Differences in the response of UCP1 mRNA to hormonal stimulation between rat and mouse primary cultures of brown adipocytes

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UCP-1 mRNA expression in rat and mice brown adipocytes

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Abstract

Uncoupling protein 1 (UCP-1), the specific marker of brown adipose tissue, is transcriptionally activated in response to adrenergic stimuli and thyroid hormones are necessary for its full expression. We describe differences in the regulation of UCP-1 mRNA expression between rat and mouse brown adipocytes in culture, using norepinephrine (NE), triiodothyronine (T3), insulin and retinoic acid (RA).

Results: NE and cAMP-elevating agents strongly increase UCP-1 mRNA levels in cultures of mouse adipocytes, but increases are low in those from rat. In rat adipocytes NE poorly increases UCP-1 mRNA expression and T3 markedly increases the adrenergic response of UCP-1, an effect not observed in mouse adipocytes. In the absence of insulin, T3 itself increases UCP-1 mRNA in rat adipocytes and enhances the response to NE, while in mouse adipocytes no effect of T3 is observed. RA by itself stimulates UCP-1 mRNA in mouse adipocytes, but not in those from rat. In rat cultures, RA requires the presence of NE and/or T3.

Conclusions: We find important differences in the hormonal regulation of UCP-1 mRNA expression in cultured preadipocytes depending on the species used as donor; those differences are observed using identical culture conditions and should be considered when doing cultures from these species.

¹ The abbreviations used are: BAT, brown adipose tissue; Ins, Insulin; NE, norepinephrine; RA, all trans-retinoic acid; T3, triiodothyronine; T4, thyroxine; UCP-1, uncoupling protein.

Introduction

Brown adipose tissue (BAT)¹ plays an important thermogenic role in hibernating, newborns and coldexposed mammals. The main function of BAT is to produce heat under adrenergic stimulation (facultative thermogenesis). This particular function is accomplished by the uncoupling protein 1 (UCP-1), a mitochondrial protein specific of BAT [1] that works as an ion channel. The activation of UCP-1 results in the uncoupling of the respiratory chain, and the dissipation as heat of the energy that otherwise would be stored as ATP [2]. The release of norepinephrine (NE) from the sympathetic nerve endings induces the expression of UCP-1 at the transcriptional level [3] and results in an increased thermogenic capacity of BAT. Adrenergic stimulation is the main stimuli of increased UCP-1 expression.

Thyroid hormones were initially thought to play a permissive role in the adrenergic stimulation of BAT [4] but studies in rodents indicate that they are also necessary for full UCP-1 mRNA expression [5,6]. Thus, the active thyroid hormone, triiodothyronine (T3) amplifies the adrenergic stimulation of rat UCP-1 mRNA expression [7], and contributes to the achievement of the maximal thermogenic capacity of BAT.

The cloning of the rat and mouse UCP-1 genes and the analysis of their promoter regions [8-11] identified cAMP-response elements (CRE) in the proximal promoter [9,12] as well as thyroid hormone and retinoic acid responsive elements (TREs and RAREs) [11,13-15], located in an "enhancer" element - 2.2/-2.5 kb upstream from the start of transcription were identified in the rat UCP-1 gene promoter. This "enhancer element" contains several CREs in the mouse UCP-1 promoter, at difference with the rat promoter. The interplay of T3 and NE in modulating the transcriptional activation of the rat UCP-1 gene has been extensively studied [16].

Although the adrenergic stimulation of UCP-1 expression in different species has been well documented in *in vivo* studies, *in vitro* experiments using cultured brown adipocytes isolated from precursor cells have produced different results with regard to the capacity of brown adipocytes to respond to adrenergic stimuli and occasionally the participation of thyroid hormones in such a process [14,17-22]. To analyze the expression of UCP-1, the specific marker of brown adipocytes, some investigators have used primary cultures of precursor cells obtained from mouse and hamster BAT [17,19]. In these experiments, UCP-1 mRNA expression was achieved by using NE, beta-adrenergic agonists or cAMP analogs. Several cell lines for brown adipocytes have been immortalized from mouse hibernomas obtained

from transgenic mice [23-26] or established by oncogene transfection of mouse cells [27]. In these mouse cells lines, induction of UCP-1 expression consistently occurs after adrenergic stimulation [20,24].

