Thyroid hormone regulation of Sirtuin 1 expression and implications to integrated responses in fasted mice

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Abstract

Sirtuin 1 (SIRT1), a NAD⁺-dependent deacetylase, has been connected to beneficial effects elicited by calorie restriction. Physiological adaptation to starvation requires higher activity of SIRT1 and also the suppression of thyroid hormone (TH) action to achieve energy conservation. Here, we tested the hypothesis that those two events are correlated and that TH may be a regulator of SIRT1 expression. Forty-eight-hour fasting mice exhibited reduced serum TH and increased SIRT1 protein content in liver and brown adipose tissue (BAT), and physiological thyroxine replacement prevented or attenuated the increment of SIRT1 in liver and BAT of fasted mice. Hypothyroid mice exhibited increased liver SIRT1 protein, while hyperthyroid ones showed decreased SIRT1 in liver and BAT. In the liver, decreased protein is accompanied by reduced SIRT1 activity and no alteration in its mRNA. Hyperthyroid and hypothyroid mice exhibited increases and decreases in food intake and body weight gain respectively. Food-restricted hyperthyroid animals (pair-fed to euthyroid group) exhibited liver and BAT SIRT1 protein levels intermediary between euthyroid and hyperthyroid mice fed *ad libitum*. Mice with TH resistance at the liver presented increased hepatic SIRT1 protein and activity, with no alteration in *Sirt1* mRNA. These results suggest that TH decreases SIRT1 protein, directly and indirectly, via food ingestion control and, in the liver, this reduction involves TR β . The SIRT1 reduction induced by TH has important implication to integrated metabolic responses to fasting, as the increase in SIRT1 protein requires the fasting-associated suppression of TH serum levels.

Key Words

- Sirtuin1 (SIRT1)
- thyroid hormones
- fasting
- calorie restriction
- thyroid hormone receptor β

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Introduction

Calorie deprivation has been proved to extend life span and reduce the rate of aging in several organisms, from yeast to rodents and primates (Weindruch & Walford

1982, Lin *et al.* 2000, Colman *et al.* 2009). It was described in some species that the mechanism by which calorie restriction promotes extended life span was dependent on

silent information regulator 2 (Sirtuin 1 (Sir2)) proteins (Lin *et al.* 2000, Rogina & Helfand 2004). However, a recent report described that the apparent life span extension effect in both *Caenorhabditis elegans* and *Drosophila* was independent of Sir2 (Burnett *et al.* 2011). In mammals, a similar role has been postulated for the Sir2 homolog, SIRT1, because of important regulatory actions on metabolism (Allard *et al.* 2008, Boily *et al.* 2008, Redman *et al.* 2011).

The SIRT1 protein is a NAD⁺-dependent deacetylase that removes acetyl groups from acetyllysine-modified proteins, such as histone (Vaquero *et al.* 2004) and non-histone proteins, like nuclear transcription factors (Vaziri *et al.* 2001, Nemoto *et al.* 2005, Li *et al.* 2007) and also cytosolic enzymes (Hallows *et al.* 2006, Mattagajasingh *et al.* 2007, Akieda-Asai *et al.* 2010). The deacetylation of histones provokes repression of gene transcription, whereas SIRT1 action on transcription factors and enzymes results in modulation of their functions. As the deacetylation mechanism requires NAD⁺, the enzyme activity is influenced by cytosolic NAD⁺/NADH ratio perturbations (Tanny & Moazed 2001), connecting SIRT1 activity to cellular metabolism.

SIRT1 is expressed in several tissues, such as liver, adipose tissues, muscle, pancreas, pituitary, and hypothalamus. Tissue-specific deletion or overexpression of SIRT1, pharmacological activators of SIRT1, and *in vitro* approaches have been used to clarify the roles of SIRT1 in each cell type. SIRT1 has been associated with decreased white fat depot accumulation (Picard *et al.* 2004), increased hepatic and muscular lipid catabolism (Feige *et al.* 2008, Purushotham *et al.* 2009), increased gluconeogenesis (Rodgers & Puigserver 2007), and improvement of insulin sensitivity (Sun *et al.* 2007, Feige *et al.* 2008, Pfluger *et al.* 2008). These punctual contributions are extremely valuable to understand the essential role of SIRT1 in adaptation to calorie restriction and fasting, conditions that are able to trigger SIRT1 protein expression in various tissues (Cohen *et al.* 2004, Kanfi *et al.* 2008), by still unknown mechanisms. On the contrary, consumption of an excessive high-fat diet is generally accompanied by reduced SIRT1 levels (Deng *et al.* 2007), and, if SIRT1 expression or activity is restored, adverse effects induced by high-fat diet ingestion are attenuated (Baur *et al.* 2006, Feige *et al.* 2008, Pfluger *et al.* 2008). However, at this point in time, our knowledge on SIRT1 protein expression regulation is limited.

