Hypermetabolism in mice caused by the central action of an unliganded thyroid hormone receptor $\alpha 1$

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Thyroid hormone, via its nuclear receptors TR α and TR β , controls metabolism by acting locally in peripheral tissues and centrally by regulating sympathetic signaling. We have defined aporeceptor regulation of metabolism by using mice heterozygous for a mutant TR $\alpha 1$ with low affinity to T3. The animals were hypermetabolic, showing strongly reduced fat depots, hyperphagia and resistance to diet-induced obesity accompanied by induction of genes involved in glucose handling and fatty acid metabolism in liver and adipose tissues. Increased lipid mobilization and β -oxidation occurred in adipose tissues, whereas blockade of sympathetic signaling to brown adipose tissue normalized the metabolic phenotype despite a continued perturbed hormone signaling in this cell type. The results define a novel and important role for the TR $\alpha 1$ aporeceptor in governing metabolic homeostasis. Furthermore, the data demonstrate that a nuclear hormone receptor affecting sympathetic signaling can override its autonomous effects in peripheral tissues.

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Introduction

Thyroid hormone plays an important role in thermogenesis, regulation of body temperature and maintenance of metabolic homeostasis. Triiodothyronine (T3) deficiency leads to increased body weight (BW) and cold intolerance (Duntas, 2002), whereas excess T3 causes heat intolerance and weight loss as a result of increased metabolic rate (Møller *et al*, 1996). T3 acts by binding to nuclear hormone receptors encoded by the TR α and TR β genes (TR $\alpha 1$ and 2, TR $\beta 1$ and 2) (Sap *et al*, 1986; Weinberger *et al*, 1986; Koenig *et al*,

1988). Ligand-bound TRs function as holoreceptors, upregulating target genes that have a positive thyroid hormone response element and downregulating those with a negative one. Importantly, unliganded TRs act as aporeceptors that exert the opposite transcriptional effects, and thereby cause the deleterious effects associated with hypothyroidism.

T3 increases free-fatty acid (FFA) levels by enhancing lipid mobilization, resulting in increased β -oxidation. Induction of gluconeogenesis and glycogenolysis by T3 causes increased free-glucose levels and an accelerated insulin-dependent glucose transport into cells (Weinstein *et al*, 1994; Dimitriadis and Raptis, 2001). T3-bound TRs activate genes such as those for phosphoenolpyruvate carboxykinase (PEPCK), malic enzyme and acetyl CoA carboxylase (ACC) in liver, muscle, as well as in white (WAT) and brown (BAT) adipose tissue, resulting in increased metabolism (Diamant *et al*, 1972; Loose *et al*, 1985; Song *et al*, 1988; Carvalho *et al*, 1993; Zabrocka *et al*, 2006). In addition to the direct effects on gene expression in peripheral tissues, thyroid hormone affects sympathoadrenal signaling by increasing tissue responsiveness to catecholamine and decreasing sympathetic activity (Silva, 2006). The interaction between the sympathetic nervous system and thyroid hormone in adaptive thermogenesis shows that intact thyroid hormone signaling is essential for heat production and adrenergic responsiveness in BAT (Silva, 1995). This is reflected by cold or heat intolerance of animals and humans with hypo- or hyperthyroidism, respectively, and hypo- and hyperthermia in extreme forms of hypo- and hyperthyroidism (Cannon and Nedergaard, 2004; Silva, 2006).

Studies of different TR knockout mice and the use of isoform-selective agonists have shown that TR α is essential for proper thermogenesis, whereas TR β regulates cholesterol metabolism (Wikström *et al*, 1998; Johansson *et al*, 1999; Ribeiro *et al*, 2001; Gullberg *et al*, 2002; Marriif *et al*, 2005). Mice lacking all T3-binding TRs have slightly decreased body temperature but are still able to increase metabolic rate in response to cold. However, total heat production is insufficient, rendering the mice cold intolerant (Golozoubova *et al*, 2004). The observed phenotype in these mice is milder than would be expected based on the observations in hypothyroid animals, emphasizing the important role of target gene repression or activation by the aporeceptor in the pathogenesis of hypothyroidism.

The present studies provide insight into the effects of aporeceptors and differentiate between the central and the tissue autonomous effects of a TR $\alpha 1$ aporeceptor on lipid and carbohydrate metabolism. For this, we studied mice heterozygous for a dominant-negative TR $\alpha 1$ mutation (R384C) that causes a 10-fold reduction in affinity to T3 (TR $\alpha 1$ + m mice). Reactivation of the mutant TR $\alpha 1$ was achieved either by T3 treatment via drinking water, or by additional TR β deletion, which causes a 10-fold increase in serum T3 levels (Tinnikov *et al*, 2002). Suppression of thyroid hormone signaling by the mutant TR $\alpha 1$ enhanced basal metabolism by increasing

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sympathetic outflow, thus causing resistance to obesity and a lean phenotype. Tissue analyses showed that BAT is the tissue targeted by the sympathetic signaling and identified the enzymatic mechanisms responsible for the elevated energy expenditure. The results show that central effects of the unliganded TR α 1 override peripheral actions of the receptor.

