TRC150094, a novel functional analog of iodothyronines, reduces adiposity by increasing energy expenditure and fatty acid oxidation in rats receiving a high-fat diet

Federica Cioffi,* Shitalkumar P. Zambad,† Laxmikant Chhipa,‡ Rosalba Senese,* Rosa Anna Busiello,§ Davinder Tuli,‡ Siralee Munshi,¶ Maria Moreno,§ Assunta Lombardi,* Ramesh C. Gupta,* Vijay Chauthaiwale,* Chaitanya Dutt,** Pieter de Lange,* Elena Silvestri,§ Antonia Lanni,* and Fernando Goglia³†

*Dipartimento di Scienze della Vita, Seconda Università di Napoli, Caserta, Italy; †Pharmacology Department; ‡Medicinal Drug Discovery, ³Cellular and Molecular Biology, ⁴Discovery Research, and **Clinical Research, Torrent Research Centre, Torrent Pharmaceuticals Ltd., Ahmedabad, Gujrat, India; §Dipartimento di Biologia, Università degli Studi di Napoli Federico II, Naples, Italy; and ¶Dipartimento di Scienze Biologiche ed Ambientali, Università del Sannio, Benevento, Italy

ABSTRACT Chronic overnutrition and modern lifestyles are causing a worldwide epidemic of obesity and associated comorbidities, which is creating a demand to identify underlying biological mechanisms and to devise effective treatments. In rats receiving a high-fat diet (HFD), we analyzed the effects of a 4-wk administration of a novel functional analog of iodothyronines, TRC150094 (TRC). HFD-TRC rats exhibited increased energy expenditure (+24% vs. HFD rats; P<0.05) and body weight (BW) gain comparable to that of standard chow-fed (N) rats [N, HFD, and HFD-TRC rats, body weight (BW) gain comparable to that of standard chow-fed (N) rats [N, HFD, and HFD-TRC rats, body weight (BW) gain comparable to that of standard chow-fed (N) rats]. These effects were independent of the AMP-activated protein kinase-acetyl CoA-carboxylase-malonyl CoA pathway but involved sirtuin 1 activation. In skeletal muscle, TRC induced a fiber shift toward the oxidative type in tibialis anterior muscle, increasing its capacity to oxidize fatty acids. HFD-TRC rats had lower (vs. HFD rats) plasma cholesterol and triglyceride concentrations. If reproduced in humans, these results will open interesting possibilities regarding the counteraction of metabolic dysfunction associated with ectopic/visceral fat accumulation.—Cioffi, F., Zambad, S. P., Chhipa, L., Senese, R., Busiello, R. A., Tuli, D., Munshi, S., Moreno, M., Lombardi, A., Gupta, R. C., Chauthaiwale, V., Dutt, C., de Lange, P., Silvestri, E., Lanni, A., Goglia, F. TRC150094, a novel functional analog of iodothyronines, reduces adiposity by increasing energy expenditure and fatty acid oxidation in rats receiving a high-fat diet. FASEB J. 24, 000–000 (2010). www.fasebj.org

Key Words: thyroid hormones • hepatic steatosis • mitochondria

Metabolic syndrome is defined as the presence of ≥5 of the following cardiovascular risk factors: increase in triglyceride levels, decrease in HDL cholesterol, moderate fasting hyperglycemia, hypertension, and increase in waist circumference. Individuals with central obesity have a higher prevalence of metabolic syndrome. Because of its major role in the development of type 2 diabetes, atherogenic dyslipidemia, cardiovascular disease, some cancers, and nonalcoholic fatty liver disease, visceral obesity has emerged as a leading cause of morbidity and mortality worldwide (1–5). Chronic overnutrition and/or an imbalance between energy expenditure and energy intake favor excessive body fat accumulation. Although we now have considerable knowledge concerning the pathways that lead to a state of energy imbalance favoring adiposity, much remains to be elucidated. Although a strategy involving a prolonged and marked reduction in caloric intake and increasing physical activity is the mainstay of the current treatment of metabolic disorders, these improvements are rarely achieved simultaneously in real life. As a consequence, the aim of much translational research is the development of new pharmacological strategies for the reduction of cardiovascular risks associated with excessive fat accumulation. One physiological/pharmacological approach to its treatment is to try to achieve a negative energy and fat balance. In particular, the peripheral stimulation of energy expenditure is an area

¹ Correspondence: Dipartimento di Scienze Biologiche ed Ambientali, Università del Sannio, Via Port’Arsa 11, 82100, Benevento, Italy. E-mail: goglia@unisannio.it doi: 10.1096/fj.10-157115
of particular scientific and therapeutic importance. Thyroid hormones (THs) are well known to stimulate cellular and mitochondrial metabolic activities while simultaneously lowering energy efficiency by influencing several aspects of energy metabolism, such as substrate cycling, ion cycling, and mitochondrial proton leaks. These effects have long been the focus of research into the potential use of THs as drugs for the stimulation of weight loss. However, the concomitant induction of a thyrotoxic state and of several adverse effects (i.e., increase in heart rate, increases in thyroid mass and heart mass, and decrease in skeletal muscle mass) has greatly limited their use as body fat- or weight-lowering agents (6, 7). The development of TH agonists/analogs that retain adequate lipid-lowering and antiobesity efficacy while being devoid of thyrotoxic effects would represent a potentially valuable therapeutic advance (7–9). Indeed, such agents should allow reductions in several important risk factors for morbidity and mortality, and unlike most previous therapeutic antiobesity strategies, which have been oriented toward appetite suppression, they should not require central nervous system involvement.

