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ORIGINAL PAPER

Uncoupling protein-3 as a molecular determinant of the action of 3,5,3′-triiodothyronine on energy metabolism

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Abstract Thyroid hormones are known to stimulate thermogenesis in rodents by exerting a permissive effect on norepinephrine that affects uncoupling protein-1 (UCP1) expression in brown adipose tissue (BAT). The aim of this study was to identify new targets of the thermogenic effects of T3 in tissues other than the BAT, such as skeletal muscle. In $\beta_1/\beta_2/\beta_3$-adrenoceptor knockout (β-less) mice, that are dramatically cold intolerant, a normal body temperature was maintained throughout 48 h of cold exposure by T3 administration. In these mice, BAT UCP1 protein expression was not modified either by cold exposure or by T3 administration. To test the possibility that T3 might act via muscle uncoupling protein-3 (UCP3), an UCP3 knockout (KO) model was used. This model exhibited a normal phenotype except that, upon T3 administration, stimulated oxygen consumption of the UCP3KO mice was significantly lower by 6% than that of the wild-type (WT) mice. This difference was observed only during the dark period (between 7.00 p.m. and 7.00 a.m.), i.e. when the mice are the most active at consuming food. Therefore, UCP3 might participate in the correction by T3 of the dramatic cold intolerance of the β-less mice. These results reactivate the idea that UCP3 might play a role in the control of energy balance.

Keywords β-less · UCP3KO · Mice · T3 · Thermogenesis

Introduction

Rodent brown adipose tissue (BAT), because it contains a unique mitochondrial uncoupling protein, termed uncoupling protein-1 (UCP1), is considered the main effector of adaptive thermogenesis. Cold exposure, via a stimulation of the BAT sympathetic nervous system activity, increases mitochondriogenesis, UCP1 expression and activity as well as the size and number of brown adipocytes resulting in a phenomenon known as cold-induced thermogenesis [1, 2].

Studies on hypothyroid rodents have shown that the active form of thyroid hormones, 3,5,3′-triiodothyronine (T3), exerts a permissive role on norepinephrine effect in the BAT and is therefore necessary for a full thermogenic response to cold exposure [3, 4]. The conversion of 3,5,3′,5′-tetraiodothyronine (T4) to T3 by type II 5′-deiodinase (D2) is the unique source of intracellular T3 in the BAT. The α- and $\beta_1/\beta_2/\beta_3$-adrenoceptors, stimulated by the sympathetic nervous system, act in a synergistic fashion to stimulate the mRNA expression and the activity of D2 [5–7]. Therefore, optimal UCP1 response to the cold requires a conversion of T4 to T3 in the BAT [5, 8, 9].

$\beta_1/\beta_2/\beta_3$-adrenoceptor knockout (β-less) mice have been created which were found to exhibit a normophagic obesity and a dramatic cold intolerance [10, 11]. BAT from these animals showed a total inhibition of the cold-induced

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induction of UCP1 protein expression [10, 11] and of D2 activity [11]. The latter observation suggested that the BAT of the β-less mice should not be able to respond to thyroid hormones.

The uncoupling protein-3 (UCP3) owes its name to its close sequence homology with the canonical UCP1. UCP3 expression is restricted to the skeletal muscle and the brown fat in the rodents and it was believed that it could play an important role in energy balance [12]. UCP3 knockout (KO) mice have been generated [13–16]. The mitochondria of their skeletal muscle displayed increased coupling of respiration to ADP availability (State 3/State 4 ratio) [14] and decreased proton leak [13, 15] as compared to those of the wild-type (WT) mice. Despite this uncoupling of oxidative phosphorylation in their muscle, the UCP3KO mice did not display phenotypic abnormalities of their energy balance. In particular, their body weights, both on a chow or a high fat diet, their exercise tolerance and their cold-induced thermogenesis were normal [13, 14]. The only exception was the observation that a long-term high fat diet induced a higher accumulation of intramuscular triglycerides in UCP3KO than in WT mice [16]. Mice with an overexpression of UCP3 protein in their skeletal muscle (UCP3-tg) generated by several laboratories [17–20] were all found to be obesity resistant. The UCP3-tg mice of Tiraby et al. [19] in which UCP3 overexpression was of moderate amplitude and restricted to the muscle glycolytic fibres revealed an uncoupling of oxidative phosphorylation and sex-specific changes in insulin sensitivity. The UCP3-tg models suggest that UCP3 should still be considered as a potential player in the control of energy balance.

There are striking data in the literature showing the privileged relationship between UCP3 thermogenic activity and thyroid hormones. T3 treatment, which increased UCP3 expression in skeletal muscle, induced a decrease in mitochondrial energy coupling in vivo in this tissue [21]. Furthermore, a study on hypothyroid rats given a single injection of T3 demonstrated an increase in UCP3 expression paralleled by an uncoupling of isolated mitochondria oxidative phosphorylation in skeletal muscle as well as an increase in the resting metabolic rate. The three responses were strictly correlated in terms of time course [22]. Finally, it has recently been shown that the increase in body temperature induced by the amphetamine-type stimulant ecstasy is strongly decreased in UCP3KO mice, suggesting that UCP3 is the molecular mediator of this response [23]. Furthermore, it has been shown that thyroid hormones can modulate the induction by amphetamine-like drugs of UCP3-dependent thermogenesis [24, 25].

