β_1 Adrenergic receptor is key to cold- and diet-induced thermogenesis in mice

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Abstract

Brown adipose tissue (BAT) is predominantly regulated by the sympathetic nervous system (SNS) and the adrenergic receptor signaling pathway. Knowing that a mouse with triple β-receptor knockout (KO) is cold intolerant and obese, we evaluated the independent role played by the β_1 isoform in energy homeostasis. First, the 30 min i.v. infusion of norepinephrine (NE) or the β_1 selective agonist dobutamine (DB) resulted in similar interscapular BAT (iBAT) thermal response in WT mice. Secondly, mice with targeted disruption of the β_1 gene (KO of β_1 adrenergic receptor (β_1KO)) developed hypothermia during cold exposure and exhibited decreased iBAT thermal response to NE or DB infusion. Thirdly, when placed on a high-fat diet (HFD; 40% fat) for 5 weeks, β_1KO mice were more susceptible to obesity than WT controls and failed to develop diet-induced thermogenesis as assessed by BAT *Ucp1* mRNA levels and oxygen consumption. Furthermore, β_1KO mice exhibited fasting hyperglycemia and more intense glucose intolerance, hypercholesterolemia, and hypertriglyceridemia when placed on the HFD, developing marked non-alcoholic steatohepatitis. In conclusion, the β_1 signaling pathway mediates most of the SNS stimulation of adaptive thermogenesis.

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Introduction

The brown adipose tissue (BAT) is a highly specialized organ capable of heat production in response to a drop in environmental temperature or feeding on a high-caloric diet (Silva 1995). While BAT's role in small and in hibernating animals is well established (Silva 1995), more recently, its presence in adult humans has pointed to a possible metabolic role and therapeutic targeting for obesity (Virtanen *et al.* 2009). BAT thermogenesis is activated by the sympathetic nervous system (SNS) with the release of norepinephrine (NE) and stimulation of α and β adrenergic receptors (Bachman *et al.* 2002). The latter are G-protein-coupled receptors that include the β_1, β_2, and β_3 isoforms, all of which are expressed in BAT (Collins & Surwit 2001). The critical role played by these receptors in BAT function and overall energy homeostasis is illustrated by the fact that mice with combined targeted disruption of the three β_1, β_2, and β_3 adrenergic receptors (TKO mice) have increased susceptibility to cold-induced hypothermia as well as diet-induced obesity (Bachman *et al.* 2002, Jimenez *et al.* 2002). However, the relative contribution of each β adrenergic receptor isoform to these processes is less understood (Collins & Surwit 2001, Lowell & Bachman 2003, Masuo & Lambert 2011).

BAT β_1, β_2, and β_3 adrenergic receptor mRNA transcripts are found at a ratio of 3:1:150 in mice acclimated at thermoneutrality, respectively, with the β_3 receptor exhibiting a much lower affinity for catecholamines than β_1/β_2 (Collins *et al.* 1994). In isolated murine BAT membrane preparations, it has been estimated that about 23% of maximally stimulated adenylate cyclase activity is mediated by the β_1/β_2 receptors, while ~77% is mediated by the β_3

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receptors (Collins *et al.* 1994). By contrast, in preparations of rat BAT membranes, about 70% of the maximally stimulated adenylate cyclase activity is via the β_1/β_2 receptors and only about 30% is via the β_3 receptor (Granneman 1992).

Studies performed on isolated brown adipocytes indicate that the β_3 adrenergic receptor plays the leading role in BAT activation (Zhao *et al.* 2011). However, mice with global β_3 receptor knockout (β_3KO) have no significant defect in basal metabolic rate, adaptive response to cold exposure (Susulic *et al.* 1995), or metabolic response to diet-induced obesity (Rohrer 1998). These animals do exhibit upregulation of β_1 (not β_2) adrenergic receptors, suggesting that either the β_3 receptor is not critical for thermogenesis or that its absence is compensated by an increase in β_1 receptor expression (Atgie *et al.* 1997, Rohrer 1998). At the same time, mice with β_1 adrenergic receptor overexpression are resistant to diet-induced obesity (Soloveva *et al.* 1997). Studies on humans confirm the relative role played by β_1 and β_2 adrenergic receptors in the determination of the basal metabolic rate, with β_1 being the most relevant isoform (Jansky 1995, Jansky *et al.* 2008).

Here, we report that infusion of the β1 adrenergic receptor selective agonist dobutamine (DB) triggers a similar interscapular BAT (iBAT) thermal response as NE. Mice with targeted disruption of the β1 gene (β_1KO) developed cold-induced hypothermia and exhibit decreased catecholamine-stimulated iBAT thermal response. They also fail to activate energy expenditure when placed on a high-fat diet (HFD) and thus are more susceptible to diet-induced obesity, indicating that the β1 signaling pathway mediates most of the SNS stimulation of adaptive thermogenesis.

