Thyroid: biological actions of 'nonclassical' thyroid hormones

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Abstract

Thyroid hormones (THs) are produced by the thyroid gland and converted in peripheral organs by deiodinases. THs regulate cell functions through two distinct mechanisms: genomic (nuclear) and nongenomic (non-nuclear). Many TH effects are mediated by the genomic pathway – a mechanism that requires TH activation of nuclear thyroid hormone receptors. The overall nongenomic processes, emerging as important accessory mechanisms in TH actions, have been observed at the plasma membrane, in the cytoplasm and cytoskeleton, and in organelles. Some products of peripheral TH metabolism (besides triiodo-L-thyronine), now termed 'nonclassical THs', were previously considered as inactive breakdown products. However, several reports have recently shown that they may have relevant biological effects. The recent accumulation of knowledge on how classical and nonclassical THs modulate the activity of membrane receptors, components of the mitochondrial respiratory chain, kinases and deacetylases, opened the door to the discovery of new pathways through which they act. We reviewed the current state-of-the-art on the actions of the nonclassical THs, discussing the role that these endogenous TH metabolites may have in the modulation of thyroid-related effects in organisms with differing complexity, ranging from nonmammals to humans.

Key Words
► thyroid hormone
► nongenomic effects
► thyroid hormone metabolism
► thyroid hormone receptors

Introduction

General notions

The thyroid gland produces two main iodothyronines: tetraiodo-L-thyronine (T_4) and triiodo-L-thyronine (T_3). In humans, T_4 is synthesized entirely within the thyroid and acts as a pro-hormone to generate T_3. Only 20% of the T_3 in circulation is secreted directly by the gland itself. The remaining T_3 derives from the peripheral monodeiodination of T_4. Deiodinase activity regulates the local and systemic availability of T_3 and other iodothyronines. Thyroid hormone (TH) deiodination is mediated by three selenoenzymes: type 1 deiodinase (D1), preferentially expressed in the liver and also expressed in the kidney, thyroid, and pituitary; D2, present in the CNS, anterior pituitary, brown adipose tissue, and placenta; and D3 in the CNS, placenta, skin, and fetal tissue. For further details on deiodinases, the reader is referred to Bianco (2011), Maia *et al*. (2011), Orozco *et al*. (2012) and Luongo *et al*. (2013).

Other biochemical pathways are involved in TH metabolism in addition to deiodination. Conjugation of phenolic hydroxyl groups with sulfate or glucuronic acid

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increases water solubility of substrates, facilitating biliary and/or urinary clearance (Visser 1990). Decarboxylation and deamination of THs lead to the formation of the so-called acetic acid-TH analogs such as triiodothyroacetic (Triac) and tetraiodothyroacetic (Tetrac) acids (Siegrist-Kaiser & Burger 1994). Several transporters contribute to the uptake of TH into the peripheral tissue, including organic anion-transporting polypeptides (OATPs), L-type amino acid transporters, monocarboxylate transporters (MCT), and bile acid transporters see for a recent review: Visser (2013).

TH actions

THs regulate cell functions through two distinct mechanisms: genomic (nuclear) and nongenomic (nonnuclear). Most effects of TH are mediated by the genomic pathway – a mechanism that requires thyroid hormone activation of nuclear receptors (TRs). This leads to a conformational change allowing interaction with specific thyroid hormone responsive elements located on the promoters of THs target genes (Bassett *et al.* 2003, Moeller & Broecker-Preuss 2011, Tata 2013, Pascual & Aranda 2013), regulating transcription rate. TRs homodimerize or interact with other nuclear receptors such as the retinoic X receptor (Forman *et al.* 1992, Bogazzi *et al.* 1994). TRs belong to a large family of ligand-dependent transcription factors, which includes nuclear hormone receptors for vitamins, xenobiotics, and sex steroids (Weitzel & Alexander Iwen 2011). They are termed as TRα and TRβ and are encoded by two genes (α and β) located on two different chromosomes (Cheng 2000) that express differently in developing and adult tissues (Oetting & Yen 2007, Cheng *et al.* 2010). Highest *Trα1* (*Thra*) expression is in the brain, with lower levels in the kidney, skeletal muscle, lungs, heart, and liver, whereas *Trβ1* is expressed predominantly in the kidneys and liver, and at lower levels in the brain, heart, thyroid skeletal muscle, lungs, and spleen (Williams 2000). TRβ isoforms are involved in lipid metabolism (Pramfalk *et al.* 2011) by reducing serum lipids (Johansson *et al.* 2005, Angelin & Rudling 2010, Shoemaker *et al.* 2012). TRβ disruption in mice impairs fatty acid (FA) oxidation (Araki *et al.* 2009) even in the presence of TRα overexpression (Gullberg *et al.* 2000, 2002). TRβ agonists have approximately tenfold greater affinity for TRβ than TRα, with a marked effect on the liver and efficacy in lowering of cholesterol (Webb 2010, Ladenson 2011). Although T_3 exerts many of its actions through canonical transcriptional regulation, an increasing amount of evidence shows that many of T_3 effects are initiated outside the nucleus and involve different signaling transduction pathways. These effects are mediated by nongenomic actions. The overall nongenomic processes are poorly understood but emerge as important accessory mechanisms in TH actions and have been observed at the plasma membrane, in the cytoplasm and cytoskeleton, and in organelles (Wrutniak-Cabello *et al.* 2001, Cheng *et al.* 2010). Membrane receptors, consisting of specific integrin alpha V beta 3 (αVβ3) receptors, have been identified (Bergh *et al.* 2005). THs on the cell surface trigger the serine–threonine kinase (MPK/ERK) pathway via the integrin receptor (Bergh *et al.* 2005, Cody *et al.* 2007), initiating complex cellular events (Lin *et al.* 2009*a*,*b*). In the cytoplasm, THs activate PI3K and thereby downstream gene transcription of specific genes. T_3 also activates PI3K from the integrin αvβ3 hormone receptor site (Lin *et al.* 2009*b*, Moeller & Broecker-Preuss 2011). Calcium is a second messenger regulated by THs through the modulation of a Ca^{2+}-ATPase (Galo *et al.* 1981). Del Viscovo *et al.* (2012) showed that THs exert short-term nongenomic effects on intracellular calcium by modulating plasma membrane and mitochondrial pathways in rat pituitary GH3 cells. Furthermore, cellular actions involving Akt/protein kinase B (shown in human fibroblasts; Moeller *et al.* 2005) and AMP-activated protein kinase (AMPK) (in mice) (Irrcher *et al.* 2008) are well known. De Lange *et al.* (2008) showed that in rat skeletal muscle, T_3 stimulates FA and glucose metabolism through rapid activation of AMPK and Akt/protein kinase B signal transduction.