Evidence published in past few years has indicated that 3,5-diiodothyronine (T2), a naturally occurring iodothyronine, is able to mimic some of the effects of triiodothyronine (T3) on energy metabolism. T2 has affinities toward TH receptors (TRα and TRβ) that are significantly lower than those of T3 (10). In addition, it has been shown that the mechanism of the metabolic effect of this biologically active iodothyronine does not involve the nuclear pathway (i.e., an interaction with the nuclear receptor for T3; refs. 11, 12). These biological differences between T2 and T3 imply that, as a therapeutic agent, T2 is likely to be effective and safer than T3.

Thus, discovery and development of therapeutic agents analogous to T2 (that is, metabolically active thyromimetics with lower affinities toward TRs) might represent a relatively safe approach for the treatment of visceral obesity and the associated comorbidities that collectively are labeled as “metabolic syndrome.” Therefore, based on the available structural information for T2, T3, and other iodothyronines, a series of novel substituted pyrazoles were designed and synthesized as T3 analogs. In this study, we focused on the metabolic properties of one of these analogs, TRC150094 (hereafter termed TRC) to gain insight into its putative identification as a novel thyromimetic functional analog. TRC was screened both for TRα1 and TRβ1 transactivation assays and for in vivo metabolic effects in rats fed a high-fat diet (HFD).

MATERIALS AND METHODS

Chemical synthesis of TRC

The chemical name of 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 Fig. 1. TRC is synthesized by the reduction of 7-methoxy-6-methyl-indan-4-carboxaldehyde using sodium borohydride in methanol, followed by treatment of the resulting benzyl alcohol with thionyl chloride to give 4-chloromethyl-7-methoxy-6-methylindane. The reaction of this chloro derivative with acetylacetone in dimethylformamide in the presence of potassium carbonate yields a diketo derivative, and further reaction with hydrazine hydrate yields 4-(7-methoxy-6-methyl-indan-4-ylmethyl)-3,5-dimethyl-1H-pyrazole. Reacting this pyrazole compound with ethyl chloropropionate in dimethylformamide in the presence of base yields the corresponding propionate derivative. Then, alkaline ester hydrolysis of this propionate derivative, followed by demethylation using boron tribromide, yields TRC.

Animals and animal care

Male Wistar rats (aged 8 wk) were purchased from Harlan Laboratories S.r.l. Italy (Udine, Italy). They were housed in individual cages in a temperature-controlled room at 28°C (thermoneutrality temperature for rats) under a 12-h light/dark cycle. Before commencement of the study, a commercial mash (Charles River Laboratories, Calco, Italy) was available ad libitum, and the animals had free access to water. At the start of the study (d 0), after 7 d of acclimatization, the rats were divided into 3 groups (each containing 8 animals). The first group (N group) received a standard diet (total metabolizable percentage of energy: 60.4% carbohydrates, 29% proteins, and 10.6% fat J/J; 15.88 kJ gross energy/g); the second group (HFD group) received an HFD (total metabolizable percentage of energy: 21% carbohydrates, 29% proteins, and 50 fat J/J; 19.85 kJ gross energy/g); the third group (HFD-TRC group) received the same HFD together with a daily intraperitoneal injection of TRC (0.750 mg/100 g body weight). N and HFD rats received a daily intraperitoneal injection of vehicle. All animals continued to have free access to water. Body weight and food intake were monitored daily throughout the entire experimental period, which lasted 4 wk. At the end of the treatment, the rats were anesthetized by intraperitoneal injection of chloral hydrate (40 mg/100 g body weight) and then were killed by decapitation. Liver, hind limb skeletal muscles (gastrocnemius and tibialis anterior), heart, and visceral white adipose tissue (either epidymal or parametrial, perirenal, mesenteric, and inguinal deposits) were removed. Tissues to be used for the preparation of mitochondria were weighed and processed immediately; other samples were immediately frozen in liquid nitrogen and stored at −80°C for further processing. All procedures were approved by the local and national institutional animal care and use committees.

Respiratory parameters of animals

Oxygen consumption (Vo2), carbon dioxide production (CO2), and respiratory quotient (RQ: VCO2/Vo2) measurements were made using a 4-chamber, indirect open-circuit
calorimeter Oxymax system (Columbus Instruments, Columbus, OH, USA), with 1 rat/chamber. This was done both at the beginning (d 0) and at the end of the treatment period. After a 1-h period of adaptation to the metabolic chamber, V0₂ and VCO₂ were measured in individual rats at 15-min intervals for 4–6 h. Then, average oxygen consumption and average CO₂ output during this period were calculated and, where appropriate, are expressed as the percent difference vs. the N and/or HFD group.

**Serum measurements**

The serum levels of cholesterol and triglycerides were determined by following standard procedures (Siemens-Bayer, Milan, Italy). Free T₃ (FT₃), free thyroxine (FT₄), and thyroid-stimulating hormone (TSH) were determined by means of radioimmunoassays. TSH was measured using materials and protocols supplied by Amersham Biosciences Corp. (Piscataway, NJ, USA), with rat TSH (rTSH) as standard [Biotrak rTSH 125I assay system]. FT₃ and FT₄ levels were determined using materials and protocols supplied by Byk-Sangtec Diagnostica (Dietzenbach, Germany).