Materials and Methods

Animals

Ninety-day-old male β_1KO mice in C57BL6/J background were evaluated. This investigation conforms to the guidelines of the committee on animal research at the Center of Biological Sciences and Health, University Presbyterian Mackenzie, and was approved by the Institutional Animal Welfare Committee. Each experiment was repeated two or three times on different sets of animals. In one set of experiments, mice were fed with chow diet and cold-induced thermogenesis was evaluated. In another set of experiments, mice were fed a HFD and diet-induced thermogenesis was evaluated.

Core body temperature during cold exposure

For cold exposure, conscious mice were housed individually in cages with no bedding in a cold room and maintained at 4 °C (Eletrolab, São Paulo, SP, Brazil) for up to 3 h with central body temperature measurements every hour. This parameter was measured using an electronic thermistor equipped with a rectal probe of 1 mm diameter (Y4000, Yellow Springs Instrument Co., Yellow Springs, OH, USA) as described previously (de Jesus *et al.* 2001).

Interscapular brown fat thermal response to NE and DB infusion in anesthetized mice

The measurements of these parameters were performed under anesthesia as described (Ribeiro *et al.* 2001). Briefly, all animals were anesthetized with urethane (560 mg/kg, i.p.) and chloralose (38 mg/kg, i.p.) on the morning of the experiment and a polyethylene (P-50) cannula was inserted into the left jugular vein. For measurement of iBAT temperatures (°C), a precalibrated thermistor probe (YSI 427; Yellow Springs Instrument Co.) was surgically placed under the brown fat pad. The iBAT temperature was measured for ∼10 min to obtain a stable baseline and then NE (2 mg/ml) or DB (600 μg/ml) was infused into the left jugular vein via infusion pump (model 2274, Harvard Apparatus, Holliston, MA, USA) at a rate of 0·643 μl/min for 30 min. After the experiment, animals were killed.

Treatment

To evaluate diet-induced thermogenesis, β_1KO male mice were placed on a HFD (Rhoster, Sao Paulo, Brazil) for 5 weeks. HFD presents 5·44 cal/g (40% lipid) while chow diet presents 1·8 cal/g. Food intake and body weight (BW) were measured daily.

Oxygen consumption

Resting oxygen consumption (VO_2) was measured in an open circuit respirometer system (O2-10, Sable System, Las Vegas, NV, USA) as described previously (17, 18). For cold exposure, mice were exposed to different temperatures (5, 15, 25, 30, 32, 34, and 36 °C) and the O_2 consumption was measured for 1 h at each temperature. The experiments begin in the late morning and finished by the end of the day (1100–1900 h). The data were collected and analyzed by the Sable Systems Software. The results are expressed as milliliters of O_2/min per g BW. For experiments performed at room temperature (25 °C), measurements were obtained over 30 min, in the afternoon (1400–1800 h) in animals fed *ad libitum*.

mRNA analysis

Total RNA of iBAT was extracted using TRIzol (Life Technologies, Inc.), according to the manufacturer's instructions, and quantified by spectrophotometry. For the reverse transcriptase reaction, 1·0 μg total RNA was used in the ImProm-II Reverse Transcription System for real-time PCR (RT-PCR; Promega) on a Robocycler thermocycler (Stratagene, La Jolla, CA, USA). Based on the reaction efficiency, about 120 ng cDNA was used for amplification.

Quantitative RT-PCR was performed using IQ SYBR Green PCR kit (Bio-Rad) on an iCycler thermal cycler (Bio-Rad). The housekeeping gene cyclophilin A was used as the internal reference. Primer sequences are available upon request. The cycle conditions were 5 min at 94 °C, 30 s at 94 °C, 30 s at 58 °C, and 45 s at 72 °C for 50 cycles followed by the melting curve protocol to verify the specificity of amplicon generation. Gene expression was determined by the $\Delta\Delta C_t$ method, as previously described by Livak (Christoffolete *et al.* 2004).

Western blot

iBAT was rapidly removed and processed for mitochondrial isolation. Uncoupling protein-1 (UCP1; Santa Cruz Biotechnology) was quantified after mitochondrial proteins were size-fractionated by 12% SDS–PAGE and identified by western blotting.

Intraperitoneal glucose tolerance test

Animals were fasted overnight and glucose (2 g/kg) was administered by i.p. injection between 0900 and 1000 h. Blood samples were collected from the tail at various times after the glucose load, as indicated, and glycemia was immediately determined on a glucose analyzer (LifeScan, Inc., Milipitas, CA, USA).

Intraperitoneal insulin tolerance test

Food was removed 6 h before the experiment was carried out between 1400 and 1500 h. Blood samples were collected from the tail at various times after insulin (0·5 U/kg) was administered by i.p. injection, as indicated, and glycemia was immediately determined on the glucose analyzer.

Blood chemistry

Total cholesterol and triglycerides were assessed via enzymatic method using a commercial kit (Roche Molecular Biochemicals) in a supernatant aliquot.

Histology

After careful dissection, tissues were immersed in buffered formol solution and fixed for 24 h. Paraffin-embedded tissues were sectioned and processed. Analyses were performed after hematoxylin–eosin or Masson staining. The area of the white adipocytes was estimated by analyzing pictures taken at 200× magnification. Picture printouts were cut, and the area of at least 40 adipocytes per animal was estimated.