THs regulates mitochondrial activity and thus it may perhaps not be surprising that the mitochondria themselves are important target for THs. THs modulate mitochondrial activity through two ways: direct or indirect. The first requires the presence inside the organelles of specific binding sites for THs that play important physiological roles in regulation of the mitochondrial transcription apparatus (see for review, Cioffi *et al.* (2013)). One of these binding sites, termed p43, has been identified as a bona fide TR that binds to the D-loop region that contains the promoters of the mitochondrial genome (Wrutniak *et al.* 1995). By contrast, the indirect way acts through increased, nuclear TR-dependent transcription of factors that modulate the expression of mitochondrial genes (see for review, Cioffi *et al.* (2013)).

The nonclassical THs

Besides T_3, nonclassical THs exist. In the present review, we summarize the highlights of their biological actions.

Tetrac and Triac

In humans, the amount of Triac produced by the liver and other tissues accounts for about 14% of T_3 metabolism (Siegrist-Kaiser & Burger 1994). Triac is weakly TRβ-selective, with a 1.5-fold affinity for TRβ (Schueler *et al*. 1990). Triac has been used to suppress thyroid-stimulating hormone (TSH) secretion in TH-resistant patients (Kunitake *et al*. 1989) and to increase metabolic rate in obese patients (Dumas *et al*. 1982). It has been shown to be more potent than T_3 as both a β-adrenergic stimulator of uncoupling protein 1 and inducer of lipoprotein lipase mRNA, D3 activity, and mRNA (Medina-Gomez *et al*. 2003). Triac inhibits expression and secretion of leptin in rat primary white and brown adipocytes with a potency similar to that of T_3 (Medina-Gomez *et al*. 2004). The use of Tetrac as a potential substitute for T_4 has been studied in the treatment of myxedema and for its ameliorating effect on peripheral lipid metabolism in humans. The effects are similar to those of T_4, but require higher dosing (Lerman 1956). Tetrac is currently used in the clinic for the treatment of TH resistance (Anzai *et al*. 2012). Therapeutic doses of Triac to treat pituitary and thyroid disorders exceed those required for T_4 and T_3 (Sherman & Ladenson 1992, Bracco *et al*. 1993), a property attributed to its short half-life in humans and rodents (Pittman *et al*. 1980, Moreno *et al*. 1994). Classic THs are transported within the cell by TH transporters (Visser 2013). Tetrac does not seem to depend on active transport, at least by the most abundant transporter MCT8. Tetrac can replace T_3 to restore normal fetal mouse brain development in MCT8-null mice (Horn *et al*. 2013).

Thyronamines

The structures of the thyronamines (TAMs), a novel class of endogenous thyroid-signaling molecules, differ from T_4 and deiodinated TH derivatives by the absence of a carboxylate group on the alanine side chain. 3-Iodothyronamine (T1AM) and T0AM have been detected *in vivo* (Scanlan *et al*. 2004, DeBarber *et al*. 2008) in the serum of rodents and humans (Saba *et al*. 2010, Hoefig *et al*. 2011), in rat liver, brain, and heart (Chiellini *et al*. 2007, Saba *et al*. 2010). Data from Piehl *et al*. (2008) present a role for deiodinases in TAM biosynthesis, defining biosynthetic pathways for T1AM and T0AM with T_4 as a pro-hormone. Seemingly in contrast, a recently developed method to detect T1AM and T0AM in tissues and plasma (Ackermans *et al*. 2010) failed to reproduce the above data. Using rats treated with (13)C-labeled T_4, the authors could detect