Heart rate was calculated at the beginning and at the end of the treatment period from the electrocardiogram obtained by means of a paper electrocardiogram recorder (Cardiotest 81; Hellige, Freiburg, Germany).

**Isolation of mitochondria from liver and skeletal muscle**

Fresh livers or hindlimb skeletal muscles (tibialis anterior, soleus, and gastrocnemius muscles, treated either separately or all together) were minced in ice-cold buffer consisting of 220 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, 1 mM EDTA, and 5 mM EGTA, pH 7.4, and then were homogenized in a Potter-Elvehjem homogenizer. Nuclei and cell debris were removed by centrifugation at 500 g for 10 min, with the resulting supernatant being centrifuged at 3000 and 8000 g for muscle and liver, respectively (13, 14). The mitochondrial pellet was washed twice and resuspended in a minimal volume of isolation medium and then kept on ice.

**Measurements of fatty acid oxidation rate and total carnitine palmitoyl transferase (CPT) activity**

The rate of mitochondrial fatty acid oxidation was assessed polarographically using a Clark-type electrode at 30°C in a final volume of 0.5 ml of 80 mM KCl, 50 mM HEPES (pH 7.0), 1 mM EGTA, 5 mM K₂HPO₄, 1% BSA (w/v), and 2.5 mM malate in the presence of ADP (120 µg/ml). The reaction was started by the addition of palmitoyl-CoA (40 µM) + carnitine (1 mM). Total CPT (CPTI plus CPT2) activity was measured spectrophotometrically by following (at 412 nm) the kinetics of carnitine-dependent CoA production in the presence of 5,5′-dithio-bis(2-nitrobenzoic acid), using palmitoyl-CoA as substrate, as reported by Alexson and Nedergaard (15). A Σ412 value of 13.6 mM⁻¹·cm⁻¹ was applied for the calculation of total CPT activity.

**Measurement of liver triglyceride content**

Lipid was extracted from frozen tissues in chloroform-methanol by the methods of Folch et al. (16). The triglyceride concentration was determined using an Infinity triglyceride kit (Sigma-Aldrich Corp., Milan, Italy).

**Determination of mitochondrial proton leak kinetics**

To detect the kinetic response of the proton leak to a change in membrane potential, 1 mg of liver mitochondria or 0.5 mg of skeletal muscle mitochondria was incubated in 1 ml of respiratory medium, consisting of 80 mM KCl, 50 mM HEPES (pH 7), 1 mM EGTA, 5 mM K₂HPO₄, and 5 mM MgCl₂, maintained at 37°C and supplemented with 1 µg/ml oligomycin (added to block protons from reentering the mitochondrial matrix via ATP synthase), 4 µM rotenone (to prevent oxidation of any endogenous NAD-linked substrates), and 80 ng/ml nigericin (to abolish the pH gradient). In addition, BSA was omitted from the respiratory medium. With the use of succinate as substrate (5 mM), the respiration rate and membrane potential were measured simultaneously using a Clark-type oxygen electrode and a triphenylmethylylphosphonium (TPMP⁺)-sensitive electrode, respectively, as described by Brown and Brand (17). The membrane potential was varied by titration with submaximal doses of malonate within the range of 0 to 2 mM. A TPMP⁺ binding correction factor of 0.4 was used.

**Determination of NAD⁺-dependent histone deacetylase sirtuin (SIRT1) activity**

Livers were dissected and minced in ice-cold isolation buffer (consisting of 220 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, 1 mM EDTA, and 5 mM EGTA, pH 7.4) and then were homogenized in a Potter-Elvehjem homogenizer. To isolate nuclei, the liver homogenate was centrifuged at 500 g for 10 min. The pellet so obtained was subsequently resuspended and spun through a 4-ml sucrose cushion (30% sucrose, 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, and 3 mM MgCl₂) at 1300 g for 10 min at 4°C. The nuclear pellet was washed with cold 10 mM Tris HCl, pH 7.5, and 10 mM NaCl. The isolated nuclei were suspended in 50–100 µl of extraction buffer (50 mM HEPES KOH, pH 7.5, 420 mM NaCl, 0.5 mM EDTA-Na₂, 0.1 mM EGTA, and 10% glycerol). The suspension was sonicated for 30 s, left on ice for 30 min, and centrifuged at 15,000 g for 10 min, after which the supernatant (crude nuclear extract) was immediately processed for immunoprecipitation studies and detection of SIRT1 activity.

SIRT1 protein was immunoprecipitated in its native form from the nuclear extracts using a polyclonal antibody against SIRT1 obtained from Cylcex Co. (Nagano, Japan) and a Catch and Release v2.0 kit from Millipore (Bedford, MA, USA). SIRT1 activity was determined using a histone deacetylase assay kit (Abnova, Taipei, Taiwan) according to the manufacturer’s instructions. SIRT1 activity was determined as the NAD-dependent and nicotinamide-inhibitable abilities of the native isolated SIRT1 protein to deacetylate a fluorometric substrate. Released substrate was detected using a fluorescence plate reader (BioTek Synergy HT: excitation, 340 nm; emission, 440 nm). All measurements (of average substrate conversion velocity) were recorded every 2 min over a period of 1 h within the linear response range of the assay (using 0.02 µg of purified SIRT1 as a control).