The aim of this study was to test the hypothesis that skeletal muscle UCP3 is a molecular determinant of the effect of T3 on energy balance. For that purpose, two models of transgenic mice generated in our laboratory were used. First, the β-less mice [10] which allowed, in the absence of a BAT reactivity, to study a possible effect of T3 on thermogenesis and second, UCP3KO mice which allowed to quantify the part played by UCP3 in the effect of T3 on oxygen consumption.

Results

β-less mice

We have previously shown that the basal colonic temperature of our β-less mice was 1.2°C lower than that of the WT mice. During the first 24 h of a cold exposure (6°C), the colonic temperature of 10 out of 13 β-less mice fell below 34°C, and after 48 h of cold exposure, only 3 out of the 13 β-less mice survived, exhibiting a colonic temperature about 4°C lower than that of the WT mice [10]. In this study, the possible effect of T3 on the β-less mouse cold intolerance was tested. WT and β-less mice were treated with an intraperitoneal injection of T3 (0.5 µg/g of body weight) immediately before cold exposure and then three times at intervals of 12 h. Surprisingly, β-less mice exposed to 6°C under the exact same conditions as those previously reported [10], but injected with T3, survived to more than 55 h and maintained a colonic temperature which was either similar or at a maximum 1°C lower than that of the cold-exposed WT mice injected with T3 (Fig. 1). The body temperature of the cold-exposed β-less mice treated with T3 was mostly equivalent to that of cold-exposed WT mice without T3 treatment [10].

In order to test if T3 could act by itself, in the absence of β-adrenergic signalling, on BAT UCP1 expression, the amount of UCP1 protein was measured in the BAT mitochondria of WT or β-less mice exposed to 6°C for 48 h with or without T3 treatment. The amount of UCP1 protein

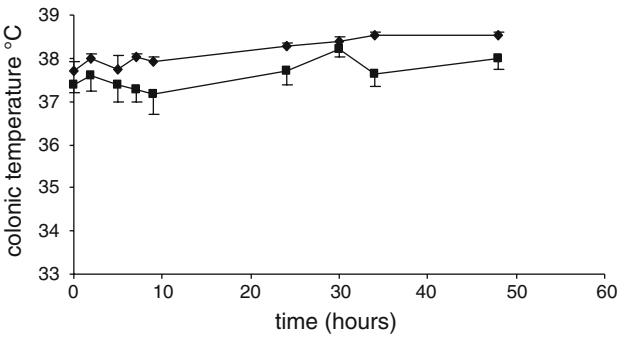

Fig. 1 Thermoregulation during exposure to 6°C of WT (◆) and β-less (■) 3-month-old female mice injected intraperitoneally with 0.5 µg/g of body weight of T3 at the times indicated in "Materials and methods". The results are the colonic temperatures expressed in °C at various periods of cold exposure. They are the means ± SEM, $n = 5$

(expressed per 50 μg of mitochondria proteins) was decreased by 18% in the β-less as compared to the WT mouse BAT. Cold exposure increased 2.3-fold the amount of UCP1 protein in the WT mouse BAT, whereas it had no effect in the β-less mouse BAT. As a result, UCP1 protein level was higher (3.3-fold) in WT than in β-less cold-exposed mouse BAT. T3 treatment decreased by 63% the amount of UCP1 protein in the WT mouse but had no effect in the β-less mouse BAT (Fig. 2).

It was interesting to test the hypothesis that T3 treatment might act via an effect on the skeletal muscle. We observed that, in the muscle of β-less mice exposed to 6°C for 12 h, T3 treatment increased the amount of UCP3 protein (expressed per 50 μg of muscle homogenate proteins) 1.3-fold (Fig. 3).

UCP3KO mice

To test the hypothesis that the T3-induced increase in muscle UCP3 expression could be a determinant of the effect of T3 on thermogenesis, an UCP3KO model was used.

UCP3KO mice were fertile and their progeny displayed a classical sex ratio of 50% males and 50% females. When UCP3KO mice were crossbred with WT mice, the allele distribution revealed a Mendelian-type transmission.

To analyse our model, we performed a phenotyping centred on energy metabolism of 2-month-old female WT and UCP3KO mice fed a standard laboratory chow diet. The food intake and body weight were similar in UCP3KO and WT mice (Fig. 4a, b). Circulating free fatty acids, leptin, glucose and insulin levels, measured after a 2 h of fast, were also similar in UCP3KO and WT mice.