in vivo conversion of T_4 to T_3 but not to T1AM in plasma or brain samples, neither any endogenous T1AM nor T0AM was detected in the plasma from rats and plasma and in thyroid tissue from humans. Indeed, iodothyronine decarboxylation to iodothyronamines has not been demonstrated directly, and the aromatic amino acid decarboxylase was shown to be unable to catalyze iodothyronine decarboxylation (Hoefig *et al*. 2012). In line with this, data from Hackenmueller *et al*. (2012) suggest that T1AM is not an extrathyroidal metabolite of T_4, yet is produced within the thyroid by a process that requires a sodium–iodide symporter and thyroperoxidase, the same biosynthetic factors necessary for T_4 synthesis. These data shed new light on the pathways potentially involved in T1AM production and imply that the enzymatic conversion of iodothyronine to iodothyronamine is not simple. Steady-state physiological T1AM serum concentrations are similar to those of T_3, and tissue concentrations of its metabolite, T0AM, exceed T_4 and T_3 metabolites by two- and 20-fold respectively (Hart *et al*. 2006, Chiellini *et al*. 2007). Physiological receptor(s) of TAMs remain to be identified. In TR receptor binding/gene activation assays, T1AM showed no affinity for TRβ and TRα, and inability to modulate nuclear TR-mediated transactivation (Chiellini *et al*. 1998). Studies surrounding TAM association with other receptors concluded that neither T0AM nor 3-T1AM activated Gαs-coupled dopamine D1 and β2 adrenergic receptors (Scanlan *et al*. 2004). T1AM, however, was found to be a potent agonist of trace amine-associated receptor 1 (TAAR1), an orphan G protein-coupled receptor (Zucchi *et al*. 2006). Rat and mouse TAAR1 are activated by T1AM, with EC50 values of 14 and 112 nM respectively. The T1AM ligand pharmacophore that activates TAAR1 was later characterized (Hart *et al*. 2006, Tan *et al*. 2007, 2008, Snead *et al*. 2008). T1AM reduces activation of the proto-oncogene *c-fos* (Manni *et al*. 2012). Ianculescu *et al*. (2009) reported that the cellular uptake of T1AM occurs via specific, saturable, and inhibitable transport mechanisms that are sodium and chloride independent, pH dependent, TAM specific, and do not involve candidate transporters of monoamines, organic cations, or THs. By a novel RNAi screening method, eight transporters of interest were identified. Knockdown resulted in T1AM transport in HeLa cells, but the physiological role of these transporters remains unknown. Studies using COS-1 cells transfected with multispecific OATPs, 1A2, 1B3, and 1C1, and the specific TH transporters, MCT8 and MCT10, proved that T1AM differentially inhibits T_3 and T_4 cellular uptake by these transporters (Ianculescu *et al*. 2010). Notably, T1AM

also inhibits both T_3 and T_4 uptake via MCT8, the most specific TH transporter. T1AM has no effect on TH transport by OATP1B3 and MCT10.

In mice, Scanlan *et al*. (2004) showed that a single i.p. injection of T1AM rapidly induced an $\sim 10\,^{\circ}\text{C}$ drop in body temperature that peaked 1 h after injection and dose dependently disappeared after 4–6 h. The same authors further showed that T1AM reduction on cardiac performance was a direct effect and independent of T1AM-induced hypothermia. In a rat working heart preparation held at 37 °C, introduction of T1AM into the perfusion buffer resulted in large and immediate decreases in both heart rate and systolic aortic pressure. Additional studies on the heart have further supported direct actions of T1AM on this organ (Chiellini *et al*. 2007, Frascarelli *et al*. 2008). A single i.p. dose of T1AM dramatically switched fuel utilization away from carbohydrates and toward lipids (Braulke *et al*. 2008). Siberian hamsters (*Phodopus sungorus*), a hibernating rodent species, and mice completely shifted their respiratory quotients (RQ) from a normal, mixed carbohydrate and lipid value (0.90 for hamsters and 0.83 for mice) to a complete and persistent lipid-related RQ value of ~ 0.7 with elevated urine ketone content. The RQ effect (4.5 h after injection) lagged behind hypothermia, bradycardia, or hyperglycemia (1 h after injection). I.v. infusion with a low T1AM dose (0.5 mg/kg) into nonfasted naive rats rapidly increased endogenous glucose production and plasma glucose, plasma glucagon, and corticosterone, but did not affect plasma insulin (Klieverik *et al*. 2009). Contrastingly, in i.c.v. injected (130 ng/100 g body weight (BW)) short-term fasted male mice (Manni *et al*. 2012), T1AM failed to ameliorate lipid profiles. It is known to possess a central effect, namely hypophagia, as well as peripheral effects of raised plasma glucose levels and reduced peripheral insulin sensitivity (the latter being also seen after i.p. injection (Braulke *et al*. 2008, Klieverik *et al*. 2009)), accompanied by pancreatic insulin production. Plasma free T_3 (fT_3) levels were also lowered. Nonfasted, drug-naive rats (Klieverik *et al*. 2009) treated with T1AM (100 μg/kg) acutely increased endogenous glucose production and hyperglucagonemia, while (in contrast to the effect in fasted mice (Manni *et al*. 2012)) plasma insulin decreased. T0AM had a similar effect that was less profound (Klieverik *et al*. 2009). Interestingly, T1AM injection in mice resulted in 12% of the injected dose in the plasma, highlighting its systemic bioavailability (Manni *et al*. 2012). Inhibition of T1AM conversion by pretreatment with a mitochondrial amine oxidase inhibitor, clorgyline (250 μg/100 g BW), prominently increased T1AM serum levels, but prevented the hyperglycemia and reduction of fT_3 levels. This led the authors to indicate that a metabolite of T1AM causes these adverse effects. Central effects of T1AM administration also included amelioration of memory and reduction in pain threshold (Manni *et al*. 2013). T1AM's enhancing effect on learning renders this compound useful in the treatment of neurodegenerative diseases.

