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RESEARCH ARTICLE | *Browning and Beiging of Adipose Tissue, Its Role in the Regulation of Energy Homeostasis and as a Potential Target for Alleviating Metabolic Diseases*

The β_3-adrenergic receptor is dispensable for browning of adipose tissues

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de Jong JM, Wouters RT, Boulet N, Cannon B, Nedergaard J, Petrovic N. The β_3-adrenergic receptor is dispensable for browning of adipose tissues. *Am J Physiol Endocrinol Metab* 312: E508–E518, 2017. First published February 21, 2017; doi:10.1152/ajpendo.00437.2016.— Brown and brite/beige adipocytes are attractive therapeutic targets to treat metabolic diseases. To maximally utilize their functional potential, further understanding is required about their identities and their functional differences. Recent studies with β_3-adrenergic receptor knockout mice reported that brite/beige adipocytes, but not classical brown adipocytes, require the β_3-adrenergic receptor for cold-induced transcriptional activation of thermogenic genes. We aimed to further characterize this requirement of the β_3-adrenergic receptor as a functional distinction between classical brown and brite/beige adipocytes. However, when comparing wild-type and β_3-adrenergic receptor knockout mice, we observed no differences in cold-induced thermogenic gene expression (*Ucp1*, *Pgc1a*, *Dio2*, and *Cidea*) in brown or white (brite/beige) adipose tissues. Irrespective of the duration of the cold exposure or the sex of the mice, we observed no effect of the absence of the β_3-adrenergic receptor. Experiments with the β_3-adrenergic receptor agonist CL-316,243 verified the functional absence of β_3-adrenergic signaling in these knockout mice. The β_3-adrenergic receptor knockout model in the present study was maintained on a FVB/N background, whereas earlier reports used C57BL/6 and 129Sv mice. Thus our data imply background-dependent differences in adrenergic signaling mechanisms in response to cold exposure. Nonetheless, the present data indicate that the β_3-adrenergic receptor is dispensable for cold-induced transcriptional activation in both classical brown and, as opposed to earlier studies, brite/beige cells.

β_3-adrenergic receptor; adipose browning; brown adipocytes; brite/beige adipocytes; UCP1

TWO TYPES OF THERMOGENESIS-COMPETENT adipocytes, brown and brite/beige (25, 38), coexist in mammals. Cold exposure, via adrenergic stimulation, leads to thermogenic recruitment of classical brown adipose tissue (10, 17, 18) and, simultaneously, to "browning" of certain (mainly subcutaneous) white adipose tissue depots (e.g., Refs. 36, 47, 59). Activation of thermogenesis in adipose tissues is mediated by the β_3-adrenergic receptor (β_3-AR) (10), an adipose tissue-selective (33) adrenergic receptor. Considering this, the reported phenotype of the β_3-AR-ablated mice is rather unexpected. In brown adipose tissue, the absence of the β_3-AR does not affect the adaptive response to cold (32, 50). In contrast, the white adipose tissue of β_3-AR knockout (KO) mice has been reported to fail to respond to cold: the induction of "thermogenic" genes, as well as the appearance of brite/beige adipocytes, was severely blunted (4, 26). Thus, remarkably, there seems to be an absolute requirement for intact β_3-AR signaling for cold-induced development of the brite/beige phenotype (browning).

The brite/beige- vs. brown-specific requirement for the β_3-AR for cold-induced responses opens the possibility to use β_3-AR KO mice as a tool to functionally distinguish between these two cell types. Furthermore, β_3-AR KO mice, due to their inability to develop brite/beige fat, would then be considered as "brite/beige fat-ablated" mice and could therefore potentially be utilized as a model for investigating functional differences between brown and brite/beige adipose tissues. This would be of additional interest considering the possibility that human brown adipose tissue could be more brite/beige than brown in character, as suggested by Wu et al. (56) and Sharp et al. (48).

Our goal here was to establish a distinction between classical brown and brite/beige adipose tissues based on the requirement for the β_3-AR for the activation of thermogenic gene expression in response to cold exposure as a first step to utilize this potentially interesting model for physiological analysis. However, in contrast to earlier observations, we found that in the absence of the β_3-AR, cold-induced activation of the thermogenic gene program was fully intact, not only in brown, but also in brite/beige adipose tissues.

MATERIALS AND METHODS

Animals

All experiments were approved by the Animal Ethics Committee of the Northern Stockholm region. Wild-type FVB/N ("sensitive to Friend leukemia Virus B strain") mice and β_3-adrenergic receptor knockout (β_3-AR KO) mice on the same background (50) were housed at 24°C on a 12:12-h light-dark cycle and had free access to water and chow diet (Labfor R70; Lantmännen, Södertälje, Sweden). At the end of the experiments, mice were euthanized with CO_2 followed by cervical dislocation. Interscapular brown adipose tissue (IBAT), inguinal white adipose tissue (ingWAT), and gonadal white adipose tissue (eWAT in male, gWAT in female mice) were dissected, snap frozen in liquid nitrogen, and kept at −80°C until further processed as described below.

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Full cold acclimation (3-wk cold exposure). For the full acclimation experiment, 8-wk-old male wild-type and β_3-AR KO mice were single caged and housed at 30°C for 3 wk. After these 3 wk, seven wild-type mice and seven β_3-AR KO mice were moved to 18°C for 1 wk (23), followed by 3 wk at 4°C, whereas seven wild-type mice and seven β_3-AR KO mice remained at 30°C.

Transient (7 day) cold exposure. Twelve-week-old male wild-type and β_3-AR KO mice that had been at 24°C were single caged and moved to either 30°C or 4°C for 7 days (4 mice per group).

Acute (48 h) cold exposure. Sixteen-week-old female wild-type and β_3-AR KO mice were single-caged and were either kept at 24°C or moved to 4°C for 48 h (3–4 mice per group).

Treatment with a β_3-AR agonist. Twelve-week-old male wild-type and β_3-AR KO mice were single-caged and housed at 30°C for 3 wk. Mice were injected intraperitoneally with either 120 μl saline or the β_3-AR agonist CL-316,243 (C5976; Sigma-Aldrich) at a dose of 1 mg/kg body wt (5–7 mice per group). Two injections were given 18 h apart, and mice were euthanized for tissue dissection 24 h after the first injection.