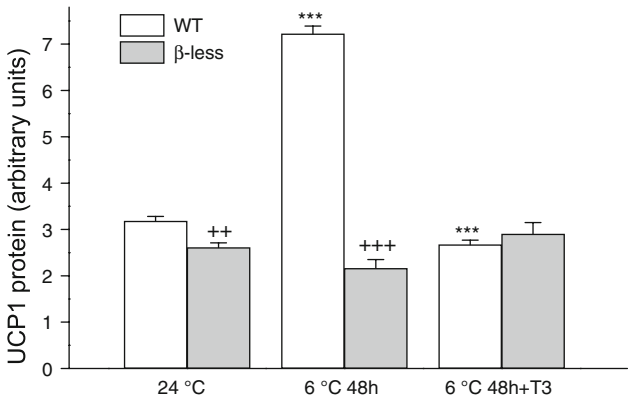

Fig. 2 Western blot quantification of the UCP1 protein/50 μg of BAT mitochondria of WT and β-less 3-month-old female mice, maintained at 24°C or exposed to 6°C for 48 h. The cold-exposed mice were injected or not intraperitoneally with 0.5 μg/g of body weight of T3 (+T3) at the times indicated in "Materials and methods". The results are expressed in arbitrary units. They are the means ± SEM, $n = 3$–6. *** $P < 0.001$ vs. respective control mice at 24°C or at 6°C without T3. $^{++}$ $P < 0.02$ and $^{+++}$ $P < 0.001$ between genotypes

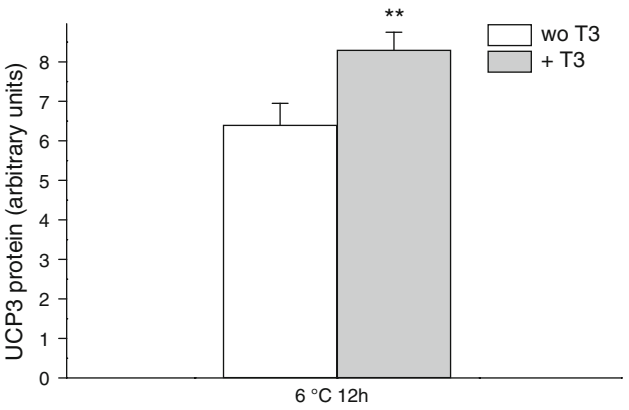

Fig. 3 Western blot quantification of the UCP3 protein/50 μg of muscle homogenate of β-less 3-month-old female mice, exposed to 6°C for 12 h. The cold-exposed mice were injected or not intraperitoneally with 0.5 μg/g of body weight of T3 at the times indicated in "Materials and methods". The results are expressed in arbitrary units. They are the means ± SEM, $n = 6$. ** $P < 0.02$ vs. controls without T3 (*wo T3*)

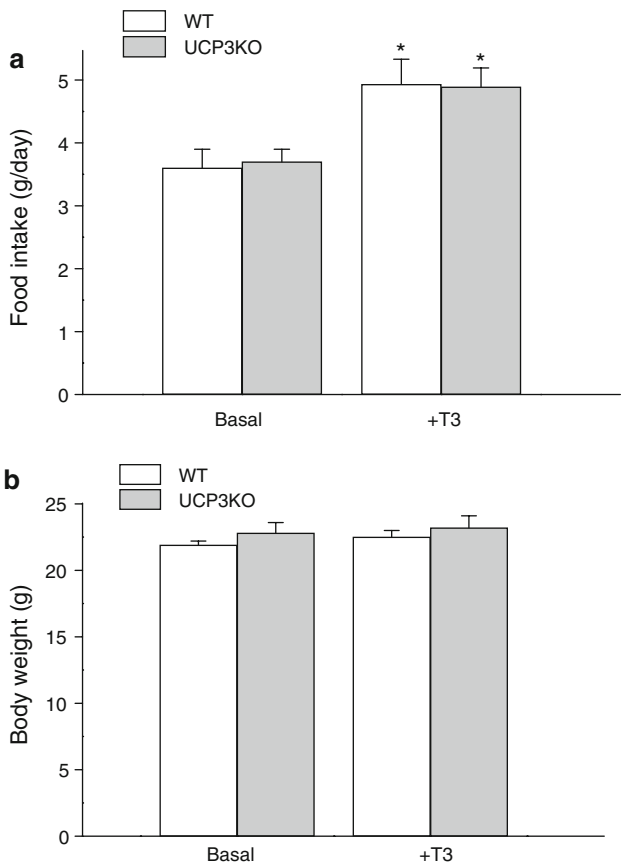

Fig. 4 (**a**) Food intake and (**b**) body weight of 2-month-old female WT and UCP3KO mice before and after implantation of a minipump delivering T3 (basal and + T3, respectively). The values are the means ± SEM of 5–6 WT and UCP3KO mice and are expressed as gram per day (g/day) of food intake and as gram (g) of body weight before and during the 3 days of T3 treatment. * $P < 0.05$ vs. respective basal values