However, fewer investigators have studied the regulation of UCP-1 expression in rat brown adipocytes [20,22]. In contrast to observations in cultured mouse cells, we have observed that adrenergic stimulation is not enough to fully induce UCP-1 mRNA in brown adipocytes from rat, and that T3 is required for a full UCP-1 response [28]. Furthermore, it has been shown that addition of T3 can increase UCP-1 expression in the absence of exogenous adrenergic stimulation in cultures of fetal rat brown adipocytes [22].

In the present study we directly compare the hormonal and adrenergic regulation of UCP-1 mRNA expression in cultured brown adipocytes derived from mouse and rat BAT. We find marked differences between both species as to their hormonal regulation of UCP-1 mRNA level, specially regarding their T3 requirements and the effect of insulin. These differences may have important implications when studying BAT adipocytes from both species, which may show differential responses.

Materials and methods

Cell isolation and culture

Animals were housed following the European Community guidelines and protocols approved by our institution. Rats and mice were fed a standard diet (SAFE A04 (Panlab) containing 16% protein, 60% carbohydrates and 3% lipids). Precursor cells were obtained from the interscapular BAT of 20-days-old rats (Sprague-Dawley, aprox. 50 g) or 30-days-old Swiss mice (about 20 g). Both genders were used, as no gender-differences were found at the ages and hormones tested in this paper (results not shown). One month-old mice were used as donors, because BAT is very small in 20-days-old mice and few precursor cells are obtained; additionally cultures are less homogeneous. But when we compared cultures obtained from 20- and 30-days-old mice we found similar patterns of responses to NE and/or T3, except for lower increases when adipocytes were obtained from younger mice. Precursor cells were isolated according to the method described by Nchad [29], with the modifications described [30]. After digestion of BAT with collagenase type I (Sigma), and filtration through 250 μ silk filters, mature adipocytes were allowed to float and discarded, the infranatant was filtrated through 25 μ silk filters and centrifuged. The precursor cells obtained were seeded in 25 cm² culture flasks (day 0), to get 1500-2000 cells / cm² at day 1 and

were grown in DMEM supplemented with 10% newborn calf serum (NCS), and 3 nM insulin, 10 mM HEPES, 50 IU penicillin and 50 µg streptomycin/ ml and 15 µM ascorbic acid. Culture medium was changed on day 1 and every second day thereafter. Precursor cells proliferated actively under these conditions (doubling time was approximately 15 h), reached confluence at the 4th or 5th day after seeding (60,000-80,000 cells / cm²) and by day 8 were fully differentiated into mature brown adipocytes. In cultures obtained from mice, confluence is reached around day 6 after seeding. Studies were performed during the differentiation period (8th day of culture) using NCS or hypothyroid serum in the presence of thyroid hormones or other treatments as specified. cAMP analogs were diluted in culture medium, and NE was prepared fresh in ascorbic acid to get the concentration required. The same batch of serum was used in the comparative experiments presented here, in an attempt to avoid differences due to serum batches.

The serums used for culture were: 1) NCS, 2) Hypothyroid serum, depleted from thyroid hormones as described [31] and that contained about 10% or less of the original amount of thyroid hormones, as assessed by RIA [32]. Before dilution in the culture medium, thyroid hormone concentrations were 77 nM T4 and 1.3 nM T3 in NCS and decreased to 2.2 nM T4 and 0.13 nM T3 in hypothyroid serum.

RNA preparation, Northern blot analysis and quantitative RT-PCR

RNA was extracted using Tri-reagent (Sigma, St.Louis, MO) or in guanidinium-HCl as described [33], using ethanol precipitation. The recovery was 50-90 µg total RNA/ 25 cm² flasks, approximately 5x10⁶ cells. For isolation of Poly(A)⁺ RNA, cells were collected and mRNA isolated using oligo-dT cellulose as described [34]. Total RNA (15-20 µg) or Poly (A)⁺ RNA (5 µg) were denatured and electrophoresed, and filters were hybridized in the conditions described [30] with specific cDNA probes that were radio labeled with [α -³²P]-dCTP using random primers. The rat UCP-1 probe was 1200bp in length (provided by Dr. D. Ricquier [35], and the mouse UCP-1 cDNA was provided by Dr. L. Kozak [36]. Autoradiograms were obtained from the filters and quantified by laser computer-assisted densitometry (Molecular Dynamics). Results in the text are representative of 2-4 different experiments. The filters were also hybridized with the rat cDNA for cyclophilin [37] to correct for differences between lanes. All the experiments were done 2-4 times using duplicates. Representative Northern blots are shown in the figures. Recently, rat and mouse UCP1 mRNAs were also quantified by qRT-PCR using specific Taqman probes for rat and mouse UCP-1 (Rn00562126m1 and Mm01244861m1; Gene expression assays, Applied Biosystems, Foster City, CA). cDNA was synthesized from 1 µg of RNA using iScript cDNA synthesis kit (BioRad, Hercules, CA).