Gene Expression Measurements

RNA isolation and cDNA synthesis. Frozen tissues were homogenized in TRI Reagent (T9424; Sigma-Aldrich), and the chloroform-isopropanol method was used to isolate RNA according to the Sigma-Aldrich TRI Reagent protocol. The RNA concentrations in the samples were measured with a Thermo Scientific NanoDrop 1000 Spectrophotometer. The High-Capacity cDNA Reverse Transcription Kit (no. 4368814; Life Technologies) was used to reverse transcribe 500 ng of total RNA into cDNA in a total volume of 20 μl, according to the manufacturer's instructions. After the reaction was completed, cDNA was diluted 10 times in water.

Real-time qPCR. All primers were validated before use to ensure good amplification efficiency (90–110%) and specificity (controlled for by melting curve analysis and inclusion of control samples in which the reverse transcriptase had been left out of the reaction). Gene-specific primers (see Table 1) and SYBR Green JumpStart Taq Ready Mix (S4438; Sigma-Aldrich) were premixed in a total volume of 11 μl. The final primer concentration used was 0.3 μM. Two microliters of the diluted cDNA were added to the premixed primer solution to a total volume of 13 μl. All samples were run in triplicate. The Bio-Rad CFX Connect Real-Time system was used to perform the real-time quantitative polymerase chain reaction. The samples were preheated 2 min at 50°C and 10 min at 95°C, after which 40 cycles of 15 s at 95°C and 1 min at 60°C were run. The real-time qPCR reaction was followed by melting curve analysis.

C_t values for general transcription factor IIb (TFIIB) (for Figs. 1, 3, and 4) or TATA-binding protein (*Tbp*) (for Fig. 5) were subtracted from the C_t values of each analyzed gene (ΔC_t method) to adjust for variability in cDNA synthesis. These ΔC_t values were antilog-transformed ($2^{-\Delta Ct}$) to determine changes in mRNA abundance. Reference gene expression was analyzed as 2^{-Ct} and was generally similar among samples (see Fig. 2). In the β_3-agonist experiment (Fig. 5), *Tbp* was used as the reference gene because TFIIB expression showed larger variation in this experiment (not shown).

Statistics. Statistical analysis was performed in GraphPad Prism version 6.0 h for MAC OS X. For each tissue, two-way ANOVA analysis was performed followed by Tukey's multiple comparison testing. Statistics for sources of variation (interaction, temperature/treatment, and genotype) are presented in Table 2. Multiple comparison *P* values are indicated in graphs and figure legends.

Table 1. *Primer sequences*

Gene	Primers	Reference
Adrb1		Roche Universal Probe Library*
Forward	CATCATGGGTGTGTTCACG	
Reverse	GAAGACGAAGAGGCGATCC	
Adrb3		Newly designed
Forward	ACTGCTAGCATCGAGACCTTG	
Reverse	AAGGGTTGGTGACAGCTAGG	
Cidea		Roche Universal Probe Library*
Forward	GCCTGCAGGAACTTATCAGC	
Reverse	GCCTGCAGGAACTTATCAGC	
Dio2		Roche Universal Probe Library*
Forward	CTGCGCTGTGTCTGGAAC	
Reverse	GGAATTGGGAGCATCTTCAC	
Pgc1a		Petrovic et al. (38)
Forward	GAAAGGGCCAAACAGAGAGA	
Reverse	GTAAATCACACGGCGCTCTT	
Tbp		Petrovic et al. (38)
Forward	ACGGACAACTGCGTTGATTT	
Reverse	TTCTTGCTGCTAGTCTGGATTG	
TFIIB		Waldén et al. (52)
Forward	TGGAGATTTGTCCACCATGA	
Reverse	GAATTGCCAAACTCATCAAAACT	
Ucp1		Waldén et al. (38)
Forward	GGCCTCTACGACTCAGTCCA	
Reverse	TAAGCCGGCTGAGATCTTGT	

*See https://lifescience.roche.com

RESULTS

As a prerequisite for using the reported difference in β_3-AR responsiveness between brown and brite/beige adipose tissue for elucidation of functional differences between these tissues, the here reported studies were undertaken. Based on the proposal that β_3-adrenergic receptor (β_3-AR) signaling is required specifically for browning of brite/beige tissues, we examined the cold-induced increase in expression of "thermogenic genes" in several adipose tissues. This was performed in mice devoid of the β_3-adrenergic receptor (β_3-AR KO mice) and in mice with intact β_3-AR signaling (wild-type mice). Mice of both genotypes were exposed to thermoneutrality (30°C) or to cold (4°C) for various time periods. The adipose depot specificity in the requirement for the β_3-AR for cold-induced gene expression was explored in three types of adipose tissue: the "classical" brown fat depot [interscapular brown adipose tissue (IBAT)], the "brite/beige" adipose depot [i.e., white adipose tissue with the highest browning capacity (inguinal white adipose tissue (ingWAT)], and in the classical white adipose tissue, possessing only very little browning capacity [epididymal white adipose tissue (eWAT)] (15, 52).

The β_3-AR Is Dispensable for Induction of Thermogenic Gene Expression in Adipose Tissues of Cold-Acclimated Mice

To achieve the greatest possible contrast between thermogenic recruitment states in adipose tissues, mice were exposed to thermoneutrality (30°C, a thermogenically nonrecruited state) or to cold (4°C) for 3 wk, i.e., until a new acclimation steady state had been achieved (a recruited state). We examined the expression of four genes generally associated with thermogenesis (uncoupling protein 1, peroxisome proliferator-activated receptor-γ coactivator-1α, cell death-inducing DFFA-like effector A, and type II iodothyronine deiodinase) and of two adrenergic receptor genes (β_1- and β_3-ARs). The highly significant cold-induced increases in uncoupling protein 1 (*Ucp1*) mRNA levels in both IBAT and ingWAT of wild-type mice (Fig. 1*A*) verified successful cold acclimation; *Ucp1* expression was not induced in eWAT. However, importantly, in β_3-AR KO mice, compared with wild-type mice, no differences were observed in the *Ucp1* expression pattern in any of the tissues examined either at 30°C

