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REVIEW

Thyroid hormone analogues and derivatives: Actions in fatty liver

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Abstract

Fatty liver or nonalcoholic fatty liver disease (NAFLD), a problem of increasing clinical significance and prevalence worldwide, is associated with increased risk for the development of cirrhosis and hepatocellular carcinoma. Although several therapeutic approaches can be used in the context of NAFLD, dietary and physical activities are still the most frequently used strategies. Some pharmacological agents show promising results although no conclusions can be drawn from recent clinical trials. Thyroid hormones [THs; thyroxine (T4) and 3,3′,5-triiodo-L-thyronine (T3)] coordinate a diverse array of physiological events during development and lipid/energy homeostasis and have some potentially therapeutic actions which include inducing weight loss, and lowering plasma cholesterol levels and tissue adiposity. The thyroid hormones exert their physiological effects by binding to specific nuclear receptors [thyroid hormone receptors (TR)] of which the TRβ isoform is liver specific and has been considered a putative target for the treatment of dyslipidemia and fatty liver. In view of this, the aim of the review is (1) to provide an overview of the action of T3 on lipid metabolism with implications for liver steatosis and (2) to provide an update on the current knowledge concerning the administration of TRβ selective thyromimetics (GC-1 and MB07811), as well as of 3,5-diiodo-L-thyronine and its novel functional analogue TRC150094 in animal models of overweight and related disorders including primarily fatty liver.

Key words: Fatty liver; Thyroid hormones; Thyromimetics; 3,5-diiodo-L-thyronine; Lipid metabolism

Core tip: Fatty liver is associated with increased risk for the development of cirrhosis and hepatocellular carcinoma. Thyroid hormones have some potentially therapeutic actions by binding to specific nuclear receptors [thyroid hormone receptors (TR)] of which the TRβ isoform is liver specific and a putative target for the treatment of dyslipidemia and fatty liver. This review provides (1) an overview of the action of T3 on lipid metabolism and (2) an update concerning the administration of TRβ selective thyromimetics (GC-1 and MB07811), as well as of 3,5-diiodo-L-thyronine and its novel functional analogue TRC150094 in animal models of overweight and fatty liver.

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INTRODUCTION

The liver plays essential roles in supporting many meta-

bolic processes and is critically involved in facilitating the maintenance of blood-glucose levels and energy homeostasis. Diet-induced obesity - commonly associated with diseases such as type 2 diabetes (T2DM), hypertension, heart failure, or cancer - also leads to fatty liver or steatosis, a histopathological condition characterized by an excess accumulation within hepatocytes of lipids, which are primarily triglycerides (TGs)[1]. Although the primary metabolic abnormalities leading to lipid accumulation within hepatocytes are still not fully understood, a decreased capacity to oxidize fatty acids, an increased delivery and transport of free fatty acids (FFAs) into the liver as well as an augmented hepatic fatty acid synthesis are likely to play significant roles in the pathogenesis of hepatic steatosis[2-4]. Moreover, steatosis is clearly, inextricably linked to modifications of mitochondrial functions[5,6]. Indeed, the mitochondrion plays an important role in the hepatocyte's metabolism because it is the primary site of fatty acid oxidation and oxidative phosphorylation. Multiple enzymes are involved in mitochondrial β-oxidation, and even partial deficiencies of these enzymes may lead to the development of hepatic steatosis[7,8] (Figure 1A). Two broad categories of hepatic steatosis have been recognized: alcoholic fatty liver disease (AFLD) and nonalcoholic fatty liver disease (NAFLD). In particular, NAFLD, commonly associated with insulin resistance (IR) and cardiovascular diseases[9], comprises a morphological spectrum of liver lesions ranging from simple triglyceride accumulation in hepatocytes (hepatic steatosis) to inflammatory and hepatocellular ballooning injury (non-alcoholic steatohepatitis; NASH), which eventually leads to fibrosis and cirrhosis[1]. The exact mechanism underlying the transition from steatosis to steatohepatitis is still unknown. According to the "two-hit" hypothesis[10]: the first hit involves the accumulation of TGs in hepatocytes that causes a vicious cycle of metabolic dysfunction; once the presence of hepatic steatosis is established, progression to steatohepatitis involves a "second hit" with oxidative stress playing a key role. Fatty liver is more susceptible to oxidative injury[1] and lipid peroxidation[11], and the chemical modification of biological molecules may be directly toxic to the cells or may stimulate host-immune response that leads to inflammation, collagen production and further disease progression[12-14].

Therapeutic interventions in NAFLD are mainly based on lifestyle changes, including diet and exercise[15,16]. Currently, there are no approved pharmacological therapies for NAFLD, but because IR is almost universally present in patients with this condition, drugs that increase insulin sensitivity are currently undergoing extensive evaluation and hold promise as therapeutically effective agents[17,18]. Several other agents, such as antioxidants and hepatoprotective compounds, have been evaluated, and the data was inconclusive or demonstrated no effects[16].

Thyroid hormones [THs; thyroxine (T4) and 3,3',5-triiodo-L-thyronine (T3)] exert a multiplicity of effects and are potent regulators of glucose and lipid metabolism and body weight. In particular, they play an important role in hepatic lipid homeostasis. They exert their physiological effects by binding to specific nuclear receptors, the thyroid hormone receptors (TR) α and β that are widely distributed throughout the body. The β isoform is the major TR expressed in the liver. The beneficial effects of TRβ activation include lowering low-density lipoprotein (LDL) cholesterol, reducing whole body adiposity and weight[19], and increasing the metabolic rate in the liver which could potentially lead to reduced lipid content. However, to date, there is a lack of data available on the specific effects elicited by T3 on liver steatosis. In a recent study, T3 was shown to exert a strong inhibitory effect on the development of steatosis and to cause a rapid regression of fully established steatosis[20].

An excess of thyroid hormone is associated with unwanted effects particularly on the heart (including tachycardia and sudden death) and also on bone and skeletal muscle[21]. Because of these adverse effects of THs, several new TH analogs (generically termed as thyromimetics) have recently been developed to generate effective and safe treatments to counteract obesity and related disorders among which hyperlipidemia and liver steatosis. These either have selective effects on the liver *vs* the heart or bind selectively to TRβ rather than to TRα without cardiac side effects[22]. Such compounds could serve as powerful new tools to address some of the largest medical problems in developed countries-obesity and related disorders[23]. Interestingly, THs also exert non-genomic effects[24] and some are attributable to naturally occurring iodothyronines apart from T4 and T3[25,26]. These THs derivatives are currently being studied to elucidate their potential biological activities and application as anti-hyperlipidemic as well as anti-steatotic agents[22].

This review, including T3 action in the liver and fatty liver, will focus on the current understanding of the actions of thyroid hormone analogues and derivatives in fatty liver in view of the development of potential future therapeutic approaches for the prevention or counteraction of liver steatosis.