Similar to SIRT1, thyroid hormones (THs) are major regulators of fuel metabolism (Yen 2001). Tri-iodothyronine (T₃) acts mainly through nuclear

TH receptors (TR), which modulate target genes' expression (Lazar 2003). Nuclear proteins also participate in this process, such as peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α), a cofactor involved in both TH and SIRT1 effects (Rodgers *et al.* 2005, Attia *et al.* 2010). Beyond that, confronting SIRT1 and TH actions, we will come across common targets, especially in lipid metabolism (Ness *et al.* 1990, Rodgers *et al.* 2005, Hashimoto *et al.* 2006, Li *et al.* 2007).

Moreover, TH is a major determinant of the basal metabolic rate of cells, accelerating metabolic processes that lead to increases in energy expenditure (Kim 2008). It is very well established that physiological adaptation to fasting involves the suppression of TH production, as a mechanism to conserve energy (Boelen *et al.* 2008). In addition, recent studies have shown the fasting-induced rise in SIRT1 expression in several tissues (Rodgers *et al.* 2005, Kanfi *et al.* 2008). Therefore, the existence of common metabolic routes regulated by TH and SIRT1, together with the inverse correlation of these factors' variation during calorie deprivation, led us to investigate whether SIRT1 expression is regulated by TH in mice and the possible interactions of TH and SIRT1 during energy deprivation.

Materials and methods

Animal care and treatments

All procedures were approved by Ethics Committee on Animal Care of the Health Sciences Center, Federal University of Rio de Janeiro (protocol: IBCCF 120). The regulation followed by the local committee is endorsed by a federal Brazilian law and is in accordance with the guidelines published in 'ARRIVE' 'Care and Use of Animals'. Animals were maintained in a controlled temperature room, with 12 h light:12 h darkness cycles (lights on from 0700 till 1900 h), and experiments were performed when male mice (mixed background strain 129Svj/C57BL/6) reached ~3 months of age. Mice were killed by decapitation after CO₂ sedation, and liver and brown adipose tissue (BAT) tissues were excised and stored at -70 °C until total RNA and protein extraction procedures. Serum was obtained from trunk blood and kept frozen at -20 °C for measurements of hormones and cholesterol.

Fasting and thyroxine reposition The fasting protocol consisted of submitting the animals to 48-h starvation. Some animals were only exposed to fasting, while others also received s.c. injections of thyroxine (T₄-reposition dose: 0.75 μ g/100 g body weight per day, Sigma; for 2 days,

starting 8 h after food withdrawal). Fasting mice and T₄-replaced fasting mice were compared to mice fed chow *ad libitum*, and all animals that did not receive T₄ reposition were submitted to saline injections to mimic stress.

Hypo- and hyperthyroidism models Mice were rendered hypo- or hyperthyroid with specific protocols. Hypothyroidism was induced by feeding animals a diet containing 0.15% 5-propyl-2-thiouracil (PTU, Sigma) for 28 days and hyperthyroidism was induced by daily s.c. injections of T₃ (supraphysiological dose: 50 µg/100 g body weight per day, Sigma) for 14 days.

Hyperthyroidism and food restriction A pair-fed protocol was performed using euthyroid and hyperthyroid mice. Serum TH excess promotes increased food intake, thus one group of hyperthyroid mice was pair-fed to euthyroid ones, named hyperthyroid-restricted. And, for comparison, one group of euthyroid animals was submitted to the same percentage of food restriction as the hyperthyroid-restricted group and called euthyroid restricted. Hyperthyroidism was induced by daily s.c. injections of T₃ (50 µg/100 g body weight per day) for 13 days, which started simultaneously to the pair-fed protocol. The amount of food offered for the hyperthyroid group was the same as the mean food intake of the euthyroid group on the previous day. Mean food ingestion of euthyroid and hyperthyroid mice was significantly different from the fifth day on T₃ treatment.

Transgenic TRβΔ337T model In addition, we analyzed a transgenic mice model bearing a dominant negative mutation (Δ337T) in TRβ, described earlier (Hashimoto *et al.* 2006). The mutant TRβ is unable to interact with T₃ but can bind to DNA and to nuclear cofactors. As TRβ participates actively in the hormonal feedback regulation, these mice are resistant to TH and exhibit elevated serum T₃ and T₄. Homozygous mice used in this study were acquired by heterozygous mating pairs, and the genotype was determined by analysis of DNA from the tail employing PCR, using the following primers: 5'-ATGGGGAAATGGCAGTGAGAG (match), 5'-ATGGG-GAAATGGCAGTGACAC (mismatch) and 3'-AGCACACT-CACCTGAAGACAT (out) (Santiago *et al.* 2011). Direct comparisons were made with littermate controls (WT).