Results were normalized to rat or mouse cyclophilin, respectively (Ppia, Rn00690933m1 and Mm02342429g1, Applied Biosystems) and the fold-change in mRNA expression was calculated by the $2^{-\Delta\Delta C_t}$ method. The coefficients of variation for cyclophilin expression by qRT-PCR are 2.4 % and 4.4% for rat and mice cultures, respectively. We also tested the coefficients of variation for 18S rRNA that were about double: 4.7 % and 10.4% for rat and mice, respectively. We consider 18S less suitable as reference gene. The use of qRT-PCR analysis disclosed clear differences in the amounts of UCP-1 mRNA, much more abundant in mouse than in rat brown adipocytes and rendered a higher sensitivity at the lower expression levels.

Determination of cAMP levels

cAMP concentrations were determined in cells by radioimmunoassay using the kit from NEN (Dupont Company, Wilmington, DE). Protein content was determined by the method of Lowry [38].

Statistical analysis

Results are means $\pm$ SEM. Statistical significant differences were determined by Student t-test ($P < 0.05$) or ANOVA analysis when applied.

Materials

DMEM was from Gibco (Uxbridge, U.K.), NCS from Flow Lab. (Irvine, Scotland) or Gibco BRL-Life Technologies (Paisley, Scotland). Most reagents were obtained from Sigma (St. Louis, MO). Anion exchange resin AG1-X8 to produce hypothyroid serum was from BioRad (Richmond, CA). Radiolabeled [α - 32 P]- dCTP (3000 Ci/mmol) and Oligo-Labeling system were from Amersham Corp (Arlington Heights, IL) and Pharmacia Inc. (Uppsala, Sweden), respectively. OligodT Cellulose was from NewEngland BioLabs (Beverly, MA). All chemicals were molecular biology grade.

Results

Preliminary results in our laboratory showed species differences in the regulation of UCP-1 mRNA in cultured brown adipocytes from rat and mouse. Fig. 1 shows the evolution of both cultures during proliferation and differentiation of the precursor cells using identical culture conditions. Both cultures are similar, though rat cultures exhibit a slightly higher degree of proliferation and accumulate more lipid droplets.

Adrenergic stimulation of UCP-1 mRNA in rat and mice adipocytes. Effect of T3

We compared the induction of UCP-1 mRNA using NE, 8Br-cAMP or Forskolin in primary cultures of brown adipocytes from rats and mice. In agreement with previous reports [17], a clear stimulation of UCP-1 mRNA is observed in mouse adipocytes after the addition of NE, or agents that increase intracellular cAMP levels (Fig. 2.A). However, UCP-1 expression after NE stimulation is very low in rat cultures and UCP-1 mRNA was barely detected by Northern analysis when stimulating with 8Br-cAMP or forskolin (Fig. 2.B).

We then assessed the effect of T3 in cell cultures of both species using identical culture conditions, which included hypothyroid serum. In these culture conditions rat adipocytes show no increase in UCP-1 expression after NE treatment (Fig. 2.C, lane 2 vs 1), but a robust adrenergic response is observed when T3 is present (Fig. 2.C, lane 3). In contrast, using the same hypothyroid conditions, mouse brown adipocytes do respond to NE (Fig. 2.D, lane 5 vs 4), and the presence of T3 modestly modifies the response to NE (Fig. 2.D, lane 6 vs 5). We further analyzed the lack of induction of UCP-1 mRNA by NE in rat cultures using a Poly (A⁺) enriched mRNA fraction from rat brown adipocytes (Fig. 2.E) or a rat UCP-1 cDNA probe (Fig. 2.F). In this way we tried to exclude the possibility that the expression of UCP-1 mRNA could lie in a low range, or that was not detectable either because we were using total RNA or because a heterologous mouse UCP-1 cDNA was used. The results clearly show that the adrenergic stimulation of rat UCP-1 mRNA requires T3 (Fig. 2.E and 2.F, lanes 9-10 vs 7-8, and lanes 13-14 vs 11-12). In this experiment the hybridization of membranes with the heterologous mouse cDNA (Lanes 7-10, E) and with the homologous rat cDNA (lanes 11-14, F) shows similar results using both probes.