ACTIONS OF T3 ON LIPID METABOLISM IN THE LIVER: IMPLICATIONS FOR FATTY LIVER

The pleiotropic effects exerted by T3 includes the maintenance of lipid homeostasis *via* regulation of gene expression in target organs such as liver and adipose tissues. Most T3 effects are mediated by the canonical, or classic, pathway which requires the nuclear T3 receptors[27-29]. Actually, T3 can also signal through non canonical pathways by binding to cytoplasmic or mitochondrial TR isoforms[24]. In mammals, two distinct genes express the TRα and TRβ isoforms. The *TRβ* gene encodes three T3-binding TRβ isoforms (β1, β2, and β3) that share high sequence homology in the DNA and T3-binding domains but differ in length and amino acid sequences in the amino-terminal A/B domain. The *TRα* gene en-

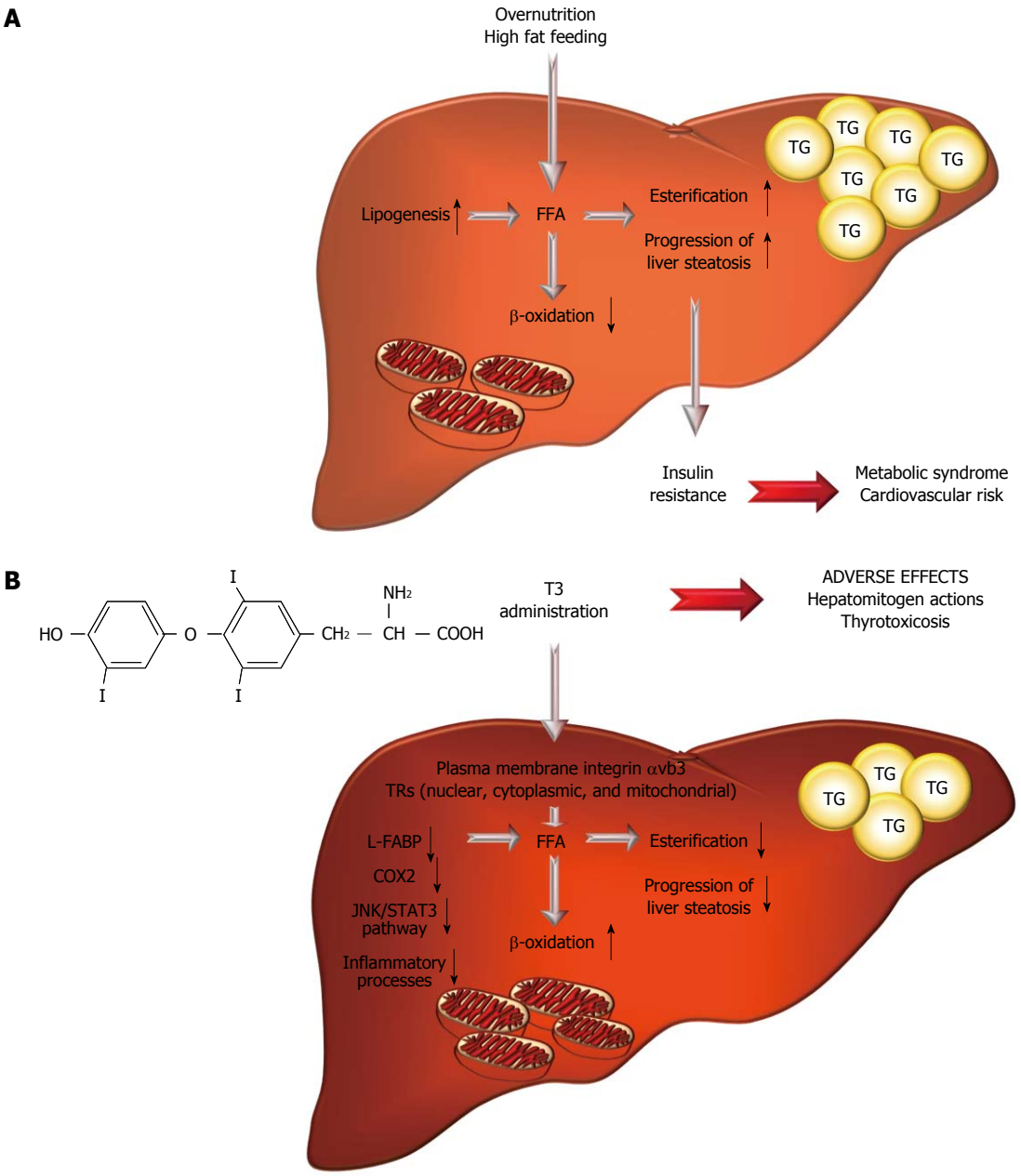

Figure 1 Hepatic lipid partitioning and liver and systemic metabolic damages in nonalcoholic fatty liver disease (A) and a schematic representation of the anti-steatotic effect of 3,3',5-triiodo-L-thyronine (B). A: Hepatic lipid partitioning and liver and systemic metabolic damages in nonalcoholic fatty liver disease. Chronic overnutrition/hyperlipidemic feeding causes fat retention in hepatocytes that, in turn, results in alteration of fat uptake, de novo synthesis (lipogenesis) and oxidation with a significant imbalance of lipid homeostasis. This can subsequently induce insulin-resistance, metabolic syndrome and cardiovascular diseases; B: A schematic representation of the anti-steatotic effect of T3: An update. T3-administration associated adverse effects are also highlighted (for details see the text). T3: 3,3',5-triiodo-L-thyronine; TRs: Thyroid hormone receptor isoforms; FFA: Free fatty acid; TG: Triglyceride; L-FABP: Liver-type fatty acid-binding protein; COX2: Cyclo-oxygenase 2; JNK: c-Jun N-terminal kinases; STAT3: Signal transducer and activator of transcription 3.

codes one T3-high affinity binding TRα1 and two splice variants (TRα2 and TRα3) which differ from TRα1 in length and amino acid sequences in the C-terminal region starting at amino acid 370, and they have no T3-binding activity[30]. While TRα1 is preferentially expressed in the heart, TRβ1 is the major isoform in the liver, kidney and thyroid. However, TRβ2 is predominantly expressed in the brain, adipose tissue and anterior pituitary gland. The liver is an important T3 target tissue[31]. T3 increases the expression of several genes involved in hepatic lipogenesis including fatty acid synthase (FAS), hepatic product spot 14 (which interacts physically and functionally with the TR to regulate malic enzyme gene expression[32]), acyl-CoA synthetase 5, fatty acid transporter protein, malic enzyme, glucose-6-P dehydrogenase (G6PDH)[33], sterol regulatory element binding protein-1c (SREBP-1c)[34]. T3 also induces genes involved in fatty acid oxidation, such as fatty acid transporter (FAT), fatty acid-binding protein (FABP), lipoprotein lipase (LPL)[33], and carnitine palmitoyltransferase-1alpha (CPT-1α), a key rate-limiting enzyme in mitochondrial fatty acid oxidation. In the liver, many of these genes (*e.g.*, malic enzyme, SREBP-1c, FAS