Our UCP3KO mice therefore did not seem to display any gross energy metabolism phenotypic differences with their WT counterparts. To test our hypothesis of a privileged relationship between T3 and UCP3 activity, we performed a series of experiments in which circulating T3 level, body weight, food intake and energy expenditure were measured in 2-month-old mice treated or not with T3. The basal oxygen consumption of 2-month-old WT and UCP3KO mice was measured for 24 h, using an indirect calorimetry chamber. Then, T3 was administered by an osmotic minipump implanted subcutaneously on the back of the mice and delivering 6 μg of T3 in NaCl 0.9% per day. The T3-induced changes in metabolic rate were monitored during the three consecutive days in the indirect calorimetry chambers. Parallel experiments were performed in WT mice in which osmotic minipumps delivering vehicle (NaCl 0.9%) alone were implanted. No effect of the implantation was observed on body weight, food intake or oxygen consumption (results not shown). The control mice were chosen as the naive animals before T3 minipump implantation. This allowed direct comparisons in the same animal in two consecutive periods of time. The basal levels of circulating T3 were similar in WT and UCP3KO mice. After 3 days of T3 treatment, the levels of circulating T3 increased significantly ($n = 4$–5, $P < 0.01$) 4.8- and 6.2-fold, reaching 6.2 ± 0.9 and 7.9 ± 0.3 nmol/l in WT and UCP3KO mice, respectively, no difference being observed between genotypes. The increase in T3 during the treatment confirmed a normal delivery of the hormone in vivo by the minipump.

During the 3 days of T3 treatment, the mean food intake increased 1.4- and 1.3-fold in WT and UCP3KO mice, respectively, but there was no difference between genotypes (Fig. 4a). An increase in food intake by T3 has already been reported [26]. This result was therefore expected and suggests that the infused hormone was physiologically active. The mouse body weights were similar between genotypes and before and after the T3 treatment (Fig. 4b). An increase in food intake with no resultant increase in body weight suggests an increase in energy expenditure.

Figure 5 shows representative oxygen consumption curves of WT and UCP3KO mice during a period of 14 h, encompassing the dark period as well as of WT and UCP3KO mice during the same period, between the second and third day after the beginning of the T3 treatment. It can be seen that T3 treatment increases oxygen consumption rate, the peak values of the WT mice being systematically higher during the dark period than those of the UCP3KO mice. The resting oxygen consumption was calculated as the mean of the five lowest values of individual oxygen consumption during the light period. These values, which measured the oxygen consumption when the mice were at rest, can be considered as the basal metabolic rate. Figure 6a shows the basal metabolic rate of WT and UCP3KO mice during the light period of the day before the T3 treatment and of the second day of treatment. Before the T3 treatment, the basal metabolic rates were similar in WT and UCP3KO mice. T3 increased the basal metabolic rates of WT mice 1.12-fold. It also increased this parameter, but not significantly, in UCP3KO mice (paired *t*-test). During T3 treatment, the basal metabolic rates of WT and UCP3KO mice were similar. The dark period, between 7.00 p.m. and 7.00 a.m., is that during which the mice were the most active at consuming food. For this period, we expressed the oxygen consumption values as areas under the curves (AUC). Figure 6b shows the AUC values before

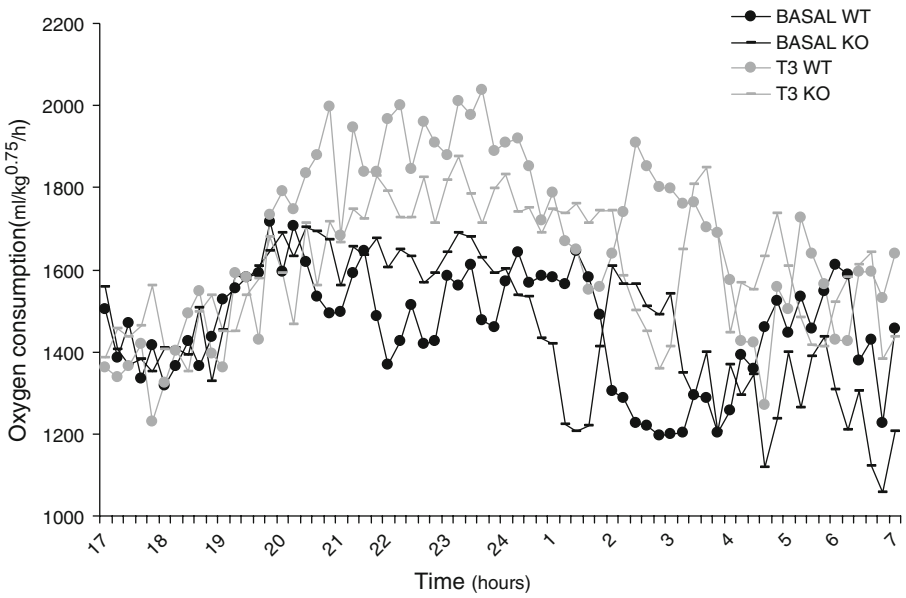

Fig. 5 Oxygen consumption of 2-month-old female WT and UCP3KO mice before and after implantation of the minipump delivering T3 (basal and T3, respectively). The mice were caged individually at 24°C with light from 7.00 a.m. to 7.00 p.m. and oxygen consumption was measured by indirect calorimetry during a period of 14 h encompassing the dark period between the second and third day of treatment. Values were collected every 12 min. They are the means of five WT and six UCP3KO mice and are expressed as ml of oxygen consumed per $kg^{0.75}$ of body weight in 1 h. SEM bars are not shown for more clarity