We also tested if the low UCP-1 mRNA adrenergic response in rat brown adipocytes could be due to a lack of increase in cAMP levels. Fig. 2.G shows that cAMP levels increase in response to NE from 20 up to 150 pmols/ mg protein at 30 min. The presence of T3 enhances the response (408 ± 18 pmols/ mg protein). Cellular cAMP levels returned to basal levels after 2 h. Treatment of rat brown adipocytes with 1 mM 8Br-cAMP or 1 μ M Forskolin for 1 h increased intracellular cAMP levels up to 501 ± 26 and 122 ± 25 pmols/ mg protein, respectively (not shown). Although the presence of T3 results in increased cAMP production after NE treatment in rat cells, the results suggest that an insufficient increase in intracellular cAMP is probably not the reason for the lack of increase in UCP-1 expression in rat adipocytes.

We have also analyzed UCP-1 mRNA expression using qRT-PCR, a more sensitive technique that

improves reliability and lowers the detection threshold. We observe clear differences in UCP-1 levels, much more abundant in brown adipocytes from mice than in those from rats. In mouse adipocytes, basal UCP-1 Ct values were ≈ 25 and Ct decreased to 18-20 using NE or NE+T3 (6 cycles, 64-fold) (the lower the Ct the higher the UCP-1 expression). In rat adipocytes, basal UCP-1 was much lower, Ct ≈ 33 -34, decreasing to 26-28 under NE and to 23-24 using NE+T3 (under present conditions). We conclude that NE increases UCP-1 mRNA levels in both species, rat and mice, but the increases are not detectable in rat adipocytes using Northern analysis due to the lower UCP-1 expression in rat adipocytes, while in mice UCP-1 levels are more abundant.

Effect of insulin on UCP-1 mRNA expression

We also analyzed in cell cultures of both species how insulin affects the adrenergic stimulation of UCP-1 mRNA in the presence or absence of T3. Depletion of insulin at the time of cellular confluence had different effects in rat and mouse cultures (Fig. 3.A and 3.B). In the presence of insulin, mouse cells exhibit low UCP-1 expression after T3 treatment (Fig. 3.A, lane 1) and the expression decreases in the absence of insulin (lane 4). In contrast, in rat cell cultures, UCP-1 is elevated by T3 >100 times in the absence of insulin (Fig. 3.B, lane 10), but not in its presence (lane 7). In the absence of T3, NE stimulation of UCP-1 is again observed only in mouse cells (Fig 3.A, lanes 2 and 5) but not in those from rat (Fig 3.B, Lanes 8 and 11), and the absence of insulin does not alter this observation in either species (Lanes 5 vs 2 and 11 vs 8, respectively). Finally, adrenergic expression of UCP-1 in the presence of T3 appears reduced (n.s.) in mouse cells when insulin is absent (Lanes 6 vs 3), but in rat cells the absence of insulin increases by 2.5-fold the effect of NE+T3 on UCP-1 mRNA expression (Lanes 12 vs 9). These results indicate that there are species differences in the regulatory effect of insulin on UCP-1 expression when T3 is present. In addition, they reveal a rat-specific effect of T3 on UCP-1 mRNA expression in the absence of adrenergic stimulation.

Direct T3 effect on UCP-1 expression in rat cultures

We further analyzed the effect of T3 *per se* on UCP-1 mRNA in cultured rat brown adipocytes using serum-free medium and hypothyroid serum (Fig. 3.C). In the absence of insulin, T3-treated cultures displayed a significant level of UCP-1 mRNA, even in the absence of an adrenergic stimuli (Fig. 3.C, lanes 1 and 5). This expression is inhibited by insulin (Lanes 2 and 6), an effect that is specially marked in serum-free medium (Lane 6 vs 5). Although UCP-1 expression is higher when both T3 and NE are added

to the cells (Lanes 3-4 and 7-8), T3-induced UCP-1 mRNA level in the absence of insulin is very significant and may have an impact on cell physiology as lower UCP-1 expression is detected if T3 is not present (Fig. 2).

