THYROID HORMONE EFFECTS ON WHOLE BODY ENERGY HOMEOSTASIS AND TISSUE-SPECIFIC FATTY ACID UPTAKE *IN VIVO*

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ABSTRACT

The effects of thyroid hormone (TH) status on energy metabolism and tissue-specific substrate supply *in vivo* are incompletely understood. To study the effects of TH status on energy metabolism and tissue-specific fatty acid (FA) fluxes, we used metabolic cages as well as ^{14}C-labelled FA and ^{3}H-labeled triglyceride (TG) infusion in rats treated with methimazole and either 0 (hypothyroidism), 1.5 (euthyroidism) or 16.0 (thyrotoxicosis) µg/100g*day of thyroxine for 11 days.

Thyrotoxicosis increased total energy expenditure (TEE) by 38% ($P < 0.05$), resting energy expenditure (REE) by 61% ($P < 0.01$) and food intake by 18% ($P < 0.01$). Hypothyroidism tended to decrease TEE (10%; $P = 0.064$), and REE (12%; $P < 0.05$), but did not affect food intake. TH status did not affect spontaneous physical activity (SPA). Thyrotoxicosis increased fat oxidation ($P < 0.01$), whereas hypothyroidism decreased glucose oxidation ($P < 0.05$). Plasma FA concentration was increased in thyrotoxic, but not in hypothyroid rats. Thyrotoxicosis increased albumin-bound FA uptake in muscle and white adipose tissue (WAT), whereas hypothyroidism had no effect in any tissue studied, suggesting mass-driven albumin-bound FA uptake. During thyrotoxicosis, TG-derived FA uptake was increased in muscle and heart, unaffected in WAT, and decreased in brown adipose tissue. Conversely, during hypothyroidism TG-derived FA uptake was increased in WAT in association with increased lipoprotein lipase activity, but unaffected in oxidative tissues and decreased in liver.

In conclusion, TH status determines EE independently of SPA. The changes in whole body lipid metabolism are accompanied by tissue-specific changes in TG-derived FA uptake in accordance with hyper- and hypometabolic states induced by thyrotoxicosis and hypothyroidism, respectively.

INTRODUCTION

Thyroid hormone (TH) is a primary denominator of energy homeostasis, reflected by the strong association between hyperthyroidism and increased energy expenditure (EE) in man. This is exemplified by the widespread clinical use of calorimetry in addition to the determination of protein bound iodine in diagnosing thyrotoxicosis (1;2) before sensitive thyroxine (T_4) and triiodothyronine (T_3) RIAs became available in the 1970s (3). Whereas modulation of resting EE by thyroid hormone status is well established in humans and rodents, it has been difficult to assess TH effects on total EE (TEE) *in vivo*. In addition, the mechanism of the increased EE induced by THs has remained incompletely understood (4). For example, few studies have addressed how THs influence spontaneous physical activity (SPA) and if changes in SPA may contribute to the alterations in EE associated with thyrotoxicosis and hypothyroidism in freely moving organisms (5;6).

We have previously studied glucose metabolism during thyrotoxicosis in rats and found increased endogenous glucose production and hepatic insulin resistance (7). Furthermore, thyrotoxicosis is associated with major changes in lipid metabolism. Fatty acids (FA) are a preferential fuel source during thyrotoxicosis (8;9), especially during the first days after the induction of thyrotoxicosis (10). These FA are provided to tissues mostly by hydrolysis (*i.e.* lipolysis) of circulating triglyceride (TG)-rich lipoprotein particles by the enzyme lipoprotein lipase (LPL), located in the capillary lumen. In addition, albumin-bound FA are provided to the tissues from the plasma, a process which is independent of LPL. Although there is evidence suggesting that LPL is regulated by TH (11), it is unknown at present how FA fluxes via these two pathways are modulated by TH in metabolically relevant tissues *in vivo*.

The aim of the present study was to examine the effects of thyrotoxicosis and hypothyroidism on whole body energy metabolism and SPA in rats. To delineate how the effects of thyroid status on whole body energy homeostasis are reflected in substrate (*i.e.* lipid) supply on the tissue level *in vivo*, we additionally studied the rates of disappearance and tissue-specific partitioning of both TG-derived and albumin-bound FA. We report effects of thyroid hormone status on TEE, resting energy expenditure (REE), SPA and substrate (*i.e.* glucose and lipid) oxidation, that are paralleled by complex and tissue-specific effects of TH on FA uptake.

MATERIALS AND METHODS

In the first experiment, 3 groups of rats were studied, i.e. hypothyroid (Hypo, n = 7), euthyroid (Eu, n = 7) and thyrotoxic rats (Tox, n = 7). All groups were treated with methimazol (MMI) in drinking water. After 7 days, all groups were implanted with subcutaneous osmotic mini-pumps (day (D) 0), delivering either vehicle (Hypo group), or thyroxine (T_4) at a dose of 1.5 (Eu group) or 16.0 µg/100 g BW*day (Tox group) (7). Rats were subsequently placed in metabolic cages for determining TEE, REE, food intake, respiratory exchange ratio (RER), and fat and glucose oxidation during a 48 h period (D9 and D10).

In the second experiment (D11), hypothyroid (Hypo, n = 9), euthyroid (Eu, n = 8) and thyrotoxic rats (Tox, n = 7) were i.v. infused with albumin-bound ^{14}C-oleate (FA) and VLDL-like

emulsion-incorporated glycerol tri[^{3}H]oleate (TG). This method enables measurement of FA turnover, tissue-specific FA partitioning and differentiation between albumin-bound and TG-derived FA uptake on the tissue level (12).

Animals

Male Wistar rats (Harlan, Horst, the Netherlands) were housed under constant conditions of temperature (21 ± 1°C) and humidity (60 ± 2%) with a 12 h/12 h light/dark (L/D) schedule (lights on 7:00 am). Animals were allowed to adapt for 6 days before the first experimental manipulations. Body weight (BW) was between 320 and 360 g. Food and drinking water was available *ad libitum*. All of the following experiments were conducted with the approval of the Animal Care Committee of the Leiden University Medical Center.