Results

Reduced BW and fat depots in TR α 1 + m mice

To determine if the unliganded TR α 1 affected metabolism, tissue analyses, gene expression profiling and measurements of oxygen consumption were done. Heterozygous male TR α 1 + m mice (4–7 months old; $n = 5$ –6 per group) had a 16% reduction in BW as compared to wild-type (wt) littermate controls (Figure 1A). Analyses of tissues showed that mutant mice had a 51% reduction in their epididymal WAT (eWAT), a 26% reduction in liver and a 33% reduction in

interscapular BAT (iBAT) weight. In contrast, soleus and extensor digitorum longus (EDL) weights were normal (Supplementary data 1), as were the adult skeletal dimensions of TR α 1 + m mice (Bassett *et al*, 2007). This shows that the reduced tissue weights are associated with a lean body mass and not dwarfism.

Histological analyses showed that adipocytes in eWAT and lipid vacuoles in iBAT were smaller in mutant as compared to wt mice (Figure 1B), a striking difference that was exacerbated by a 16-h fast. The WAT capsule surrounding the iBAT showed an increased number of multi-ocular adipocytes in mutant mice, whereas wt animals had large lipid vacuoles (Figure 1B). To determine if the adipose tissue phenotype was caused by aporeceptor activity of the mutant TR α 1, we studied TR α 1 + m mice also lacking TR β . Such TR α 1 + m β – mice have a 10-fold increase in serum thyroid hormone levels that activates the mutant TR α 1 (Forrest *et al*, 1996; Tinnikov *et al*, 2002). Figure 1C shows that, as expected,

Figure 1 Metabolic phenotype of TR α 1 + m mice. (A) Body weight (BW), liver, epididymal white adipose tissue (eWAT) and interscapular brown adipose tissue (iBAT) weight in TR α 1 + m mice under fed and fasted conditions on a control diet ($n = 5$ –6 per group). Data are presented as mean $\pm$ s.e.m.; statistical differences between TR α 1 and wt mice are indicated, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (two-way ANOVA). (B) H&E staining in eWAT and iBAT of TR α 1 + m and wt mice. (C) eWAT and iBAT morphology in TR α 1 + m mice with an additional deletion of TR β . This causes an increase in T3 levels that reactivates the mutant TR α 1. Fat cell necrosis is present in +m/– and + + / + –, but not + m / + – mice. + + / + – = control mice, + m / + – = mutant mice, + m / – = mutant mice with additional TR β deletion. (D) H&E and Oil Red O staining in liver. Scale bars represent 50 μ m in iBAT and liver, 100 μ m in eWAT. (E) Increased oxygen consumption in TR α 1 + m mice.

eWAT and iBAT cell sizes were comparable to that in control animals (TR α 1 + + β + -) and that fat cell necrosis was present in both TR α 1 + m β - - mice and control animals. In addition, deletion of TR β normalized BW. Histological liver analysis showed that mice carrying the TR α 1 mutation had lower glycogen content in liver, and that this was normalized by the TR β -null allele (Figure 1D). The morphological changes in the mutant mice suggested an increased metabolism that could be resolved through reactivation of the mutant TR α 1 by high levels of thyroid hormone.

To elucidate the cause of the tissue weight differences, we determined the metabolic rate: O₂ consumption was increased by approximately 20% over an 18-h period in the mutant mice (Figure 1E). A control experiment showed that this difference was not due to increased overall locomotor activity (Supplementary data 2). The data thus indicate that the reduction in adipose tissue mass is caused by an increased metabolic rate.

Resistance to diet-induced obesity

Next, we tested if the BW of the mutant mice could be normalized by increasing their caloric intake through a high-fat diet (HFD). A 12-week treatment failed to normalize BW in the mutant mice, despite their increased caloric intake (Figure 2A–C, Supplementary data 3), indicating a resistance to diet-induced obesity. This was further supported by morphological analysis of iBAT, which showed moderate activation in the TR α 1 + m mice, whereas wt mice showed typical signs of inactive tissue such as increased adipocyte size and scattered fat cell necrosis in eWAT.