and CPT-1α) are directly regulated by T3/TR as the thyroid hormone response elements (TREs) have been reported in their promoters[34,35]. Importantly, T3 transcriptional activity also depends on several other factors including the type of TREs located on the promoters of target genes, the developmental- and tissue-dependent expression of TR isoforms, and a number of nuclear co-regulatory proteins. TRs bind to TREs not only as homodimers but also as heterodimers with other members of the receptor superfamily, such as retinoic X receptors (RXRs), vitamin D receptor, and all subtypes of the retinoic acid receptors. Heterodimerization with RXR dramatically increases the binding of TRs to TREs, the responsiveness of TR to T3, transcriptional activation[30] and, due to promiscuity of RXR in heterodimerization with many members of the receptor superfamily, allows TR to crosstalk with other receptors. Crosstalk with peroxisome proliferator-activated receptor (PPAR) signaling *via* heterodimerization with RXR by TR is a well-known example[30,36].

Moreover, in the liver, activation of the NAD^+-dependent deacetylase sirtuin 1 (SIRT1) facilitates fatty acid oxidation[37]. Indeed, in hepatocytes isolated from mice lacking SIRT1, fatty acid oxidation rates are reduced, and these mice accumulate lipids within the liver[38]. Recently, SIRT1 has been reported to interact directly with TRβ1, contributing to the T3-mediated stimulation of hepatic genes *via* the activation of several factors such as PPARα, estrogen-related receptor α (ERR-α), and peroxisome proliferator-activated receptor γ coactivator (PGC-1α)[39]. In the liver, PGC1-α promotes expression of genes involved in hepatic fatty acid oxidation[40] and drives the expression of genes involved in hepatic gluconeogenesis *via* interactions with HNF4α (hepatic nuclear factor-4) and FoxO1 (forkhead transcription factor)[41,42]. In particular, PGC-1α appears to be another key player in T3-signaling as it is able to coactivate the TRβ[43] and its overexpression enhances the induction T3-mediated of CPT-1α[44] and PDK4 (pyruvate dehydrogenase kinase, isozyme 4)[45,46]. Interestingly, T3 can also signal in a TRβ-independent manner by binding to surface receptor such as integrin αvb3 receptor, thus activating the MAPK/ERK and PI3K/Akt/mTOR-C1 pathways[24]. Recent studies have highlighted that such effects in hepatocytes have the potential to modulate lipogenesis and cholesterologenesis[47].

Normal serum THs levels are essential for the maintenance of a sufficient pool of cholesterol to meet the body's requirements and to regulate the critical steps of cholesterol synthesis, uptake and metabolism[19]. T3 signaling: (1) stimulates 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG CoA) reductase and farnesyl pyrophosphate (FPP), favoring cholesterol synthesis; (2) up-regulates the LDL receptor (LDLR; low density lipoprotein receptor), increasing cholesterol uptake; and (3) stimulates cholesterol 7α-hydroxylase (CYP7A1), enhancing the metabolism of cholesterol into bile acids[48-50].

Although the T3-mediated actions involved in the regulation of serum lipid homeostasis as well as in hepatic fat metabolism are quite well established, the T3-specific effects on NAFLD are still not fully elucidated. A study using a nutritional model of NAFLD [treating rats with high-fat choline-methionine deficient (CMD) diet] revealed that co-feeding T3 (4 mg/kg of diet) with a CMD diet exerts a strong inhibitory effect on the development of steatosis. Indeed, T3 prevents accumulation of TGs by inducing fatty acid oxidation with subsequent impairment of TGs hepatic synthesis/accumulation, and decreases the expression of liver-type fatty acid-binding protein (L-FABP), an abundant protein in the cytosol of hepatocytes that facilitates fatty acid transport and utilization[20]. Furthermore, the same study showed that T3 administration for only 1 wk, following 10 wk on a CMD diet, caused a rapid regression of fully established steatosis by: (1) dramatically reducing liver TGs levels and cyclooxygenase 2 (COX2) expression; (2) down-regulating pathways, such as JNK (c-Jun N-terminal kinase) and STAT3 (signal transducer and activator of transcription 3) pathways, usually activated in inflammatory processes; and (3) reducing the severity of liver injury as determined by serum levels of transaminases (AST and ALT; aspartate aminotransferase and alanine aminotransferase)[20] (Figure 1B).

Of note, T3 is also a potent mitogen that, in the liver, induces Cyclin-D1 expression[51]. Very recently, it has been shown that it exerts hepatocyte mitogenic response by PKA-dependent β-catenin activation, thus eliciting a potent liver regeneration action[52]. This has suggested that T3 can have therapeutic relevance in the treatment of selected cases of hepatic insufficiency.

However, long term treatment with T3, both in animals and humans, can produce several adverse effects including systemic thyrotoxicosis. Thus, extensive research is dedicated to the identification of new effective and safe active molecules (T3-derivatives and analogues) for the treatment of dyslipidemias, NAFLD, obesity and related disorders.

In particular, the development of synthetic thyroid hormone analogues which have tissue-selective hormone actions (*i.e.*, selective thyromimetics) has been pursued.

THYROMIMETICS AND LIVER STEATOSIS

As mentioned previously, many of the T3 actions are tissue-specific and are primarily mediated by a panel of TR isoforms that are expressed in different ratios in various tissues. Thus, there is a rationale to pursue approaches that selectively modulate TRs function, and several agents have been shown to have some β-selective, hepatic selective and/or cardiac sparring activities. The possibility of selectively targeting the TRβ was suggested by the findings that the TRα-forms may preferentially regulate the heart rate, whereas many other actions of T3 are mediated by the TRβ. X-ray crystal structures of the TRα and TRβ ligand-binding domains (LBDs) suggested that a