Hormonal treatment; block and replacement

At D0 of the protocol animals were placed in individual cages and treated with methimazole 0.025% (MMI, Sigma, the Netherlands) in drinking water containing 0.3% saccharin. At D7, osmotic minipumps (OMP, Alzet 2ml2, Durect Corp., Cupertino, USA) loaded with L-thyroxine (T_4, Sigma, the Netherlands) solved in 6.5 mM NaOH and 50% propylene glycol, were implanted under the dorsal skin during the surgical procedure. OMPs delivered either vehicle (hypothyroid rats), or T_4 at a dose of 1.5 µg (replacement dose; euthyroid group) or 16.0 µg (thyrotoxic group) /100 g BW*day, as described previously (7).

Surgery

In all animals an intra-atrial silicone cannula was implanted through the right jugular vein for infusion and sampling (13) during anesthesia (7). The cannula was tunneled to the head subcutaneously, fixed with dental cement to 4 stainless-steel screws inserted into the skull. A mixture of 60% amoxicillin, 20% heparin and 20% saline in polyvinylpyrrolidone (Sigma, the Netherlands) was used to fill the cannula and prevent inflammation and occlusion.

Energy expenditure, fat oxidation, spontaneous physical activity and food intake

At D7, animals were placed into an 8-cage combined, open circuit indirect calorimetry system (LabMaster system, TSE Systems, Bad Homburg, Germany, for the remainder of this manuscript referred to as "metabolic cages"), measuring food and water intake, O_2 uptake and CO_2 production, as well as SPA. Although the cages including bedding were identical to the cages in which the rats were housed the first 7 days (only the cover of the metabolic cage differs), animals were adapted to this environment before the start of the actual measuring periods (D9 and D10) for approximately 48 h. EE, RER and fat oxidation were calculated from the O_2 uptake and CO_2 production relative to individual body weights (14). O_2 uptake and CO_2 production were measured with 10 min intervals. Food and water intake and physical activity were measured continuously. Activity monitoring and detection of animal location was performed with infrared sensor pairs arranged in strips for horizontal (X level) and vertical (Z level) activity, detecting every ambulatory movement. Spontaneous physical activity (SPA or XA), high-frequent activity (XF; equivalent of breathing activity), total activity (XT=XA+XF)

and rearing (Z) were monitored. The infrared sensors for detection of movement allowed continuous recording in both light and dark phases.

Radiolabeled FA infusion

At D11, rats were restrained from access to food from 5h prior to the labelled lipid infusion (i.e. from 9:00 am onwards). Rats were connected to a metal collar attached to polyethylene tubing (for blood-sampling and isotope infusion) which was kept out of reach of the animals by a counterbalanced beam. This allowed all subsequent manipulations to be performed outside the cages without handling the freely moving animals. After obtaining a blood sample for measurement of plasma TH, FA, and TG concentrations (800 µL), rats received a primed (500 µl in 5 min), continuous (500 µl/h) infusion of albumin-bound ^{14}C-oleate (FA) and VLDL-like emulsion-incorporated glycerol tri[^{3}H]oleate (TG) i.v. for 2 h. At the end of the 2 h infusion period we obtained another blood sample (800 µL) for measurement of plasma [^{3}H]-FA and [^{14}C]-FA radioactivity. Rats were sacrificed and striated muscle (M triceps brachii), heart, liver, three white adipose tissue (WAT) depots (gonadal (epididymal), subcutaneous, visceral) and infrascapular brown adipose tissue (BAT) were harvested, snap frozen and stored at -20°C for subsequent analysis.

Preparation of radiolabeled emulsion particles

Protein-free VLDL-like TG-rich emulsion particles were prepared from 100 mg total lipid at a weight ratio of triolein (Sigma, St. Louis, MA, US): egg yolk phosphatidylcholine (Lipoid, Ludwigshafen, Germany): lyso phosphatidylcholine (Sigma, St. Louis, MA, US): cholesteryl oleate (Janssen, Beersse, Belgium): cholesterol (Sigma, St. Louis, MA, US) of 70: 22.7: 2.3: 3.0: 2.0 in the presence of 800 µCi of glycerol tri[9, 10(n)-^{3}H]oleate ([^{3}H]TG) (Amersham, Little Chalfont, UK), as reported previously (15). Lipids were hydrated in 10 mL of 2.4M NaCl, 10 mM Hepes, 1 mM EDTA, pH 7.4, and sonicated for 30 min at 10 µm output using a Soniprep 150 (MSE Scientific Instruments, UK) equipped with a water bath for temperature (54°C) maintenance. The emulsion was separated into fractions with a different average size by density gradient ultracentrifugation. Intermediate (80 nm) [^{3}H]-TG particles were mixed with a trace amount of [^{14}C]-oleic acid (Amersham, Little Chalfont, UK) complexed to bovine serum albumin (BSA).

Tissue uptake analysis

Tissues were dissolved in 5 mol/l KOH in 50% (vol/vol) ethanol. After overnight saponification, protein content was determined in the various organs using BCA kit (BCA Protein Assay Kit, Thermo Scientific). Radioactivity was measured in the saponified organs and corrected for the corresponding protein concentration and plasma specific activities of [^{3}H]-FA and [^{14}C]-FA. Calculations of tissue FA uptake and rate of disappearance were performed as described previously (12).

