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$\beta_1/\beta_2/\beta_3$-adrenoceptor knockout mice are obese and cold-sensitive but have normal lipolytic responses to fasting

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Abstract **Catecholamines are viewed as major stimulants of diet- and cold-induced thermogenesis and of fasting-induced lipolysis, through the β-adrenoceptors ($\beta_1/\beta_2/\beta_3$). To test this hypothesis, we generated $\beta_1/\beta_2/\beta_3$-adrenoceptor triple knockout (TKO) mice and compared them to wild type animals. TKO mice exhibited normophagic obesity and cold-intolerance. Their brown fat had impaired morphology and lacked responses to cold of uncoupling protein-1 expression. In contrast, TKO mice had higher circulating levels of free fatty acids and glycerol at basal and fasted states, suggesting enhanced lipolysis. Hence, β-adrenergic signalling is essential for the resistance to obesity and cold, but not for the lipolytic response to fasting.**

Key words: β-Adrenergic; Obesity; Uncoupling protein; Fasting; Thermogenesis

1. Introduction

Epinephrine and norepinephrine are the main effectors of the adrenal medulla and of the sympathetic nervous system. They mediate their effects through α- and β-adrenoceptors. The β-family consists of the β_1-, β_2- and β_3-subtypes [1]. β-Adrenoceptors are involved in development, behaviour, heart function, smooth muscle tone and energy metabolism. The three β-subtypes coexist in both white adipose tissue (WAT) and brown adipose tissue (BAT). In WAT, β-adrenoceptor signalling is thought to stimulate lipolysis in response to fasting. In BAT it mediates heat production in response to cold exposure or overfeeding via activation of the uncoupling protein-1 (UCP1) [2]. Surprisingly, none of the β-adrenoceptor knockout (KO) mice so far generated, i.e., β_1-, β_2-, β_1/β_2-KO [3–5] and β_3-KO [6,7] were cold-sensitive or became overtly obese. These weak metabolic phenotypes suggested a functional redundancy of the β-adrenoceptor subtypes or compensations by pathways other than the β-adrenoceptor system. To investigate these hypotheses, we generated $\beta_1/\beta_2/\beta_3$-adrenoceptor triple-KO (TKO) mice. Here we show their metabolic phenotype characterised by a normophagic obesity and a dramatic cold-intolerance, but a normal lipolytic response to fasting.

2. Materials and methods

2.1. Mice

Animals were treated in accordance with our institutional guidelines. TKO ($\beta_1^{-/-}\beta_2^{-/-}\beta_3^{-/-}$) and wild type (WT) ($\beta_1^{+/+}\beta_2^{+/+}\beta_3^{+/+}$) strains were obtained by inter-crossing our β_3-KO mice [7] and β_1/β_2-KO mice [5], kindly provided by Dr B.K. Kobilka (Howard Hughes Medical Institute, Stanford, CA, USA). $\beta_1^{+/-}\beta_2^{+/-}\beta_3^{+/-}$ Offspring were crossed to generate $\beta_1^{+/+}\beta_2^{+/+}\beta_3^{+/+}$ and $\beta_1^{-/-}\beta_2^{-/-}\beta_3^{-/-}$ mice, from which were established WT and TKO colonies on the same, mixed genetic background (129 Sv/ev, 129 SvJ, FVB/N, C57BL/6J and DBA/2). Genotypes were determined by Southern blot [7,5]. Mice were maintained at 24°C on a 12 h/12 h light/dark cycle (7:00–19:00 h), with free access to water and standard laboratory chow diet (Nordos, Cergy, France) unless otherwise indicated. Mice were housed individually 10 days before and throughout the energy balance experiments. Food intake was measured twice a week and corrected for spillage. Each experiment was repeated two to three times on different offspring from different mating pairs.

2.2. Western blots

BAT mitochondria were prepared and Western blot performed as previously described [8,9], using a sheep polyclonal primary anti-UCP1 antibody (generously provided by Dr D. Ricquier, Meudon, France). The cytochrome oxidase (COX) protein was detected as previously described [9].

2.3. Colonic temperature

The colonic temperature was measured as an index of body temperature, in animals accustomed to handling, to minimise any artifactual 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, Denmark).

2.4. Body composition

Carcasses of mice were dried at 90°C and then homogenised. Total fat content was determined by the Soxhlet extraction method using light petroleum benzine. The fat-free mass was obtained by subtraction of body fat content from dry weight.