3,3′,5′-Triiodo-L-thyronine

3,3′,5′-T_3 (reverse T_3, abbreviated as rT_3), a product of 5-deiodination of T_4 by D1 and D3, is a potent initiator of actin polymerization in astrocytes. It portrays similar effectiveness to T_4 and much more than T_3 (Farwell *et al*. 2006). In hypothyroid rodents, neurons and astrocytes develop poor actin cytoskeletons that T_3 replacement cannot rescue. However, rT_3 initiates reappearance of filamentous actin within minutes without altering total actin mRNA or protein content (Farwell *et al*. 1990, Siegrist-Kaiser *et al*. 1990). This rT_3 property is attributed to TRΔα1, a native TR isoform that lacks a nuclear localization signal and is present in the extranuclear compartment of astrocytes and neurons. This isoform has the ligand affinity and specificity required for of actin polymerization by rT_3. A study of the astrocytes of the developing mouse cerebellum deprived of both TRs showed that TRΔα1 rescued the actin cytoskeleton's response to rT_3 (Flamant & Samarut 2003). Thus, THs may require TR regions that are not necessarily canonical DNA-binding regions. rT_3 also inhibits free FA levels in chickens stimulated with dexamethasone or adrenaline (Bobek *et al*. 2002).

3,5-Diiodo-L-thyronine

Several studies have indicated 3,5-diiodo-L-thyronine (T_2), an endogenous metabolite of T_3, as a peripheral mediator of several TH metabolic effects. Although conversion of T_3 to T_2 has not been demonstrated *in vitro*, indirect evidence indicates that T_2 is indeed formed from T_3 *in vivo* through deiodination (Moreno *et al*. 2002). Serum concentrations of T_2 in humans are within the picomolar range (16 pM in healthy individuals to 50 pM maximum in individuals with nonthyroidal illness; Pinna *et al*. 1997). Rat intra-hepatic T_2 concentrations are 1.5 fmol/100 mg (Moreno *et al*. 2002). To date, results in hypothyroid rats suggest that T_2 has specific actions on resting metabolic rate (RMR) that are distinct from those of T_3: they are more rapid and not attenuated by actinomycin D (see Fig. 1A and B; Lanni *et al*. 1996).
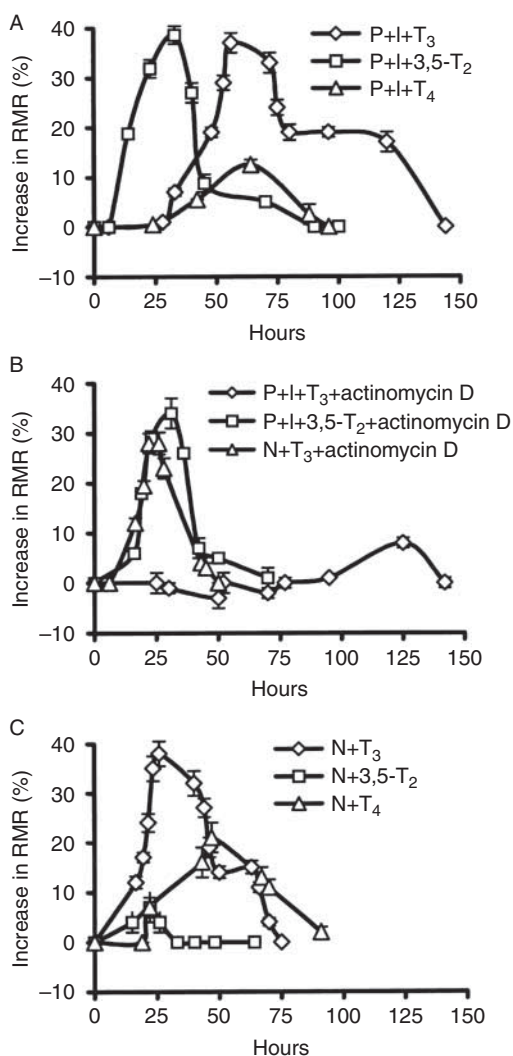
Figure 1