**Preparation of total lysates and Western immunoblot analysis**

For Western blotting analysis, gastrocnemius muscle, tibialis anterior muscle, or liver was homogenized in lysis buffer containing 20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA, pH 7.4 and 5 mM EDTA-Na₂, 0.1 mM EGTA, and 10% glycerol. The suspension was sonicated for 30 s, left on ice for 30 min, and centrifuged at 15,000 g for 10 min, after which the supernatant (crude nuclear extract) was immediately processed for immunoprecipitation studies and detection of SIRT1 activity.

For Western blotting analysis, gastrocnemius muscle, tibialis anterior muscle, or liver was homogenized in lysis buffer containing 20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 2.5 mM Na₂HPO₄; 1 mM β-CH₃H₂O₂PNa₂; 1 mM Na₂VO₃; 1 mM PMSF; 1 mg/ml leupeptin; and 1% Triton X-100 (all from Sigma-Aldrich Corp., St. Louis, MO, USA) using an Ultra-Turrax and then were centrifuged at 13,400 g
for 10 min at 4°C (Beckman Optima TLX; Beckman Coulter S.p.A., Milan, Italy). For determination of muscle fiber content, supernatants were used without further processing. For the determination of other cytosolic proteins, supernatants were ultracentrifuged at 86,000 g for 10 min at 4°C (Beckman Optima TLX). Myosin heavy chain (MHC) Ib protein levels were determined in crude muscle lysates using a monoclonal antibody (Chemicon International, Temecula, CA, USA). The protein levels of AMP-activated protein kinase (AMPK) and those of phosphorylated AMPK (Thr-172) and phosphor-

TABLE 1. Effect of TRC, T2, and T3 on human TRα1 and human TRβ1 transcriptional activity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Human TRα1 (fold)</th>
<th>Human TRβ1 (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 μM</td>
<td>35.0 ± 0.9</td>
<td>30.8 ± 7.8</td>
</tr>
<tr>
<td>0.02 μM</td>
<td>17.2 ± 1.6</td>
<td>14 ± 2.6</td>
</tr>
<tr>
<td>T2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 μM</td>
<td>20.4 ± 1.7</td>
<td>27.4 ± 1.3</td>
</tr>
<tr>
<td>2 μM</td>
<td>13.9 ± 1.9</td>
<td>15.9 ± 2.1</td>
</tr>
<tr>
<td>TRC, 20 μM</td>
<td>2.4 ± 0.4*#</td>
<td>2.5 ± 0.5*#</td>
</tr>
</tbody>
</table>

Representative data of 3 independent experiments; all samples done in duplicate. Values are means ± sd. *P < 0.0001 vs. T3, #P < 0.0001 vs. T2.

Assessment of the TR transactivation potential of TRC

TRs exert their major effects by modulating the transcription of target genes via binding to specific nuclear hormone receptors TRα1 and TRβ1. To assess the effect of TRC on transcriptional activity of human TRs, cells overexpressing human TRα1 and human TRβ1 were used. A COS7 cell line (ATCC CRL-1651; American Type Culture Collection, Man-

TABLE 2. Body weight gain, daily food intake, oxygen consumption, and RQ of N, HFD, and HFD-TRC rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>N</th>
<th>HFD</th>
<th>HFD-TRC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain (g)</td>
<td>97.63 ± 3.99</td>
<td>139.94 ± 6.20*</td>
<td>98.0 ± 6.33**</td>
</tr>
<tr>
<td>Daily food intake (g)</td>
<td>21.15 ± 0.59</td>
<td>22.42 ± 0.97</td>
<td>21.00 ± 0.95</td>
</tr>
<tr>
<td>Oxygen consumption (L O2/h/kg0.75)</td>
<td>0.75 ± 0.05</td>
<td>0.70 ± 0.03</td>
<td>0.87 ± 0.04*</td>
</tr>
<tr>
<td>RQ</td>
<td>0.94 ± 0.03</td>
<td>0.82 ± 0.02**</td>
<td>0.77 ± 0.02**</td>
</tr>
</tbody>
</table>

Values are means ± se for 8 different animals. *P < 0.01, **P < 0.001 vs. N rats; *P < 0.05, **P < 0.01 vs. HFD rats.
suggesting that HFD-TRC livers contained relatively less fat (Fig. 2). Accordingly, HFD rats exhibited a significantly higher level of hepatic triglycerides than N rats, and this level was significantly reduced (−35% vs. HFD rats) after TRC administration (Table 4).

Notably, no significant difference was found in serum FT₃, FT₄, or TSH levels, among the three groups (Table 4). Moreover, there is no increase in heart weight in HFD-TRC rats but rather a normalization of it (Table 4). This finding indicates that at the given dose, TRC does not induce thyrotoxic effects, which are known to be mediated through the TR. This conclusion is in agreement with the data relating to the lack of TR transactivation potential of TRC (Table 1).

Analysis of whole-body O₂ consumption (V₀₂) showed that in HFD-TRC rats energy expenditure was significantly higher (+24%) than in HFD rats (Table 2). Furthermore, the RQ, which reflects the ratio of carbohydrate to fatty acid oxidation, was significantly lower in HFD rats than in N rats, with TRC treatment further decreasing it (Table 3), indicating that HFD-TRC rats used a relatively greater ratio of fatty acids as a fuel source.

TRC treatment did not decrease the skeletal muscle mass, indicating a sparing of lean mass in the metabolic effect of this compound. This result is in direct contrast with the observed adverse effect of TH. TRC treatment induced a significant increase in muscle mass of the tibialis anterior vs. that in both N (+16%) and HFD (+32%) rats (Table 3). TRC administration did not alter the gastrocnemius and soleus muscle mass of HFD rats.

