quality control data for T_2 assay averaged as follows, without apparent differences between isomers: accuracy 88–104%, precision 95–97%, recovery 78%, matrix effect +8%. For T_3 and T_4, recovery and precision were 104–128 and 85–94%, respectively. Recovery, matrix effect and precision averaged 67, −15, and 115% for T_3; 44, −7, and 90% for T_4. The lower limit of detection, determined with a signal to noise ratio of 3, was 11.5 pg/mL (22 pmol/L) for T_2, 9.1 pg/mL (14 pmol/L) for T_3, and 8.5 pg/mL (11 pmol/L) for T_4.

Representative chromatograms obtained in two patients are shown in **Figure 1**. 3,5-T_2 and 3,3′-T_2 were present in all samples, while 3′,5′-T_2 was never detected. Peak identity was determined by comparison with appropriate standard based on retention times and ratio between the three transitions.

Analyte concentration was determined by appropriate calibration curves and was based on the first transition, namely Q transition in **Table 2**. Scatter plot of the results obtained for 3,5-T_2 and 3,3′-T_2 are shown in **Figure 2**. 3,5-T_2 concentration averaged 41±5 pg/mL, i.e., 78 ± 9 pmol/L. In the same samples the concentration of 3,3′-T_2 was about 3-fold higher and averaged 133±15 pg/mL, i.e., 253±29 pmol/L.

As shown in **Figure 3**, 3,5-T_2 concentration was significantly related to 3,3′-T_2 concentration ($r = 0.540$, $P < 0.01$), while no significant correlation was observed with either T_3 or T_4 in the subset of patients in which these hormones were assayed.

DISCUSSION

Due to the emerging role of 3,5-T_2 in lipid and glucose metabolism, the adequate measurement of 3,5-T_2 and related compound is a challenging aim of thyroid hormone research. This is necessary to identify the clinical variables correlated with 3,5-T_2 concentrations and the effect of physiological and pathophysiological interventions. It would also be crucial to determine the effective concentrations achieved in experimental animals after the administration of exogenous 3,5-T_2, and to compare them with the endogenous levels.

In the past, different immunoassays have provided variable results. Recently, a novel immunoassay has been developed and used in a large clinical series (7, 13, 14), but endogenous levels appear to be below the lower detection limit (200 nmol/L) in about one third of the subjects. In addition, the

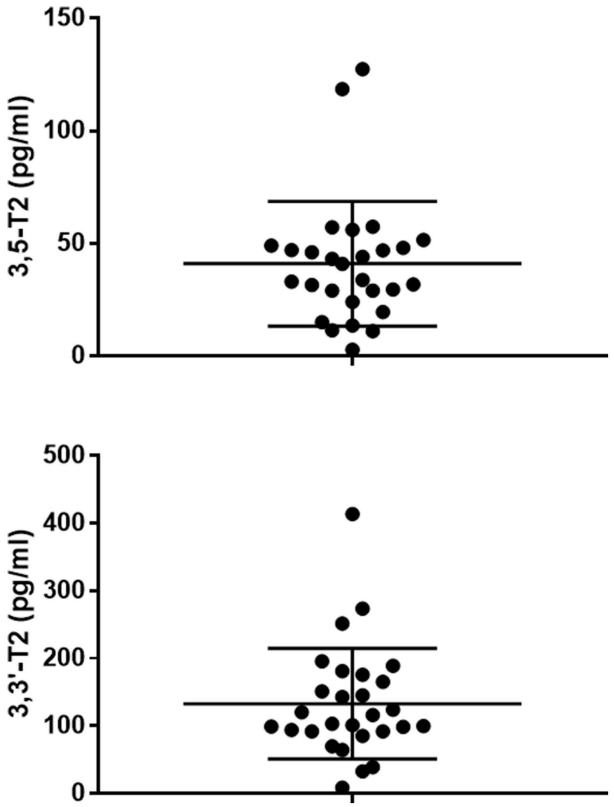

FIGURE 2 | Scatter plots showing the serum concentrations of 3,5-T_2 (upper panel) and 3,3′-T_2 (lower panel) in the 28 patients included in our series. The horizontal lines represent mean ± SD. Please note a different scale was used in the two panels.