However, activation of the mutant receptor by treating the adult mice with pharmacological doses of T3 in combination

with an HFD caused a rapid increase in BW in TR α 1 + m mice and markedly reduced their resistance to diet-induced obesity (Figure 2A). In an independent experiment, we measured free T3 levels in mice that received T3 via drinking water, which revealed no difference between the groups (wt 61.6 ± 7.9 ; TR α 1 + m 68.2 ± 3.3 pmol/l). The increase in BW upon T3 treatment was paralleled by a partial normalization of caloric intake (Figure 2C). Furthermore, T3 treatment in combination with control diet (CD) normalized white and brown adipocyte morphology in the mutant mice (Figure 2D). We thus conclude that the resistance to diet-induced obesity and in part also the hyperphagia were caused by the unliganded, mutant TR α 1.

Accelerated glucose and lipid handling

To determine if carbohydrate level balancing also was affected in TR α 1 + m mice, we performed intraperitoneal glucose tolerance tests (ipGTTs). Figure 3A shows an accelerated glucose clearance, while the insulin response was unexpectedly unaltered. HFD treatment increased glucose concentrations in an ipGTT to the level of wt animals on a CD (Figure 3B). Higher substrate utilization was further supported by increased insulin-mediated glucose uptake in isolated soleus and EDL muscles of TR α 1 + m mice (Figure 3C). Under fed conditions, glucose levels were comparable in wt and mutant mice, whereas insulin tended to be lower in mutants (Supplementary data 4). The increased insulin requirement that is associated with high thyroid hormone levels was reflected by a strong elevation in insulin levels in T3-treated wt animals on an HFD, whereas the mutant mice were protected from this effect (Supplementary data 4). These data indicate that the TR α 1 + m mice have enhanced

Figure 2 Resistance to diet-induced obesity. (A) Body weight (BW) in TR α 1 + m mice increases upon T3 treatment (arrowhead indicates start of treatment). Data are presented as mean $\pm$ s.e.m.; indication of statistical differences: *wt HFD versus TR α 1 + m HFD, [§]TR α 1 + m HFD versus TR α 1 + m CD, [‡]TR α 1 + m CD versus wt CD. (B) Body fat in TR α 1 + m versus wt mice on an HFD. (C) Increased caloric intake in TR α 1 + m mice. (D) Adipose tissue morphology upon T3 treatment and CD visualized by H&E staining. Scale bars represent 100 μ m for eWAT and 50 μ m for iBAT.

Figure 3 Glucose handling. (A) Glucose clearance and insulin levels in TR α + m mice in intraperitoneal glucose tolerance test (ipGTT). (B) ipGTT in TR α 1 + m mice on a control and high-fat diet (CD and HFD). (C) Basal and insulin-stimulated glucose uptake in EDL and soleus muscle of TR α 1 + m mice. Data are presented as mean $\pm$ s.e.m.; * P < 0.05, ** P < 0.01, *** P < 0.001.

insulin sensitivity that improves glucose handling, which may be related to their reduced body fat.

Serum parameters were analyzed as a first step to identify the cause of the observed metabolic phenotype. The expected decrease in total T3 (TT3) and total T4 (TT4) in response to a 16-h fast was present, although TT3 and TT4 levels upon fasting remained somewhat higher in mutant mice (Figure 4A). Increased lipid utilization in mutant mice was reflected by lower serum FFAs, triglycerides and cholesterol, independent of diet and feeding status (Figure 4B). These differences were effectively normalized by T3 treatment (Figure 4B). β -Hydroxybutyrate levels were normal in TR α 1 + m mice, but remained lower upon fasting, a difference that was not ameliorated by T3 treatment (Figure 4B).

Figure 4 Blood hormone and lipid parameters. (A) Thyroid hormone levels. Total T3 and T4 levels in TR α 1 + m and wt mice under fed and fasted conditions. (B) Blood lipid parameters. Data are presented as mean $\pm$ s.e.m.; * P < 0.05, ** P < 0.01, *** P < 0.001.

We conclude that these results reflect an increased energy demand in TR α 1 + m mice.