T3 treatment and during the dark period between the second and third day of treatment. Before T3 treatment, the mean oxygen consumption AUCs were similar in WT and UCP3KO mice. T3 increased the mean oxygen consumption AUCs of WT and UCP3KO mice 1.18- and 1.12-fold, respectively. The difference between the T3 effects was not significant (paired *t*-test). However, the absolute mean oxygen consumption value of the T3-treated WT mice was significantly higher by 6% than that of the T3-treated UCP3KO mice (unpaired *t*-test). The VCO_2 and the respiratory quotient were measured and no difference could be detected between the two genotypes under both basal and T3-stimulated conditions.

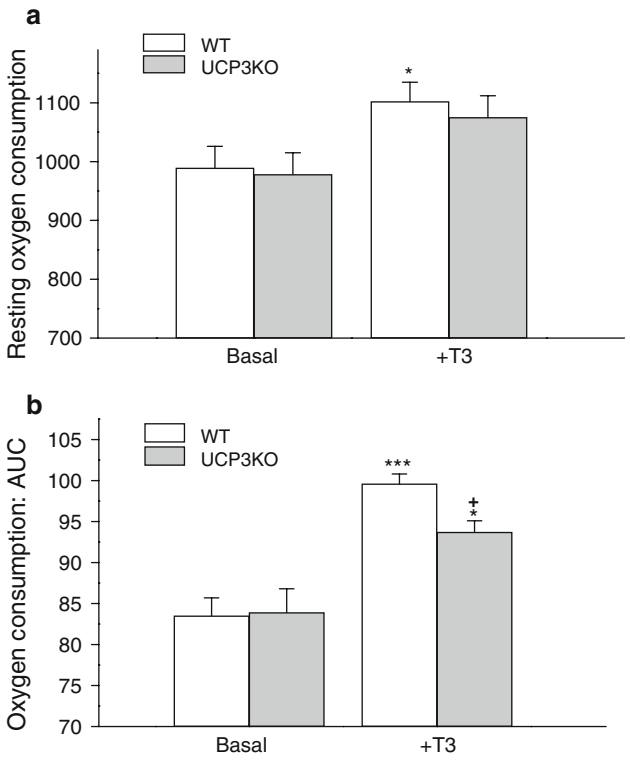

Fig. 6 **a** Basal metabolic rate of 2-month-old female WT and UCP3KO mice before and after implantation of the minipump delivering T3 (basal and + T3, respectively). The values are those obtained during the light period (7.00 a.m. to 10.00 a.m. and 3.00 p.m. to 7.00 p.m.) of the day before the treatment and of the second day of treatment, respectively. They are the means ± SEM of minimal oxygen consumption values of five WT and six UCP3KO mice (five data per mouse) and are expressed as ml of oxygen consumed per $kg^{0.75}$ of body weight in 1 h. * $P < 0.05$ vs. basal value. **b** Oxygen consumption of 2-month-old female WT and UCP3KO mice before and after implantation of the minipump delivering T3 (basal and + T3, respectively). The values are those obtained during the dark period (7.00 p.m. to 7.00 a.m.) between the second and third day of treatment. They are the means ± SEM of the areas under the curve (*AUC*) of five WT and six UCP3KO mice and are expressed as ml of oxygen consumed per $kg^{0.75}$ of body weight per hour. * $P < 0.05$ and *** $P < 0.001$ vs. respective control mice. $^{+}$ $P < 0.05$ between genotypes

Neither the lack of UCP3 nor our 3-day T3 treatment modified BAT UCP1 mRNA expression (results not shown).

Discussion

This study shows that the administration of T3 rescues the dramatic cold intolerance of β-less mice, suggesting that T3 has effects independent of the sympathetic nervous system. These effects and the possible mechanisms of the rescue have been analyzed. Cold exposure induces, among others, an increase in BAT mitochondrial protein content as well as in UCP1 expression and activity, which is mediated by the sympathetic nervous system [2]. Our results show only a slight reduction in the level of BAT UCP1 protein in β-less as compared to WT mice maintained at 24°C. This suggests that, at this temperature slightly below thermoneutrality, an adrenergic signalling pathway other than the β-, potentially the α-adrenergic system, compensates for the lack of β-adrenoceptors. Our results also confirm previous observations showing that the cold-induced increase in BAT UCP1 protein is blunted by the absence of β-adrenoceptors [10, 11] suggesting that, under an acute cold-stress condition, the compensation system becomes insufficient. Unexpectedly, T3 administration decreased the level of UCP1 protein in cold-exposed WT mouse BAT. It has been reported, in rats treated with thyroxine, that the induced rise in obligatory thermogenesis reduces the demand for non-shivering thermogenesis [27]. In this study, T3 administration, by increasing obligatory thermogenesis, induced the observed decrease in WT BAT UCP1 expression. Finally, T3 treatment had no effect on the amount of UCP1 protein in the β-less mouse BAT. Altogether, these results suggest that the effects of T3 on WT and on β-less mouse thermogenesis are not mediated by an effect of the hormone on BAT UCP1 expression.