### Effects of TRC on serum cholesterol and triglycerides

Increased fat oxidation might be expected to result in alterations of some serum parameters related to fat metabolism, such as the levels of cholesterol and triglycerides. In fact, compared with their values in the HFD group, each of these parameters was decreased by TRC administration (Table 4). The serum cholesterol level was 62% higher in HFD rats than in N rats, and TRC administration significantly reduced it, although it did not reach the value seen in N rats. The serum triglyceride level was 99% higher in HFD rats than in N rats. TRC administration reduced serum triglyceride levels by 23% compared with the HFD group level, although the values are not significantly different from those for the HFD rats.

![Figure 2](image-url). Macroscopic view of livers (white arrow) and visceral adipose tissues (black arrow) of N, HFD, and HFD-TRC rats.

---

**TABLE 3. Tissue weights for N, HFD, and HFD-TRC rats**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>N</th>
<th>HFD</th>
<th>HFD-TRC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>1.07 ± 0.01 (0.28)</td>
<td>1.25 ± 0.06 (0.29)*</td>
<td>1.07 ± 0.04 (0.27)**</td>
</tr>
<tr>
<td>White adipose tissue</td>
<td>22.00 ± 0.86 (3.7)</td>
<td>33.42 ± 2.69 (7.8)**</td>
<td>26.31 ± 2.28 (6.5)*</td>
</tr>
<tr>
<td>Liver</td>
<td>11.40 ± 0.36 (3.0)</td>
<td>13.56 ± 0.43 (3.2)**</td>
<td>11.91 ± 0.18 (3.0)**</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>1.62 ± 0.09 (0.60)</td>
<td>2.03 ± 0.2 (0.48)*</td>
<td>2.14 ± 0.10 (0.53)**</td>
</tr>
<tr>
<td>Tibialis anterior</td>
<td>1.06 ± 0.07 (0.28)</td>
<td>0.93 ± 0.03 (0.22)</td>
<td>1.23 ± 0.05 (0.30)*</td>
</tr>
<tr>
<td>Soleus</td>
<td>0.23 ± 0.02 (0.06)</td>
<td>0.24 ± 0.01 (0.063)</td>
<td>0.22 ± 0.02 (0.054)</td>
</tr>
</tbody>
</table>

Values in parentheses represent percentage ratio tissue weight/body weight. Values are means ± se for 8 different animals. *P < 0.05, **P < 0.005, ***P < 0.001 vs. N rats; *P < 0.05, **P < 0.01, ***P < 0.001 vs. HFD rats.
Effects of TRC on mitochondrial fatty acid import and oxidation rate

Having established that the above effects were exerted by TRC on hepatic lipid content, on serum cholesterol and triglyceride levels, and on RQ, we next investigated the mitochondrial capacity to oxidize fatty acid in both liver and muscle in rats subjected to HFD or HFD-TRC treatment. Compared with the value obtained for N rats, the liver mitochondrial capacity to oxidize fatty acids tended to be slightly (+16%) higher in HFD animals. Administration of TRC to HFD rats significantly enhanced the ability of liver mitochondria to oxidize fatty acids (+32%) (Table 5). To investigate whether this TRC-induced stimulation of fatty acid oxidation in liver mitochondria might be dependent on their import into mitochondria, we also measured total CPT activity. Although total CPT activity was not affected by HFD, it was significantly increased by TRC (+56% vs. HFD).

Concerning the ability of skeletal muscle mitochondria to oxidize fatty acids, no differences were observed among the three groups either in the oxidation rate or in total CPT activity when assessed in hind limb muscle (Table 5). However, when individual muscles were separated according to their structural/metabolic characteristics (fiber composition and oxidative/glycolytic metabolism), the results were different. In particular, neither CPT activity nor the fatty acid oxidation rate was influenced by TRC administration in soleus muscle, a slow oxidative muscle. The values for CPT activity were 12.2 ± 3.2, 13.8 ± 3.8, and 14 ± 4.2 nmol O/min/mg protein for N, HFD, and HFD-TRC rats, respectively; the values for fatty acid oxidation were 53 ± 3.2, 55 ± 3.6, and 54 ± 4.2 nmol CoA/min/mg protein, for N, HFD, and HFD-TRC rats, respectively. In gastrocnemius muscle, a predominantly fast glycolytic muscle, TRC induced slight, but not significant, increases in total CPT activity and the fatty acid oxidation rate. The values for CPT activity were 14.2 ± 3.7, 14.8 ± 3.8, and 14.9 ± 3.2 nmol O/min/mg protein, for N, HFD, and HFD-TRC rats, respectively; the values for fatty acid oxidation were 41 ± 3.1, 45 ± 3.4, and 44 ± 3.7 nmol CoA/min/mg protein for N, HFD, and HFD-TRC rats, respectively.

Interestingly, in tibialis anterior, a predominantly fast oxidative glycolytic muscle, TRC induced a significant increase (vs. HFD) in total CPT activity (+42%) and also in the fatty acid oxidation rate (+35%) (Table 5).