To identify which tissues contributed to the observed changes in serum parameters, gene expression profiling was performed on eWAT, iBAT, liver and soleus muscle tissues. The results revealed strong induction of genes involved in lipolysis, lipogenesis and glucose handling in eWAT (Figure 5A; Supplementary data 5A and B). The effects were less pronounced in iBAT and liver, and absent in soleus muscle (Figure 5B and C; Supplementary data 5C–H). In eWAT, target gene expression reflected an increase in both lipogenesis and β -oxidation: ACC1 and fatty acid synthase (FAS) were induced six-fold and were paralleled by a four-fold induction of ACC2 (Figure 5A). In concordance with this, PGC1 α was three-fold and PPAR α four-fold increased in TR α 1 + m mice (Figure 5A), whereas no changes were observed in PPAR γ expression (Supplementary data 5A–H). Similar but less dramatic changes were seen in iBAT and liver (Figure 5B and C). In the liver, we found evidence for increased gluconeogenesis; PEPCK showed a three-fold increase (Figure 5D). GLUT4 was doubled in eWAT, but remained unaffected in iBAT (Figure 5D). A full overview of all

Figure 5 Gene expression profiles. (A–C) Relative gene expression measured by real-time PCR in fed and fasted mice on a control diet. (D) Gene expression levels indicative of glucose handling. (E) Leptin concentrations measured by ELISA in TR α 1 + m mice. (F) β -Oxidation and lipid mobilization as measured by ACC and MCD enzyme activity in epididymal WAT and interscapular BAT. ND = not determined. Data are presented as mean $\pm$ s.e.m.; * P < 0.05, ** P < 0.01, *** P < 0.001. For statistical analysis of panels A–D, see Supplementary data 5.

genes analyzed and subsequent statistical analyses are available in Supplementary data 5A–H. The differences between adipose tissues and liver may be partially related to local differences in T3 concentrations, since these tissues rely on different T3 sources. eWAT is dependent on circulating T3, whereas in liver a four-fold induction of type I deiodinase (Dio1) mRNA indicated an increase in local T4 to T3 conversion, suggesting higher local T3 concentrations that would allow partial reactivation of the mutant TR α 1 (Figure 5C). Type II deiodinase (Dio2) mRNA levels in the fed status in iBAT were similar in wt and mutant mice, whereas in the fasted status Dio2 mRNA increased approximately 12-fold in the mutants as compared to four-fold in the wt mice (Figure 5B). Our data indicate that the TR α 1 aporeceptor affects glucose and lipid handling via distinct mechanisms and that local differences in deiodination may contribute to the differences between tissues.

In view of the substantial differences in tissue weights and morphology, we performed ELISA on serum leptin levels to confirm that altered tissue mRNA levels were reflected in relevant serum changes. We confirmed that TR α 1 + m mice

have lower leptin levels, which are consistent with their increased food intake (Figure 5E). Importantly, lipid handling measured in eWAT and iBAT by enzyme activity assays revealed increased ACC activity in both eWAT and iBAT and malonyl CoA decarboxylase (MCD) activity in iBAT (Figure 5F). The increased lipid mobilization was substantiated by the increased β -oxidation seen specifically in iBAT of the mutant mice, which indicated that this tissue is responsible for the increased energy demand in TR α 1 + m mice and therefore for their lean phenotype.

Altered sympathetic outflow

Intriguingly, the observed lean phenotype resembles a state of hyper- rather than hypothyroidism. We therefore tested the possibility that increased sympathetic signaling would be overriding the effects of the TR α 1 aporeceptor at the tissue level. Since iBAT showed the most hallmarks of hypermetabolism, 8-week-old mice were acclimated to 30°C to inhibit sympathetic stimulation of facultative thermogenesis. Figure 6A shows that this resulted in normalized eWAT and iBAT weights in mutant mice, although body and liver

Figure 6 Functional denervation of BAT through rearing at 30°C. **(A)** Normalization of epididymal WAT and interscapular BAT weight. **(B)** Decreased food intake and oxygen consumption. **(C)** Body weight increase in mice reared at 30°C. **(D)** Normalized gene expression in eWAT as measured by real-time PCR. **(E)** H&E staining shows uniform white adipocyte sizes, while brown adipocytes are characterized by a single large lipid droplet, suggesting inactivity. Scale bars represent 100 μ m for eWAT and 50 μ m for iBAT. Data are presented as mean $\pm$ s.e.m.; * P < 0.05, ** P < 0.01, *** P < 0.001.

weights remained lower. Food intake was decreased below wt levels and O₂ consumption was normalized (Figure 6B). The mutant mice gained weight rapidly (Figure 6C), which was associated with normalization of gene expression levels of ACC, CPT1 β , PGC1 α , PPAR α and GLUT4 in eWAT (Figure 6D). The adipose tissue morphology of the mutant mice normalized during the acclimation to 30°C (Figure 6E).