Sirt1 mRNA and protein analysis

Total RNA was isolated from liver using Trizol reagent (Invitrogen Corp.). Subsequently, 1 µg of total RNA was

submitted to reverse transcription using Superscript III kit (Invitrogen Corp.). Products were amplified on Applied Biosystems 7500 Real-Time PCR System (Life Technologies Co.) using iQ SYBR Green Supermix. Cycle parameters were 50 °C for 2 min and 95 °C for 10 min, followed by 44 cycles at 95 °C for 15 s, 53 °C for 30 s, and 72 °C for 45 s.

The intron spanning primers used in this manuscript have been described earlier by our own group and by others (Rodgers *et al.* 2005, Akamine *et al.* 2007, Machado *et al.* 2009, Paula *et al.* 2010, Faustino *et al.* 2011, 2012, Santiago *et al.* 2011). *Sirt1* primers: forward, 5'-CAG-GTT-GCA-GGA-ATC-CAA-A-3'; and reverse, 5'-CAA-ATC-AGG-CAA-GAT-GCT-GT-3' (Rodgers *et al.* 2005). 36B4 (Rplp0, Genbank MGI:1927636) primer was used as control, forward: 5'-CCG-AGG-CAA-CAG-TTG-GGT-A-3' and reverse: 5'-TGT-TTG-ACA-ACG-GCA-GCA-TTT-3' (Akamine *et al.* 2007). Primers used for the amplification of cDNAs of interest were synthesized by Integrated DNA Technologies, Inc, Coralville, IO, USA. One hundred nanograms of RNA of each sample were evaluated in duplicate in the same assay.

Efficiency of each reaction was calculated using a serial dilution and varied from 94% for SIRT1 to 98% for 36B4, and products' purity was confirmed by agarose gel analysis. Both primer sets were verified to provide a single peak on a melting curve analysis. Relative mRNA expression was calculated by comparing the PCR cycle threshold (C_t) between the groups, after correcting for 36B4 using the ΔΔC_t method (Livak & Schmittgen 2001). Results are expressed relative to values of control group, which was set to 1.

Protein expression was determined by western blotting. Liver and BAT samples were homogenized in lyses buffer (pH 6.4; 50 mM HEPES, 1 mM MgCl₂, 10 mM EDTA, and 1% Triton X) with protease inhibitor cocktail Complete (Roche/DSM Nutritional). Thirty micrograms of total protein extract of each sample were resolved by SDS-PAGE on 10% gel and transferred onto a polyvinylidene difluoride membrane (Westran; Whatman, Maidstone, Kent, UK). The membrane was blocked with 5% nonfat dry milk (Molico; Nestle, São Paulo, SP, Brazil) and incubated overnight at room temperature with anti-SIRT1 polyclonal antibody (1:500 dilution; Santa Cruz Biotechnology) and anti-cyclophilin antibody (1:70 000 dilution for liver and 1:6000 for BAT, Affinity Bioreagents, Golden, CO, USA), used as internal control. Membranes were then washed and incubated with peroxidase-labeled anti-rabbit IgG antibody (1:7500 dilution; Amersham Biosciences) for 3 h at room temperature. All blots were then washed and incubated with a luminogen

detection reagent (ECL; Amersham Biosciences) for further exposure on an autoradiograph film (Kodak). Protein bands were evaluated by densitometry using the software Kodak 1D3.5. The membranes were stained with Rouge Ponceau and submitted to densitometry analysis. Densities of protein bands were normalized for that of cyclophilin and also for that of Ponceau staining and they were expressed in relation to control groups. Both methods of loading correction gave the same results.