2.5. Blood parameters

Serum was obtained from tail or retro-orbital bleeds. Glucose was measured using a 'One touch' glucosemeter (Lifescan, Johnson and Johnson, Milpitas, CA, USA). RIA kits were used for insulin, ^{125}I tracer from Sorin Diagnostics (Saluggia, Italy) and guinea pig anti-rat insulin serum from Linco (St. Charles, MD, USA), for glucagon (Linco) and for free T3 (ICN Pharmaceuticals, Orangeburg, NY, USA). Free fatty acids (FFAs) were measured using the NEFA-C kit (Wako Chemicals, Neuss, Germany) and glycerol as described by Wieland et al. [10].

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2.6. DNA content

BAT tissues were homogenised [8] and DNA content was measured by fluorimetry [11].

2.7. Statistical analysis

Data are expressed as means ± S.E.M. Unless otherwise indicated, significance was evaluated using the unpaired Student's *t*-test. Data were also analysed using a one-way analysis of variance (ANOVA; $P < 0.05$) by the computer software STATISTIX, version 4.0 (Analytical Software, St. Paul, MN, USA).

3. Results and discussion

3.1. Validation of TKO mice

TKO mice were viable and fertile with normal litter sizes. The model was validated by Northern blot on tissues expressing high levels of β_1-, β_2- and β_3-adrenoceptors (heart, lung and BAT, respectively). As expected, no expression of these receptors was observed in TKO mice (data not shown).

3.2. TKO mice are not intolerant to fasting

WAT is the main source of circulating FFAs and glycerol, and β-adrenergic stimulation is considered a major determinant of the fasting-induced increase in lipolysis [12]. We therefore expected the levels of FFAs and glycerol during fasting to be lower in TKO than in WT mice. Instead, TKO mice exhibited 30–60% higher levels of FFAs and glycerol in both the fed and fasted states (Fig. 1A,B). The paradoxical increase of FFAs levels may reflect a decreased peripheral fatty acid utilisation. However, the simultaneous increase in glycerol levels rather suggests an increased lipolysis in TKO mice. These results for the first time show that β-adrenergic signalling is not essential in the control of lipolysis. In addition to β-adrenergic signalling, stimulation of lipolysis during fasting may involve the effect of other lipolytic hormones as well as the decrease of the antilipolytic effect of insulin [13]. The paradoxical increase of FFAs in TKO mice interestingly occurs despite normal insulin and glucose levels (Table 1). Increased lipolysis might hence be due to an over-compensation by lipolytic hormones other than catecholamines. The possible implication of glucagon [14], glucocorticoids [15] or adrenocorticotropin hormone [16] deserves further study. In this respect it is interesting to note that TKO fasted mice exhibited 23% higher glucagon levels than WT controls (Table 1).

3.3. TKO mice are dramatically cold-intolerant

The thermogenic capacity of small mammals depends on shivering and on BAT function. Cold exposure stimulates the BAT sympathetic nervous system, which, via the β-adrenoceptors, mediates a rapid increase in UCP1 expression and thermogenesis and later an increase in BAT size. This phenomenon is called cold-induced thermogenesis [17].