T3 has been shown to induce an increase in muscle UCP3 expression in WT rodents [22]. These data are corroborated by our observation that T3 increases the level of UCP3 protein expression in cold-exposed β-less mice. Since UCP3 expression is increased by T3, it might as a consequence be involved in the rescue of the dramatic cold intolerance of β-less mice by T3. This hypothesis, however, is in contradiction with the findings that challenge a role of UCP3 in adaptative thermogenesis [28]. However, the hypothesis that UCP3 is a target of the thermogenic effect of T3 was supported by the study of de Lange et al. [22] showing that the increase in muscle UCP3 expression by T3 was paralleled by an increase in metabolic rate in WT rodents. To support unequivocally this hypothesis, the use of our UCP3KO mouse model seemed most appropriate.

The metabolic phenotyping of our UCP3KO mice confirmed previous studies, on similar models, showing that the lack of UCP3 does not modify food intake and body weight [13–15]. It also confirmed that the lack of UCP3 does not affect the levels of circulating glucose, insulin, FFA [14] and leptin [13]. It is noteworthy that a slight decrease in circulating glucose and FFA was reported by Gong et al. [13] which was observed neither in our study nor in that of Vidal-Puig et al. [14].

Indirect calorimetry measurements have been performed in the three UCP3KO models and it was unanimously reported that the lack of UCP3 does not affect oxygen consumption, either during the light or the dark period [13–15]. In one of the UCP3KO models, indirect calorimetry detected an increase in the respiratory quotient, indicating impaired fatty acid oxidation [15].

Gong et al. [13] compared the effects of T3 on the oxygen consumption of WT and UCP3KO mice. Their T3 treatment consisted in an injection of 1 mg/kg per day for 4 days. After the fourth injection, indirect calorimetry was performed and T3 was found to stimulate oxygen consumption during the dark period similarly in WT and UCP3KO mice. In this study, the protocol chosen for T3 administration was more physiological as far as the mode of administration and the dosages were concerned. As a consequence, the T3-induced increases in oxygen consumption were small and subtle inter-genotype differences in response to T3 could be revealed. This trend was confirmed during the dark period where the difference between the two genotypes became significant, suggesting that UCP3 is needed for a total effect of T3. The fact that the difference between genotypes in T3-stimulated mice is only observed during the dark period suggests that UCP3 might become uncoupling during metabolically active periods combining physical activity and high fuel disposal.

Under our experimental conditions, the T3-induced increase in energy expenditure in both WT and UCP3KO mice probably compensated for the increase in food intake and thus prevented an increase in body weight. The difference in energy expenditure between WT and UCP3KO mice is probably not sufficient to result in differences in body weight between the two genotypes. It is not possible to quantify the part played by UCP3 in the rescue by T3 of the β-less mouse cold intolerance. An elegant way to answer this question would be to generate a β-less-UCP3KO mouse.

It has already been reported that UCP3KO mice do not display compensatory up-regulations of UCP1 mRNA in their BAT and of UCP2 mRNA in their skeletal muscle [14]. The same absence of UCP1 compensation was observed in our UCP3KO mice. Therefore, the inter-genotype difference in oxygen consumption observed in this study upon T3 treatment cannot be ascribed to changes in BAT UCP1 expression. It has also been reported that the lack of UCP3 does not modify the physical activity [13]. Therefore, the inter-genotype difference in oxygen consumption upon T3 treatment should not be ascribed to changes in physical activity.

Altogether, our indirect calorimetry studies are in line with the idea that UCP3 may be involved in the rescue of the dramatic cold intolerance of β-less mice by T3. It should be kept in mind that the effects of T3 in β-less mice might also be mediated by effects of the hormone on futile cycles and shivering.

Altogether, our results suggest that UCP3 is involved in a non-negligible part of the effect of T3 on oxygen consumption and, as a consequence, that UCP3 might be involved in the correction by T3 of the dramatic cold intolerance of the β-less mice. These results reactivate the idea that UCP3 might play a role in the control of energy balance.

Materials and methods

Animals

The mice were treated in accordance with our institutional guidelines and all procedures used were approved by the Office Vétérinaire Cantonal (Geneva, Switzerland). They were maintained at 24 ± 1°C or at 6 ± 1°C for the cold-exposure experiments on a 12 h/12 h light cycle (7.00 a.m. to 7.00 p.m.) with free access to water and standard laboratory chow diet (Nordos, Cergy, France). Mice were housed individually 10 days before and throughout the experiments. In UCP3KO mouse experiments, food intake was measured every third day and corrected for spillage. At the end of the experiments, the mice were killed by cervical dislocation.