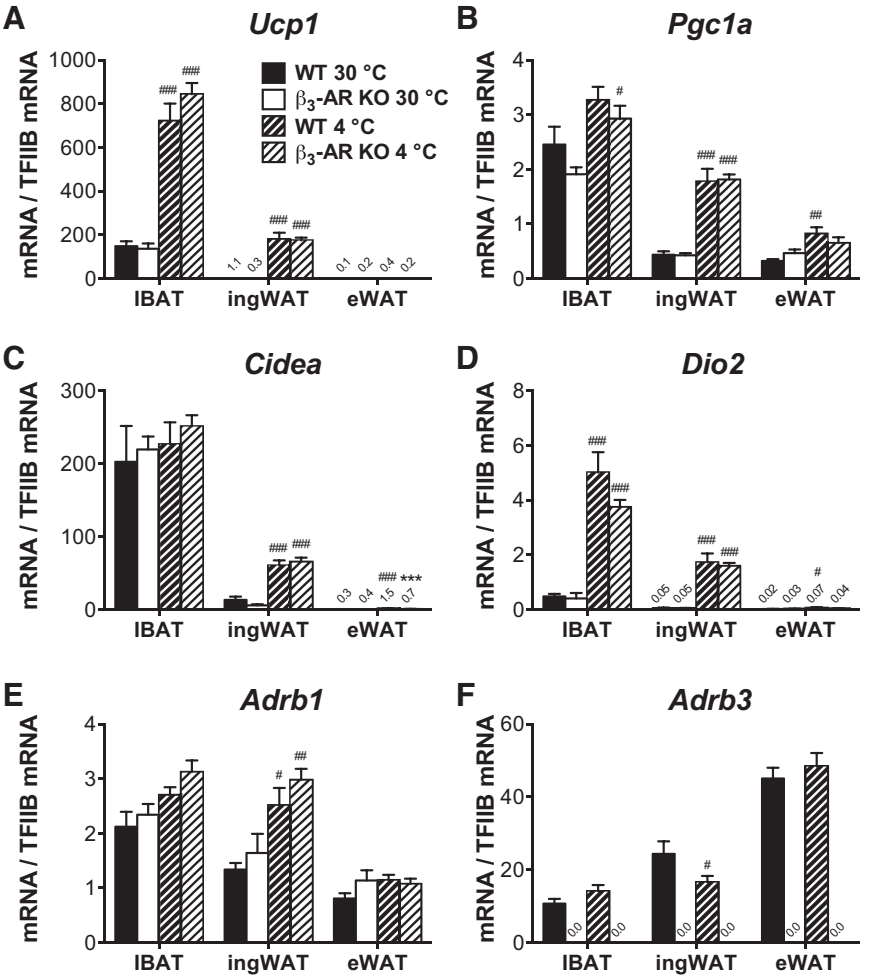

Fig. 1. The β_3-adrenergic receptor is dispensable for "thermogenic" gene expression induction in adipose tissues in cold-acclimated mice. mRNA levels of uncoupling protein 1 (*Ucp1*; *A*), peroxisome proliferator-activated receptor gamma coactivator 1-α (*Pgc1a*; *B*), cell death-inducing DFFA-like effector A (*Cidea*; *C*), type II iodothyronine deiodinase (*Dio2*; *D*), β_1-adrenergic receptor (*Adrb1*; *E*), and β_3-adrenergic receptor (*Adrb3*; *F*) in interscapular brown adipose tissue (IBAT), inguinal white adipose tissue (ingWAT), and gonadal white adipose tissue (eWAT) of male wild-type and β_3-AR KO mice after 3-wk exposure to thermoneutrality (30°C) or cold (4°C) (7 mice per group). Values are means ± SE. Where bars were invisible, only numeric values for means are presented. Data were analyzed using two-way ANOVA (see MATERIALS AND METHODS). ANOVA statistics are given in Table 2. *Significant difference between wild-type and β_3-AR KO mice for each tissue using two-way ANOVA followed by Tukey's multiple comparison test. #Significant difference between 30 and 4°C calculated similarly. *#$P < 0.05$, **##$P < 0.01$, ***###$P < 0.001$.

or at 4°C (Fig. 1*A*). Thus it may be concluded that under these conditions the β_3-AR is dispensable for cold-induced expression of *Ucp1* in all of the adipose tissues examined. Of note is the absence of adipose tissue specificity in this requirement, which thus is in contrast with previous studies: significantly diminished induction of *Ucp1* mRNA levels in ingWAT of cold-exposed β_3-AR KO mice has earlier been observed in two independent studies (4, 26).

Additionally, we examined the requirement of the β_3-AR for cold induction of other classical thermogenic genes. Peroxisome proliferator-activated receptor-γ coactivator-1α (*Pgc1a*), a thermogenic gene regulating mitochondriogenesis (43, reviewed in 42), demonstrated the same trend as *Ucp1*, although the magnitude of *Pgc1a* cold induction in IBAT was much lower compared with *Ucp1* (Fig. 1*B*). Again, no effects of the absence of the β_3-AR were observed in IBAT, ingWAT, and eWAT (Fig. 1*B*), in contrast to Barbatelli et al. (4).

Cell death-inducing DFFA-like effector A (*Cidea*) is a gene mainly expressed in brown but not in white adipocytes (1, 35, 62), but it is induced in white adipose depots upon cold acclimation (5, 15, 20). *Cidea* expression in IBAT was not induced by cold, as reported earlier (5, 15, 20) [but see Barbatelli et al. (4)]. Also, no genotype effect (wild-type vs. β_3-AR KO mice) was observed for *Cidea* expression in IBAT. As expected (e.g., Refs. 4, 15, 20), *Cidea* mRNA levels were markedly induced in both ingWAT and eWAT upon cold acclimation (Fig. 1*C*). However, no differences in *Cidea* expression levels were observed in ingWAT between wild-type and β_3-AR KO mice at either temperature (Fig. 1*C*). In eWAT, the expression levels of *Cidea* were far lower than in IBAT and ingWAT (Fig. 1*C*) (1, 15).

Type II iodothyronine deiodinase (*Dio2*), the enzyme-converting thyroid hormone T_4 into the active T_3 form, is another gene the expression of which is remarkably increased in adipose tissues upon cold acclimation (27, 49). *Dio2* expression was indeed upregulated in IBAT and ingWAT of cold-acclimated mice, but this occurred in both wild-type and β_3-AR KO mice to the same extent (Fig. 1*D*). In eWAT, *Dio2* expression was far lower than in IBAT and ingWAT (Fig. 1*D*).