Analysis of lipoprotein lipase (LPL) and hepatic lipase (HL) activity

Striated muscle, heart, liver and three WAT depots were cut into small pieces and put in 1 mL 2% BSA-containing DMEM medium. Heparin (2 units) was added and samples were incubated at 37°C for 60 minutes. After centrifugation (10 min at 13.000 rpm), the supernatants were

taken and snap-frozen until analysis. Total LPL and HL activity was determined as modified from Zechner et al. (16). In short, the lipolytic activity of tissue supernatant was assessed by determination of [^{3}H]oleate production upon incubation of tissue supernatant with a mix containing an excess of both [^{3}H]triolein, heat-inactivated human plasma as sources of the hydrolase coactivator apoC2 and FA-free BSA as FFA acceptor.

Plasma analysis

Plasma concentrations of the thyroid hormones T_3 and T_4 were determined by an in-house RIA, with inter- and intra-assay CV of 7-8% and 3-4% (T_3), and 3-6 and 2-4% (T_4), respectively. Detection limits for T_3 and T_4 were 0.3 nmol/l and 5 nmol/l, respectively. Plasma TSH concentrations were determined by a chemiluminescent immunoassay (Immulite 2000, Diagnostic Products Corp., Los Angeles, CA), using a rat-specific standard (17). The inter- and intra-assay CV for TSH were less than 4% and 2% at ± 3.5 mU/l, respectively, and the detection limit was 0.2 mU/l. Blood samples were kept in chilled paraoxon-coated Eppendorf tubes to prevent *ex vivo* lipolysis. The tubes were placed on ice and immediately centrifuged at 4°C. Plasma levels of TG and FA were determined using commercially available kits and standards according to the manufacturer's instructions (Instruchemie, Delfzijl, The Netherlands) in 96-wells plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands). Lipids were extracted from plasma according to Bligh and Dyer (18). The lipid fraction was dried under nitrogen, dissolved into chloroform/methanol (5:1 [vol/vol]) and subjected to TLC (LK5D gel 150; Whatman) using hexane:diethylether:acetic acid (83:16:1) [vol/vol/vol]) as mobile phase. Standards for FA and TG were included during the TLC procedure to locate spots of these lipids. Spots were scraped, lipids dissolved in hexane and radioactivity measured.

Statistics

Both energy homeostasis and FA uptake data were analysed by non-parametric Kruskall-Wallis (KW) test, and a Mann Whitney U *post hoc* test was performed if KW revealed significance to determine which experimental groups differed from each other. Cosinor analysis was performed on the metabolic cage data of individual animals (48 h). Curve fitting was performed using constrained nonlinear regression analysis (SPSS 16.0). Subsequently, only if the significance level (*P* value) of the fitted curve was less than 0.05, data were used to calculate mesor, amplitude and acrophase of the individual curve. Significance was defined at $P \leq 0.05$. Data are presented as mean ± SEM.

RESULTS

Effects of thyroid status on energy homeostasis

Body weight and eating behaviour

At the time of starting hormonal (T_4) treatment (osmotic mini-pump implantation; day 0), there were no differences in bodyweight (BW) between groups (Hypo 339 ± 13, Eu 340 ± 5, Tox 345 ± 4 g, ns). At the time of placement in the metabolic cages (day 7), BW was decreased by 13 ± 3 g in thyrotoxic rats, compared with an increase of 2 ± 5 g and 13 ± 9 g in euthyroid and

hypothyroid rats, respectively (Hypo *vs*. Eu ns, Eu *vs*. Tox $P < 0.05$, Hypo *vs*. Tox $P = 0.053$). However, during the whole period in the metabolic cages (day 7-10), BW increased to a similar extent in all groups (Hypo 12 ± 1, Eu 14 ± 3, Tox 12 ± 1 g, ns).

After placement in the metabolic cages animals were allowed to adapt to this new environment for 48 h (day 7-8). Subsequently, we gathered energy homeostasis data for 48 h (day 9-10). During this time period, thyrotoxic rats showed increased cumulative food intake by 18% as compared with euthyroid rats. Hypothyroid rats ate less than euthyroid rats, although this did not reach statistical significance (Hypo 44 ± 2 g, Eu 48 ± 2 g, Tox 57 ± 2 g, Eu vs Tox $P < 0$, 01, Hypo *vs*. Eu $P = 0.128$).

Plasma thyroid hormones

Plasma concentrations of T_3, T_4 and TSH following the 48 h measurement of energy homeostasis are given in Table 1. Plasma T_3 and T_4 concentrations were 163% and 30% higher, respectively, in thyrotoxic rats as compared with euthyroid rats. In hypothyroid rats, plasma T_3 and T_4 concentrations were decreased to 44% and 15%, respectively, of euthyroid levels. Plasma TSH was 12.9 ± 2.2 mU/l in hypothyroid rats, and showed similar values in euthyroid and thyrotoxic rats (0.3 ± 0.1 and 0.2 ± 0.0 mU/l, ns, respectively).

Table 1. Plasma thyroid hormone concentrations after 48 h measurement of energy homeostasis (day 11) in hypothyroid (Hypo), euthyroid (Eu) and thyrotoxic (Tox) rats. * $P < 0.01$ *vs*. Eu.

	Hypo n = 7	**Eu n = 7**	**Tox n = 7**
T_3 (nmol/l)	0.50 ± 0.11 *	1.14 ± 0.07	3.00 ± 0.27 *
T_4 (nmol/l)	20 ± 2 *	136 ± 8	177 ± 8 *
TSH (mU/l)	12.9 ± 2.2 *	0.3 ± 0.1	0.2 ± 0.0

Total EE, physical activity and resting EE

Total EE (TEE) showed a clear diurnal rhythm in all treatment groups, with a rise in the dark (*i.e.* active) period (Fig. 1A). As expected, this was paralleled by a similar rhythm in SPA in all groups (Fig. 1B). There was a marked, 37% increase in mean TEE/kg in thyrotoxic relative to euthyroid rats ($P < 0.05$). This increase persisted when mean TEE was not corrected for BW ($P < 0.05$, data not shown). Hypothyroid rats showed a trend ($P = 0.064$) towards decreased (-10%) mean TEE relative to euthyroid rats. Cosinor analysis revealed similar changes in the mesor of the fitted curves. In addition, there was a decrease in the amplitude of the rhythm in EE by 46% in hypothyroid relative to euthyroid rats ($P < 0.05$) as well as a ~1 h phase-advance of the acrophase relative to euthyroid and thyrotoxic rats ($P < 0.05$). There were no differences in the mean levels of SPA between groups (Kruskall-Wallis, ns). Also mesor, amplitude and acrophase of the fitted curves of SPA exhibited no differences between thyrotoxic, hypothyroid and euthyroid rats (Table 2). Likewise, there were no differences in high-frequency activity (equivalent of breathing; XF), rearing (Z) or total activity (XT) between groups, nor in mesor, amplitude or acrophase of the fitted curves (data not shown).