SIRT1 activity

SIRT1 activity was measured in liver samples, processed to obtain nuclear extract as described earlier (Setsuie *et al.* 2009). Briefly, frozen livers were ground into powder using liquid nitrogen and suspended in lysis buffer (10 mM Tris-HCl, pH 7.5; 10 mM NaCl; 15 mM MgCl₂; 250 mM sucrose; 0.5% Nonidet P-40; and 0.1 mM EGTA) in the absence of protease inhibitor. This preparation was submitted to a sucrose cushion (30% sucrose; 10 mM Tris-HCl, pH 7.5; 10 mM NaCl; and 3 mM MgCl₂) and centrifuged at 1300 *g* for 10 min. The pellet was washed once (10 mM Tris-HCl, pH 7.5) and suspended in extraction buffer (50 mM HEPES-KOH, pH 7.5; 420 mM NaCl; 0.5 mM EDTA; 0.1 mM EGTA; and 10% glycerol). The suspension was centrifuged at 15 000 *g* for 10 min, and supernatant was stored in -70°C . Forty micrograms of nuclear extracts were used for Sirt1 activity analysis using commercial kit CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay (MBL International, Woburn, MA, USA), following manufacturer's recommendations. The assay was based on SIRT1 ability to deacetylate a fluorometric substrate, in the presence of NAD⁺, and the reaction product was detected using a fluorescence plate reader (Viktor X2: excitation, 355 nm; emission, 460 nm). Measurements were recorded every 2 min, and the 30-min record was used for comparisons.

Serum measurements

Serum T₃ and T₄ concentrations were determined using a commercial RIA kit (ICN Pharmaceuticals, Costa Mesa, CA, USA) according to the manufacturer's instructions. Serum TSH was measured using a specific mouse TSH RIA, with reagents acquired from the National Hormone and Pituitary Program (Torrance, CA, USA), as detailed previously (Oliveira *et al.* 2006). The minimum assay detection value was 25 ng/dl for T₃, 1 µg/dl for T₄, and 30 ng/ml for TSH. The samples that were compared were analyzed in the same assay and the intra-assay variation

was <6%. Serum cholesterol was determined using commercial kit (Applied Biosystems) following the recommendations of the manufacturer.

Statistical analyses

Results are presented as mean $\pm$ S.E.M. One-way ANOVA, followed by the Student–Newman–Keuls multiple comparisons test, was used for comparisons between three or more groups. The Student's *t*-test was used for comparisons between two groups (data from the mutant TR mice). For TSH analysis, nonparametric tests were employed: Kruskal–Wallis or Mann–Whitney test. The analysis was performed using the software GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). Differences were considered to be significant at $P < 0.05$.

Results

Increased SIRT1 expression induced by fasting depends on TH suppression

As fasting is associated with suppression of TH secretion and to increased SIRT1 protein expression, we wondered about a possible relationship between these two events. Expectedly, 48 h-fasted mice presented lower serum TH levels related to fed animals, T₄ concentration was below detection level, and serum T₃ was reduced by 26% (Fig. 1D, $P < 0.01$). As shown in Fig. 1A, compared with fed mice, SIRT1 protein content was 21% higher in the liver of 48-h-fasted mice ($P < 0.005$), whereas T₄ replacement in physiological dose prevented the fasting-induced increment in SIRT1 protein levels. SIRT1 activity mirrored the protein expression profile, being increased by 28% in fasted animals (Fig. 1C, $P < 0.01$) and similar to fed mice in the liver of fasted mice replaced with T₄. These results indicate that hepatic SIRT1 protein expression induced by fasting is dependent on the decline of serum TH. In BAT, fasting promoted a 2.6-fold increase in SIRT1 expression (Fig. 1B, $P < 0.01$), and T₄-replaced fasting mice presented intermediary values of SIRT1 expression, between fed and fasted groups, being statistically different from both. Thus, BAT SIRT1 protein regulation in fasting is partly dependent on reduction of TH levels, but other mechanisms may contribute.

TH negatively regulates SIRT1 protein expression and activity

To further investigate the role of TH in the regulation of SIRT1 expression, we studied hypothyroid and

Figure 1

Increased SIRT1 expression induced by fasting depends on TH suppression. (A) Liver and (B) BAT SIRT1 protein expression in fed, 48-h-fasted (F48), and T₄-replaced fasted (F48 h + T₄) mice. Representative autoradiograph of SIRT1 protein expression (~120 kDa) and cyclophilin (19 kDa) obtained by western blotting analysis. The dotted line means that one neighbor lane was excluded. Data from fasted and T₄-replaced fasted groups are relative

to the fed group; * $P < 0.005$ vs fed group and # $P < 0.005$ vs T₄-replaced fasted group respectively, $n = 8$ mice per group. (C) SIRT1 enzymatic activity of liver nuclear extract samples. Data from fasted and T₄-replaced fasted groups are relative to fed group, presented as mean $\pm$ s.e.m. $P < 0.01$, $n = 4$ mice per group. (D) Serum T₃ and T₄ concentration. ND, no detectable values. * $P < 0.01$ vs fed group, $n = 8$ mice per group.

hyperthyroid mice. As depicted in Table 1, serum concentrations of TH and TSH, as well as the evaluated physiological parameters, were consistent with pharmacological modification of thyroid status. In hypothyroid mice, total serum T₃ was reduced by 52%, T₄ concentration was undetectable, and TSH levels were 81-fold higher than in euthyroid group. T₃-treated mice exhibited T₃ concentrations about three times higher than the euthyroid group, while T₄ and TSH were below the detection limit. As expected, serum cholesterol levels were increased in hypothyroid mice and decreased in

hyperthyroid ones. Food intake was diminished in hypothyroidism (~33%) and augmented in hyperthyroidism (~25%) in relation to the euthyroid group. The body weight gain during treatment was about 25% for hyperthyroid, 10% for euthyroid mice, and 0.01% for hypothyroid group.