Effect of TRC on AMPK phosphorylation in liver and skeletal muscle

We next investigated the biochemical mechanisms that might be involved in the TRC-induced stimulation of fatty acid oxidation in the liver. AMPK (a key kinase driving lipid oxidation), when activated by an increase in the AMP/ATP ratio, inhibits ACC through phosphorylation. A decrease in ACC activity reduces the

<table>
<thead>
<tr>
<th>Parameter</th>
<th>N</th>
<th>HFD</th>
<th>HFD-TRC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
<td>N</td>
<td>HFD</td>
<td>HFD-TRC</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitoyl CoA oxidation (nmol O/min/mg protein)</td>
<td>40.50 ± 3.61</td>
<td>46.86 ± 3.12</td>
<td>61.85 ± 2.99***,##</td>
</tr>
<tr>
<td>CPT activity (nmol CoA/min/mg protein)</td>
<td>4.41 ± 0.22</td>
<td>4.86 ± 0.43</td>
<td>7.60 ± 0.51**##</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitoyl CoA oxidation (nmol O/min/mg protein)</td>
<td>49.08 ± 2.33</td>
<td>49.55 ± 4.55</td>
<td>47.79 ± 1.96</td>
</tr>
<tr>
<td>CPT activity (nmol CoA/min/mg protein)</td>
<td>15.11 ± 12</td>
<td>14.21 ± 0.81</td>
<td>15.60 ± 1.18</td>
</tr>
<tr>
<td>Tibialis anterior</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitoyl CoA oxidation (nmol O/min/mg protein)</td>
<td>32.02 ± 2.40</td>
<td>39.15 ± 4.55</td>
<td>52.59 ± 1.96***,##</td>
</tr>
<tr>
<td>CPT activity (nmol of CoA/min mg of protein)</td>
<td>10.18 ± 1.11</td>
<td>11.08 ± 0.61</td>
<td>15.73 ± 0.73***,##</td>
</tr>
</tbody>
</table>

Values are means ± se for 8 rats. *P < 0.05, **P < 0.01, ***P < 0.001 vs. N rats; *P < 0.05, **P < 0.01 vs. HFD rats.
intracellular malonyl-CoA level and stimulates CPT1, leading to an increased influx of long-chain fatty acids into the mitochondria, where they are oxidized (18).

Liver and muscle responded differently to the present treatments. In the liver, the HFD and HFD-TRC animals had equally lowered AMPK phosphorylation levels compared with the N controls (Fig. 3A). Because TRC stimulated fatty acid oxidation in the liver, we also measured the expression of ACC in this tissue. The phosphorylation of both isoforms was reduced in the HFD livers, and this reduced level was unaffected by TRC (Fig. 3B), thus excluding the involvement of the AMPK-ACC-CPT pathway in the observed effect of TRC on hepatic fatty acid oxidation.

In the gastrocnemius muscle, a slight increase in AMPK phosphorylation tended to be observed in HFD rats, and the HFD value was not altered by TRC (Fig. 3C). On the other hand, no differences were observed in tibialis anterior muscle whatever the treatment (Fig. 3D). These data indicate no involvement of AMPK in muscle metabolic shift, at least at the time point investigated.

**Effect of TRC on mitochondrial proton leak kinetics in liver and skeletal muscle**

Next, to examine whether TRC might affect the efficiency of mitochondria, we evaluated the kinetic response of their proton leak to a change in membrane potential. For this, we used both liver and skeletal muscle mitochondria from N, HFD, and HFD-TRC rats.

Administration of the HFD to rats induced mitochondrial uncoupling in the liver. With the phosphorylation of ADP to ATP inhibited, HFD liver mitochondria exhibited an enhanced flux of protons across the inner membrane toward the matrix compared with N mitochondria. Indeed, HFD liver mitochondria had to respire at a higher level than N mitochondria to maintain the same membrane potential (Fig. 4A). Administration of TRC to HFD rats apparently restored the proton leak kinetic observed in N rats (Fig. 4A), because the proton leak kinetics detected in N and

**Figure 3.** Effects of TRC on AMPK and ACC phosphorylation (P-ACC) in liver and on AMPK phosphorylation in gastrocnemius and tibialis anterior muscles. A) AMPK phosphorylation in liver. B) ACC phosphorylation in liver. C) AMPK phosphorylation in gastrocnemius muscle. D) AMPK phosphorylation in tibialis anterior muscle. Measurements were made by Western blotting using ultracentrifuged total lysates (see Materials and Methods), with each lane containing 30 μg of protein from a single rat. Even protein loading was reassessed after blotting by probing the blot with a β-actin antibody. Quantified data are means ± se from 6 independent experiments. *P < 0.05 vs. N rats.

**Figure 4.** Effects of TRC on proton-leak kinetics in liver (A) and skeletal muscle (B) mitochondria from N, HFD, and HFD-TRC rats. Data are means ± se from 4 independent experiments.
HFD-TRC liver mitochondria overlapped, suggesting that administration of TRC to HFD rats may prevent the enhancement of the proton leak induced in liver by the HFD.

Administration of the HFD to rats also induced mitochondrial uncoupling in skeletal muscle, as seen in Fig. 4B, but administration of TRC to HFD rats did not alter the proton leak kinetics.

Effect of TRC on SIRT1 activity in liver nuclei

To investigate further the molecular mechanism responsible for the hepatic fat depletion that occurs due to increased fatty acid oxidation after TRC treatment (despite a reduction in AMPK phosphorylation), we measured the SIRT1 protein level and activity level in liver nuclei from N, HFD, and HFD-TRC rats. SIRT1 is crucial for hepatic energy homeostasis, its absence from the liver leading to steatosis (19, 20). As shown in Fig. 5A, the nuclear protein level of SIRT1 did not differ among the three groups. Interestingly, SIRT1 nuclear activity, although not different between N and HFD livers, was strongly activated in HFD-TRC livers (Fig. 5B), a finding that is in line with the nonsteatotic phenotype observed in these animals.