Changes in RMR in hypothyroid (P+I) and euthyroid (N) rats after administration of T_4, T_3, and the nonclassical thyroid hormone T_2, with or without simultaneous administration of actinomycin D. (A) Hypothyroidism was chronically induced by combined treatment of propylthiouracil (PTU) and IOP (P+I). N and P+I rats were acutely injected with 25 μg iodothyronine/100 g body weight (BW). Each data-point shows the mean±s.e.m. from four rats. Values are expressed as a percent change from the value at time 0 (i.e. immediately before injection). Actual RMRs (IO2 kg/0.75 per h) at time 0 were 1.01±0.12, 0.99±0.10, and 0.94±0.08 for P+I+T_3, P+I+3,5-T_2, and P+I+T_4 groups respectively. (B) Actinomycin D (8 μg/100 g BW) was injected in combination with 25 μg/100 g BW of either T_3 or 3,5-T_2. Each data-point is the mean±s.e.m. from four rats. Values are expressed as a percent change from the time 0 value (i.e. immediately before injection). Actual RMRs (IO$_2$/kg$^{0.75}$/h) at time 0 were 1.06±0.15, 0.98±0.12, and 1.44±0.04 for P+I+T_3+actinomycin D, P+I+ 3,5-T_2+actinomycin D, and N+T_3+actinomycin D groups respectively. (C) RMRs (IO2 kg/0.75 per h) at time 0 were 1.41±0.03, 1.39±0.04, and 1.42±0.03 for N+T_3, N+3,5-T_2, and N+T_4 groups respectively. Figure adapted from Moreno *et al*. (2002), republication approved by the Endocrine Society.

T_3 injection to euthyroid animals resulted in combined T_3 and T_2 patterns on RMR (see Fig. 1C). T_3, propylthiouracil (inhibiting type 1 deiodinase and thyroid peroxidase activity), and iopanoic acid (inhibiting the activity of all three deiodinases) combined treatment showed a long-term induction of RMR. T_2 is thus predominantly responsible for short-term induction of RMR (Fig. 1A; Moreno *et al.* 2002). The metabolic effects of T_2 also underlined the ability of this hormone to improve survival of hypothyroid rats to cold with persistent increased energy expenditure (Lanni *et al.* 1998). Furthermore, upon T_2 administration to hypothyroid rats, the RMR and FA oxidation increases in the muscle mitochondria are accompanied by mitochondrial translocation of the FA transporter FAT/CD36, ensuring effective increases in metabolic rate (Lombardi *et al.* 2012). Several data support the ability of T_2 to stimulate mitochondrial activities in a very rapid manner (Lanni *et al.* 1992, 1993, 1994, Goglia 2005, Cavallo *et al.* 2011), excluding short-term genomic action of T_2. Chronic treatment of hypothyroid rats with T_2 revealed a different picture: T_2 upregulated protein levels of ATP synthase subunits (alpha, beta, F(o)I-PVP, and OSCP; Mangiullo *et al.* 2010). Increase in β-subunit mRNA accumulation suggested indirect transcriptional regulation by T_2 through activation of the transcription factor, GA-binding protein/nuclear respiratory factor-2 (Mangiullo *et al.* 2010). Moreover, D1 activity in the rat anterior pituitary has been shown to be increased transiently after a single injection of T_2, while in a reaggregate culture of anterior pituitary, T_2 has been demonstrated to stimulate D1 at 24 h after its application, dose dependently (Baur *et al.* 1997).

T_2, as a nonclassical TH, is able to prevent BW gain when administered i.p. to rats fed a high-fat diet without inducing T_3-related undesirable side effects (tachycardia, cardiac hyperplasia, and decreased TSH levels), at least at the administered dose (25 μg/100 g BW for 4 weeks) (Lanni *et al.* 2005, De Lange *et al.* 2011, Moreno *et al.* 2011). At this dose, by almost doubling hepatic FA oxidation rate, T_2 efficiently prevented HFD-induced i) hepatic fat accumulation, ii) insulin resistance, and iii) increase in serum triglycerides (TGs) and cholesterol levels (Lanni *et al.* 2005). T_2 stimulated mitochondrial uncoupling, decreased ATP synthesis, and increased fat burning, thus counteracting obesity (Lanni *et al.* 2005).

An important consequence of the above-described effects of T_2 included increased skeletal muscle insulin sensitivity, due to an increased response to insulin of Akt/PKB phosphorylation, and sarcolemmal glucose transporter 4 accumulation (Moreno *et al.* 2011). Importantly, T_2 also induced biochemical and structural shifts

toward glycolytic myofibers (Moreno *et al.* 2011). For an overview of T_2's effects on metabolism, see Fig. 2. Proteomic analysis of intracellular and mitochondrial proteins (Silvestri *et al.* 2010) revealed that mitochondria were the principal targets of T_2's normalizing effects on changes induced by high-fat diet (HFD). BN-page analysis revealed that T_2 partially restored individual OXPHOS complex (predominantly complexes I and II) levels induced by HFD. Additionally, T_2 augmented the activities of respiratory complexes, with respect to both HFD (except complex V) and N animals (except complex IV). In rats pre-fed a HFD, subsequent T_2 administration reduced hepatic fat and hyperlipidemia via the same biochemical pathways described previously (Mollica *et al.* 2009).