Effects of Retinoic acid (RA) in the presence of T3 and insulin

RA *per se* has been reported to increase UCP-1 mRNA in primary cultures of mouse brown adipocytes [14]. We have examined the effects of RA alone or combined with T3 and/or NE on the induction of UCP-1 mRNA in cultures of rat and mouse brown adipocytes in the presence or absence of insulin. After RA treatment, mouse cells show significant UCP-1 mRNA expression (Fig. 4.A lane 1) that is further increased in the presence of T3, NE or both (Lanes 2 to 4). However, if insulin is not present, we observe a marked, general decrease in UCP-1 expression in mouse cells after all treatments (Fig. 4.A, lanes 5 to 8 versus lanes 1 to 4).

In contrast, rat adipocytes do not exhibit UCP-1 expression after the addition of RA (Fig. 4.B, lane 9), and a significantly higher UCP-1 mRNA levels are reached when T3, NE or both have also been added to the cells (Lanes 10 to 12 vs lanes 2 to 4). Again, a completely opposite pattern of insulin regulation for UCP-1 is observed in rat adipocytes. In rat cells, the absence of insulin leads to a dramatic increase of UCP-1 expression when T3 is part of the treatment (Lanes 15 and 16 vs 11 and 12), in sharp contrast to the results obtained in mouse adipocytes after the same treatments (lanes 6 and 8 vs 2 and 4). These results in the presence of RA further underscore the differences that exist between both species in the effects of regulation of UCP-1 mRNA level by T3 and insulin.

Given that we did not observe effects of RA alone on UCP-1 mRNA expression in rat cell cultures (in contrast to mouse cultures), we tested different RA doses, both alone or combined with NE at two different exposure times. RA treatment alone did not increased UCP-1 expression in rat adipocytes at any of the doses or exposure times used (Fig. 4.C, Lanes 2 to 7). In the presence of NE, RA was more effective when added at the highest dose used (Fig. 4.C, lanes 8 and 11). The combined treatment of RA with NE and T3 increased UCP-1 mRNA even at low RA doses (Lanes 14 and 15), although in view of the previous results the contribution of RA signaling to this induction is probably minimal.

Discussion

UCP-1 expression is critical for BAT function in mammals and plays an important role in facultative thermogenesis and energy balance, especially in rodents. Cell cultures of brown adipocytes from different species, either in primary culture [17,19,20,29,39,40] or in an established cell line [23-26], have been an attractive and convenient model to study the regulation of UCP-1 expression, as well as that of other genes involved in brown adipose differentiation. Achieving UCP-1 expression in some of these models to demonstrate that they are true brown fat cells has not been a simple task, especially in immortalized cell lines [41,42] and rat cell cultures. Furthermore, some studies on the hormonal and adrenergic regulation of UCP-1 have shown inconsistent results in culture models of different cell types or from different species. Using the same type of cell culture model, we show herein that there are substantial differences between the mouse and rat species in how adrenergic and/or hormonal stimuli regulate UCP-1 expression *in vitro*. These differences are not due to differences in age, gender or developmental stage of mice and rats used as donors, because cultures obtained from male vs. female mice or from 20 vs. 30-days old mice gave similar patterns of responses to NE, T3, RA and insulin, though showed lower increases (see Methods). In fact, we have used 30-days-old mice -10 days older than rat donors- because few precursor cells are obtained from 20-days-old mice and cultures became scarce and non-homogeneous. We are aware that donor rats are used at weaning (3 weeks), when lactation is ending and a switch from milk (10% fat, 3% lactose) to a solid diet (3% fat, 60% CHO) is occurring, metabolism changes for the adaptation to a high carbohydrate diet and possibly the responses to insulin as well, which may influence the responses obtained, as further discussed below. It might be considered that due to the metabolic changes taking place at weaning, epigenetic changes may occur in the precursor cells obtained, which may affect the response of the cells in culture, or the signalling transduction pathways

In the present paper we also compared the results obtained by Northern blots with the analysis using a more sensitive technique, qRT-PCR. The higher sensitivity of qRT-PCR allows us to quantify increases that would be undetectable using Northern blots, especially in rat cultures in which basal UCP-1 mRNA levels are lower. But the differences we find between species do not depend on the sensitivity of the technique used, as the results obtained by qRT-PCR confirmed the results obtained by densitometry of Northern blots, though with a higher sensitivity.