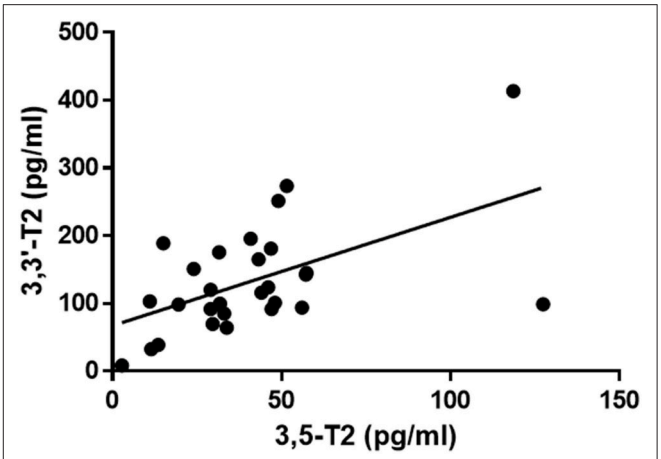

FIGURE 3 | Scatter plot of the relationship between 3,5-T_2 and 3,3′-T_2 serum concentration in the 28 patients included in our series. Linear regression analysis yielded $r = 0.540$ with $P = 0.003$. The regression line is plotted ($y = ax + b$, with $a = 1.600 \pm 0.489$ and $b = 67.29 \pm 24.06$ pg/mL).

experience acquired with thyroid hormones assays suggests that immunological methods cannot be easily employed to determine tissue concentrations.

Mass spectrometry is a very promising analytical technique, since it displays significant advantages in terms of accuracy, sensitivity and specificity (15–17) and it has been successfully applied to tissue homogenates (18). So far, only a few papers reported 3,5-T_2 measurements in humans or in animal models by mass spectrometry-based techniques.

Wang et al. (8) developed a LC-MS-MS method for the simultaneous measurement of 3,5-T_2, 3,3′-T_2, T_3, rT_3, and T_4 in bovine serum samples and human serum from Standard Reference Materials. Both in bovine and in human serum the concentration of 3,5-T_2 and 3,3′-T_2 was below the lower detection limit of the method, namely 740 pg/mL (1.41 nmol/L) for 3,5-T_2 and 920 pg/mL (1.75 nmol/L) for 3,3′-T_2.

In 2014 Jonklaas et al. (19) published the results of a pilot study in which 3,3′T_2 concentrations were evaluated by LC-MS-MS in 100 patients, with the primary aim to correlate T_2 and clinical variables. Mean detected 3,3′-T_2 levels were around 0.013–0.040 pmol/L and they could demonstrate an association between poor clinical conditions and low levels of T_2 (19).

In a subsequent investigation, Hansen et al. (20) succeeded in detecting T_2 in adult frog (*Xenopus laevis*) plasma and tadpole (*Rana catesbeiana*) serum. They developed a new analytical method by optimizing solid phase extraction, eventually choosing hydrophilic-lipophilic balanced mixed-mode polymeric materials (Plexa) and C18-materials to measure 11 thyroid hormone metabolites, including 3,5-T_2 and 3,3′-T_2, in 50 μl of serum or plasma, with a lower detection limit of 250 pg/mL (476 pmol/L). 3,5-T_2 and 3,3′-T_2 were detected in 80 and 30% of the samples, respectively, and their concentrations averaged 660 and 640 pmol/L, respectively.

Quite recently, Min Li et al. (21) optimized an LC-Q-TOF-MS method to determine thyroid hormone metabolites in placenta tissue. With this method, it was possible for the first time to track the presence of T_2 (either 3,3′-T_2 and 3,5-T_2) in human and mice placenta at the concentration of 160–260 and 70–130 pg/g, respectively.