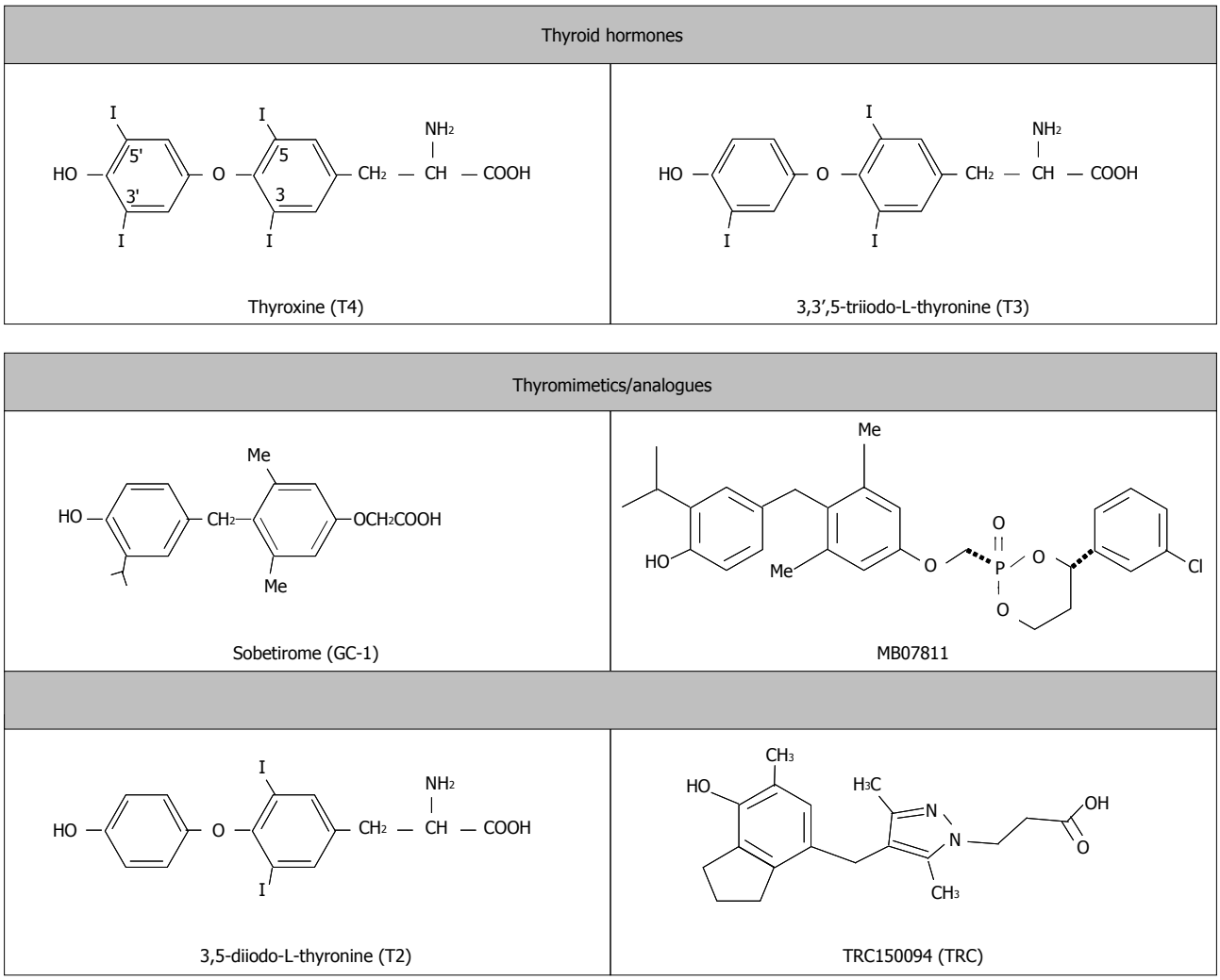

Figure 2 Chemical structure of thyroid hormones and thyromimetics/analogues with reported anti-steatotic effects.

single amino acid difference in the ligand-binding cavities of the two receptors could affect hydrogen bonding in the receptor region where the ligand's 1-position substituent fits and might be exploited to generate β-selective ligands[53].

The development of a TRβ-selective agonist has prompted a number of studies addressing whether such molecules could be used to trigger the metabolic effects of T3 while preserving the TRα-expressing tissues[54-58]. Essentially, these studies have been encouraging as it has been shown that the use of TRβ-selective agonists can prevent or improve metabolic parameters and/or complications resulting from high-fat feeding, NAFLD[20], or genetic hypercholesterolemia[59,60] with the liver being their major target. Indeed, tissue distribution analyses suggest that these molecules achieve TRβ selectivity by virtue of being concentrated predominantly in liver[55,61]. TRβ activation in the liver also favorably affects plasma cholesterol and lipoprotein levels by multiple mechanisms, which include increasing: (1) LDL clearance through increased expression of LDLR, (2) high-density lipoprotein (HDL) uptake through SR-B1 (scavenger receptor class B type 1); and (3) bile acid synthesis *via* CYP7A1[62].

To exploit the favorable consequences of hepatic TRβ activation, a variety of synthetic TRβ agonists have been prepared and tested on a variety of experimental models[63-73]. Ideally, these selective TRβ agonists, would cause modest increases in the metabolic rate without tachycardia[22]. However, it has been reported that most of thyromimetics could suppress thyroid axis and lower serum T4/T3 levels, especially at high doses.

Here, we will discuss two thyromimetics, namely GC-1[74] and MB07811[57,75,76] (Figure 2), for which these effects are less marked and which, at the same time, elicit anti-steatotic effects. In particular, MB07811, being specifically targeted to the liver, reduces serum T4 levels (-50% in Sprague-Dawley rats) probably by enhancing T4 to T3 conversion through deiodinase 1[53,57]. As far as it concerns GC-1, it has been shown a dose-dependent ability to reduce thyroid-stimulating hormone (TSH) levels, being this action 20-fold less potent than that of T3[55].

GC-1 (or sobetirome) is a halogen-free thyroid hormone agonist[74]. Although the structural changes it contains with respect to the natural hormone T3 (*i.e.*, replacement of the three iodines with methyl and isopro-