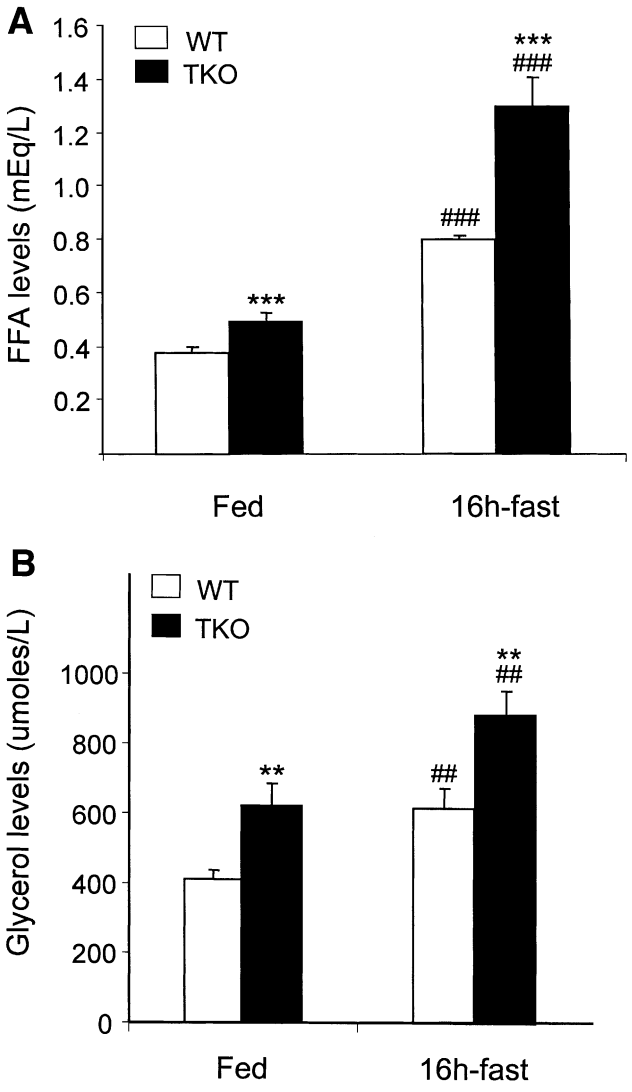

Fig. 1. Circulating FFAs and glycerol in fed and fasted WT and TKO female mice. Fed values were measured 4 h after the beginning of the dark cycle and fasted values after 16 h of fasting (started at 18.00 h). (A) FFAs, $n = 15$, two separate experiments, ANOVA. (B) Glycerol, $n = 4$–6. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ in fasted vs fed, respectively; $^{**}P < 0.01$, $^{***}P < 0.001$ between genotypes. Results are expressed as means+S.E.M.

At 24°C, the colonic temperature of TKO mice was 1.2°C lower than that of the WT (36.7°C ± 0.2 vs 37.9°C ± 0.1; $P < 0.005$). This slight hypothermia was not due to a decrease in thyroid hormone levels (Table 1).

Upon exposure to 6°C, most TKO mice (10 out of 13) exhibited a clear cold-intolerance during the first 24 h

Table 1
Blood parameters

	Fed		Fasted	
	WT	TKO	WT	TKO
Glucose (mg/dl)	94 ± 3	80 ± 5	48 ± 3$^{\#\#\#}$	48 ± 2$^{\#\#\#}$
Insulin (pg/ml)	1005 ± 20	992 ± 6	570 ± 4$^{\#\#\#}$	538 ± 1$^{\#\#\#}$
Glucagon (pg/ml)	113 ± 12	131 ± 13	124 ± 5	152 ± 8*
T_3 (pmol/l)	6.3 ± 0.2	6.7 ± 0.2	–	–

Results are from 3-month old female mice. $^{\#\#\#}P < 0.005$ in fasted vs fed, $^{*}P < 0.05$ between genotypes. ANOVA. Fed values were measured 4 h after the beginning of the dark cycle and fasted values after a 16 h, overnight fasting. $n = 5$–10.

(Fig. 2A). After 48 h of cold exposure, the few surviving TKO mice had a colonic temperature about 4°C lower than that of WT mice (Fig. 2A) and performed intense shivering, as assessed by visual inspection. Furthermore, their food intake was only 50% of that of the cold-exposed WT mice (1.3 ± 0.1 vs 2.5 ± 0.2, $P < 0.005$).

BAT of TKO mice were heavier than that of WT animals (171 ± 12 mg vs 83.0 ± 6.8 mg; $n = 10$; $P < 0.005$), with an abnormal light-brown colour (data not shown). In vitro, β_1- and β_3-adrenoceptors control, respectively, the proliferation and differentiation of brown adipocytes into multilocular cells expressing UCP1 [18]. The total number of cells in TKO BAT was not lower than in WT, as assessed by DNA content (900 ± 62 vs 747 ± 55 μg per BAT, $n = 6$; $P = 0.1$), but the brown adipocytes, hypertrophied and unilocular, looked like white adipocytes (Fig. 3). TKO BAT also lacked adaptive responses to 48 h cold exposure, such as increased UCP1 protein expression (Fig. 2B). A similar phenotype was recently observed in another model of TKO mice [19].

Taken together, these results demonstrate that β-adrenergic signalling is essential for the control of body temperature and for normal adaptive responses of the BAT to cold exposure.