T_2 carries out its antilipidemic effects via activation of two important factors involved in lipid metabolism: AMPK and nuclear deacetylase sirtuin 1 (SIRT1). AMPK is a known sensor of cellular ATP levels (Ruderman *et al.* 2013), and SIRT1 regulates metabolic balance in response to increases in cellular NAD^+:NADH ratios. It has recently been identified to be a crucial target abating, diet-induced obesity in rodents (Rodgers *et al.* 2005, Lagouge *et al.* 2006, Gerhart-Hines *et al.* 2007). After simultaneous administration to rats receiving a high-fat diet, rapid induction of hepatic FA oxidation by T_2 (within 6 h) was concomitant with hepatic activation of SIRT1, an activity persisting over time. Increased phosphorylation of AMPK was observed after 4 weeks of treatment (De Lange *et al.* 2011).
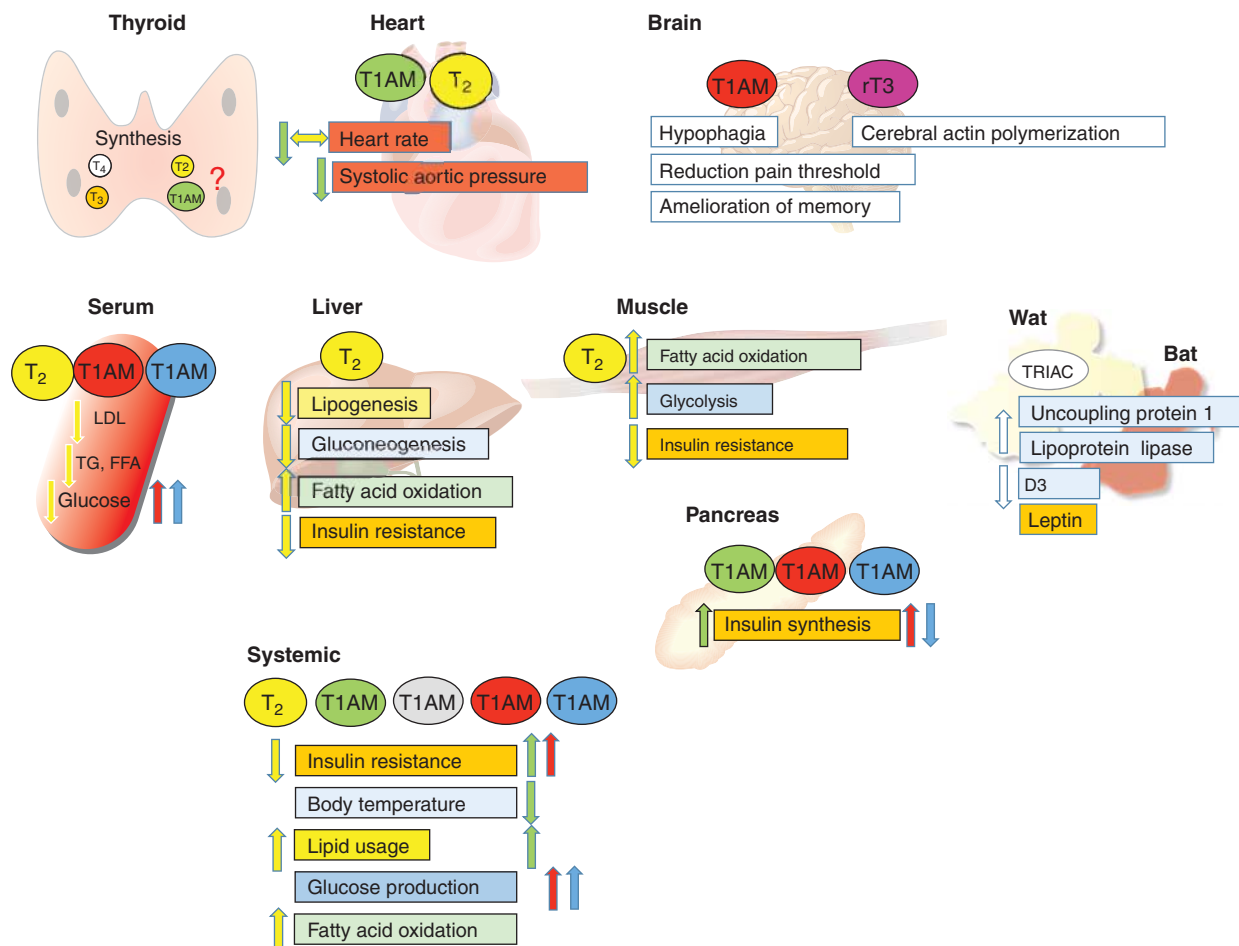

Figure 2

Thyroid synthesis of thyroid hormones (THs) and administration effects of nonclassical THs. Question mark, thyroid synthesis of T_2 and T1AM is currently still under debate. Peripheral synthesis of nonclassical THs by deiodinase activity (see text) is not indicated. Administration of nonclassical THs (for explanations of treatments, see text) indicated in color. White, Triac incubation in rat primary cultures; yellow, i.p. administration of T_2 in rats on a high-fat diet; green, i.p. or i.v. administration of T1AM in mice; grey, i.v. administration of T1AM in mice; red, i.c.v. administration of T1AM to fasted mice; blue, i.c.v. administration of T1AM to mice; and purple, rT_3 administration to mice. Arrows pointing upward, upregulation; arrows pointing downward, downregulation; double horizontal arrows, no change; and absence of arrows, no effect reported to date.