SIRT1 protein content in liver and BAT of hypo- and hyperthyroid mice are depicted in Fig. 2. Liver SIRT1 protein content was increased in hypothyroid and decreased in hyperthyroid mice related to euthyroid ones (Fig. 2A, $P < 0.0001$). SIRT1 expression was

Table 1 Physiological parameters of hypo-, eu-, and hyper-thyroid mice. Data are presented as mean $\pm$ S.E.M.

	HYPO	HYPO EU	HYPER	P value
T ₃ (ng/dl)	56.74 $\pm$ 5.66 ^a	107.8 $\pm$ 4.32	352.8 $\pm$ 35.96 ^a	<0.001 ^a
T ₄ (μ g/dl)	ND	1.55 $\pm$ 0.15	ND	–
TSH (ng/ml)	8.713 $\pm$ 10 ^a	107.5 $\pm$ 10.7	–	<0.0001 ^a
Cholesterol (mg/dl)	141.50 $\pm$ 3.63 ^a	127.30 $\pm$ 3.63	98.17 $\pm$ 4.85 ^a	<0.0001 ^a
Food intake (g/day)	4.03 $\pm$ 0.19 ^a	6.11 $\pm$ 0.22	7.62 $\pm$ 0.60 ^a	<0.001 ^a
Body weight variation (g)	–0.03 $\pm$ 0.65 ^a	2.34 $\pm$ 0.84	5.88 $\pm$ 0.33 ^a	<0.0001 ^a

HYPO, hypothyroid; HYPO EU, euthyroid; HYPER, hyperthyroid.

^avs euthyroid. *n* = 8–9 mice per group.

diminished in BAT of hyperthyroid animals when compared with euthyroid and hypothyroid groups (Fig. 2D, *P* < 0.01). Therefore, the protein expression of SIRT1 was downregulated by TH in tissues such as liver and BAT and upregulated by TH deficiency in the liver. As hepatic SIRT1 expression was differently altered in the absence and excess of TH, we also verified a significant negative correlation between SIRT1 content in the liver and serum T₃ levels (data not shown, *r* = –0.78, *P* < 0.001). Furthermore, as shown in Fig. 2B, liver SIRT1 activity mirrored the protein content, being significantly higher in hypothyroid than in hyperthyroid mice (*P* < 0.05).

To have some insight into the mechanism underlying the regulation of SIRT1 expression by TH, we proceeded with the investigation at the mRNA level. Liver *Sirt1* mRNA was not significantly modified by hypo- or hyperthyroidism (Fig. 2C).

Both TH level alterations and their secondary effect on food ingestion have a role in SIRT1 regulation

Hyperthyroidism and hypothyroidism were associated with increased and decreased food intake and body weight respectively (Table 1). Therefore, to evaluate the participation of an indirect effect of ingestion and weight gain variations on SIRT1 expression, we studied pair-fed groups. Mice were submitted to daily saline or T₃ injections simultaneously to pair-fed protocol. As depicted in Fig. 3C, euthyroid-restricted mice exhibited decreased serum T₃ (*P* < 0.005), while T₄ concentration reduction did not reach statistical significance. As expected, serum T₃ was increased and T₄ levels were undetectable in hyperthyroid (T₃-treated) mice and food restriction did not alter the hormonal profile of hyperthyroid mice. The hyperthyroid mice's food ingestion was about 40% higher than that of euthyroid ones (Fig. 3B, *P* < 0.0001). The hyperthyroid-restricted group received the same amount of chow as the euthyroid mice, and euthyroid-restricted group was submitted to the same percentage of

chow restriction (40%). Hyperthyroid mice gained weight from the beginning till the end of the treatment (11% increase), while food restriction in hyperthyroid and euthyroid mice induced a similar weight loss of about 18 and 13% respectively (Fig. 3A, *P* < 0.0001 vs free-feeding controls). Euthyroid-restricted mice exhibited no alteration in liver SIRT1 content in relation to euthyroid free-fed mice. As expected, liver and BAT SIRT1 expression was decreased in hyperthyroid mice with free access to food (Fig. 3D and E, *P* < 0.01). However, hyperthyroid animals pair-fed to euthyroid ones presented intermediary expression between hyperthyroid and euthyroid groups, not being statistically different from any of them. These data indicate that TH-induced increases in food ingestion and body weight are only partly responsible for the SIRT1 protein expression regulation observed in hyperthyroid mice.