The method we developed has the advantage of a lower detection limit, which allowed 3,5-T_2 and 3,3′-T_2 detection in all our samples. This improvement is probably due to the higher sample volume and to the lower matrix effect allowed by the extensive washing steps included in the pre-analytical procedure. While this is promising, several drawbacks should be acknowledged: the needed samples volume is still too high for routine clinical use; the method is time consuming; the pre-analytical steps are too complex to be implemented on-line with the MS equipment. In addition, the human factor may be crucial, since each operator needed a significant training period before becoming able to get reproducible results. For these reasons, we believe that further developments are necessary before starting large-scale clinical investigations. In particular, sample preparation should be improved to make it easier and increase its reproducibility, throughput and ruggedness. If this can be achieved, it will become possible to establish a proper age- and gender-related reference range, and to investigate the effects of the different thyroid states and/or thyroid hormone supplementation.

While the present investigation should be regarded as a feasibility study, it may help to get further insight into T_2 biochemistry and biology. Our results confirm that previous reports based on mass spectrometry-based techniques may have missed endogenous T_2 because of their lower sensitivity. On the other hand, immunological techniques, particularly the recent method developed by Lemphul et al. provided control values for 3,5-T_2 that are about 3-fold higher than we observed. The simplest explanation of this difference is the hypothesis that the different techniques allow identification of different T_2 fractions. In general, immunological techniques rely on long incubation times and limited sample processing. In the presence of a high affinity antibody the analyte is expected to dissociate from its endogenous binding sites, and a good estimate of its total amount is obtained. On the other hand, mass spectrometry requires extensive sample processing and protein precipitation, making it likely that protein-bound analyte is lost.

T_3 and T_4 are known to be largely bound to plasma proteins, with a free/total ratio lower than 1/100, and a good agreement is usually observed between the results of mass spectrometry-based assays and the total amounts detected by immunological methods (9). Extensive binding to plasma protein has also be reported for 3-iodothyronamine, whose assay is complicated by additional pitfalls, such as oxidation by serum enzymes and adduct formation, further accounting for a large discrepancy between mass spectrometry and immunological detection (22). At present, very little is known about T_2 binding to plasma proteins and/or serum metabolism. Adding T_2 to reconstituted systems including specific plasma proteins and comparing the results of immunological and mass spectrometry-based assays might help to clarify this issue.

Our investigation was not aimed at evaluating the relationships between T_2 level and other clinical variables. We could simply test the association between different iodothyronines. Interestingly, while 3,5-T_2 and 3,3′-T_2 levels were significantly related, neither T_2 isomer showed any significant correlation with T_3 or T_4. This is consistent with the observation reported by Lemphul et al. (7) and it supports the hypothesis that T_2 production may be specifically regulated. Because of this association, it would be interesting to investigate whether 3,3′-T_2 assay may be used in the clinical field as an indirect index of 3,5-T_2 availability.

In conclusion, we developed a method to measure 3,5-T_2 and 3,3′-T_2 in human serum samples, that significantly reduced matrix effect and showed high sensitivity and specificity. The method is promising, and further developments aimed at reducing its technical complexity might eventually provide a novel diagnostic tool amenable to large-scale clinical use.

ETHICS STATEMENT

Serum samples were obtained from 28 patients, including 8 healthy volunteers and 20 women undergoing endocrinological screening and found to be euthyroid. Serum was obtained by using the remaining part of samples obtained for independent clinical indications. All subjects gave informed consent and since no additional blood drawings were performed, ethical committee approval was not required.

AUTHOR CONTRIBUTIONS

LL, NN, GS, ES, and MB performed the experiments. EC and TS collected the human samples. FS and SG analyzed the data. RZ, LL, and AS wrote the manuscript draft.

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