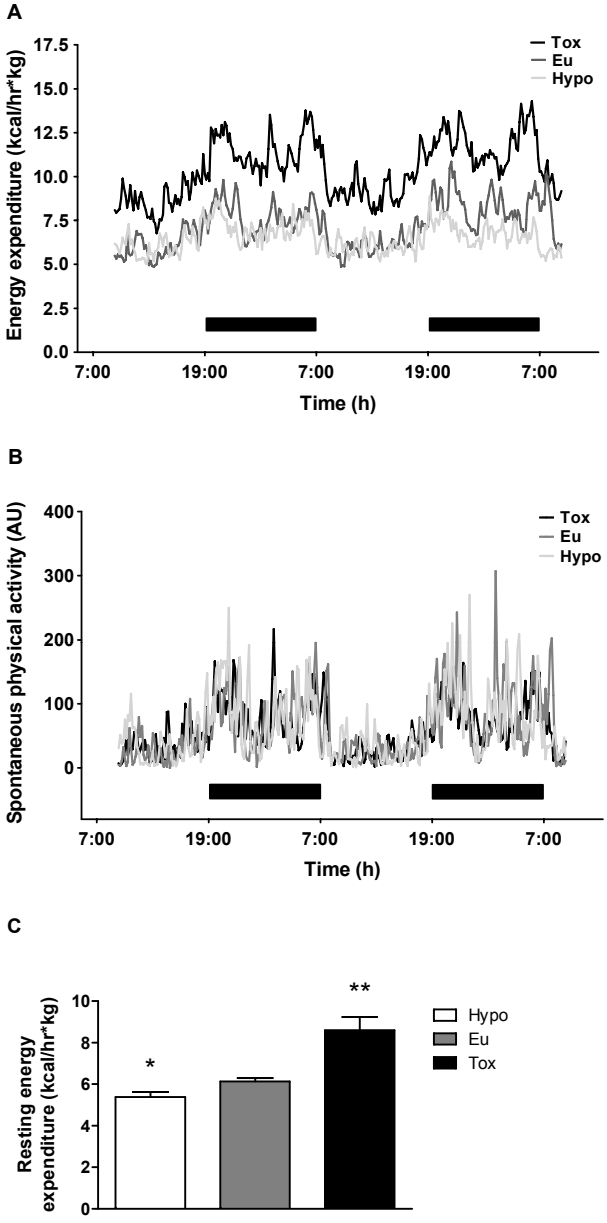

Fig. 1. Forty-eight-hour total energy expenditure (TEE, A) and spontaneous activity (SPA, B) in hypothyroid (Hypo), euthyroid (Eu) and thyrotoxic (Tox) rats entrained to a regular 12/12-h L/D cycle. Horizontal black bars indicate the dark phase of the L/D cycle. Data are mean of 7 animals per group at each time point and the interval between time points was 10 min. Cosinor data and statistical analysis are given in Table 2. Resting energy expenditure (REE, defined as the mean energy expenditure during 10 min intervals of inactivity (see text) in each individual animal, C) in hypothyroid (Hypo), euthyroid (Eu) and thyrotoxic (Tox) rats. Note the increase of REE in Tox *vs.* Eu rats (** $P < 0.01$) that was more pronounced than the decrease of REE in Hypo *vs.* Eu rats (* $P < 0.05$). Data are mean ± SEM of 7 animals per group.

Table 2. Data derived from cosinor curve-fit of all individual animals with statistical analysis. * $P < 0.05$ *vs.* Eu, " $P = 0.073$ *vs.* Eu, ^ $P < 0.05$ *vs.* Tox.

	Energy Expenditure (kcal/h*kg)			
	Mesor	**Amplitude**	**Acrophase (h)**	**n**
Hypo	6.52 ± 0.26 "^	0.64 ± 0.15 *^	23.15 ± 0.16 *^	7
Eu	7.24 ± 0.21	1.19 ± 0.09	00.23 ± 0.18	7
Tox	10.16 ± 0.67 *	1.70 ± 0.32	00.39 ± 0.22	7
	$P = 0.001$	$P = 0.009$	$P = 0.015$	
	Spontaneous physical activity (AU)			
	Mesor	**Amplitude**	**Acrophase (h)**	**n**
Hypo	63 ± 6	37 ± 8	00.26 ± 0.15	7
Eu	62 ± 5	38 ± 5	01.09 ± 0.35	7
Tox	60 ± 7	33 ± 6	00.13 ± 2.25	7
	$P = 0.901$	$P = 0.780$	$P = 0.248$	
	Respiratory exchange ratio			
	Mesor	**Amplitude**	**Acrophase (h)**	**n**
Hypo	0.94 ± 0.01 *	0.0123 ± 0.0014 ^	20.44 ± 0.30 *^	7
Eu	0.97 ± 0.01	0.0120 ± 0.0010	22.27 ± 0.24	7
Tox	0.91 ± 0.01*	0.0306 ± 0.0110 *	03.35 ± 2.48	7
	$P = 0.014$	$P = 0.032$	$P = 0.018$	

In each animal we determined the total number of 10 min intervals in which the activity sensors did not detect any activity (activity units (AU) = 0). Hypothyroid rats tended to spend more time inactive than euthyroid rats (533 ± 72 *vs.* 359 ± 40 min, $P = 0.064$), whereas thyrotoxic rats showed a trend towards less inactivity time compared to euthyroid rats (256 ± 33 min, $P = 0.073$). During these periods of inactivity, mean EE, termed resting EE (REE), was markedly higher in thyrotoxic as compared with euthyroid rats ($P < 0.01$), and lower in hypothyroid rats ($P < 0.05$ *vs.* Eu, Fig. 1C). REE/TEE ratios showed no differences between groups (Hypo 0.83 ± 0.02, Eu 0.85 ± 0.02, Tox 0.82 ± 0.01, KW, ns).