SIRT1 regulation by TH involves TR β

Considering that TR β is the major TR isoform expressed in the liver, with an essential role in the regulation of hepatic metabolism, we reasoned that TR β might participate in SIRT1 regulation. Therefore, we employed an animal model of transgenic mice that possesses the germline mutation TR β Δ 337T, which abrogates the ability of the receptor to bind T₃, leading to resistance to TH action. Because of the resistance to TH at hypothalamus and pituitary, these animals have high serum TH (Fig. 4D, *P* < 0.0001), but even so, the liver protein content of SIRT1 in homozygous mutant mice has the same pattern as in hypothyroid animals, being 26% increased in comparison with WT mice (Fig. 4A, *P* < 0.05). SIRT1 activity was also 40% augmented in homozygous mice (Fig. 4B, *P* < 0.05) and, at the mRNA level, *Sirt1* expression was similar between groups (Fig. 4C). These results are consistent with the hypothesis that SIRT1 protein expression is downregulated by TH in the liver by a mechanism involving TR β .

Figure 2

TH negatively regulates SIRT1 protein expression and activity. (A) Liver and (D) BAT SIRT1 protein expression in hypothyroid (HYPO), euthyroid (EU), and hyperthyroid (HYPER) mice. Representative autoradiograph of SIRT1 protein expression (~120 kDa) and cyclophilin (19 kDa) obtained by western blotting analysis. The dotted lines mean that one lane on each side was excluded. Data on SIRT1 expression analyzed by densitometry are expressed relative to

euthyroid group. * $P < 0.005$ vs euthyroid group, $n = 6-12$ mice per group. (B) SIRT1 enzymatic activity in liver nuclear extract samples. # $P < 0.05$, hypothyroid group vs hyperthyroid group, $n = 7-9$ mice per group. (C) Liver relative expression of *Sirt1* mRNA (normalized by 36B4, as internal control). Data from hypothyroid and hyperthyroid groups are relative to euthyroid group, presented as mean $\pm$ S.E.M. $n = 5-8$ mice per group.

Discussion

SIRT1 and THs are major regulators of the metabolism in response to energy availability. This study shows that increased TH levels in mice reduce SIRT1 protein

expression in liver and BAT and that fasting-induced suppression of TH axis plays a major role in the concomitant elevation of SIRT1.

The physiological relevance of the negative regulation of SIRT1 protein abundance by TH can be envisaged by

Figure 3

TH alterations and secondary effects on ingestion have a role in SIRT1 regulation. Physiological parameters and hepatic SIRT1 protein expression of euthyroid and hyperthyroid animals with free access to food (EU and HYPER) or submitted to 40% food restriction (EU+R and HYPER+R). (A) Body weight variation during the experimental protocol. * $P < 0.0001$ vs euthyroid group, $n = 5-9$ mice per group. (B) Cumulative food ingestion. * $P < 0.0001$ vs euthyroid group, $n = 5-9$ mice per group. (C) Serum T₃ and T₄

concentrations. ND, no detectable values. * $P < 0.005$ vs euthyroid group. (D) Liver and (E) BAT SIRT1 protein expression. Representative autoradiograph of SIRT1 protein expression (~120 kDa) and cyclophilin (CYCLO; 19 kDa) obtained by western blotting analysis, and data on SIRT1 expression analyzed by densitometry are expressed relative to euthyroid group and presented as mean $\pm$ s.e.m. * $P < 0.01$ vs euthyroid group, $n = 5-9$ mice per group.

the results on experiments in fasting mice. Nutritional limitation is accompanied by activation of specific metabolic pathways, which ensure adaptation and survival to these conditions. We observed that 48-h-fasting induced decreased T₃ and T₄ levels and increased hepatic SIRT1 content. Other reports in the literature have already described, independently, fasting-induced decreased TH levels (Diano *et al.* 1998, Hashimoto *et al.* 2001) and hepatic SIRT1 content increment (Rodgers *et al.* 2005, Kanfi *et al.* 2008). Reduced TH concentration serves to conserve energy, as these hormones are important

stimulators of basal metabolism and thermogenesis (Klieverik *et al.* 2009). Simultaneously, increased SIRT1 activity and decreased TH action avoid metabolic processes that consume ATP, such as lipogenesis and other anabolic pathways (Oppenheimer *et al.* 1991, Picard *et al.* 2004). Concomitantly, SIRT1 increases gluconeogenesis rates and mobilizes other energy sources, avoiding hypoglycemia (Rodgers & Puigserver 2007). Here, we demonstrated that TH replacement in a fasting condition prevented SIRT1 protein increment observed in liver of fasted mice. This result indicates that TH suppression