β-less mice

β-less and WT strains were obtained by inter-crossing our β_3-KO mice [29] and β_1/β_2-KO mice [30], kindly provided by Dr. B.K. Kobilka (Howard Hughes Medical Institute, Stanford, CA, USA). $\beta_1^{+/-}\beta_2^{+/-}\beta_3^{+/-}$ offsprings were crossed to generate $\beta_1^{+/+}\beta_2^{+/+}\beta_3^{+/+}$ (WT) and $\beta_1^{-/-}\beta_2^{-/-}\beta_3^{-/-}$ (β-less) mice, from which were established WT and β-less colonies on the same, mixed 129 Sv/ev, 129 SvJ, FVB/N, C57BL/6J and DBA/2 genetic background. Genotypes were determined by Southern blot [10]. Studies were performed on 3-month-old female mice.

UCP3KO mice

As shown in Fig. 7a, the targeting vector was constructed by deleting 6 kb of the Ucp3 gene consisting in a part of

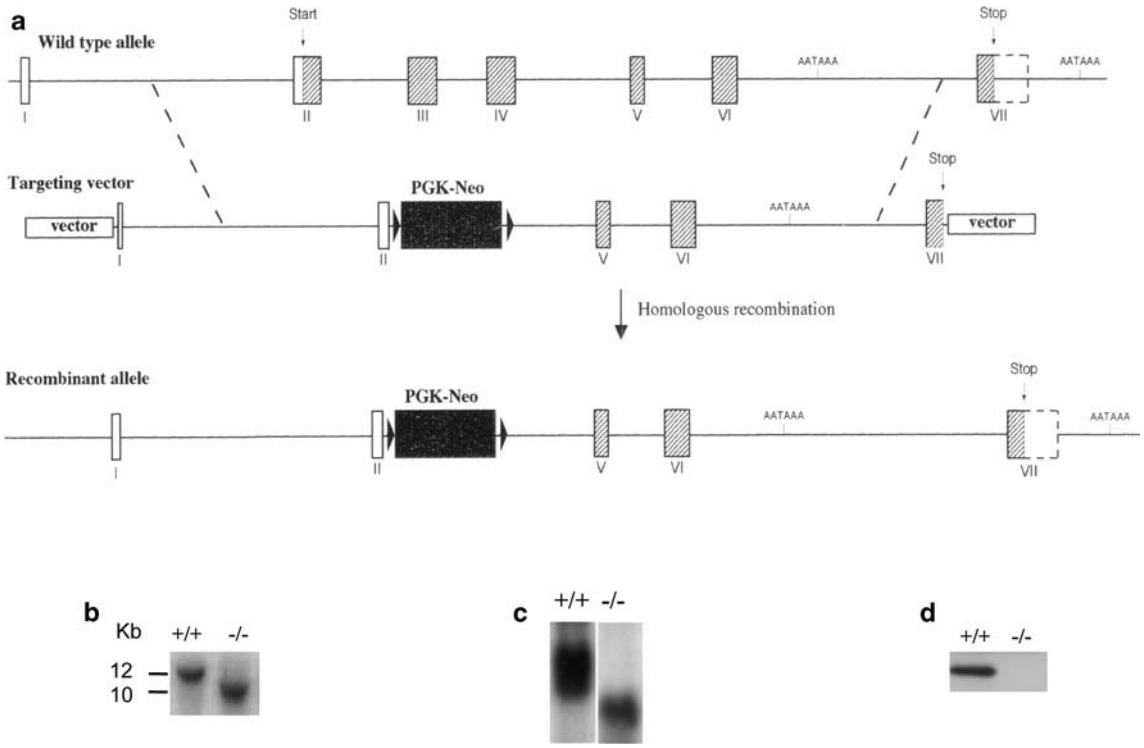

Fig. 7 **a** Schematic representation of the strategy used for UCP3 gene disruption. Wild-type UCP3 gene, targeting vector (UCP3KO construct) and recombinant allele are shown. The targeting vector was constructed by deleting 6 kb of the Ucp3 gene consisting in a part of exon II, from the ATG start codon and downstream, and in the totality of exons III and IV. The deleted fragment was replaced by a neomycin-resistance gene driven by a PGK promoter (PGK-Neo) surrounded by two loxP sequences (*black triangles*). These loxP sequences could be used to delete the neomycin-resistance gene. C57BL/6J mouse embryonic stem cells were electroporated with the targeting vector and homologous recombinant alleles were obtained. **b** Genotyping of the UCP3KO mice by Southern blot. The *Sac*1-digested DNAs of the WT and UCP3KO mice were analysed by Southern blot, using an UCP3 probe. The *Sac*1 digestion fragment of the WT allele displayed the expected size of 12 kb, whereas that of the truncated allele, in which the deleted 6 kb fragment was replaced by the 4 kb neomycin-resistance gene, displayed the expected size of 10 kb. **c** Northern blot detection of an UCP3 RNA signal in skeletal muscle mRNA of wild-type (+/+) and UCP3KO mice (−/−). The signal observed in the +/+ lane is replaced in the −/− lane by a smaller one. This short messenger, that lacks the start codon, was unable to translate into a protein, as shown in the Western blot. **d** Western blot detection of UCP3 protein in skeletal muscle mitochondria of wild type (+/+) mice. No UCP3 protein can be detected in UCP3KO (−/−) mice