No Upregulation of β_1-AR mRNA in β_3-AR KO Mice

Thus our data clearly demonstrate that the induction of the thermogenic gene program in adipose tissues, even including ingWAT, does not require β_3-AR signaling. As it has been reported that in BAT the absence of the β_3-AR leads to an increase of β_1-AR levels, which functionally compensates for the loss of the β_3-AR (11, 32, 50), we examined whether an increase in the expression of the β_1-AR (*Adrb1*) in adipose tissues of β_3-AR KO mice could explain the absence of genotype effects on thermogenic gene expression. In the three tissues analyzed, no differences in *Adrb1* expression were

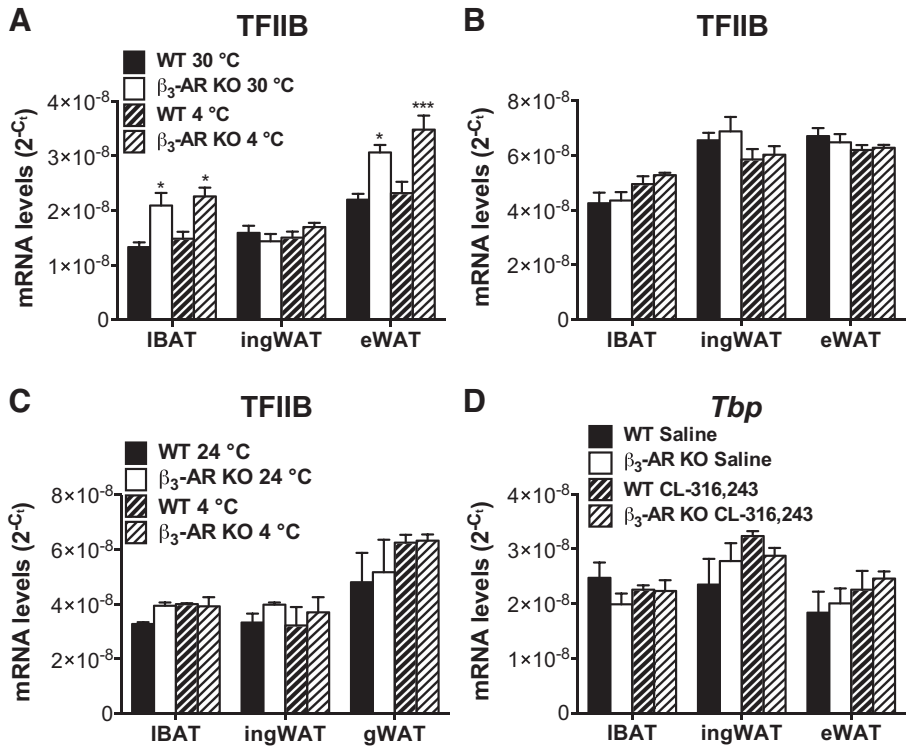

Fig. 2. Reference gene expression in adipose tissues. Reference gene expression levels in adipose depots of wild-type and β_3-AR KO mice, related to data in Figs. 1*A*, 3*B*, 4*C*, and 5*D*. Values are antilog-transformed C_t values (2^{-Ct}) presented as means ± SE. Conditions are indicated in the graph legends [legend in *A* (3 wk) corresponds also to *B* (7 days)]. Two-way ANOVA analysis indicated no significant interaction between genotype and temperature in any of the experiments (Table 2). In *A*, genotype was a significant source of variation in IBAT and eWAT, and in *B*, temperature was a significant source of variation in IBAT (Table 2). For further statistics see legend to Fig. 1. These reference gene expression data did not affect the conclusions drawn from the data in Figs. 1 and 3–5. *Significant difference between wild-type and β_3-AR KO mice for each tissue using two-way ANOVA followed by Tukey's multiple comparison test. $*P < 0.05$, $***P < 0.001$.

observed between wild-type and β_3-AR KO mice (Fig. 1*E*). However, in ingWAT, *Adrb1* expression was increased upon cold acclimation, although this occurred in both wild-type and β_3-AR KO mice (Fig. 1*E*). Thus we did not observe a compensatory increase in β_1-AR mRNA levels (i.e., an increase occurring only in the KO mice, having the potential to take over the function). Due to the unavailability of reliable antibodies against mouse β_1-AR (we tested antibodies from various suppliers), we examined only its mRNA levels.

We also verified the absence of a functional β_3-AR gene (*Adrb3*) product in the β_3-AR KO mice using primers that were designed to be within the sequence that was deleted (Table 1). *Adrb3* mRNA was not detectable in β_3-AR KO mice (Fig. 1*F*). However, using primers that were designed to be within the sequence that was not deleted, we were able to amplify the remaining fragment of the transcript; its expression levels were similar to the levels in wild-type mice (data not shown).

The data above were all expressed relative to TFIIB as reference gene. As seen in Fig. 2*A*, the differences in TFIIB expression were not such that they qualitatively affected the outcome of the experiment.

No Qualitative Differences Due To Various Experimental Designs

The results above demonstrate that in mice devoid of the β_3-AR, cold acclimation led to an induction of thermogenic genes to levels remarkably similar to those in mice possessing intact β_3-AR signaling, not only in IBAT (as reported earlier), but also in ingWAT and eWAT. Therefore, the initially observed depot-specific requirement for the β_3-AR cannot be used to distinguish between classical brown and brite/beige adipose depots under these conditions. However, the discrepancy with earlier studies (4, 26) could theoretically originate from details in the experimental procedures. To examine this possibility, we tested several other experimental conditions.

No transient effects of the absence of the β_3-AR. It may be hypothesized that during prolonged cold exposure (i.e., cold acclimation), mechanisms compensating for the absence of β_3-AR signaling could develop in ingWAT of β_3-AR KO mice. To evaluate this possibility, we exposed mice to 30°C or to 4°C for only 7 days.

After 7 days of cold exposure, *Ucp1* expression was induced in IBAT and ingWAT in both wild-type and β_3-AR KO mice (Fig. 3*A*). Again, no principal differences between wild-type and β_3-AR KO mice were observed (Fig. 3).

Pgc1a expression was significantly increased in IBAT and ingWAT of cold-exposed mice, but no differences were observed between genotypes (Fig. 3*B*). *Cidea* expression remained mostly unchanged in all tissues (Fig. 3*C*) while *Dio2* expression was induced in both IBAT and ingWAT but to the same extent in mice of the two genotypes (Fig. 3*D*). Thus no difference in induction of thermogenic genes was observed in adipose tissues of β_3-AR KO and wild-type mice.

Similarly to the case in cold-acclimated mice, expression levels of *Adrb1* were measured. No differences in *Adrb1* expression between wild-type and β_3-AR KO mice were observed (Fig. 3*E*). The absence of *Adrb3* expression in β_3-AR KO mice again validated the experiment (Fig. 3*F*), and the reference gene expression was not significantly different in the different conditions (Fig. 2*B*).

Thus, even when mice are exposed to 4°C for 7 days, no impairment in the cold-induced increase in expression of the analyzed genes in the β_3-AR KO mice was observed. The necessity for the β_3-AR for cold-induced browning is thus not a transient phenomenon.