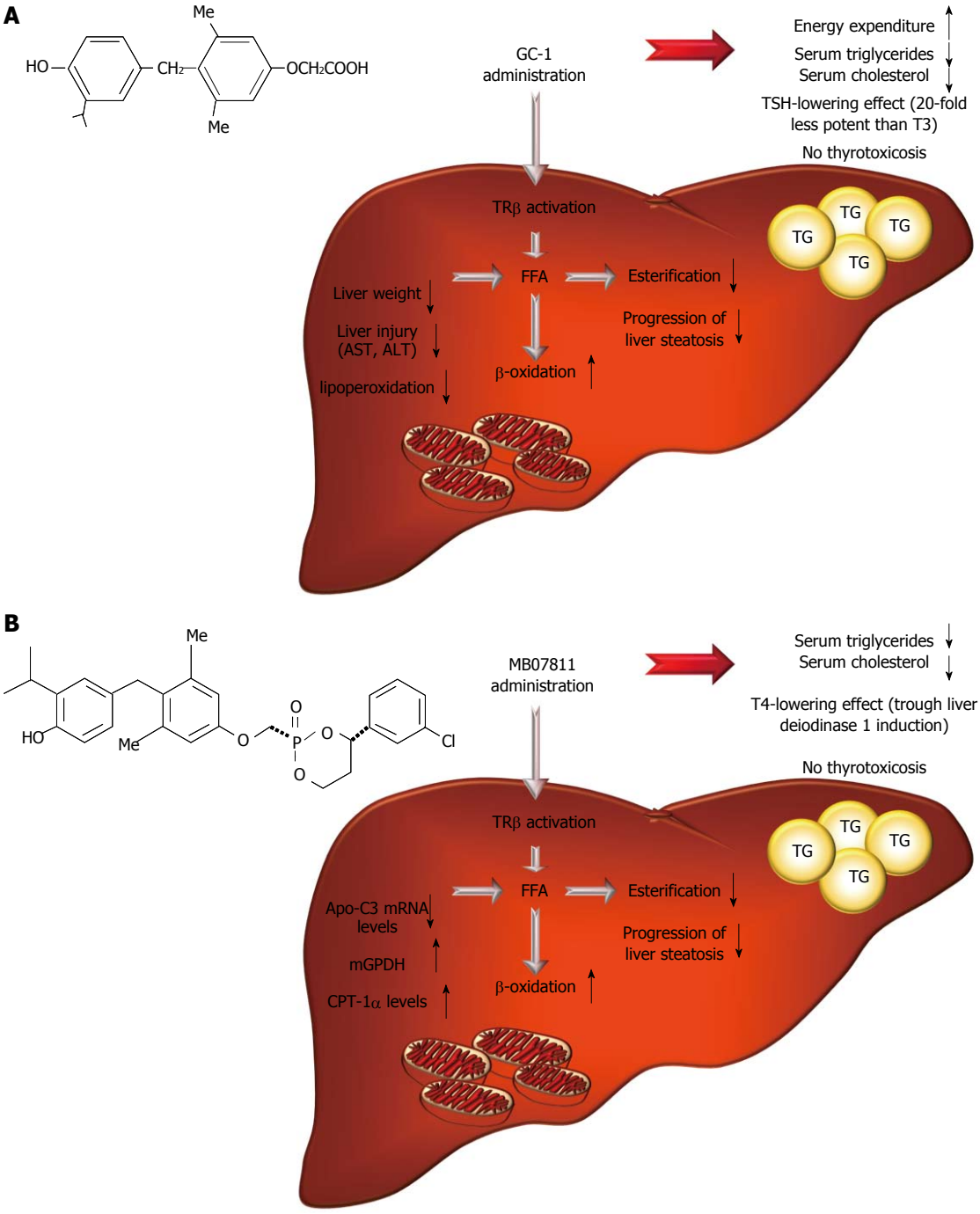

Figure 3 A summary of key events and molecular pathways underlying GC-1 (A) and MB07811 (B) anti-steatotic and hypolipidemic effects (for details see the text). TSH: Thyroid-stimulating hormone; T3: 3,3',5-triiodo-L-thyronine; TRβ: Thyroid hormone receptor β isoform; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; FFA: Free fatty acid; TG: Triglyceride; T4: Thyroxine; Apo-C3: Apolipoprotein C3; mGPDH: Mitochondrial glycerol-3-phosphate dehydrogenase; CPT-1α: Carnitine palmitoyltransferase-1α.

pyl groups, replacement of the biaryl-ether linkage with methylene linkage, and replacement of the amino acid side chain with an oxyacetic acid side chain)[77], it binds the TRβ with an affinity that is comparable to that of T3[22]. Functionally, when GC1 is administered to rats undergoing a CMD diet prevents and reverses the hepatic steatosis much like T3[20]. Moreover, similar to T3, GC-1 can reduce liver weight, liver weight/body weight ratio, and serum TGs levels. GC-1 also causes a reduction of CMD-induced TGs accumulation in the liver, with the disappearance of hepatic TGs being accompanied by a concomitant decrease of lipoperoxidation, and of liver injury as indicated by the significant reduction in AST and ALT levels[20]. These findings made GC-1 an ideal molecule for therapies against fatty liver disease. Interestingly, GC-1 also stimulates energy expenditure[78] and mitochondrial oxidative processes but to a lesser extent compared to T3[79,80] (Figure 3A). Notably, animal studies[55] revealed that treatment with GC-1 also induces a reduction of cholesterol levels similar to that obtained

with equimolar doses of T3 and even higher than that achieved with the most common drugs currently available on the market for the treatment of hypercholesterolemia, such as the inhibitors of HMG CoA reductase (statins)[77]. In doing so, GC-1 regulates key steps in the reverse cholesterol transport pathway[62], increasing the expression of HDL receptor SR-B1 in the liver, stimulating the activity of CYP7A1 and inducing the expression of hepatic ATP-binding cassette proteins G5/G8 (ABCG5/G8), which promote biliary cholesterol secretion. Consequently, treated animals displayed an increased turnover of plasma HDL cholesterol, and an increased amount of fecal excretion of bile acids and cholesterol[70]. Phase 1 clinical studies tested the therapeutic concept of lowering cholesterol and found GC-1 to be generally well tolerated at all doses studied[81,82].

In a recent study, GC-1 has also been shown to be capable of markedly reducing serum cholesterol in mice devoid of functional LDLRs by inducing CYP7A1 expression[60]. These results, having elucidated the possibility that a LDLR-independent mechanism could underlie GC-1 action, potentiated the idea that GC-1 may represent a promising cholesterol-lowering therapeutic with a specific application for the treatment of diseases such as homozygous familial hypercholesterolemia. Currently, there are only limited treatment options for this disorder because most therapeutics are only minimally effective.

Another agent being studied is MB07811 which exhibits increased TR activation in liver relative to other tissues[57]. By using several experimental approaches, MB07811 was shown to have anti-steatotic activity and was able to reduce hepatic triglyceride levels in both normal and metabolically-challenged animal models, including ob/ob mice, Zucker rats, and mice with diet-induced obesity (rodent models of NAFLD)[83]. The main mechanism underlying MB07811 effects appears to be an augmented metabolic rate in the liver and, specifically, an increased rate of mitochondrial β-oxidation. MB07811 increases: (1) the levels of CPT-1α[35] and short and intermediate length acyl-carnitine species in plasma and (2) the liver mitochondrial respiration rates as well as the activity of hepatic mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH), an enzyme which is important for energy production and dissipation. Decreased mRNA levels of apolipoprotein C3 (Apo-C3), an inhibitor of hepatic lipase activity, might also contribute to the activation of fatty acid oxidation pathways. Additionally, MB07811 can also lower both serum cholesterol and triglyceride levels[83,84] (Figure 3B). These data in rodents confirm that MB07811 represents a novel class of liver-targeted TR agonists with beneficial LDL-lowering properties, and suggest that these compounds may provide additional therapeutic benefit to hyperlipidemic patients with concomitant NAFLD[83]. The human Phase 1b clinical trial showed reduced LDL cholesterol and TGs levels in both normolipidemic and hyperlipidemic subjects without severe adverse events[23].

Together, these data demonstrate that selective activation of hepatic TR prevents or reverses fatty liver and reveals a new approach to treat NAFLD based on selectively burning hepatic fat.

Now, the question is whether other molecules such as naturally occurring TH metabolites, even deprived of TR selectivity or characterized by low binding affinity for TRs, can have uses as therapeutic applications.