Induction of SIRT1 led to deacetylation of peroxisome proliferator-activated receptor γ coactivator-1α and sterol receptor element binding protein-1c (SREBP-1c), associated with induction and reduction of expression of genes involved in FA oxidation and lipogenesis respectively (De Lange *et al.* 2011). These findings provide a clue to explain T$_2$'s effectiveness in lowering hepatic fat accumulation and counteracting insulin resistance with respect to T$_3$. In a similar system, T$_3$ has increased hepatic lipogenesis (Cable *et al.* 2009).

A second clue indicating that T$_2$ has contrasting effects on hepatic lipogenesis involving SREBP-1c was found *in vitro*. T$_3$ increases an active precursor of SREBP-1c in HepG2 cells without modulating SREBP-1c transcription (Gnoni *et al.* 2012). T$_2$, however, blocks proteolytic cleavage and thus activation of SREBP-1c (Rochira *et al.* 2013) independent of transcription. Consequently, FA synthase expression reduced. The resulting inhibitory effect of T$_2$ on lipogenesis is concordant with *in vivo* findings (De Lange *et al.* 2011) and mechanistically complementary to SIRT1-dependent deacetylation of SREBP-1c (De Lange *et al.* 2011). The liver is not the only organ in which T$_2$ activates SIRT1: T$_2$ has been shown to act through SIRT1 activation in the kidney (Shang *et al.* 2013). Treatment with T$_2$ prevented diabetic nephropathy (DN) in a diabetic rat model via SIRT1-dependent deacetylation and p65, a subunit of nuclear factor-κB, inactivation, thus inhibiting the inflammatory process crucial to this pathology. In rat mesangial cells, the DN phenotype was induced by exposure to high glucose, and treatment with T$_2$ under these conditions counteracted the DN phenotype. Co-treatment with T$_2$ and sirtinol – a specific SIRT1 inhibitor (Shang *et al.* 2013) – abolished deacetylation of p65. T$_2$ leads to dephosphorylation of JNK independent of SIRT1, and did not associate with abating the DN phenotype (Shang *et al.* 2013). Thus, SIRT1 activation plays a crucial role in T$_2$'s relieving effect on DN.

As a first step in projecting rodent data to study T$_2$'s metabolic effects in humans, two healthy volunteers were administered T$_2$ (1–4 μg/kg BW) acutely. After 6 h, a significant increase in RMR was detected in both patients. Daily T$_2$ administration for 28 days increased RMR by $\sim$15% and decreased BW by about 4 kg. Ultrasonography revealed that one subject showed reductions in steatosis. Additionally, total serum cholesterol levels were lowered, and no side effects were recorded (Antonelli *et al.* 2011). A mechanism by which T$_2$ reduces cholesterol in the serum (Lanni *et al.* 2005, Antonelli *et al.* 2011, De Lange *et al.* 2011) was recently called into question. T$_2$'s LDL-lowering effects are independent of the LDL receptor (Goldberg *et al.* 2012),

as determined by feeding a western type diet to LDL receptor-deficient mice (Ldlr$^{-/-}$) and treating with T$_2$. The diet was chosen because dietary absorption of cholesterol and TG drives hepatic apoB production, especially in Ldlr$^{-/-}$ mice. These mice develop much higher levels of cholesterol and atherosclerosis. T$_2$ had no effect on TG levels, probably due to increased lipolysis, but led to marked reductions in liver apoB and circulating apoB48 and apoB100 (Goldberg *et al.* 2012).

To study whether the lipid-lowering effects of T$_2$ were directly acting on the liver, or if they were secondary to changes in endocrine or metabolic pathways, primary rat hepatocytes, overloaded with lipids (to obtain 'fatty hepatocytes') and treated with T$_2$ (10^{-5} M) have been employed (Grasselli *et al.* 2011a). This experimental setup demonstrated that T$_2$ reversed the effects induced by lipid overload in these cells, thus supporting a direct effect of T$_2$. Moreover, rat hepatoma (FAO) cells defective for functional TRs were used to answer whether T$_2$-mediated lowering of hepatic lipid profiles even requires TR action (Grasselli *et al.* 2011b). Exposure to pharmacological doses of T$_2$ (10^{-5} M) for 24 h reversed the effects induced by FAs and increased mitochondrial uncoupling, thus indicating that the actions of T$_2$ in these cells are independent of transcriptionally functional TRs.

Intracellular action of T$_2$ also is described in avian cells during fetal development and cell differentiation (Incerpi *et al.* 2002). T$_2$ regulates DNA synthesis, cell-cycle proteins (Alisi *et al.* 2004), and several membrane-associated transport systems, whose activity is related to cell proliferation (Incerpi *et al.* 2002, 2005). T$_2$'s effect on the Na$^+$–H$^+$ exchanger was identified for 14-day- and 19-day-old cells, whereas the effect on amino acid transport was present at late stages of embryo development. Both transport systems were activated through a signal transduction pathway involving the PKC, MAPK, and PI3K pathways (Incerpi *et al.* 2002). Moreover, T$_2$ exerts a short-term inhibitory effect on the Na$^+$–K$^+$-ATPase, the magnitude of which strongly correlates to the developmental age of the isolated cells (Scapin *et al.* 2009). The Na$^+$–K$^+$-ATPase inhibition is mediated through the activation of PKA, PKC, and PI3K (Scapin *et al.* 2009). Signal transduction pathways contributing to the modulation of the sodium pump by T$_2$ are involved in the control exerted on cell proliferation.