Table 2. *Two-way ANOVA P values for interaction, temperature/treatment, and genotype*

	IBAT			ingWAT			eWAT		
	Interaction	Temperature	Genotype	Interaction	Temperature	Genotype	Interaction	Temperature	Genotype
Acclimation									
TFIIB	0.973	0.329	<0.0001*	0.150	0.462	0.862	0.432	0.160	<0.0001*
Ucp1	0.195	<0.0001*	0.284	0.882	<0.0001*	0.844	0.353	0.352	0.996
Pgc1a	0.698	0.001*	0.082	0.848	<0.0001*	0.917	0.081	0.001*	0.852
Cidea	0.903	0.378	0.511	0.231	<0.0001*	0.830	0.001*	<0.0001*	0.007*
Dio2	0.161	<0.0001*	0.115	0.700	<0.0001*	0.689	0.057*	0.013*	0.313
Adrb1	0.639	0.004*	0.141	0.754	<0.0001*	0.157	0.122	0.282	0.303
Adrb3	0.133	0.132	<0.0001*	0.052	0.053	<0.0001*	0.489	0.491	<0.0001*
Transient									
TFIIB	0.704	0.016*	0.476	0.849	0.067	0.528	0.536	0.163	0.755
Ucp1	0.048*	<0.0001*	0.975	0.546	<0.0001*	0.456	0.104	0.245	0.023*
Pgc1a	0.484	0.0002*	0.449	0.479	<0.0001*	0.721	0.078	0.023*	0.012*
Cidea	0.066	0.002*	0.083	0.928	0.055	0.150	0.034*	0.056	0.038*
Dio2	0.881	<0.0001*	0.946	0.393	<0.0001*	0.432	0.385	0.002*	0.370
Adrb1	0.505	0.064	0.095	0.771	0.001*	1.000	0.610	0.893	0.030*
Adrb3	0.798	0.797	0.0003*	0.021*	0.020*	<0.0001*	0.650	0.649	<0.0001*
Acute									
TFIIB	0.129	0.153	0.223	0.867	0.707	0.280	0.850	0.119	0.775
Ucp1	0.649	<0.0001*	0.721	0.311	0.016*	0.489	0.437	0.093	0.802
Pgc1a	0.100	<0.0001*	0.096	0.418	0.003*	0.958	0.106	0.027*	0.011*
Cidea	0.584	0.008*	0.432	0.792	0.307	0.397	0.270	0.661	0.298
Dio2	0.011*	<0.0001*	0.023*	0.221	0.002*	0.676	0.754	<0.0001*	0.068
Adrb1	0.011*	0.016*	0.018*	0.544	0.256	0.608	0.725	0.926	0.593
Adrb3	0.091	0.094	<0.0001*	0.107	0.109	<0.0001*	0.028*	0.027*	<0.0001*
CL-316,243									
Tbp	0.251	0.945	0.210	0.161	0.086	0.899	0.950	0.140	0.519
Ucp1	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
Pgc1a	0.001*	0.001*	0.038*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
Cidea	0.011*	0.044*	0.044*	0.005*	0.001*	0.031*	0.041*	0.029*	0.002*
Dio2	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
Adrb1	0.073	0.001*	0.076	0.392	0.679	0.325	0.391	0.085	0.383
Adrb3	0.705	0.663	<0.0001*	0.007*	0.006*	<0.0001*	<0.0001*	<0.0001*	<0.0001*

Values were calculated as described in MATERIALS AND METHODS. IBAT, interscapular brown adipose tissue; ingWAT, inguinal white adipose tissue; eWAT, gonadal white adipose tissue. *P values <0.05.

Mimicking of experimental conditions used in earlier studies. The studies that were in contrast to our results were performed in female mice (4, 26). To exclude the possibility that the effects of the β_3-AR absence could be sex dependent, we analyzed female mice under conditions mimicking those described in Barbatelli et al. (4) (i.e., female mice exposed to either 24 or 4°C for 48 h).

Female wild-type and β_3-AR KO mice were housed at 24°C and were then either acutely exposed to 4°C for 48 h or kept at 24°C. Gene expression levels of *Ucp1*, *Pgc1a*, and *Dio2* in IBAT increased after 48 h at 4°C in both wild-type and β_3-AR KO mice (Fig. 4, *A*, *B*, and *D*). Expression levels of these genes in ingWAT showed the same pattern as in IBAT, but were induced to a lower extent (Fig. 4, *A*, *B*, and *D*). In gonadal white adipose tissue (gWAT; the female equivalent of eWAT), the cold-induced changes were even smaller than in ingWAT (Fig. 4, *A*, *B*, and *D*). Thus, again, no differences were observed in thermogenic gene expression between wild-type and β_3-AR KO mice in the tissues investigated. After 48 h of cold exposure, *Cidea* expression in ingWAT was not increased (Fig. 4*C*).

Similarly to the previous experiments, practically no increased expression of *Adrb1* was observed in β_3-AR KO mice (Fig. 4*E*). Again, the absence of detectable *Adrb3* mRNA in β_3-AR KO mice validated the experiment (Fig. 4*F*) and no differences were seen in reference gene expression (Fig. 2*C*).

Thus the observed absence of a requirement for β_3-AR-mediated induction of cold-sensitive genes in ingWAT was not due to sex or time differences.

β_3-AR KO Mice Are Devoid of a Functional β_3-AR Response

The absence of *Adrb3* expression validated the genotypes of all mice used. However, it cannot theoretically be excluded that the mutated β_3-AR gene product in the β_3-AR KO mice retained the ability to respond to β_3-AR agonists, i.e., norepinephrine, in vivo. To examine whether the β_3-AR KO mice were really devoid of functional β_3-AR signaling, the response to the β_3-AR agonist CL-316,243 was examined in wild-type and β_3-AR KO mice.

Male mice acclimated to 30°C were injected with CL-316,243 (1 mg/kg ip) 24 and 6 h before death. *Ucp1*, *Pgc1a*, *Cidea*, and *Dio2* expression were all strongly induced in all three investigated tissues in the CL-316,243-treated wild-type mice (Fig. 5, *A–D*). In β_3-AR KO mice, no response to CL-316,243 treatment was observed (Fig. 5, *A–D*), demonstrating the absence of a functional β_3-AR response in these mice.