The adrenergic stimulus is the main determinant of UCP-1 mRNA expression as demonstrated *in vivo* in rats [3,7,43-45] as well as *in vitro*, using primary cultures or cell lines of brown adipocytes from mouse

[17,20,26]. This regulatory effect is mediated through beta-adrenergic receptors, mainly the beta3 subtype [17,19,20,24-26,40,46]. Stimulated receptors translate the signal into increased intracellular cAMP level, which then activate transcription of UCP-1 at the gene promoter level through cAMP responsive elements or CREs [12].

In the present work, we also observe induction of UCP-1 mRNA expression as a result of adrenergic stimulation or treatment with cAMP elevating agents, especially when the adipocytes are from mouse. UCP-1 expression in rat brown adipocytes respond less to these agents, and UCP-1 mRNA is low (as clearly shown when using Poly (A⁺) enriched mRNA fraction), regardless of the adrenergic stimuli used. It is only when T3 is present that UCP-1 is fully induced in rat adipocytes by adrenergic stimulation. This phenomenon is observed at any time during the differentiation process [28] and is consistent with the enhancement of adrenergic or cAMP induction of UCP-1 expression achieved by T3 in rats [6] and in floating or cultured rat brown adipocytes [47,48].

In the absence of T3, rat adipocytes express low UCP-1 levels even though NE treatment leads to significant increases in intracellular cAMP that are very similar to those found in mouse adipocytes after similar adrenergic stimulus (35 amols cAMP / cell = 150 pmols cAMP / mg protein) [49]. Treatment of rat adipocytes with 8BrcAMP or Fork leads to a much higher intracellular level of cAMP, but still a low induction in UCP-1 mRNA is observed if T3 is not present. Our data show that T3 treatment leads to higher cAMP production, suggesting that insufficient cAMP production in the rat adipocyte is not responsible for the low UCP-1 induction upon adrenergic stimulation. In addition, the reproduction of this observation both in serum and serum-free media further supports the hypothesis that specific limiting factors may exist in the rat species, probably at the gene promoter level. The fact that T3 exposure overcomes these limiting factors indicates a rat-specific role for T3 in UCP-1 expression, which probably involves the expression, recruitment or interaction of other factors acting on UCP-1 transcription at the gene promoter level as further discussed below.

A more prominent and specific role of T3 in the rat brown adipocyte is further supported by our experiments in the absence of adrenergic stimulation. In this case, T3 treatment leads to an increased basal level of UCP-1 mRNA in rat, but not in mouse adipocytes. These differences between species extend to the response to RA and insulin. While RA treatment markedly increase UCP-1 expression in mouse brown adipocytes, such increase is not observed in those from rat. Significant differences in the UCP-1 response

to RA between species are also observed in the presence of T3, NE or both. These observations do not mean that T3 is unimportant for mice BAT, as exemplify by the cold intolerance of *dio2* null mice, in which the adrenergic input is extremely high; possibly the adrenergic stimuli is more important in mice than in rats due to the smaller size of mice and to its higher demand of facultative thermogenesis.

Taken together, these results reveal significant differences between mouse and rat in the hormonal and adrenergic stimulation of UCP-1, a critical protein for BAT function. The basis for these differences might lie in how the promoter of the UCP1-1 gene is regulated by various stimuli in rat and mouse. The mechanism for the synergistic action of T3 with NE in rat adipocytes has been identified in the enhancer region located 2.2-2.5 kb upstream the start of transcription in the rat promoter. This region contains two TREs that seem to mediate the synergistic action of T3 and NE [13,50,51]. Other response elements for RA or PPAR gamma have also been identified in this genomic region [14,15]. We thus hypothesize that the differences in the regulation of UCP-1 in mouse and rat adipocytes may be due to differences in the sequence of this regulatory region, particularly concerning the effects of T3 and NE. On this regard, we should note that while several CREs are present in this enhancer region of the mouse UCP-1 gene (BRE, -2.3 to -2.5 kb), in which a single TRE has been postulated but not tested functionally [9,52], several thyroid response elements (TREs) "in tandem" have been identified in the enhancer region of the rat UCP-1 gene which act synergically [11,13,51]. It is thus possible that the differential effects of T3, RA and NE in adipocytes of both species are mediated by different responses, interactions and/or recruitment of transcription factors and co-factors through this genomic region that may also involve response elements not yet identified.