Figure 4

SIRT1 regulation by TH in liver involves TR β , but it is not at a transcriptional level. (A) Hepatic SIRT1 protein expression in WT and homozygous TR β Δ 337T mutant mice (TR β Δ 337T unable to bind T₃). Representative autoradiograph of SIRT1 protein expression (~120 kDa) and cyclophilin (CYCLO; 19 kDa) obtained by western blotting analysis, and data on SIRT1 expression analyzed by densitometry are expressed relative to WT. **P* < 0.05 vs WT group, *n* = 4 mice per group. (B) SIRT1 enzymatic activity analyzed in

liver nuclear extract samples. **P* < 0.05 vs WT group, *n* = 6–7 mice per group. (C) Relative expression of *Sirt1* mRNA (normalized by 36B4, as internal control) from liver samples. Data from homozygous TR β Δ 337T group are relative to WT group, presented as mean $\pm$ S.E.M. *n* = 6–8 mice per group. (D) Serum T₃ and T₄ concentration of WT and TR β Δ 337T mutant mice. **P* < 0.0001 vs WT group, *n* = 5 mice per group.

during fasting is required for the upregulation of SIRT1 in the liver. We also observed the same regulatory pattern for BAT, another important metabolic tissue, which SIRT1 expression response to fasting was attenuated by TH replacement. To the best of our knowledge, there is no previous study of SIRT1 protein in BAT during fasting. It is possible that SIRT1 in BAT, similar to its action in white adipose tissue, may induce lipid oxidation pathways. Therefore, the data suggest that the suppression of thyroid

function during fasting is responsible in great part for the induction of SIRT1 expression in this condition.

In support to this hypothesis, our study shows that excess or deficiency of TH modifies SIRT1 expression in mice. Hyperthyroid mice exhibited lower SIRT1 protein content in liver and BAT, while hypothyroid mice presented higher SIRT1 protein expression in the liver, suggesting that TH regulation of SIRT1 protein expression exhibits tissue specificities. In the liver, even though

the magnitude of the variation was small, it was observed, consistently, in the classical pattern of a negative TH-regulated target protein: decreased protein expression in the presence of TH excess and increased protein content in TH deficiency. However, the mechanism underlying the regulation of SIRT1 protein content by TH is not clear, and the unchanged steady levels of *Sirt1* mRNA do not support a transcriptional mechanism. Other reports had demonstrated altered levels of the protein in the presence of unchanged *Sirt1* mRNA expression (Kanfi *et al.* 2008, Cakir *et al.* 2009), and post-transcriptional and post-translational mechanisms have been shown to be involved (Zschoernig & Mahlknecht 2008, Gao *et al.* 2011). However, in our study, we are not able to exclude the possibility that *Sirt1* mRNA level might have preceded the significant increase in SIRT1 protein, as we did the analysis just by the end of the hypo- and hyperthyroidism induction protocols.

TH controls body weight gain by stimulating food ingestion and energy expenditure (Kong *et al.* 2004, Klieverik *et al.* 2009). In rodents, hypothyroidism is frequently associated with decreased food intake and lower body weight gain, whereas the opposite phenotype is observed in moderate hyperthyroid mice or rats (Karakoc *et al.* 2004, Hernandez *et al.* 2007, Calvino *et al.* 2012). In our experiment, hypothyroid mice lost body weight possibly by its lower food intake, which was not due to a unpalatable PTU diet, as other hypothyroid mice models induced by methimazole and potassium perchlorate also achieved body weight loss (Hernandez *et al.* 2007). On the contrary, in our hyperthyroid model, T₃ stimulus on food intake might have exceeded the stimulus on energy expenditure and by this, mice gained weight, which was also observed by others (Kong *et al.* 2004, Hernandez *et al.* 2007). As SIRT1 expression may vary according to nutrient availability (Cohen *et al.* 2004, Kanfi *et al.* 2008), we questioned whether decreased SIRT1 content observed in hyperthyroidism was a direct effect of TH, or whether it was secondary to increased food ingestion and body weight gain induced by the hormone excess. Hyperthyroid mice submitted to food restriction, receiving an amount of chow equalized to the mean consumption of euthyroid group, exhibited attenuation of the decrease in SIRT1 expression induced by TH, suggesting a role for the higher ingestion and body weight in the SIRT1 repression observed in hyperthyroid mice. However, the same percentage of food restriction and body weight lost in euthyroid mice did not change SIRT1 protein levels, which suggests that thyroid and nutritional status, in conjunction, regulate SIRT1 protein levels in the liver and BAT.