RER and substrate oxidation

Euthyroid rats showed a diurnal rhythm in RER (Fig. 2A), although less evident than the rhythm in TEE and SPA. The nocturnal acrophase fits with a relative increase in glucose oxidation in the dark (*i.e.* feeding) period. Both thyrotoxic and -although to a lesser extent- hypothyroid rats showed a decrease in mean RER levels relative to euthyroid rats (Tox: 94% of Eu levels, $P < 0.01$, Hypo: 96% of Eu levels, $P < 0.05$). In addition, cosinor analysis revealed that the amplitude of the day-night rhythm in RER was markedly increased in thyrotoxic rats (155%, $P < 0.01$). Although the RER phase difference between the euthyroid and thyrotoxic groups did not reach statistical significance ($P = 0.128$), there appeared to be an inverse rhythm in thyrotoxic relative to hypothyroid rats ($P < 0.05$). Hypothyroid animals showed

a RER increase in the early part of the dark period, whereas thyrotoxic rats showed a pronounced trough in RER during the feeding periods at the beginning and end of the dark period. Cosinor analysis confirmed that hypothyroid rats exhibit a decrease ($P = 0.053$) in the mesor of their RER day-night rhythm relative to euthyroid rats, but less pronounced than in thyrotoxic animals ($P < 0.01$). The acrophase of the RER rhythm in hypothyroid animals was ~2 h phase-advanced relative to euthyroid ($P < 0.05$), and almost 7 h relative to thyrotoxic animals ($P < 0.05$).

Substrate oxidation is depicted in Fig. 2B-C. In euthyroid animals, mean levels of glucose oxidation were ~30-fold higher than fat oxidation, in line with *ad libitum* access to carbohydrate-rich chow. Thyrotoxic animals showed no difference in glucose oxidation relative to euthyroid rats, whereas hypothyroid rats showed a mild decrease in mean level of glucose oxidation relative to euthyroid (19%, $P < 0.05$) and thyrotoxic animals (23%, $P < 0.05$, Fig. 2B). Mean levels of fat oxidation were markedly (479%) increased in thyrotoxic relative to euthyroid rats ($P < 0.01$), but there was no difference in fat oxidation between hypothyroid and euthyroid rats (ns, Fig. 2C). Thus, RER showed a decrease in both thyrotoxic and (to a lesser extent) hypothyroid animals relative to euthyroid rats. However, the mechanism of this decrease was different between groups, *i.e.* a decrease of glucose oxidation in hypothyroid animals, and a pronounced increase in fat oxidation in thyrotoxic animals.

Effects of thyroid status on lipid turnover, uptake and partitioning

In order to determine how whole body alterations in fat oxidation induced by thyrotoxicosis and hypothyroidism translated into substrate (*i.e.* FA) uptake at the tissue level, we applied a dual FA-isotope infusion technique that permits differentiation between plasma TG-derived and plasma albumin-bound FA uptake.

Plasma thyroid hormones

Plasma T_3, T_4 and TSH concentrations in thyrotoxic, euthyroid and hypothyroid rats are shown in Table 3.

FA and TG plasma concentrations and rate of disappearance

Plasma FA concentrations were 118% higher in thyrotoxic relative to euthyroid rats ($P < 0.01$). Plasma TG concentrations tended to increase in thyrotoxic ($P = 0.059$) rats, and showed a significant decrease in hypothyroid ($P < 0.05$) compared with euthyroid rats (Table 3).

Rate of disappearance (Rd) of ^{14}C-FA was 59% increased in thyrotoxic relative to euthyroid rats ($P = 0.054$). There were no differences in Rd of ^{3}H-TG between groups (Table 3).

Table 3. Plasma thyroid hormone concentrations before radio-labeled FA infusion (day 11) in hypothyroid (Hypo), euthyroid (Eu) and thyrotoxic (Tox) rats. * P < 0.01 *vs.* Eu.

	Hypo n = 9	**Eu n = 8**	**Tox n = 7**
T_3 (nmol/l)	0.41 ± 0.08 *	1.21 ± 0.09	2.78 ± 0.27 *
T_4 (nmol/l)	19 ± 2 *	139 ± 7	187 ± 5 *
TSH (mU/l)	10.9 ± 1.8 *	0.2 ± 0.0	0.2 ± 0.0