Recently, it has been shown that T$_2$ exerts short-term effects on intracellular calcium concentrations and NO release by modulating plasma membrane and mitochondrial pathways in pituitary GH3 cells (Del Viscovo *et al.* 2012). In particular, T$_2$ facilitates physiological Ca^2

Table 1 Involvement of TRs or alternative receptors in non-classical thyroid hormone action

Non-classical thyroid hormone	Affinity for/transactivation through TRα or TRβ	Affinity for other receptors
Triac	High (Schueler *et al*. 1990)	None yet identified
T1AM	Absent (Chiellini *et al*. 1998)	High (trace amine-associated receptor 1 (TAAR1); Zucchi *et al*. 2006)
rT$_3$	High[a] (TRΔα1 (native TR isoform); Flamant & Samarut 2003)	None yet identified
T$_2$	Weak (human TRα; Cioffi *et al*. 2010)	None yet identified
	Weak (human TRβ; Ball *et al*. 1997, Cioffi *et al*. 2010, De Lange *et al*. 2011, Mendoza *et al*. 2013)	
	High (short TRβ of tilapia fish; Mendoza *et al*. 2013, Navarrete-Ramirez *et al*. 2014)	

[a]Nongenomic action.

efflux from mitochondria through activation of mt-NCX by interacting with different mitochondrial complexes (Del Viscovo *et al*. 2012).

T$_2$'s biological effects are not restricted to mammalian species. Indeed, in the goldfish *Carassius auratus*, stimulation of pyruvate-fueled liver and muscle mitochondrial respiration was observed 5 min after 0.3 nM T$_2$ incubation (Leary *et al*. 1996). García *et al*. (2004) examined the effects of short-term T$_4$, T$_3$, and T$_2$ exposure (0.1 μM; 12 or 24 h) on D1 and D2 activities and mRNA in killifish (tilapia) liver and showed that these hormones decreased D2 activity, the effect of T$_2$ being relatively more rapid. T$_2$ also regulates thermal acclimation in zebra fish (*Danio rerio*) with an efficiency comparable to T$_3$ (Little *et al*. 2013). The conserved role for T$_2$ on regulating metabolic efficiency in many species validates the motion to learn more about biological actions of this nonclassical TH.

Mechanism of action of T$_2$

Questions surrounding the cellular-molecular mechanism of action of T$_2$ remain. Both TR- and non TR-mediated actions may be elicited by T$_2$. Ball *et al*. (1997) reported that T$_2$ exerted selective thyromimetic effects. In the same report, T$_2$ showed a 60 times weaker affinity for TRβ than T$_3$. Mendoza *et al*. (2013) reported that in teleosts, effects of T$_2$ may be mediated by an isoform of one of the two known TRβs, namely TRβ1 that contains a 9-amino-acid insert in its ligand-binding domain (long TRβ1), whereas T$_3$ binds preferentially to a short TRβ1 isoform lacking this insert. Moreover, the authors confirmed that T$_2$ has a weak affinity for human TRβ (about 40-fold less than T$_3$) and a similarly weak transactivation capacity compared with T$_3$ (Ball *et al*. 1997, Cioffi *et al*. 2010, De Lange *et al*. 2011, Mendoza *et al*. 2013). In tilapia, both T$_3$ and T$_2$ are

important in growth, a process, however, mediated by different TRβ1 isoforms (Navarrete-Ramirez *et al*. 2014). An overview of TR or alternative receptor involvement in the action of nonclassical THs is shown in Table 1.

Non TR-mediated effects of T$_2$ are evident. It is known that T$_2$ specifically stimulates the activity of isolated cytochrome *c* oxidase (COX) from bovine heart mitochondria. T$_3$ barely stimulates COX. T$_2$ binding to COX induces conformational changes. Studies show specific binding of labeled T$_2$ to the subunit Va of COX from bovine heart. T$_2$, and to a small extent T$_3$, but not thyroxine and thyronine, abolished allosteric inhibition of ascorbate respiration of reconstituted COX by ATP. Inhibition is rescued by a monoclonal antibody to the subunit Va. (Goglia *et al*. 1994, Arnold *et al*. 1998). T$_2$ directly activates SIRT1 (De Lange *et al*. 2011), influencing downstream pathways and inducing benefits. Shang *et al*. (2013) showed that this interaction mitigates a DN by using the Sirtuin inhibitor Sirtinol.

Conclusion and perspectives

It is clear that the so-called 'nonclassical THs' can induce various biological actions. TH derivatives exert important actions on metabolic parameters and on growth. At the cellular–molecular level, several pathways are affected, the most intriguing of which are related to lipid metabolism and signaling pathways. Beneficial effects of these molecules require more considerations due to their potential to modulate human health.

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