NATURALLY OCCURRING IODOTHYRONINES AND LIVER STEATOSIS: 3,5-T2

An increasing amount of data indicates that there are at least four natural iodothyronines with significant, but not identical, biological activities, namely T4, T3, rT3 (reverse T3), and 3,5-diiodo-L-thyronine (T2). T2 is particularly intriguing because of its effects on metabolism[22,25,26]. T2 has been estimated, in euthyroid rats, to reach serum concentrations of approximately 5 pM and is present in liver at concentrations of approximately 1.0 fmol/100 mg[85]. In humans, serum T2 levels consistently elevated in disease states, with the mean T2 serum level being 16.2 ± 6.4 pM in healthy subjects, 21.6 ± 4.8 pM in patients with brain tumors and 46.7 ± 48.8 pM in patients with sepsis[86].

T2 measurements are routinely taken using methods based on immunoassays, an approach with high sensitivity but which lacks specificity for many analytes[87,88]. In recent years, mass spectrometry (MS) techniques have drawn attention to the analyses of T4 and T3 because they provide high mass accuracy, structural information, and have the ability to quantify the hormones[89-93]. A recently developed methodology revealed that electrospray ionization tandem mass spectrometry (ESI-MS/MS) can be used for identification and quantification of mixtures of isomers and has been applied to identify and quantify T3 and rT3 isomers as well as T2 isomers[94,95]. Currently, however, intrinsic instrumental limits restrain the application of such approaches as routine tools for biological samples analysis and slow the advancements in understanding T2 metabolism[96,97].

T2 is a product of a currently unknown peripheral enzymatic process most probably utilizing T3 as its precursor[85] and has 50-1000 times lower affinity for TR than T3[98]. Thus, it is unlikely that TR activation represents a central mechanism in its effects on metabolism, at least in physiological conditions. However, more recent studies have reported new data concerning T2 binding to TRβ isoforms in teleosts[99,100]. Specifically, T2 has been shown to bind and transactivate both the human and the long tilapia TRβ1 isoform whereas T3 preferentially binds the short isoform. These results prompted a reevaluation of the mechanisms of action of thyroid hormone metabolites.

Several studies on T2 effects in mammals revealed its ability to stimulate cellular/mitochondrial respiration by pathways with mitochondria and bioenergetic mecha-

nisms being the major targets[26,80,101-103]. Outside the mitochondria, T2 also has effects on carriers, ion-exchangers, and enzymes, and may affect the transcription of some genes, but again the underlying mechanisms appear to be different from those elicited by T3[26].

In 1998, Arnold *et al*[104] identified the Va subunit of the mitochondrial respiratory chain complex cytochrome-*c* oxidase (COX) as a specific binding site for T2 using photoaffinity labeling procedures. T2 binding to the COX complex abolishes the allosteric ATP inhibition of COX which leads to a decrease in the respiratory control ratio of the complex[105] thus rendering the oxidative phosphorylation more inefficient.

The biological and pharmacological importance of T2 has become a topic of considerable interest to researchers during the past few years, and now represents a significant and promising issue in the field of metabolism and THs.

The effects and mechanisms underlining the beneficial actions of T2 have so far been studied with both *in vivo* and *in vitro*[106-108] models. *In vivo* studies from different laboratories have shown that acute or chronic administration of T2 to rats results in significant changes in mitochondrial activities and resting metabolic rate[22,26,109].

A recent study reported an increased basal metabolic rate and decreased body weight also in humans chronically administrated with T2 with no deleterious side effects on the thyroid axis or at the cardiac level[110].

Of the currently described *in vivo* stimulatory and beneficial effects of T2, a particular physiological and pharmacological relevance appears to be associated with those effects that we can define as hypolipidemic and anti-steatotic effects which have been described in several animal models[111-114]. Among the methods to study liver metabolism and physiology, the variation in the nutritional status is a widely used approach because of its ability to affect several signaling pathways and regulatory mechanisms[115-118]. High fat feeding (HFD) in animals, in particular, has the advantage to mimic most features of human fat overload and overnutrition and allows the study of obesity and related disorders such as ectopic fat accumulation.

Specifically, the administration of T2 to rats subjected to HFD is able to prevent and reduce the visceral fat accumulation as well as hepatosteatosis, serum levels of triglycerides and cholesterol, and the onset of IR without inducing thyrotoxicosis[111,112,114,119]. Moreover, T2 has been reported to elicit additional beneficial effects on lipid metabolism by reducing LDL-cholesterol in a LDLR independent way in a mouse model of familial hypercholesterolemia[120].

The simultaneous administration of T2 (25 μg/100 g body weight) to rats receiving a HFD for 4 wk can prevent liver steatosis by stimulating hepatic fatty acid oxidation and increasing mitochondrial uncoupling[111]. This leads to a less efficient utilization of lipid substrates, and helps to prevent body-weight gain, hepatic fat accumulation, hypertriglyceridemia and hypercholesterolemia levels without inducing changes in T3 and T4 serum levels or affecting the hypothalamus-pituitary-thyroid (HPT) axis[111,119].

The T2 effects on liver fatty acid oxidation are paralleled by an increased entry of activated fatty acids into the mitochondria *via* activation of the CPT system. This leads to a strong reduction in the fatty acids inside the cell and a strong activation of AMP-activated protein kinase (AMPK), enhancing the cycle of fat uptake and fat burning and the disappearance of lipid droplets (LDs)[111].

Importantly, T2 administration prevents the HDF-induced lipid peroxidation, as well as the increase in H_2O_2 metabolism counteracting both lipid accumulation and oxidative stress associated with increased fat metabolism[121].

In a more recent study, to further investigate how T2 affects lipid and glucose metabolism in HFD rats eliciting beneficial effects on liver, independently of AMPK, the involvement of another important regulator of metabolic balance[122-127], SIRT1, was studied[114]. In HFD rats, T2 was demonstrated to (1) rapidly increase hepatic nuclear SIRT1 activity and (2) through SIRT1-activation, deacetylate PGC-1α and SREBP-1c with a concomitant upregulation of genes involved in mitochondrial biogenesis and downregulation of lipogenic genes[114]. Moreover, the obtained data added new information on the time-latency of the anti-steatotic effect of T2 which within 6 h after administration, rapidly and directly activates hepatic SIRT1 (affecting β-oxidation and mitochondrial biogenesis) and later (4 wk) promotes AMPK phosphorylation/activation thus profoundly modulating liver expression pattern of genes and proteins.

A proteomic study[113] showed that the steatotic effect of HFD, and the anti-steatotic effect of T2-treatment are strictly associated with altered expression levels of several proteins and enzymes involved in key liver metabolic (canonical and non-canonical) pathways across different subcellular compartments (*i.e.*, cytoplasm, mitochondria and nuclei). These pathways included: fatty acid metabolism, ketone-bodies and energy metabolism, amino acid and nitrogen metabolism, the urea cycle and the stress response and protein turnover.