To study if the facultative thermogenesis in BAT was responsive to a norepinephrine (NE) challenge, the ability of TR α 1 + m mice to defend their body temperature was tested. Both in a short-term (1.5 h) and long-term (6 h) experiment, TR α 1 + m mice successfully defended their body temperature (Figure 7A and B). However, the mutant mice had a significant increase in their lower critical temperature (LCT) (which is the ambient temperature below which basal metabolic rate becomes insufficient to balance heat loss), and the defended body temperature was higher (37°C versus 36°C in wt), as calculated by extrapolation of the temperature defense curve (Figure 7A). Furthermore, their body temperature, when kept at 21°C, was approximately

1°C lower than normal (37.3°C versus 38.2°C in wt mice; Figure 7B). This contrasts the subsequent iBAT gene expression analysis. The mutant mice that were exposed to cold for 6 h showed reduced UCP1 and PGC1 α mRNA levels, whereas Dio2 and mitochondrial transcription factor 1 (TFAM1) were normal (Figure 7C). This demonstrates a minor but significant impairment in iBAT function of TR α 1 + m mice, which is in agreement with their lower body temperature despite the increased calculated theoretical defended body temperature.

BAT sensitivity to β -adrenergic receptor-induced thermogenesis was studied by measuring O₂ consumption after injection of NE. We found that the increase in O₂ consumption caused by NE was delayed in TR α 1 + m mice, even though the O₂ consumption during basal thermogenesis and the maximum response to NE were unaltered (Figure 7D, left panel). The NE challenge was subsequently performed in mice acclimated to 30°C to exclude that increased basal sympathetic tone in mutant mice affected the response to acute NE stimulation. Surprisingly, the BAT response to NE was even further impaired in these mutant mice (Figure 7D,

Figure 7 Sympathoadrenal signaling. (A) TR α 1 + m mice have increased lower critical temperature (LCT) and a higher theoretical defended body temperature (arrows). (B) Body temperature at 4°C. (C) Gene expression measured by real-time PCR in iBAT of mice that were kept at 4°C for 6 h. Data are presented as mean $\pm$ s.e.m.; * P < 0.05, ** P < 0.01. (D) NE challenge in mice reared at 21°C and mice reared at 30°C.

right panel). In addition, TR α 1 + m mice acclimated to 30°C showed lower O₂ consumption during basal thermogenesis, which is in agreement with the lower O₂ consumption in TR α 1 + m mice above the LCT. Our data thus indicate that impaired sympathetic signaling in the BAT due to the mutant TR α 1 is partially compensated through increased basal sympathetic tone.

Discussion

In the present study, we show that, contrary to expectation, the mutant TR α 1R384C with aporeceptor activity causes hypermetabolism associated with increased O₂ consumption, hyperphagia and resistance to diet-induced obesity. Treatment with thyroid hormone has two effects since the mutant TR α 1 represses wt TR α 1 and TR β function: (i) reactivation of the mutant TR α 1 and (ii) restoration of wt TR α and TR β signaling. Thus, it is unlikely that the normalization, as observed by additional deletion of TR β , can be ascribed to the absence of the β receptor *per se*. Rather, the rescue of the metabolic phenotype results from the subsequent 10-fold elevation in thyroid hormone levels. The demonstration that reactivating the aporeceptor with high levels of T3 administered via the drinking water ameliorated the hypermetabolic phenotype in TR α 1 + m mice with intact TR β alleles confirms that the phenotype was caused by the unliganded TR α 1.

The evidence for BAT being the tissue responsible for the high energy expenditure rests on several observations: the histology indicated a metabolic activation, the ACC and MCD expression levels and enzyme assays pointed to prominent activities, and the high β -oxidation seen in BAT but not in WAT or liver (unpublished) further supported the concept. The normalization by acclimation to 30°C firmly established BAT as the tissue responsible for the high energy expenditure, and indicates that the mutant TR α 1 mediates the hypermetabolism by interfering with sympathetic signaling. Thus, our findings suggest that a TR α 1 aporeceptor acting centrally can predominate over its antagonistic effects in peripheral tissues. It is possible that similar mechanisms of action should be considered when studying the effects of other nuclear hormone receptors on peripheral lipid and glucose metabolism.

The data also allow the interpretation of why the other tissues examined exhibited signs of elevated metabolism. High activity in BAT will first deplete the local energy stores, as was evident in our histological analyses of BAT tissue. Secondly, lipid stores in WAT will be mobilized, requiring elevated lipogenesis and the type of increases in expression of, for example, ACC1, FAS, LPL and HSL we observed; eventually, WAT content in the animal will decrease. That both the liver and the serum were devoid of or had reduced lipids is likely to be a further consequence of supplying BAT with energy.

The mutant mice also showed an increased glucose tolerance. Muscle had a rapid uptake of glucose, whereas in particular the liver overexpressed genes involved in glucose handling and lacked histological signs of glycogen. Interestingly, the liver weights failed to normalize and the expression of Dio1 remained high after acclimation to 30°C, whereas the lipid and glycogen stores were similar to those of controls (unpublished). An interpretation of this is that carbohydrate stores also become depleted by the metabolically active BAT. However, the failure of the liver to fully normalize indicates either a residual, tissue autonomous effect of the mutant TR α 1 or that it affects the size of the liver during development.