3.4. TKO mice are obese

As shown in Table 2, TKO mice developed a progressive obesity at adulthood. Three-month old TKO female mice had a normal body weight, but a percentage body fat 33% higher than that of WT mice. Onset of obesity occurred later in TKO male mice. At 5 months, they had a 16% higher body weight and a 64% higher percentage body fat than WT controls. Interestingly, the absolute fat-free dry mass was normal in TKO mice. This is surprising, since β_2-adrenoceptors mediate anabolic effects on muscle protein synthesis [20].

Development of obesity may have various origins: increased food intake, impaired lipid mobilisation from fat stores, or decreased energy expenditure. Strikingly, obesity in TKO mice is not due to hyperphagia, since food intake was normal in the period during which mice developed obesity (Table 2). It can neither be ascribed to impaired lipid mobilisation from fat stores, since lipolysis may in fact be increased in TKO mice (see above).

A meal or a period of overeating stimulates, via the β-adrenoceptors, UCP1 expression in BAT and thermogenesis, a phenomenon called diet-induced thermogenesis [21]. The higher adiposity despite similar food intake reflects a higher food efficiency in TKO as compared to WT mice. This could be explained, in part, by hypothermia of TKO mice at 24°C and/or by a defect in diet-induced thermogenesis. Indeed, obesity was recently associated with defective diet-induced thermogenesis in another model of TKO mice [19]. A contribution of impaired BAT function to the phenotype is likely, since mice with a genetic ablation of BAT are cold-sensitive and obese [22]. Nevertheless, UCP1 KO mice, which also have a defect in BAT function, are cold-sensitive but not obese [23]. Therefore obesity in TKO mice cannot be ascribed solely to a defect in BAT thermogenesis.

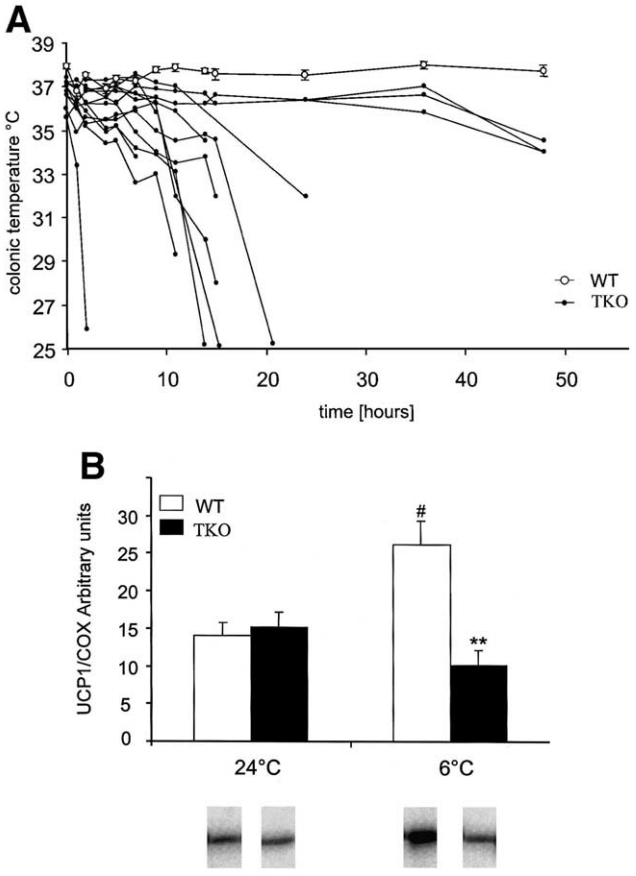

Fig. 2. Thermoregulation during cold-exposure (6°C) in WT and TKO female mice. (A) Colonic temperatures of individual TKO female mice (●) compared to the mean ± S.E.M. temperature of WT mice (○); $n = 13$, two separate experiments. (B) Western blot quantification of UCP1 in BAT of WT and TKO mice kept at 24°C or exposed 48 h to 6°C. Results are expressed as means ± S.E.M. of arbitrary values normalised with corresponding COX values; $n = 3$. Insets, representative signals. $^{\#\#}P < 0.05$ in cold-exposed vs 24°C, respectively; $^{**}P < 0.01$ between genotypes.