A puzzling difference between mouse and rat in the regulation UCP-1 expression arises from the presence of insulin in the culture medium. Our results reveal a general effect of insulin in enhancing UCP-1 expression in mouse brown adipocytes under several treatments. However, the response of rat adipocytes to insulin is the opposite, as it tends to diminish UCP-1 mRNA level. Our studies in progress shows that insulin, through *erk* signaling inhibits UCP-1 expression in rat adipocytes in culture. The enhancing effect of insulin on UCP-1 in mouse brown adipocytes is observed using both, 20- and 30-days-old mice as donors, suggesting that insulin changes at weaning might not be the cause of the differential response of mouse vs rat adipocytes. The differential responses to insulin in rat and mouse deserve further investigation, and we cannot exclude that insulin changes (nutrition, diet) may affect and modulate the

response of adipocytes in culture. This species-specific effect of insulin is even more dramatic on the actions of T3 when no adrenergic stimulation is present.

Particularly unexpected is the different effect that insulin exerts in adipocytes of both species on the T3- and NE-dependent regulation of UCP-1. Insulin has a great impact on lipogenesis and the overall process of adipose conversion and is used by many investigators to facilitate adipose differentiation *in vitro* in various cell culture models. Mice lacking insulin receptors in BAT (BATIRKO) shows a loss of BAT and glucose intolerance, though UCP1 increases in BAT along life [53]. UCP-1 is reduced in diabetic states [54-56]. Insulin induces UCP-1 in rat fetal adipocytes [57,58], an effect not observed in brown adipocytes from adult rats. However, no insulin response elements have been identified in the UCP-1 gene promoter. It is possible that these differential effects of insulin between species may be mediated by unidentified regulatory regions targeted by insulin-activated pathways. The presence of additional regulatory regions is supported by the observation that UCP-1 expression is restored in diabetic rats by L-arginine administration [56]. Further research is required to elucidate the role of insulin and its pathways in rat cultures, and the discrepancy found between UCP-1 increases in insulin-depleted rat adipocytes and UCP-1 decreases in diabetic or fasting rats.

In summary, we show that there are important differences in the adrenergic and hormonal regulation of UCP-1 mRNA between mouse and rat adipocytes in culture. Further investigations are required to elucidate the molecular mechanisms underlying these findings. These results do not question *in vivo* findings, but enrich them. Considering the importance of BAT function for adaptative thermogenesis and energy balance, our results suggest we should proceed with caution when comparing the hormonal regulation of UCP-1 expression in adipocytes from different mammalian models, including humans, even when those models are as similar as the rat and mouse.

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Legends to Figures

Fig. 1. Microphotographs of mouse (A-C) and rat (D-F) brown adipocytes in primary culture during proliferation and differentiation from their precursor cells. Precursor cells were isolated from mouse or rat BAT and allow to proliferate and differentiate in 10% NCS. Microphotographs were taken on days 1, 4 and 8 after seeding for mouse (A-C) and rat adipocytes (D-F).

Fig. 2.A.B. *Adrenergic stimulation of UCP-1 mRNA in mouse and rat brown adipocytes.* Rat or mouse brown precursor cells were grown in standard conditions (10% NCS) until day 7. Cells were maintained during the last 20 hours in medium containing 1% NCS and NE (3 μ M), 8Br-cAMP (BrcAMP, 1 mM) or Forskolin (F, 5 μ M) were added during the last 6 hours before recollection. Fifteen μ g of total RNA per lane were used. The specific mouse or rat UCP-1 cDNA probes were used to hybridize the respective mouse or rat samples. Hybridization with cyclophilin cDNA was used to correct for differences between lanes. A representative Northern and quantification of UCP-1 mRNA by qRT-PCR are shown (n=3 for mice and n=6 for rat). All increases were significant ($P<0.05$) vs basal values. **Fig. 2. C-F.** *Effect of triiodothyronine (T3) on the adrenergic stimulation of UCP-1 mRNA in mouse and rat brown adipocytes.* Rat (C, E and F) or mouse (D) brown preadipocytes were grown in 10% NCS until day 7. Cells were maintained during the last 20 hours in medium containing 2% hypothyroid serum, supplemented or not with 5 nM T3. NE (1 μ M) was added during the last 7.5 h before recollection. Twenty μ g of total RNA (C, D) or 10 μ g of poly A⁺ RNA (E, F) were used per lane. Cyclophilin cDNA was used to correct for differences between lanes. A mouse (C, D and E) or a rat (F) UCP-1 cDNA was used. A representative experiment is shown (n=2). **Fig. 2.G.** *Time course increases in cellular cAMP levels in rat brown adipocytes.* Rat brown adipocytes were grown in standard conditions until day 7. Cells were maintained during the last 20 hours in medium containing 1% hypothyroid serum, supplemented or not with 5 nM T3. NE (2 μ M) was added and cells collected at 0.5, 1, 2 and 4 hours in 0.1 N HCl for cAMP determination. Data represent the mean $\pm$ SD of triplicate determinations in three different cultures.