In a previous report (Tinnikov *et al*, 2002), we described normal adipose content in 7 to 8-week-old TR α 1 + m mice, which contrasts the results obtained in this study

using 4 to 7-month-old animals. The distinct results may be due to the fact that the mutant mice have a delayed postnatal development and reach full maturation 2–4 weeks later than wt controls, but may also reflect genotype-specific changes in metabolism that occur during aging. The developmental delay affects, for example, weight gain, bone mineralization, eye opening (Tinnikov *et al*, 2002) as well as brain maturation (K Wallis, M Sjögren and B Vennström, unpublished data). It is thus possible that the defective neuronal development also contributes to aberrant central signaling and subsequent hypermetabolism.

Hypermetabolism in TR α 1 mice: the result of increased sympathetic signaling

Obligatory thermogenesis is sufficient to sustain core temperature over a limited range of environmental temperatures, below which facultative thermogenesis is required to stay warm. Hence, cold exposure elicits a complex response marked by increases in thermogenic capacity of BAT, oxygen consumption and food intake. The thermogenic response in BAT is induced by sympathetic stimulation resulting in NE release, which acts synergistically with T3 (Silva, 2006). Increased sympathetic activity causes increased local T3 concentrations via induction of Dio2, brown adipocyte proliferation, increased UCP1 expression and activity and mitochondrial biogenesis, a process known as recruitment (Cannon and Nedergaard, 2004). Morphological analyses of adipose tissues of TR α 1 + m mice indicated interconversion of WAT into BAT, which was in accordance with BAT activation reflected by increased PGC1 α , LPL and Dio2 mRNA expression upon fasting. However, there were no differences in UCP1 expression, or in TFAM or NRF1, which are involved in mitochondrial biogenesis. These data are in line with observations in mice lacking all thyroid hormone receptors that are able to recruit BAT and have normal UCP1 expression (Golozoubova *et al*, 2004).

Fatty acid synthesis and oxidation occur in separate cell compartments to prevent immediate oxidation of newly synthesized fatty acids. ACC1 converts acetyl CoA into a malonyl CoA pool, which serves as a substrate for fatty acid elongation by FAS, whereas the malonyl CoA produced by ACC2 serves as a potent inhibitor of CPT1 and thus of β -oxidation (Abu-Elheiga *et al*, 2001). Upregulation of both fatty acid synthesis and β -oxidation is stimulated by sympathetic signaling and occurs during cold exposure, when fatty acid synthesis secures fat stores to provide fuel for heating the body during continued periods of increased β -oxidation. The underlying mechanism may be explained by distinct intracellular malonyl CoA pools and selective increased malonyl CoA turnover caused by increased MCD activity, resulting in increased CPT1 activity (Yu *et al*, 2002). The increased levels of ACC1, ACC2 and FAS mRNA and increased MCD activity and β -oxidation in TR α 1 + m mice are in accordance with such a state of increased sympathetic signaling.

At thermoneutrality, when obligatory thermogenesis is able to meet the body requirements to maintain body temperature, TR α 1 + m mice have a lower metabolic rate (Figure 7), which is congruent with a receptor-mediated hypothyroidism. However, at lower ambient temperatures, TR α 1 + m mice had an increased metabolic rate, which necessitated a study of their thermoregulation. Previously, TR isoform-specific actions were identified in facultative

thermogenesis: transcriptional induction of UCP1 requires TR β , whereas TR α is essential for the synergistic effect between sympathetic signaling and thyroid hormone action (Ribeiro *et al*, 2001). Upon a 6-h cold exposure, the TR α 1 + m mice failed to increase UCP1 and PGC1 α expression levels to the same extent as wt mice. This may be due to interference by the mutant TR α 1 with TR β function in BAT. The ability of TR α 1 + m mice to successfully defend their body temperature, despite impaired UCP1 stimulation, indicates that the TR α 1 + m mice rely in part on different mechanisms to maintain body temperature, possibly shivering thermogenesis.

The defective sympathetic stimulation of facultative thermogenesis observed in hypothyroid mice (Ribeiro *et al*, 2001) was also present in TR α 1 + m mice, as was reflected by their delayed increase in O₂ consumption in response to an NE challenge. The O₂ response was even further impaired in mice acclimated to 30°C, suggesting that increased sympathetic outflow at 21°C is part of a compensatory mechanism to keep body temperature sufficiently high. In addition, the lower body temperature observed at 21°C was in contrast with the higher theoretical defended body temperature. This discrepancy suggests that facultative thermogenesis is unable to produce enough heat through uncoupling to reach the defended body temperature. This is likely caused by a BAT-specific effect of the mutant TR α 1, causing increased hypothalamic outflow that would stimulate thermogenesis through alternative mechanisms and causing energy expenditure through a futile cycle.