Expression levels of *Adrb1* were increased by CL-316,243 treatment only in IBAT of wild-type mice (Fig. 5*E*) while the expression of *Adrb3* in IBAT was not affected. Strikingly, *Adrb3* expression levels in ingWAT and eWAT of wild-type

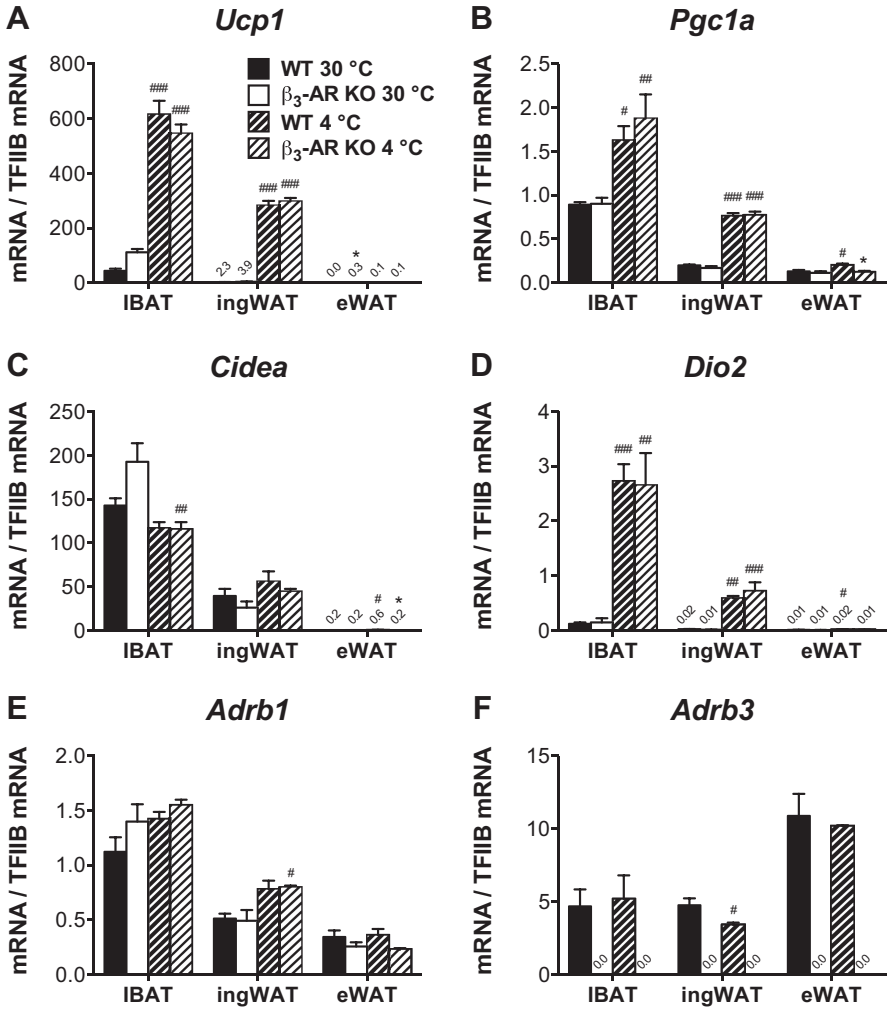

Fig. 3. No transient effects of the absence of the β3-adrenergic receptor on cold-induced gene expression. Expression levels of genes as in Fig. 1 in IBAT, ingWAT and eWAT of male wild-type and β3-AR KO mice after 7 days' exposure to thermoneutrality (30°C) or cold (4°C) (4 mice per group). See legend to Fig. 1 for details of full gene names and statistics. *Significant difference between wild-type and β3-AR KO mice for each tissue using two-way ANOVA followed by Tukey's multiple comparison test. #Significant difference between 30°C and 4°C calculated similarly. *#$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$.

mice were notably decreased after CL-316,243 injections (Fig. 5*F*) (14). Again, reference gene expression did not qualitatively affect the outcome of the experiment (Fig. 2*D*). In this experiment, *Tbp* was used as a reference gene since the expression of TFIIB was affected by the treatment (not shown).

Thus the absence of a functional β3-AR response in the β3-AR KO mice fully validated the results obtained in cold exposure experiments. Therefore, the general requirement of the β3-AR for the induction of the thermogenic gene program in ingWAT in response to cold must be brought into question.

DISCUSSION

Earlier studies had suggested a qualitative difference between brown and brite/beige adipose tissues in that only in brite/beige adipose tissue was there an absolute requirement for β3-AR for cold-induced browning. In the present study, we intended to utilize the suggested brite/beige adipose tissue specificity to functionally distinguish brite/beige from brown adipose tissue depots. However, we found that the β3-AR is dispensable for cold-induced gene expression not only in BAT but also in ingWAT and gWAT. The dispensability of β3-AR was expected for BAT (32, 45, 50) but was entirely unexpected for the brite/beige and white adipose depots (4, 26). Thus our results disappointingly suggest that the requirement of the β3-AR for cold-induced gene expression cannot be used either to distinguish between classical brown and brite/beige adipose depots or to examine functional differences between these two tissues.

Mouse Model Differences

We have performed experiments under conditions leading to full thermogenic recruitment of adipose tissues and also under conditions attempting to mimic those in Barbatelli et al. (4). In none of these experiments did we see any indication of a requirement for β3-AR for ingWAT browning.

The main difference between our and earlier studies is the use of independently generated β3-AR KO mouse models, which were also on different genetic backgrounds. In the present study, the mice generated by Susulic et al. (50) on the FVB/N background were used. Susulic et al. (50) used a rather uncommon approach for obtaining KOs by injecting the targeting vector directly into zygotes. The zygotes and thus the resulting knockout mice were derived from FVB/N inbred mice and the strain has been maintained on the same genetic background. The targeted mutation comprises a deletion of 306 nucleotides covering amino acids 120 (in the third transmembrane domain) to 222 (at the end of the fifth transmembrane domain) (50). We were able to amplify the remaining fragment