All the analyzed liver subcellular compartments were significantly affected, in terms of protein expression, by both HFD and long-term T2-treatment. However, mitochondria appeared to be a major target for the metabolic and energy adaptations induced by fat-overload, and displayed a significant response, in terms of their proteome, to T2-treatment. These data supported the concept that T2-supplementation while having hypolipidemic and anti-steatosis effects, may provide protection against diet-induced liver damage, possibly by counteracting the alterations in the expression of several cellular proteins, reducing oxidative stress and impairing the mitochondrial respiratory chain[113].

T2 administration to rats is also able to reduce pre-existing hepatic fat accumulation (that had already been induced by feeding with a HFD)[112] by eliciting systemic

and tissue specific effects. In particular, T2, without suppressing TSH, decreases body weight gain, metabolic efficiency and serum levels of cholesterol, triglycerides and ALT. In the liver, T2 increases hepatic mitochondrial oxygen consumption and fatty acid oxidation and activates mitochondrial proton leak reducing mitochondrial oxidative stress[112].

In vitro studies have been performed to address whether the anti-steatotic effect of T2 is due to the direct action of T2 on the liver or if it is a secondary effect due to upstream changes in endocrine or metabolic pathways. Primary cultures of rat hepatocytes overloaded with lipids ("fatty hepatocytes") and then treated with T2 showed a reduction in: (1) lipid content and LD diameter; (2) PPARs expression; and (3) activities of acyl-CoA oxidase (AOX) and antioxidant enzymes. These data support a direct role of T2 in reducing the excess fat in cultured hepatocytes[128]. The putative involvement of TRs in mediating such lipid-lowering effects of T2 has been elucidated using the rat hepatoma FaO cellular model, which is defective for functional TRs[128]. The addition of T2 to lipid-overloaded cells resulted in: (1) reduction in lipid content; (2) downregulation of PPARα, PPARγ, and AOX expression; (3) increase in PPARδ expression; and (4) stimulation of mitochondrial uncoupling[128]. These data demonstrate, for the first time that the *in vitro* lipid-lowering actions of T2 may be not mediated by TRs.

All the utilized approaches, both in animal models and humans, successfully highlighted metabolic actions and potential pharmacological use of T2. Notably, T2, without thyrotoxic side effects, increasing resting metabolic rate, decreasing adiposity and body weight, could be considered an active agent in preparation for treatment of metabolic disorders, such as T2DM, overweight and NAFLD (Figure 4A).

THYROID HORMONE FUNCTIONAL ANALOGUES: TRC150094 (TRC)

T2 research has been recently focused on the discovery and development of T2 functional analogues with therapeutic potentials. In particular, a series of novel substituted pyrazoles were designed and synthesized as T2 analogs with lower affinities toward TRs. Among these molecules, TRC150094 (TRC) has attracted particular attention emerging as a novel thyromimetic and functional T2-analogue linking fat consumption with the pathogenesis of hepatic steatosis. The chemical name for TRC is 3-[4-(7-hydroxy-6-methyl-indan-4-ylmethyl)-3,5-dimethyl-pyrazol-1-yl]-propionic acid, and its chemical structure is shown in Figure 2. TRC was synthesized by the Torrent Research Centre, Torrent Pharmaceuticals Ltd., Ahmedabad, Gujarat, India and has a much lower potency toward both TRα1 and TRβ1 isoforms' activation than T3. Therefore, it is devoid of adverse effects on the heart classically associated with TR hyperactivation [the rank order of potencies for TR (α1/β1) transcriptional activation being T3 > T2 >>TRC[129]].

When screened for its *in vivo* metabolic effects, TRC (injected, at a dose of 0.750 mg/100 g body weight, in rats simultaneously undergoing a HFD for 4 wk) counteracts the hepatic pathological condition observed in HFD rats without any thyrotoxic effects[129]. The anti-steatotic activity of TRC is due to increased rates of mitochondrial fatty acid uptake and oxidation, respiratory chain activity and resting metabolic rate, and to the activation of the CPT system[129,130]. Importantly, TRC significantly increases SIRT1 activity although the SIRT1 protein level remains unaltered. These results strongly suggested that a TRC-induced increase in SIRT1 activity might underlie the increase in fatty acid oxidation and the prevention of liver steatosis observed in the TRC-treated rats[129].

An integrated functional study performed by combining *in vivo* and *ex vivo* metabolic assays with proteomic and bioinformatic analyses showed that TRC administration to rats with pre-existing body fat accumulation significantly altered the expression levels of several proteins and enzymes involved in key liver metabolic pathways, including amino acid, nitrogen, fructose and mannose metabolism, and RXR activation and function[130]. Consistent with the increased oxidation of mitochondrial fatty acids and the unaltered mitochondrial efficiency, numerous mitochondrial enzymes associated with fatty acid oxidation and energy metabolism were increased in livers from HFD-TRC[130].

Oral administration of TRC to obese Zucker rats (obese ZSF1, spontaneously hypertensive fatty rats) decreased hepatic steatosis[131] by inducing a significant increase in mitochondrial respiration as well as an increased fatty acids oxidation. All the above mentioned studies have so far provided the characterization of the pharmacological/metabolic effects of TRC and highlighted its potential utility as a new compound with effectiveness for prevention/amelioration of certain key biochemical parameters altered during feeding on a HFD-regimen and a cluster of multiple cardiovascular risk factors associated with visceral obesity, steatosis and metabolic derangements (Figure 4B). If reproduced in humans, these results could determine whether TRC150094 represents an attractive therapeutic agent for the treatment of overweight dysglycemic patients.

Indeed, clinical trials are currently in progress to translate these effects into approaches for the treatment of human obesity.

THYROID HORMONES AND MITOCHONDRIAL FUNCTIONS

As already stated, mitochondria play a fundamental role in the development, perpetuation and worsening of liver steatosis and NAFLD. Indeed, the generation of NAFLD, apart from involving defects or polymorphisms in mitochondrial DNA, can be an important consequence of damages to the respiratory chain complexes impairing mitochondrial oxidative capacity, particularly critical when fatty acid supply to the hepatocytes is increased as