Effect of TRC on MHCIIb protein expression in skeletal muscle

Because the fiber type profile plays a role in muscle metabolic capacity, we wondered whether the above differences among muscles types in the effects of TRC on CPT activity and fatty acid oxidation rate might be paralleled by differential effects of TRC on the muscle structure, in terms of shifts in fiber composition. For this analysis, we selected the gastrocnemius and tibialis anterior muscles, which differed in the metabolic and trophic effects of HFD and TRC (see Tables 3 and 5). As shown in Fig. 6A, in gastrocnemius muscle the

Figure 5. Effects of TRC on nuclear SIRT1 protein expression and activity in liver. A) SIRT1 nuclear protein expression level. Measurements were made by Western blotting using nuclear protein (see Materials and Methods), with each lane containing 30 μg of protein from a single rat. Even protein loading was reassessed after blotting by probing the blot with a β-actin antibody. B) Activity of SIRT1 nuclear protein. SIRT1 activity was measured by a fluorimetric assay using native immunoprecipitated SIRT1 protein from freshly isolated nuclei (see Materials and Methods). Quantified data are means ± s.e. from 4 independent experiments. *P < 0.05 vs. N and HFD rats.

Figure 6. Effect of TRC on MHCIIb protein expression in muscles. A) MHCIIb protein levels in gastrocnemius muscle. B) MHCIIb protein levels in tibialis anterior muscle. Measurements were made by Western blotting using total crude lysates (see Materials and Methods), with each lane containing 15 μg of protein from a single rat. Even protein loading was reassessed after blotting by probing the blot with a β-actin antibody. Quantified data are means ± s.e. from 4 independent experiments. *P < 0.05 vs. N and HFD rats.
DISCUSSION

Overweight, hepatic steatosis, and associate metabolic derangements are now global problems, and although a prolonged and marked reduction in caloric intake is known to alleviate these pathological conditions, this is rarely achieved in real life. Therefore, an effective pharmacological therapy would be useful. Targeting mitochondria by stimulating their metabolism to improve mitochondrial function may represent an important way to counteract those “modern” pathological conditions that are due to excessive calorie intake/HFD coupled with a sedentary way of life. In this study, we investigated the possible metabolic properties of TRC, a functional analog of THs, and we extended our investigation from the whole animal to the activities of two metabolically very active tissues: liver and skeletal muscle.

Whole animals

TRC stimulated in vivo oxygen consumption and induced a decrease in RQ when administered simultaneously with an HFD. These data clearly indicate that TRC concomitantly stimulates energy expenditure and the oxidation of lipids. RQ is indeed considered a good index of a shift in metabolic substrate toward either glucose (when RQ approximates to 1.0) or lipid utilization (when RQ~0.7) (21). The above conclusion as to the effects of TRC is strongly supported by our data showing stimulation of fatty acid oxidation in both the liver and the tibialis anterior muscle as well as a fiber shift toward the oxidative phenotype in that muscle (this aspect will be discussed below). An important consequence of the TRC-induced stimulation of oxygen consumption and decrease in RQ is the observed marked reduction in hepatic steatosis and in the weight of the visceral fat pad. Previous studies have shown that effects very similar to these may be observed after the administration of the THs T₃ (8) and T₂ (22, 23), and our initial intention was to examine whether the effects reported here might be mediated by affecting levels of THs or by an interaction of TRC with TRs. In that case, we should expect undesirable side effects, such as increases in heart mass and rate and changes in the serum levels of TSH and T₃. However, because no changes were observed in the serum levels of TSH, T₃, or T₂ and as the interactions of TRC with TRs were much weaker than those of T₃, we can conclude that the effects observed after TRC administration are not mediated through interaction with TRs. Importantly, no undesirable effects were observed on heart rate or heart weight, neither of which appeared to be adversely affected by TRC. Another important aspect is that the THs modulate the hypothalamic thyroid axis and appetite through their central (CNS) effect. To study the possibility that TRC crosses the blood-brain barrier, we performed a tissue kinetics and distribution study (data not shown) showing that the levels of TRC in the brain are >100 times lower than plasma levels, which shows relatively very low permeability to brain.

Liver

The liver, an important contributor to both energy expenditure (24) and lipid/glucose homeostasis, appears to be a major target for TRC. Indeed, HFD induced a significant increase in the liver content of triglycerides, as revealed both by measuring the content itself and by the lighter color of the organ in HFD rats (Fig. 2). Simultaneous administration of TRC to HFD rats restored both the triglyceride level and the color to those observed in the livers of N rats. The antisteatotic activity of TRC appears to be attributable to increased rates of mitochondrial fatty acid uptake and oxidation. This conclusion is supported by the TRC-induced increase in the activity of the hepatic CPT system, which is involved in the fatty acid oxidation cycle. Indeed, CPT is rate-limiting for the transport of fatty acids into the mitochondrial matrix, and, thus, for their oxidation (25, 26). At the mechanistic level, we searched for the biochemical pathways involved in the effects of TRC on the CPT system and liver fatty acid oxidation. The AMPK-ACC-malonyl CoA signaling pathway plays a major regulatory role in the activation of CPT1 (the outer enzyme of the CPT system). Because of this, it seemed possible that AMPK activation might be an important mediator of the actions of TRC in the liver. Because, however, both the HFD and HFD-TRC animals (after 4 wk of treatment) had equally lowered hepatic AMPK phosphorylation levels vs. the N controls, we can exclude the involvement of the AMPK-ACC-CPT pathway in the observed stimulatory effect of TRC on hepatic fatty acid oxidation. We next tested the possibility that TRC might affect mitochondrial efficiency and consequently lipid oxidation. During mitochondrial respiration, the transfer of protons along the respiratory chain in the inner mitochondrial membrane is coupled to the translocation (pumping) of protons from the mitochondrial matrix into the intermembrane space, thus generating an electrochemical proton gradient across the inner membrane. This gradient is used to drive the protons back into the matrix through the ATP synthase complex, thus resulting in ATP synthesis. The system is not completely efficient, however, and part of the gradient may be dissipated as heat via a leak of protons, during reentrance into the matrix while bypassing the ATP-synthase complex. An augmentation of this proton leak would thus lead to a secondary increase in substrate oxidation and to the burning of fat. Such a mechanism is, for example, operative with T₂ (20). Comparison of the proton leak kinetic plots (Fig. 3) obtained for mitochondria isolated from N, HFD, and