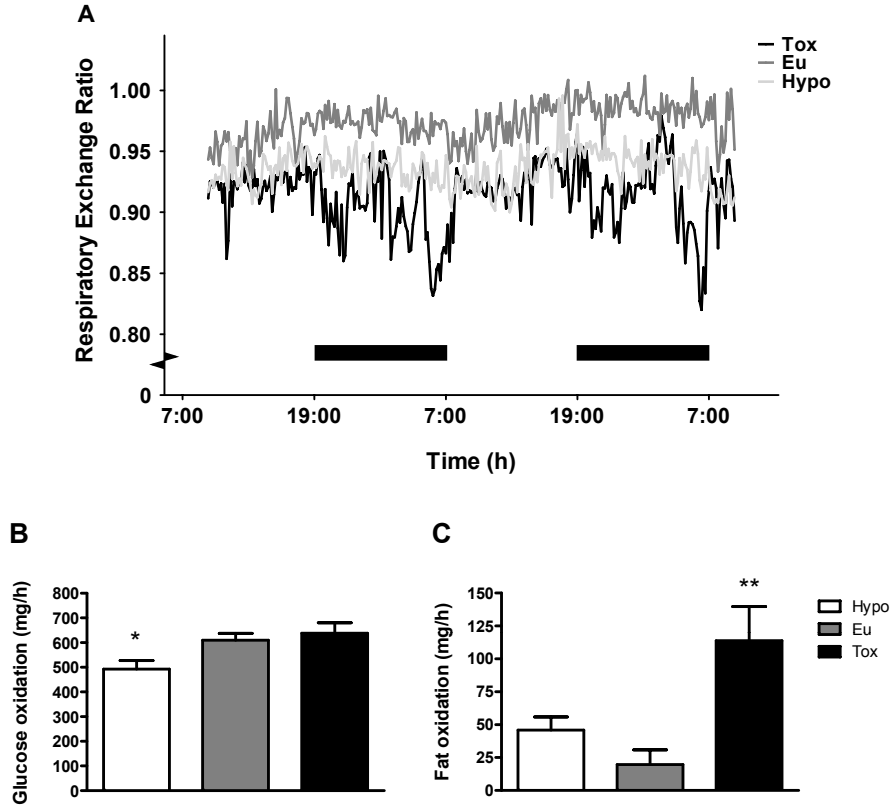

Fig. 2. Forty-eight-hour respiratory exchange ratio (RER, A) in hypothyroid (Hypo), euthyroid (Eu) and thyrotoxic (Tox) rats entrained to a regular 12/12-h L/D cycle. Horizontal black bars indicate the dark phase of the L/D cycle. Data are mean of 7 animals per group at each time point, and the interval between time points was 10 min. Cosinor data and statistical analysis are given in Table 2. Mean 48 h glucose oxidation (B) in hypothyroid (Hypo), euthyroid (Eu) and thyrotoxic (Tox) rats. Note that Hypo rats exhibit decreased glucose oxidation as compared with Eu animals (* $P < 0.05$). Mean 48 h fat oxidation (C) in hypothyroid (Hypo), euthyroid (Eu) and thyrotoxic (Tox) rats. Note that Tox rats exhibit increased fat oxidation as compared with Eu animals (** $P < 0.01$).

Tissue-specific TG-derived FA uptake, lipoprotein lipase, hepatic lipase activity and albumin-bound FA uptake

Thyrotoxicosis induced an increase of TG-derived FA uptake in striated muscle (58%, $P < 0.05$, Fig. 3A), and tended to increase TG-derived FA uptake in heart (78%, $P = 0.059$) relative to euthyroid rats, but did not induce alterations in muscle or heart LPL activity (Fig. 3B). Thyrotoxicosis induced a pronounced decrease in TG-derived FA uptake to 21% of euthyroid levels in BAT ($P < 0.01$). It should be noted that FA uptake was approximately 30-fold higher in BAT as compared to oxidative striated muscle, in line with the high mitochondrial density and high FA oxidative capacity of brown adipocytes. Thyrotoxicosis had no effect on TG-derived FA uptake in any of the WAT depots, although it induced a modest decrease in LPL activity in gonadal WAT (48%, $P < 0.05$). In contrast, hypothyroid rats showed a pronounced increase of

TG-derived FA uptake in gonadal and visceral WAT (184%, $P < 0.01$ and 75%, $P < 0.05$, respectively, Fig. 3A), associated with an increase in LPL activity both in gonadal and visceral WAT (234%, $P < 0.01$ and 306%, $P < 0.05$, respectively, Fig. 3B). In liver, hypothyroid rats showed decreased