exon II, from the ATG start codon and downstream, and in the totality of exons III and IV. The deleted fragment was replaced by a neomycin-resistance gene driven by a PGK promoter (PGK-Neo) surrounded by two loxP sequences. These loxP sequences could be used to delete the neomycin-resistance gene. The UCP3KO mice were generated using the technique described by Revelli et al. [29]. We chose to generate the mice in the C57BL/6J obesity-prone genetic background. Thus, C57BL/6J mouse embryonic stem cells were electroporated with the targeting vector and homologous recombinant clones were injected into C57BL/6J blastocysts. Genotypes were determined by Southern blot. Genomic DNA was isolated from the mouse tail by sodium dodecylsulphate/proteinase K digestion followed by salt precipitation. The DNA was digested with *Sac*1 and analysed using a standard Southern blot technique. The UCP3 probe used recognized a part of the 3′-untranslated region of the Ucp3 gene absent from the targeting vector. It therefore allowed distinguishing between random insertion and homologous recombination events. The probe was labelled by random priming with [α-^{32}P]-dCTP to a specific activity of approximately 1×10^9 dpm/μg of DNA. Hybridizations were performed using Quickhyb^{TM} solution as previously described [31]. Blots were exposed at −80°C to Hyperfilm ECL films. Total gastrocnemius muscle RNA was isolated by the method of Chomczynski and Sacchi [32], and UCP3 mRNA was quantified by Northern blot, using mouse UCP3 and human β-actin full-length cDNA probes as previously described [33]. Gastrocnemius muscle mitochondria were prepared as previously described [34] and the pellet suspended in 400 μl of distilled water. The Western blots were performed as described below.

Body temperature and T3 treatment

The colonic temperature was measured as an index of body temperature in mice accustomed to handling to minimize any artefactual increase in body temperature. A lubricated

digital probe was inserted 2 cm into the colon and held until a steady peak temperature was measured (Ellab thermometer, Copenhagen). For T3 treatment, 15 µg of the hormone was dissolved in 50 µl of a solution containing NaCl 0.9% and mouse serum 1% and injected intraperitoneally immediately before cold exposure, and then three times at intervals of 12 h (8.00 a.m. to 8.00 p.m.). Each dose of T3 injected/g of body weight was between 50% and 2.5-fold doses previously used in the rat [3, 27].

Blood thyroid hormones

Serum was obtained from tail bleeds. T4 and T3 were measured by radioimmunoassay (Buhlmann, Basel, Switzerland).

Western blots

BAT mitochondria were prepared as previously described [34]. Gastrocnemius muscle homogenate was prepared in five volumes (ml/g) of RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% Na deoxycholate, 0.1% SDS, 1:200 protease inhibitor cocktail, Sigma Chemical Co, St Louis, MI and 50 mM Tris/HCL pH 8.0). Protein concentrations were measured according to Bradford [35] using the Bio-Rad protein assay, with bovine serum albumin as a standard. Exactly 50 µg of proteins were dried under vacuum and, after dissolution in 10 µl of a loading buffer containing 20% glycerol, 6% SDS, 0.02% bromophenol blue and 15% 2-mercaptoethanol in 0.25 M Tris/HCL pH 6.8, loaded on the gel. Western blots were performed using a sheep polyclonal primary anti-UCP1 antibody generously provided by Dr. D. Ricquier (Meudon, France) and a rabbit anti-human UCP3 antibody, CabrX (Research Diagnostics, Inc, San Antonio, TX). The signals were detected by chemiluminescence using a standard ECL kit and developed on a Hyperfilm ECL film.

Indirect calorimetry

Oxygen consumption of WT and UCP3KO mice was measured using the OXYMAX system 4.93 (Columbus Instruments, Columbus, OH). Mice were caged individually in four 1-l chambers at 24°C with free access to food and water. Settling and measuring times were both of 120 s. Room air served as reference air. The resting oxygen consumption was estimated as the mean of the five lowest values of individual oxygen consumption during the light period. Total oxygen consumption was calculated as the area under the curve of the oxygen consumption for the dark period. T3 was dissolved as described for T4 by Cettour-Rose et al. [36]. Six WT and five UCP3KO 2-month-old female mice were placed in the indirect calorimetry chamber for 24 h and their oxygen consumption was measured. Then, osmotic minipumps (Alzet, model 2001, Cupertino, Canada) delivering 6 µg of T3 in 90 µl, or the same volume of the vehicle (NaCl 0.9%) per day for 3 days, were implanted subcutaneously on the back of the mice under isofluorane (Abbott Laboratories, Abbott Park, IL) anaesthesia. Animals were reinstalled in the chamber and the oxygen consumption was measured during 3 days. At the end of the experiment, the mice were killed by cervical dislocation and the BAT were dissected and immediately frozen in liquid nitrogen. At the same time, blood samples were collected and 100 µl of serum was used for total T3 quantification.

Statistical analyses

Significance was evaluated using unpaired or paired Student's *t*-tests and the computer software STATISTIX, version 4.0 (Analytical Software, St. Paul, MN).

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