Calorie restriction-based studies have been reported to increase SIRT1 protein levels in several tissues (Cohen *et al.* 2004); even though in the liver, the opposite regulation has also been proposed (Chen *et al.* 2008). However, the experimental paradigms vary in duration and intensity of food restriction leading to particular adaptation mechanisms (Wang *et al.* 2006). In our study, 2 weeks of 40% food restriction of euthyroid mice did not result in significant changes in liver or BAT SIRT1. Others, employing the same percentage of food restriction, but for a longer period of time, found either increases or decreases in liver SIRT1 protein levels (Cohen *et al.* 2004, Chen *et al.* 2008), and, as far as we know, there is no report on SIRT1 variations on BAT during food restriction. Additionally, serum THs are also expected to reduce in response to food restriction protocols (Araujo *et al.* 2009), although there is disagreement among reports that have shown decreased or unaltered TH levels (Araujo *et al.* 2009, Luvizotto *et al.* 2010), which seems to be related not only to duration but to intensity of food deprivation. In our study, serum T₃ was decreased, but T₄ reduction did not reach statistical significance. Therefore, the short-term duration of food restriction (2 weeks) may explain the unaltered levels of liver and BAT SIRT1 and the borderline suppression of thyroid function.

Our data suggest that TR β seems to participate in the mechanism by which SIRT1 is downregulated by TH as mice bearing the Δ 337T TR β , unable to bind T₃, exhibited increased liver SIRT1 protein content, even in the presence of high serum TH levels, and characterizing a phenotype similar to TH deficiency. Hence, unbound TR β may interfere in cellular SIRT1 protein abundance, but the mechanisms involved remain to be elucidated. As *Sirt1* mRNA levels were unchanged, we wondered about the involvement of non-transcriptional mechanisms. It has already been demonstrated that proteasomes participate in hypothalamic regulation of SIRT1 protein levels exerted by nutrient availability (Sasaki *et al.* 2010). Beyond that, other reports described the importance of unbound TR β in stabilizing cytoplasmic proteins and, by contrast, when TR β interacts with T₃, proteasomal degradation was no longer avoided (Furuya *et al.* 2007, Guigon *et al.* 2008). Therefore, it is possible that unbounded TR β may reduce SIRT1 protein degradation rates; however, more experiments are necessary to give support to this hypothesis. Another possibility is that the increased T₃ levels in mutant TR β mice could generate an augmented 3,5-diiodothyronine (T₂) pool, which has been demonstrated to be able to increase SIRT1 activity (de Lange *et al.* 2011).

Other pieces of evidence suggest the interplay between the actions of SIRT1 and TH. Transgenic mice with moderate overexpression of SIRT1 (SirBACO) and hypothyroid mice have a phenotype of energy conservation, exhibiting lower energy expenditure, lower body temperature, and lower food intake (Banks *et al.* 2008), although, to the best of our knowledge, the TH levels were not measured in those animals. On the other hand, SRT1720, a SIRT1-specific activator that attenuated the deleterious effects of high-fat diet intake, mostly by triggering lipid oxidation in metabolic active tissues, as skeletal muscle, liver, and BAT (Feige *et al.* 2008), and, specifically in BAT, SRT1720 were also able to increase TR α and TR β mRNA expression (Feige *et al.* 2008). In addition, both T₂ and TRC150094, a pharmacological functional analog of T₂, reduced deleterious metabolic effects of high-fat diet in rats, and the mechanism may be related to their ability to increase SIRT1 activity in the liver (Cioffi *et al.* 2010, de Lange *et al.* 2011). Interestingly, pituitary SIRT1 is preferentially co-localized with TSH cells and SIRT1 overexpression and knockdown in pituitary cells resulted in increased and decreased TSH secretion respectively (Akieda-Asai *et al.* 2010). These data, in conjunction with ours in this study, strongly suggest that TH signaling pathways and SIRT1 seem to have physiologically important relationships in the control of energy homeostasis.

In conclusion, our study points to TH as an important negative regulator of SIRT1 protein expression, and moreover, the study reveals a new role for TH suppression during fasting, which is to allow the upregulation of SIRT1, with important consequences to the integrated responses in adaptation to fasting.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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