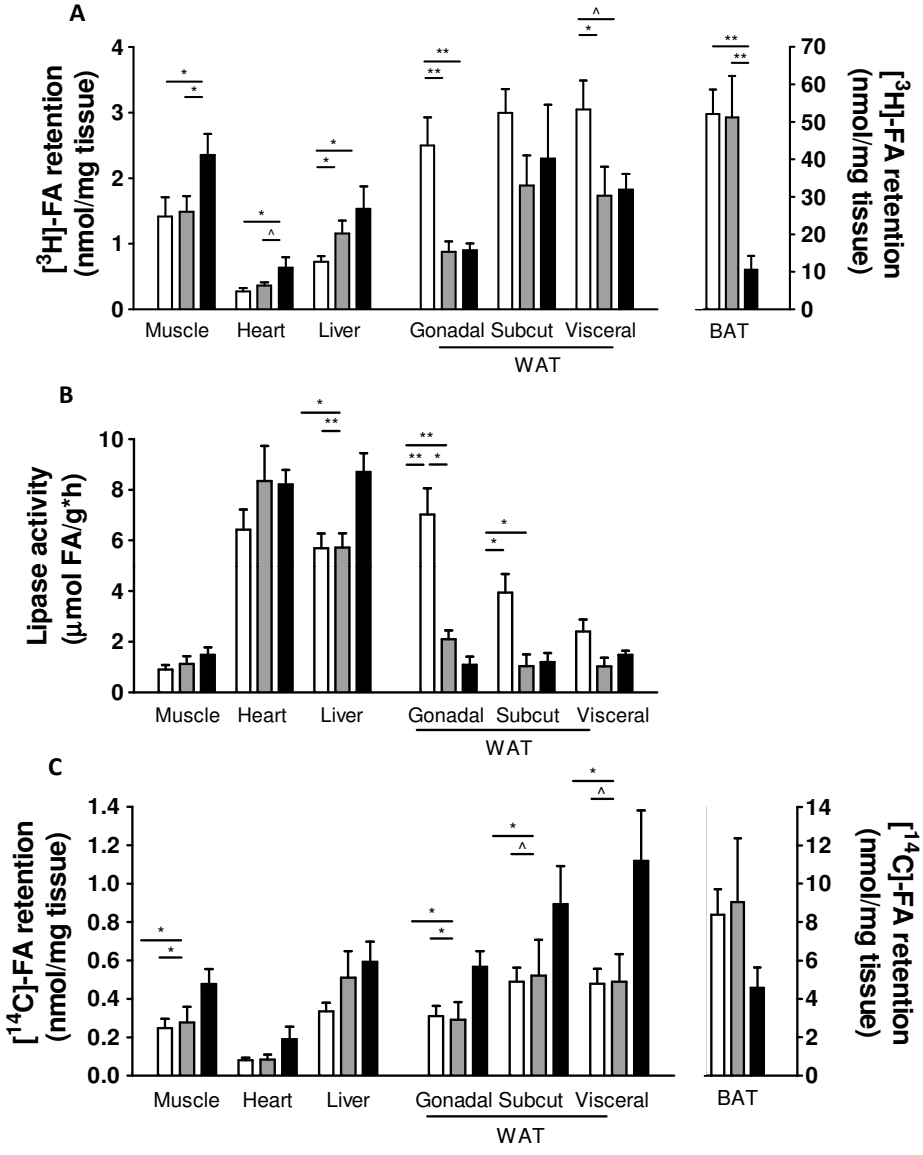

Fig. 3. Triglyceride (TG)-derived fatty acid (FA) uptake (A), lipoprotein lipase activity (B) and albumin-bound FA uptake (C) in striated muscle, heart, liver, three white adipose tissue (WAT) depots (gonadal, subcutaneous, visceral) and brown adipose tissue (BAT) of hypothyroid (Hypo, white bars), euthyroid (Eu, grey bars) and thyrotoxic (Tox, black bars) rats. ^ $0.05 < P < 0.10$, * $P \leq 0.05$, ** $P < 0.01$ vs. Eu.

TG-derived FA uptake relative to euthyroid rats (37%, $P < 0.05$), but no change in HL activity. Conversely, thyrotoxic rats showed no effect on TG-derived FA uptake but an increase in HL activity (52%, $P < 0.01$).

Thyrotoxicosis induced an increase in albumin-bound FA uptake in striated muscle (71%, $P = 0.054$, Fig. 3C), a 97% increase of albumin-bound FA uptake in gonadal WAT ($P < 0.05$), and it similarly tended to increase albumin-bound FA uptake in subcutaneous and visceral WAT (71%, $P = 0.059$ and 129%, $P = 0.059$, respectively). There was no difference in albumin-bound FA uptake between hypothyroid and euthyroid rats in any of the tissues studied.

DISCUSSION

We studied the changes in whole body energy metabolism associated with thyroid hormone status and we delineated how these changes translate into substrate (*i.e.* FA) uptake at the tissue level. Our main findings are that thyrotoxicosis induces a hypermetabolic phenotype (increased TEE, REE, and fat oxidation) as well as increased food intake favouring substrate replenishment. Interestingly, thyrotoxicosis did not increase SPA, indicating that changes in SPA do not contribute to increased TEE. Moreover, hypermetabolism was associated with increased TG-derived FA uptake in most oxidative tissues, whereas TG-derived FA uptake was unaltered in WAT. Conversely, hypothyroidism induced a hypometabolic phenotype with a mild decrease in REE, a trend towards decreased TEE, and a decrease of glucose oxidation. In addition, TG-derived FA uptake was increased in lipid storing WAT, concomitantly with increased LPL activity. However, during hypothyroidism TG-derived FA uptake in oxidative tissues was unaltered. These alterations in TG-derived FA uptake during thyrotoxicosis and hypothyroidism indicate that FA uptake from TG-rich lipoproteins is differentially regulated by thyroid hormones in a tissue-specific manner.

The mechanism of the increase of TEE induced by thyrotoxicosis is incompletely understood. It has been known for many years that REE is highly responsive to thyroid hormones (1). In addition, many thyrotoxic patients show a characteristic resting tremor and self-reported increased physical activity. Conversely, many hypothyroid patients complain of slowness (19). However, there is little experimental evidence indicating that thyroid status modulates locomotor behaviour or SPA, and it is unclear how this relates to the alterations in energy homeostasis induced by hypothyroidism and thyrotoxicosis. This is of particular interest, since accumulating evidence suggests that EE associated with SPA, termed non-exercise activity thermogenesis (NEAT), is an independent (negative) determinant of (development of) obesity in humans and rodents. As thyroid hormone is a principal regulator of energy metabolism, it may also be involved in the regulation of NEAT. The present study shows that although moderate hyperthyroidism increases TEE by 37%, it induces no alterations in SPA. In contrast, resting REE, defined as the energy expended during time periods when no activity was detected, is increased by 61% in thyrotoxic rats. Moreover, hypothyroidism induces a significant 12% decrease of REE, but it does not affect SPA either, and REE/TEE ratios are unaffected by both hypothyroidism and thyrotoxicosis. Together, our data strongly suggest that the increased energy requirements of SPA are not determined by thyroid hormone status

and do not explain increased TEE associated with thyrotoxicosisOur data are in contrast with those of Levine et al. (5) who reported increased SPA during thyrotoxicosis in rats, suggesting that NEAT was a significant component of the increase in TEE. This discrepancy is most likely explained by the pharmacological dose of T_3 used by Levine et al. to induce thyrotoxicosis, resulting in a ~13-fold increase in plasma T_3. In the present study serum T_3 was increased only 2.5-fold, which is within the range of plasma T_3 often found in patients with thyrotoxicosis. Interestingly, this was paralleled by a relatively mild, 30% increase in plasma T_4 concentrations. In thyrotoxic patients, a relative overproduction of T_3 giving rise to increased plasma T_3/T_4 ratios may be observed (20). Deiodinase type 1 (D1), which is mainly expressed in liver and kidney, is positively regulated by T_3 (21). Therefore, D1-mediated T_3 production is thought to be a major source of extra-thyroidal T_3 during hyperthyroidism (22). Indeed, the increased T_3/T_4 ratio in our rat model of thyrotoxicosis is paralleled by an induction of hepatic D1 expression (7), which may underlie the relatively mild increase of plasma T_4 relative to T_3.