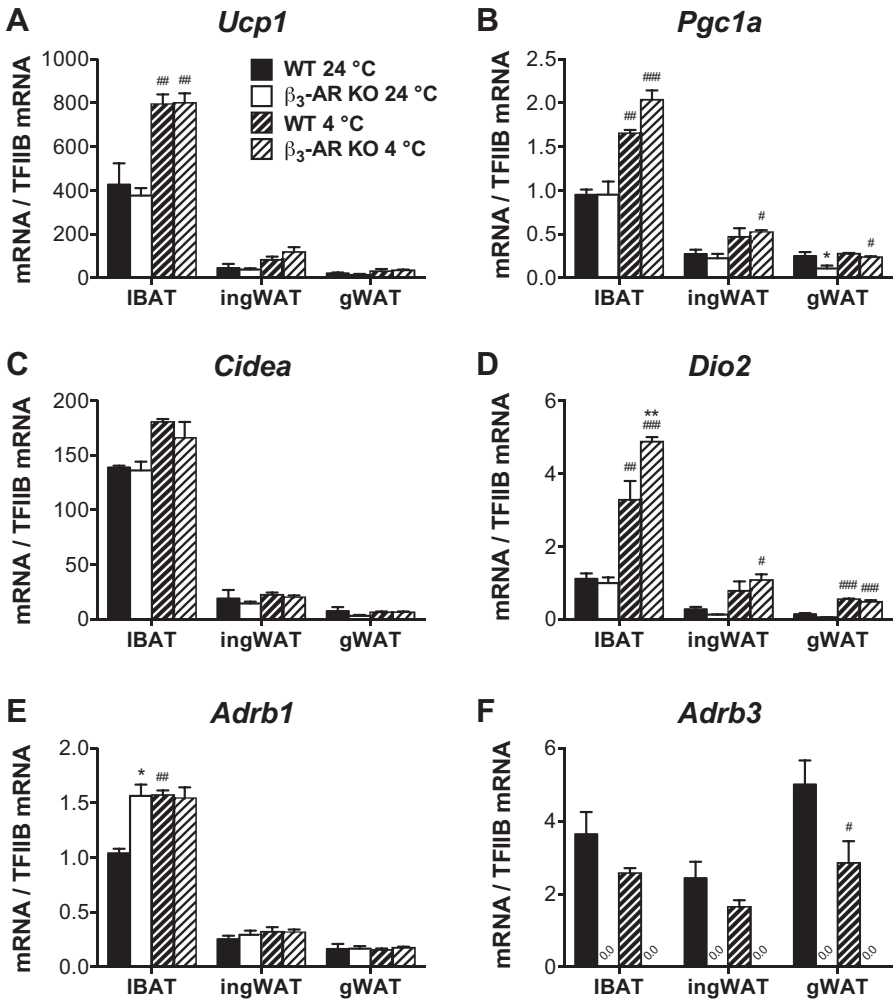

Fig. 4. Mimicking the experimental conditions used in earlier studies. Expression levels of genes as in Fig. 1 in IBAT, ingWAT and gWAT of female wild-type and β_3-AR KO mice after 48 h exposure to cold (4°C) or that remained at 24°C (3–4 mice per group). See legend to Fig. 1 for details of full gene names and statistics. *Significant difference between wild-type and β_3-AR KO mice for each tissue using two-way ANOVA followed by Tukey's multiple comparison test. #Significant difference between 30°C and 4°C calculated similarly. *#$P < 0.05$, **##$P < 0.01$, ###$P < 0.001$.

of the transcript; its expression levels were similar to the levels in wild-type mice (data not shown).

Revelli et al. (45) generated a knockout mouse by the conventional method; the targeting vector was electroporated into embryonic stem cells originating from the 129Sv strain that were then microinjected into blastocysts of C57BL/6J mice. The mutant allele contains a neomycin-resistance cassette introduced in exon I and thus interrupts the coding sequence. The insertion site corresponds to the end of the fifth transmembrane domain, which precedes the third intracellular loop of the receptor, which is of vital importance for signal transduction. Thus, regardless of the genetic method applied for the β_3-AR ablation, both mouse models lack a functional response to β_3-AR agonists (40, 50, and Fig. 5). We are unable to see that differences in the generation of the knockout mice could explain the differences between the observations.

In addition to the differences in the process of generation of the β_3-AR KO mice, the two β_3-AR KO mouse models were also on different genetic backgrounds. The mice used by Jimenez et al. (26) and Barbatelli et al. (4) were on the C57BL/6J or 129Sv background. It is of importance to note that various mouse strains display significant differences in several metabolic parameters, including adrenergic signaling and cold-induced changes in brown and white adipose tissues (12, 16, 19, 24, 41, 47, 53, 55, 57). Also, the phenotype or viability of numerous genetically modified mouse models is dependent on the genetic background of the mouse (2, 29, 30, 37, 51).

Thus, in FVB/N mice (as shown here), but according to the earlier reports not in C57BL/6J and 129Sv mice (4, 26), cold-induced browning of white adipose depots relies on β_3-AR-independent signaling mechanisms. This could be related to the historical background thereof; C57BL/6 and 129Sv mice, although metabolically very different, share a more similar origin than FVB/N mice (6). We have compared the expression levels of the β_1-AR and β_3-AR between these three strains but found no qualitative differences (not shown). Further examination of the effects of the different genetic alterations (mutations) of the β_3-AR in the context of different genetic backgrounds could result in new important insights into the signaling mechanisms in these strains. Irrespective of the genetic background impact, it is clear from the data presented here that the β_3-AR dependence is not a universal trait for brite/beige adipocyte formation. It cannot therefore be used to distinguish between tissues and it cannot a priori be expected that brite/beige-like tissues in other species are β_3-AR dependent, since this is not the case even within the mouse species.