Metabolic characteristics of TR α 1-mediated resistance to thyroid hormone

More than 300 patient families with resistance to thyroid hormone syndrome, RTH, have been found. The patients have an inherited mutation in the TR β gene (Weiss and Refetoff, 1996, 2000). However, no patients harboring an equivalent mutation in the TR α gene have been described. This may be related to the absence of obvious aberrancies in thyroid hormone levels, unlike those found in the RTH patients.

Based on the results presented here, a hypothesis regarding the metabolic characteristics of patients with a TR α 1 mutation indicates hypermetabolism. However, the phenotypes of two other mouse strains with mutant TR α 1 genes differ from what is described here: dwarfism accompanied by reduction in WAT by the TR α 1PV allele (Liu *et al*, 2003; Ying *et al*, 2007), or obesity in combination with impaired catecholamine-stimulated lipolysis by the TR α 1P398H mutant (Liu *et al*, 2003; Ying *et al*, 2007). In cultured WAT cells, the TR α 1PV mutant was described to repress the ability of PPAR γ to activate its target genes, and the expression of ACC, FAS and PPAR γ was found to be reduced in the mutant animals, in contrast to what was seen with the TR α 1R384C allele in our study. The TR α 1P398H mutation strongly inhibits liver PPAR α expression and reduces expression of genes involved in fatty acid oxidation by interfering with PPAR α action (Liu *et al*, 2007), whereas TR α 1R384C mutation led to a strong induction of PPAR α gene expression, no change in PPAR γ levels and overexpression of genes involved in lipid handling. Taken together, this indicates that the position of a mutation may determine how the mutant receptor interacts with

different hormonal response elements, other nuclear receptors and/or their coregulators.

Nevertheless, our results as well as those by Liu *et al* (2007) support the concept that the TR α 1R384C receptor is akin to a wt aporeceptor: it confers on the CPT1 α and ACO response elements the same moderate PPAR α -interfering properties as wt TR α 1 (Liu *et al*, 2007), and its activation by high levels of T3 leads to normalization of the hypermetabolism as well as most other phenotypic aberrancies (Tinnikov *et al*, 2002; Venero *et al*, 2005; Bassett *et al*, 2007). The normalization of metabolism by acclimation to 30°C furthermore argues against mechanisms involving, for example, constitutive binding by TR α 1R384C to the coactivator PCG1 α or the corepressor RIP140, events that could lead to a phenotype very similar to the one we have described.

Hypothalamic signaling

The dramatic improvement of the metabolic phenotype by functional denervation of sympathetic signaling to the BAT points to the importance of the hypothalamus in regulating metabolic rate and the ability of central signaling to override peripheral effects. Intriguingly, increased sympathetic outflow resembles a state of hyperthyroidism, rather than of hypothyroidism. Discrepancies between peripheral and central thyroid hormone signaling have also been reported during critical illness in humans and fasting in rodents (Lechan and Fekete, 2004, 2006; Fliers *et al*, 2006). During severe illness, thyroid hormone levels decrease without giving rise to high levels of TSH or TRH, and the effects are mediated by the hypothalamus (Fliers *et al*, 1997; Wiersinga, 2000). During fasting and in experimental models of critical illness, a relative state of hyperthyroidism is present in the hypothalamus as a result of increased local deiodination (Diano *et al*, 1998; Boelen *et al*, 2004; Fekete *et al*, 2004; Coppola *et al*, 2005). It is feasible that the mutant TR α 1 is involved in regulating hypothalamic T3 levels under these conditions and thereby affects sympathetic outflow to BAT and thus could contribute to the hypermetabolic phenotype. The possibility that altered hypothalamic signaling also can contribute to metabolic wasting, such as that observed in cancer cachexia, merits further study.

Materials and methods

Animals

The mouse strain carrying the dominant-negative R384C mutation in TR α 1 and the combination with a TR β -null allele have been described previously (Tinnikov *et al*, 2002).

The TR α 1R384C mice used in the experiments here had been backcrossed to C57BL/6Ncrl for 3–4 or 8–10 generations. Experiments done in both cohorts produced similar results. Littermate male mutant and wt mice aged 4–7 months were kept at 21°C on a 12 h light/12 h dark cycle. For thermoneutrality studies, mice were transferred to 30°C at the age of 2 months and kept at this temperature for at least 4 weeks. Control and HFD mice were obtained from Research Diets (New Brunswick, NJ) (D12450B: 3.85 kcal/g, 10% kcal fat; D12451: 4.73 kcal/g, 45% kcal fat). Animal care procedures were in accordance with the guidelines set by the European Community Council Directives (86/609/EEC). Required animal permissions were obtained from the local ethical committees.