Altogether, our results demonstrate that, whereas β-adrenergic signalling is not a key component of the response to fasting, it plays an essential role in the control of body tem-

Table 2
Body weight, food intake and body composition

	Females		Males	
	WT	TKO	WT	TKO
Body weight (g)	21.4 ± 0.3	20.6 ± 0.9	32.0 ± 0.8	37.0 ± 1.3***
Food intake (g/day)	3.7 ± 0.2	3.7 ± 0.4	4.1 ± 0.2	4.1 ± 0.2
Body fat content (g)	3.4 ± 0.1	4.1 ± 0.1***	5.9 ± 0.5	11.5 ± 0.9***
% Body fat	15.3 ± 0.6	20.3 ± 0.6***	18.0 ± 1.4	29.6 ± 1.4***
Fat-free dry mass	4.7 ± 0.1	4.1 ± 0.1	7.5 ± 0.1	7.5 ± 0.2
% Fat-free dry mass	21.2 ± 0.3	20.3 ± 0.5	22.7 ± 0.2	19.6 ± 0.5***

Results are from 3 month-old female and 5 month-old male mice. Food intake was measured between the ages of 2 to 3 month in female and of 2 to 5 months in male mice. $^{***}P < 0.005$ between genotypes; $n = 15$ for body weight and food intake and 5–6 for body composition.

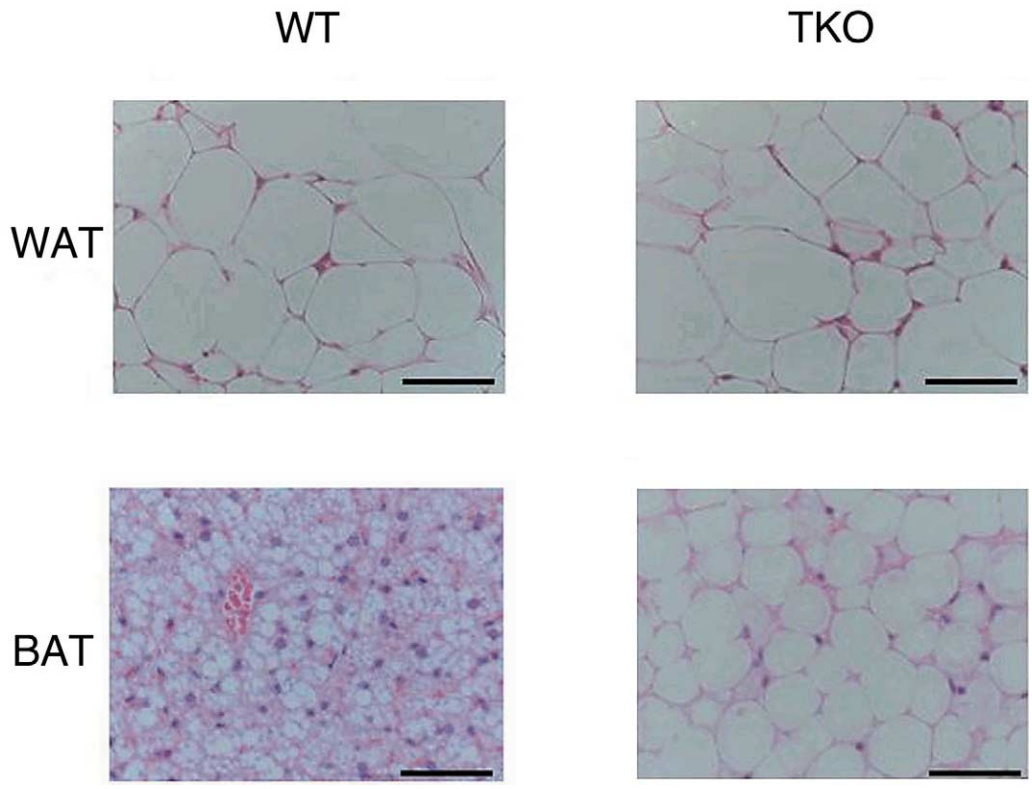

Fig. 3. Morphology of adipose tissues in WT and TKO female mice. Hematoxylin–eosin-stained sections (5 μm) of (a) WAT and (b) BAT. $n = 3$, representative microphotographs are shown. Scale bar = 50 μm.

perature and energy balance. Our study should further stimulate the interest in searching for new thermogenic β-agonists, which have been considered during the last decades as a major strategy to treat obesity in humans.

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