Our experimental approach does not allow for measurement of other components of TEE, such as diet-induced thermogenesis (absorption, digestion and metabolism of food) and facultative thermogenesis (energy expended to maintain body temperature during cold exposure in homeothermic species). However, it is reasonable to assume that the 18% increase in 48 h cumulative food intake led to an increase of diet induced thermogenesis in thyrotoxic rats, although this component generally comprises only a minor part (~10-15%) of TEE. Facultative thermogenesis is unlikely to have played a role in our study, as it is generally negligible under thermo-neutral circumstances.

TH is known to play a role in regulating seasonal adaptations in several species, for example reproduction and maintenance of body weight (23;24), but its possible involvement in modulating rhythms of shorter phase, *i.e.* circadian rhythms, has received less attention. This possibility is theoretically supported by thyroid hormone receptor α1 mRNA expression in the region of the main circadian oscillator, *i.e.* the suprachiasmatic nuclei (SCN) (25), although this has not been confirmed at the protein-level (26;27). Previous studies have shown lack of an effect of hypothyroidism on rhythms of locomotor (wheel running) activity (28). Therefore, the subtle effects of hypothyroidism on the acrophase of the daily TEE and RER rhythms we did observe, are most likely occurring downstream of the SCN. Euthyroid rats exhibited a through in RER during the light period, fitting with relatively high fat oxidation during the inactive period when little food is consumed. The shift in acrophase of RER in thyrotoxic rats appears to be mainly due to increased fat oxidation during the nightly feeding periods (data not shown), suggesting that during thyrotoxicosis, high energy demands require mobilization of energy stores on top of the nutrients supplied by increased food intake during the active period.

Lipoprotein lipase (LPL) is the key enzyme regulating tissue-specific FA disposal by hydrolyzing triglycerides (TG) in circulating TG-rich lipoprotein particles. LPL has been proposed as a metabolic "gatekeeper" (29), directing substrate to tissues dependent on the body's metabolic status (30;31). In the present study, we explored TH effects on tissue FA uptake, and we were able to differentiate between TH effects on TG-derived (*i.e.* LPL-dependent) and albumin-bound (*i.e.* LPL-independent) FA uptake on the tissue level. In addition, we measured

tissue-specific LPL activity. In keeping with the observed hypermetabolic state associated with thyrotoxicosis, we found that thyrotoxicosis increases TG-derived FA uptake in major oxidative tissues such as striated muscle and the heart, without affecting TG-derived FA uptake in lipid-storing WAT depots. This increase in TG-derived FA uptake in oxidative tissues was not paralleled by increased local LPL activity. It has been previously reported that the linear relationship between muscle TG-derived FA uptake and LPL activity in euthyroid animals is lost after experimental alterations in thyroid status (32). This may be explained by TH effects on additional determinants of the process of TG-derived FA-uptake. In addition, TH induced stimulation of local blood flow (33;34) may have interfered with FA-uptake, independently of LPL activity.

Conversely, hypothyroidism increased TG-derived FA uptake in WAT. Indeed, earlier studies in rats have also reported increased LPL activity in WAT during hypothyroidism (35) that could be reversed by tri-iodothyronine (T_3) administration (36;37). However, hypothyroidism had no effect on TG-derived FA uptake in oxidative tissues. In the liver, hypothyroidism decreased TG-derived FA uptake but not hepatic lipase (HL) activity, whereas thyrotoxicosis increased HL activity, but not TG-derived FA uptake. Taken together, the present evidence suggests that during thyrotoxicosis, hypermetabolism and increased FA oxidation are facilitated by preferential shuttling of TG-derived FA's to oxidative tissues. Conversely, during hypothyroidism TG-derived FA's are shuttled to lipid storing WAT, away from the liver and oxidative tissues, via increased tissue-specific LPL activity.

Circulating FA that are not incorporated in TG-rich lipoprotein particles are bound to albumin in plasma. We found that thyrotoxicosis increases albumin-bound FA uptake in muscle as well as in the WAT depots, whereas hypothyroidism had no effect on albumin-bound FA uptake in any of the tissues studied. Plasma FA concentration were increased in thyrotoxic, but not in hypothyroid relative to euthyroid animals. This is in line with the notion that tissue uptake of albumin-bound FA is mainly driven by the concentration gradient between the capillary lumen and the intracellular space (30). Interestingly, in contrast to the increase in FA-uptake in oxidative tissues like striated muscle and heart, thyrotoxicosis induced a pronounced decrease of TG-derived FA uptake in BAT. BAT is the main site for adaptive thermogenesis in rodents. During cold exposure, sympathetic stimulation of BAT induces local conversion of T_4 to T_3, thereby generating heat via induction of mitochondrial uncoupling (38). Simultaneously, LPL is markedly induced via a β-adrenergic mechanism, enabling replenishment of the FA used for mitochondrial oxidation (39). During thyrotoxicosis, increased thermogenesis has been proposed to evoke a compensatory decrease in sympathetic tone to BAT (40;41). We now speculate that such a decrease in sympathetic tone may explain the marked decrease in TG-derived FA uptake in the present study, possibly via decreased LPL activity.

In conclusion, our data indicate that FA uptake from TG-rich lipoproteins is regulated by TH in a tissue-specific manner. Thyrotoxicosis increases TG-derived FA uptake in all oxidative tissues except BAT, whereas hypothyroidism increases TG-derived FA uptake in lipid storing WAT via increased LPL activity, and decreases uptake in liver. In contrast, albumin bound FA uptake during hypothyroidism and thyrotoxicosis appears to be merely mass, *i.e.* concentration gradient, driven.

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