Fig. 3.A and B. *Effect of T3 and insulin depletion on the adrenergic induction of UCP-1 mRNA in mouse and rat brown adipocytes.* Rat or mouse brown preadipocytes were grown in 10% NCS until day 7. Half of the flasks from both cultures were depleted from insulin from day 4. Cells were maintained during

the last 20 hours in medium containing 1% NCS supplemented or not with 2 nM T3. NE (3 μ M) was added during the last 6 hours. Mouse or rat UCP-1 cDNA probes were used to hybridize the respective mouse or rat samples. Cyclophilin cDNA was used to correct for differences between lanes. A representative experiment is shown. Below, the Figures show mouse or rat UCP-1 mRNA analysis using qRT.PCR and specific Taqman probes for rat or mouse UCP-1, using rat or mouse Cy as reference gene (n=2-7 points/bar). Results are means $\pm$ SEM. * P <0.05 vs its respective T3. # P <0.05 vs +insulin. **Fig. 3.C.** *Direct effect of T3 on UCP-1 mRNA induction in rat brown adipocytes. The role of insulin.* Cultures of rat brown preadipocytes were grown in standard conditions (10% NCS+ insulin) until day 7. Cells were maintained during the last 24 hours in 10% hypothyroid serum or in serum-free medium, with no insulin, supplemented as specified with 10 nM T3, 10 μ M NE or 4 nM insulin. All treatments lasted 24 hours. A rat UCP-1 cDNA probe was used and rat cyclophilin cDNA was used to correct for differences between lanes. A representative experiment is shown and quantification of UCP-1 mRNA was done using qRT.PCR as described above. Results are means $\pm$ SEM (n=4). * P <0.05 vs T3 in each of the conditions.

Fig. 4. A and B. *Effect of RA and T3 on the adrenergic stimulation of UCP-1 mRNA in mouse and rat brown adipocytes. Effect of insulin depletion.* Rat or mouse brown preadipocytes were grown in 10% NCS until day 7. Half of the flasks from both cultures were depleted from insulin from day 4. Cells were maintained during the last 20 hours in medium containing 1% NCS, supplemented or not with 2 nM T3 and/or 1 μ M RA. NE (3 μ M) was added during the last 6 hours. The specific mouse or rat UCP-1 cDNA probes were used to hybridize the respective mouse or rat cultures. Cyclophilin cDNA was used to correct for differences between lanes. A representative experiment is shown and quantification of UCP-1 mRNA was done using qRT.PCR and specific Taqman probes. Results are means $\pm$ SEM (n=3-5 for mouse and n=2-4 for rat). * P <0.05 vs RA, # P <0.05 vs +insulin. **Fig. 4.C.** *Effect of RA on the stimulation of UCP-1 mRNA using rat brown adipocytes.* Rat brown preadipocytes were grown in 10% NCS until day 7. Cells were maintained during the last 20 hours in medium containing 1% NCS supplemented with 0.1 nM, 10 nM or 1 μ M RA for 6 or 20 hours. T3 was added during the last 20 hours as specified. NE (3 μ M) was added during the last 6 hours. Rat UCP-1 cDNA probe was used for Northern analysis and Cyclophilin cDNA was used to correct for differences between lanes. A representative experiment is shown and quantification of UCP-1 mRNA was done using qRT.PCR (n=2).

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MOUSE

RAT

Proliferation

Confluence

Differentiation

Figure 1

Fig 2

Fig 3

Fig 4