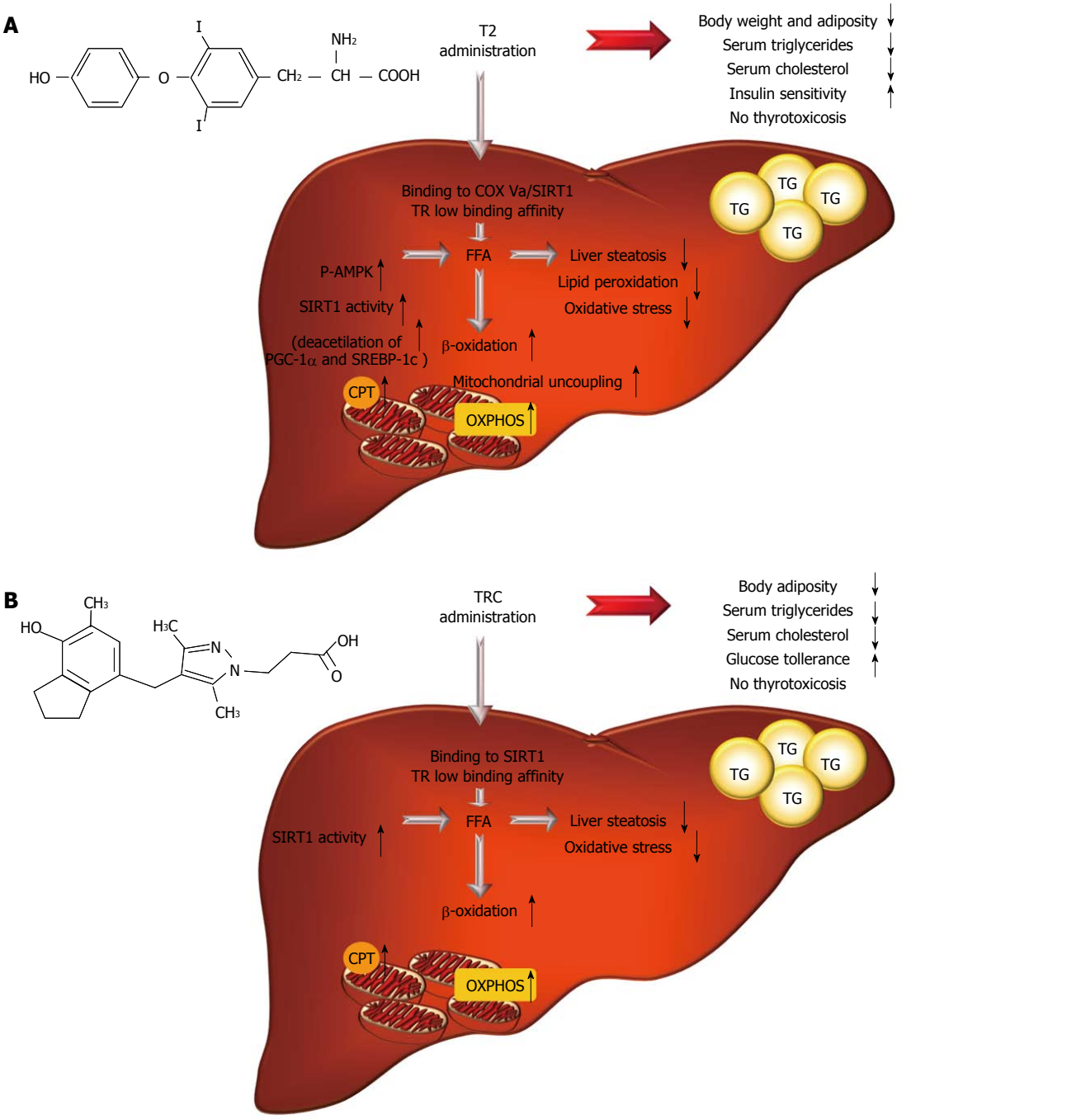

Figure 4 A summary of key events and molecular pathways underlying T2 (A) and TRC (B) anti-steatotic and hypolipidemic effects (for details see the text). T2: 3,5-diiodo-L-thyronine; TR: Thyroid hormone receptor; COX Va: Cytochrome-*c* oxidase Va subunit; FFA: Free fatty acid; TG: Triglyceride; P-AMPK: Phosphorylated AMP-activated protein kinase; SIRT1: NAD^+-dependent deacetylase sirtuin 1; PGC-1α: Peroxisome proliferator-activated receptor γ coactivator; SREBP-1c: Sterol response element binding protein-1c; CPT: Carnitine palmitoyltransferase system; OXPHOS: Oxidative phosphorylation system.

in calorie-rich diets[132].

Mitochondrial bioenergetics deficits may be the consequence of: (1) a progressive decay of oxidative capacity with impairments of β-oxidation; and (2) major changes in the redox balance with increased reactive oxygen species production.

It is widely recognized that mitochondria are central targets for THs actions. Indeed, mitochondria, providing about the 90% of the cellular energy supply, likely may be a major player of the so called calorigenic effects of THs[133]. In particular, T3 stimulates mitochondriogenesis and thereby augments cellular oxidative capacity and induces, at the same time, substantial modifications in mitochondrial inner membrane protein and lipid compositions, activating uncoupling of oxidative phosphorylation[133].

In terms of time latency, two types of effects of T3 on mitochondria have been described: (1) a rapid stimulation of respiration, which is evident within minutes/hours after hormone treatment; (2) a delayed induction

of mitochondrial biogenesis and changes in mitochondrial mass, which occur one to several days after hormone treatment. The first effect is probably due to extranuclear/non-genomic mechanisms; the second one involves both T3-responsive nuclear genes and a direct action of T3 at mitochondrial level[109,133]. In other words, this second effect allows T3 to modulate mitochondria activity in two different ways: direct or indirect. The direct action requires the presence inside the organelles of specific binding sites for the hormone[134-136] while the indirect one possibly requires T3 binding to extramitochondrial sites and the modulation of the expression of either nuclear-encoded mitochondrial proteins or intermediate factors (*e.g.*, nuclear respiratory factors 1 and 2; mitochondrial transcription factor A)[137,138].

T3, by the regulation of these pathways, allows the coordinated expression of both the nuclear and the mitochondrial genome that in turn modulates mitochondrial biogenesis, turnover and bioenergetics.

Although the network of factors and cellular events involved in T3 signaling remains incompletely understood, the so far described mechanisms can justify, at least in part, the above reported hypolipidemic and antisteatotic effects elicited by T3 in NAFLD.

CONCLUSION

Considering the impact of THs on the maintenance of lipid homeostasis but also of their adverse effects in TH excess states, recent efforts to identify effective and safe treatments for the counteraction of metabolic disorders (such as liver steatosis), has led to the development and characterization of thyromimetics. Some of these analogs are undergoing further examinations for possible clinical applications. However, despite their original promise, it is unlikely that any first generation synthetic ligands (*i.e.*, GC-1 and MB07811) which already reached human clinical trials will develop into therapeutics. Thus, attention should be focused on other molecules, such as T2 or TRC which could have therapeutic applications. Indeed, such compounds could serve as powerful new tools to address some of the largest over-nutrition associated medical problems as they are able to reduce, at least in animal models of diet-induced obesity, body adiposity, serum triglycerides and cholesterol. They also preserve glucose homeostasis without thyrotoxic side effects. Notably, the hypolipidemic effect of T2 is associated with a potent ability in both preventing and reducing fatty liver. Increasing evidence supports TH derivatives and analogues as attractive active agents that could be taken into consideration for the establishment of new treatments in the counteraction of metabolic disorders, such as T2DM, obesity and NAFLD, thus clinical trials are desirable.

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