Thyroid hormone treatment and serum parameter measurements

For determination of serum parameters, fed or overnight fasted (16 h) animals were killed by decapitation (after 11 weeks on diet, when applicable), after which trunk blood was collected and tissues dissected for further analyses. Serum was obtained after centrifugation of blood samples and stored at –80°C until assayed for serum parameters.

Animals were placed on control or HFD, and BW and food intake were determined weekly. Animals received T3 for 12 days via drinking water (0.01% albumin, T3 concentration 0.5 µg/ml). TT3 and TT4 were measured by radioimmuno assay (TKT31 and TKT41; Diagnostic Products Corporation, Los Angeles, CA). FFA, triglycerides, cholesterol, β -hydroxybutyrate and insulin were assayed according to the manufacturer's instructions (FFA: Wako Chemicals GmbH, Neuss, Germany; triglycerides: Sigma Diagnostics Inc., St Louis, MO; cholesterol and β -hydroxybutyrate: STANBIO Laboratory, Boerne, TX; insulin: Mercodia AB, Uppsala, Sweden).

Oxygen consumption and thermoregulatory metabolism

O₂ consumption was measured using the OxyMax System (Columbus Instruments, Columbus, OH) or the Somic Inca System (Somedic Sales AB, Hörby, Sweden). To determine the thermoregulatory metabolism in the mutant TR α 1 mice, O₂ consumption was studied as a function of ambient temperature. Measurements were performed at temperatures ranging between 7 and 34°C during a 1.5-h period. Metabolic rates at the different temperatures were defined as the lowest, stable metabolic rate observed for at least 4 min. A minimum interval of 3 days was present between measurements at different temperatures. For the NE challenge studies, mice were anesthetized with sodium pentobarbital (70 mg/kg BW) and injected with (NE 1 mg/kg BW). Defense of body temperature over a 6-h period was tested by measurements using a rectal probe with a 1-h interval ($n = 6$ mice per group).

Glucose handling

2-Deoxyglucose uptake was measured on isolated EDL (fast-twitch glycolytic) and soleus (slow-twitch oxidative) muscles as described elsewhere (Shashkin *et al*, 1995). For ipGTT, glucose was injected (2 g/kg BW: 20% solution) after a 16-h fast. For determination of glucose levels, blood samples were obtained from the tail at 0, 15, 30, 60 and 120 min after injection and analyzed using an Accu-Chek Sensor glucose meter (Roche Diagnostics). For insulin levels, blood samples were taken at 0, 30, 60 and 120 min after glucose injection.

Histology

Tissues were fixed in 10% formalin rinsed in PBS, dehydrated through increasing concentrations of ethanol, cleared and embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). To demonstrate intracellular lipids, formalin-fixed tissue was cryoprotected with sucrose, and frozen sections were stained with Oil Red O.

Real-time PCR

RNA was isolated from snap-frozen tissues using an RNeasy mini kit or RNeasy lipid kit (Qiagen, Sweden) according to the manufacturer's instructions. cDNA was obtained after reverse transcription and used for real-time PCR using the ABI 7300 system and the ABI Prism 7000 (Applied Biosystems, Sweden). Quantification was performed using a standard curve and HPRT was used as a reference gene. Primer sequences are listed in Supplementary data 6.

β -Oxidation and enzyme activity assays

Fat tissue (eWAT and iBAT) was incubated in low-glucose DMEM (Gibco, Sweden) containing 2% (w/v) fatty acid-free BSA, 0.30 mM L-carnitine and ³H-palmitic acid (3 µCi per well). Excess palmitic acid was removed by trichloroacetic acid precipitation. After extraction with chloroform/methanol (2:1), ³H₂O production was determined (Wang *et al*, 2003). ACC activity was determined by an NADH-coupled assay and normalized to protein content (Wagner *et al*, 1998). Background phosphatase activity was determined in samples without acetyl CoA and used for correction. MCD activity was determined using a carnitine acetyltransferase-linked assay (Antinozzi *et al*, 1998).

Statistical analysis

Prism 4 for Macintosh and In Stat 3 for Macintosh software was used for statistical analysis. Data were analyzed using the Student's *t*-test or two-way ANOVA followed by a Bonferroni or Tukey test to compare between groups. Differences were considered significant if *P* < 0.05. All data are represented as their mean value $\pm$ s.e.m.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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