ANTIOBESITY EFFECT OF IODOTHYRONINE’S FUNCTIONAL ANALOG
HFD-TRC rats suggested that HFD mitochondria exhibited an enhanced proton leak compared with N rats and, moreover, that administration of TRC restored the normal values. Thus, the metabolic effects of TRC may not be through an enhancement of the mitochondrial proton leak.

Sirtuins make up a small gene family, with seven members in mammals, of which SIRT1 is the founding member (27). It now seems evident that SIRT1 is a key molecule that promotes longevity at least partly through its effect on metabolic homeostasis, which may be expected to have a great impact in the prevention/treatment of metabolic disorders such as obesity and dyslipidemia. In the liver, activation of SIRT1 facilitates fatty acid oxidation (28). In addition, it has recently been shown that in hepatocytes isolated from mice lacking SIRT1, fatty acid oxidation rates are reduced and also that such mice accumulate lipids within the liver (19). Therefore, we tested for possible involvement of SIRT1 in the effects induced by TRC. When we measured the activity level of the native SIRT1 protein immunoprecipitated from liver nuclei, we observed a significant increase in HFD-TRC rats vs. HFD rats, although the SIRT1 protein level remained unaltered. It is thus conceivable that a TRC-induced increase in SIRT1 activity underlies the increase in fatty acid oxidation and the prevention of liver steatosis observed in the HFD-TRC rats.

Skeletal muscle

Skeletal muscle, quantitatively the largest organ in the body, contributes 30–40% of the resting metabolic rate in adults and is a major site for the oxidation of fatty acids and glucose. Indeed, it accounts for ∼80% of insulin-stimulated glucose uptake. Our data on β-oxidation in skeletal muscle seemed to indicate a lack of effect of TRC on the lipid oxidative capacity of muscle when the tissue samples examined contained soleus, gastrocnemius, and tibialis anterior muscle lumped together. However, skeletal muscle displays a marked flexibility in its usage of fuel in response to different stimuli, a flexibility characterized by structural, biochemical, and functional modifications (29). We therefore investigated the possibility that there might be morphological and functional modifications after administration of TRC by analyzing the soleus, gastrocnemius, and tibialis anterior muscles separately. TRC was ineffective in stimulating fatty acid uptake and oxidation in the soleus, tended to stimulate them slightly in the gastrocnemius, and stimulated them significantly in tibialis anterior without involving AMPK, at least at the time point investigated. These data were in good agreement with the results of our fiber shift analysis, which showed a TRC-induced shift toward MHCIb in tibialis anterior but not in gastrocnemius, supporting a shift toward the use of fatty acids over glucose as fuel in the former muscle. It has been reported that morbidly obese subjects have a lower percentage of type I oxidative fibers than do their lean counterparts (i.e., ∼42 vs. 55%) (30), which could contribute to the lower substrate oxidation capacity of skeletal muscle and insulin resistance observed with obesity. However, the results reported here showing the capacity of TRC to increase the oxidative muscle fibers (particularly in muscle having relatively low oxidative capacity) may have clinical importance especially when coupled with the other biochemical changes induced by TRC. The evidence of translation of effects seen in animals to humans in clinical trials of thymomimetics such as DIPTA and KB2115 is encouraging (31).

CONCLUSIONS

Our study shows that simultaneous systemic administration of TRC to rats receiving an HFD results in a reduction in fat accumulation within the liver and a marked reduction in adipose tissue mass. A further point of interest is that TRC induced a reduction in the serum levels of triglycerides and cholesterol. At the organ level, although both liver and skeletal muscle proved to be targets for TRC, the effects of this compound were quite different between the two tissues. Of course, clinical trials will be needed to translate these effects to the treatment of human obesity. If reproduced in humans, these results may offer an interesting perspective on the possible pharmacological approaches to the above-mentioned lifestyle-related dysfunctions.

The authors thank Dr. Onorato Goglia (Azienda Ospedaliera Rummo-Benevento) for performing cholesterol and triglycerides assays. This study was funded by Torrent Pharmaceuticals Ltd. (Ahmedabad, Gujarat, India). TRC150094 is a molecule patented under a patent owned by Torrent Pharmaceuticals Ltd. S.P.Z., L.C., D.T., S.M., R.C.G., V.C., and C.D. are employees of Torrent Pharmaceuticals Ltd. No other potential conflict of interest relevant to this article was reported.

REFERENCES