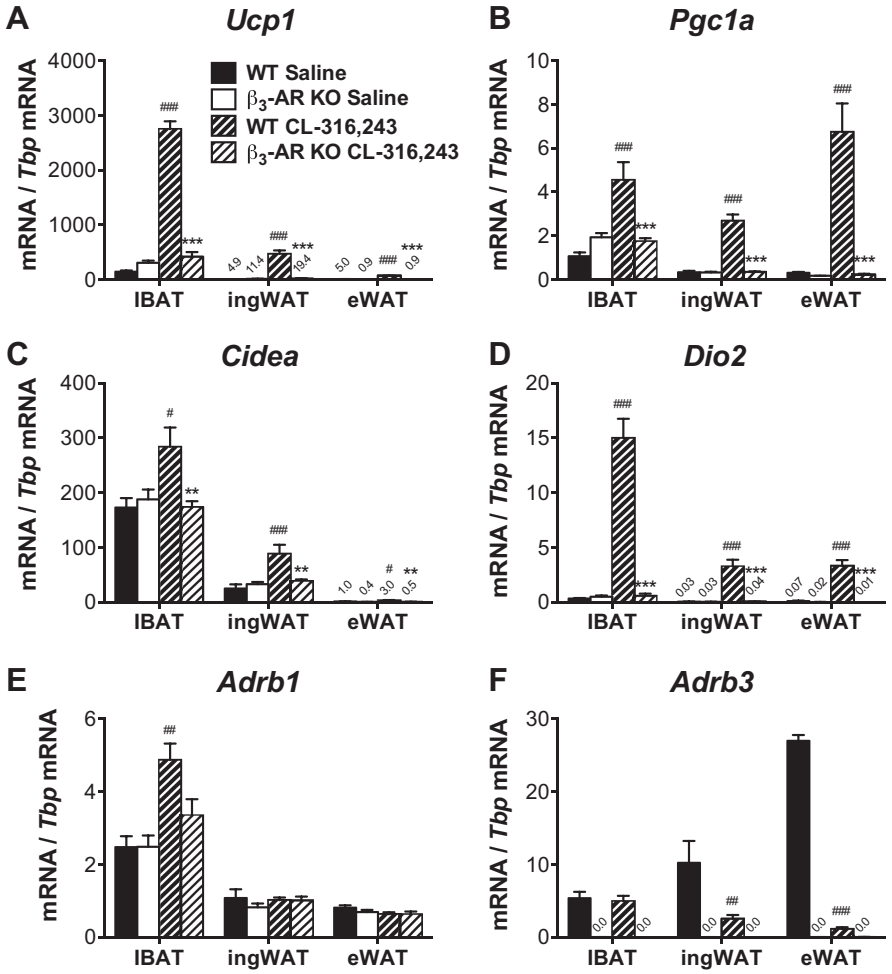

Fig. 5. β3-AR KO mice are devoid of functional β3-AR-response. Expression levels of genes as in Fig. 1 in IBAT, ingWAT and eWAT of male wild-type and β3-AR KO mice after saline or CL-316,243 injections. See legend to Fig. 1 for details of full gene names and statistics (in the present figure # is to indicate a significant difference between saline and CL-316,243). *Significant difference between wild-type and β3-AR KO mice for each tissue using two-way ANOVA followed by Tukey's multiple comparison test. #Significant difference between saline and CL-316,243 calculated similarly. #$P < 0.05$, **##$P < 0.01$, ***###$P < 0.001$.

Contribution of Other Adrenergic Receptors

In the absence of the β3-AR, other signaling mechanisms (adrenergic or nonadrenergic) might be able to compensate in FVB/N mice.

In adipose tissues, physiological responses to sympathetic stimulation are predominantly mediated via β1- and β3-ARs (10); brown fat responds to cold with increased proliferation of precursor cells in a β1-dependent manner (8, 9, 28) while the thermoregulatory response is generally ascribed to the β3-AR (60, 61). As both receptors share the same intracellular signaling pathway, a conceivable explanation for fully preserved functionality of brown adipose tissue in β3-AR KO could be a compensatory increase in amount of the β1-AR (11, 32, 50). However, the effects were modest, and Revelli et al. (45) in their β3-AR KO mouse model even observed a significant decrease of β1-AR expression in BAT but nonetheless, as discussed above, unaltered function of brown fat. In our data, we did not observe consistent alterations of β1-AR expression in β3-AR KO mice.

As we demonstrated here that ingWAT of β3-AR KO mice also competently responded to cold, it may be hypothesized that in ingWAT of FVB/N mice, the β1-AR compensates for the function of β3-AR. Data in support of this hypothesis have been reported by several groups (11, 13, 32, 50). We demonstrated that β1-AR expression levels in ingWAT (the depot that was not previously investigated) of β3-AR KO mice were not different from the levels in wild-type mice (Figs. 1*E*, 3*E*, 4*E*, and 5*E*). However, protein levels of the receptor or the β1-AR receptor activity may still be increased in β3-AR KO mice. However, as validation experiments of several commercial β1-AR antibodies did not result in identification of a suitable antibody, we were unable to examine β1-AR protein abundance [validation examination was performed on β1-AR expressing tissues (brown fat and heart) and, in parallel, on the same tissues from β1-AR KO mice (included as a negative control) (not shown)]. To our understanding, brite/beige adipocyte formation has not yet been investigated in β1-AR-deficient mice (46). Therefore, with the current body of data we can neither accept nor exclude a compensatory role of the β1-AR for the browning of adipose tissues.

Nonadrenergic Signaling Mechanisms

Although alternative (non-β3)-adrenergic signaling mechanisms are likely to be responsible for the observed unaltered cold-induced gene expression in the FVB/N β3-AR KO mice examined here, it cannot be excluded that nonadrenergic signaling mechanisms could also play a role.

Studies using mice lacking all three β-ARs (β-less mice) (3) have implied the presence of nonadrenergic signaling pathways contributing to recruitment and activation of brown and brite/

beige adipocytes (44, 58). Several nonadrenergic signaling molecules, such as adenosine (22, 44), cardiac natriuretic peptides (7), FGF21 (21), prostaglandins (31, 54), and many other compounds (34), have been proposed to induce browning of white adipose tissues.

The Remaining Importance of the β_3-AR

The β_3-AR, in the presently used model, is clearly dispensable for cold-induced browning (32, 50, and the data presented here). However, there are processes that are impaired in the β_3-AR KO mice. The β_3-AR KO model of Revelli et al. (45) has been reported to be more susceptible to high fat diet-induced weight gain than wild-type mice. Based on the proposal that brite/beige adipose depots could only be recruited through β_3-AR signaling (4, 26), it could be inferred that brite/beige depots are of special significance to counteract obesity. However, Preite et al. (39) have recently observed that even in the model of Susulic et al. (50), the lack of β_3-AR makes mice susceptible to high-fat diet-induced obesity. Since we have shown here that brite/beige depots are recruitable even in the absence of the β_3-AR, these experiments, taken together, do not indicate that a specific antiobesity effect can be ascribed to brite/beige adipose depots.

Distinguishing Brown from Brite/Beige

The initial aim of this study was to use the suggested requirement for the β_3-AR for cold-induced browning to distinguish functionally between classical brown and brite/beige adipose cells and tissues. However, the results obtained indicate that this is not possible in this particular model and this shows that the necessity for the β_3-AR is not universal for the formation of brite/beige adipocytes. Thus the question of how to properly distinguish classical brown from brite/beige adipocytes remains. Whether this can be achieved by searching for further molecular markers, developmental origins, or functional differences is still an open question. Our observations further imply that any such future findings might be strain dependent, which should be taken into account if extrapolations are to be made to humans.

In conclusion, our results demonstrate that the browning of WAT in mice is not necessarily dependent on the β_3-AR, in contrast to what has been reported earlier.

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AUTHOR CONTRIBUTIONS

J.M.d.J., R.T.W., and N.P. conceived and designed research; J.M.d.J., R.T.W., N.B., and N.P. performed experiments; J.M.d.J., R.T.W., J.N., and N.P. analyzed data; J.M.d.J., R.T.W., B.C., J.N., and N.P. interpreted results of experiments; J.M.d.J., R.T.W., and N.P. prepared figures; J.M.d.J., J.N., and N.P. drafted manuscript; J.M.d.J., B.C., J.N., and N.P. edited and revised manuscript; J.M.d.J., R.T.W., N.B., B.C., J.N., and N.P. approved final version of manuscript.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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