Statistical analysis

Statistical analyses were done by ANOVA followed by Student's and Newman–Keuls post-test when $P<0{\cdot}05$.

Results

β_1 adrenergic receptors are key to cold-stimulated adaptive thermogenesis

To evaluate the role of β_1 adrenergic receptor in cold-stimulated adaptive thermogenesis, mice were housed individually with no bedding at 4 °C for up to 3 h and core body temperature measured hourly. All animals experienced a slight decrease in core temperature 1 h into the cold exposure, with the wild-type (WT) controls returning to baseline values at the end of the experiment (Fig. 1A). However, β_1KO animals experienced a more prolonged and intense hypothermia, with a substantial drop in core temperature by the end of the experiment ($-6{\cdot}2\pm0{\cdot}7$ °C). A similar contrasting response between the two groups of animals was observed while monitoring VO_2 during cold exposure, with WT animals exhibiting a much more pronounced acceleration of VO_2 when compared with β_1KO mice (Fig. 1B).

To test the hypothesis that β_1KO animals have defective BAT adaptive thermogenesis, we tested their iBAT thermal response to a 30-min infusion with NE. While WT animals exhibited an increase in ~2 °C in iBAT temperature, β_1KO mice exhibited a much reduced response (only about 0·55 °C; Fig. 1C). The relevance of the β_1 adrenergic signaling pathway in this process was further explored during infusion with the β_1 receptor selective agonist DB (Aikawa *et al.* 1996, Huang *et al.* 1998). In WT, the increase in iBAT temperature in response to DB infusion was not different from the response

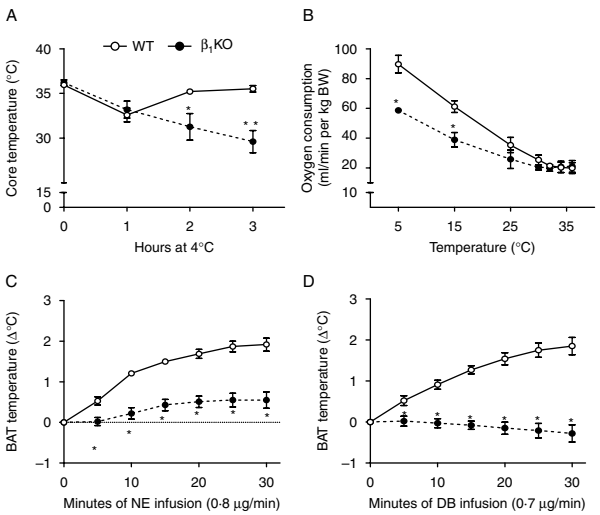

Figure 1 Lack of β_1 adrenergic receptor on cold-induced thermogenesis. (A) Central body temperature during cold exposure (4 °C) for 3 h of C57 (WT) and β_1KO; *$P<0{\cdot}05$ and **$P<0{\cdot}001$ vs WT; (B) oxygen consumption during decreasing temperature exposure of C57 (WT) and β1KO mice; *$P<0{\cdot}001$ vs WT; (C) BAT thermogenic response to NE infusion of C57 (WT) and β1KO mice; *$P<0{\cdot}004$ vs WT; (D) BAT thermogenic response during infusion of DB of C57 (WT) and β1KO mice; *$P<0{\cdot}004$ vs WT; entries are mean±S.E.M. of six animals per group.

to NE, supporting the major role played by the β_1 adrenergic signaling pathway (Fig. 1D). By contrast, there was no response to DB infusion in β_1KO animals (Fig. 1D). As a whole, these data indicate that the β_1 adrenergic signaling pathway mediates most of the iBAT response to NE infusion and is critical in the acute BAT activation during cold exposure.

Role of β_1 adrenergic receptor in diet-induced thermogenesis

To further evaluate the role of the β_1 adrenergic receptor in energy homeostasis, WT and β_1KO mice were fed with a HFD for 5 weeks. The integrated caloric intake was approximately two times higher in animals fed with a HFD (Fig. 2A), resulting in a greater weight gain in both WT and β_1KO mice when compared with the respective controls kept on chow diet (Fig. 2B). However, in response to high-fat feeding, β_1KO animals gained significantly more BW (Fig. 2B) and exhibited increased white adipocyte area (Fig. 2C) when compared with WT animals placed on the same diet. Remarkably, whereas in the WT animals fed a HFD resulted in an ~50% increase in VO_2, this induction in VO_2 was blunted in the β_1KO mice (Fig. 2D), explaining their increased susceptibility to diet-induced obesity. This is further supported by the observation that the HFD-induced increase in BAT UCP1 levels was blunted in the β_1KO animals (Fig. 2E). Notably, no compensatory changes in the expression of the other two BAT β adrenergic receptor isoforms were observed in the β_1KO animals (Fig. 2F) and the HF-induced increase in BAT β_2 adrenergic receptor mRNA was similar in both the groups of animals (Fig. 2F).

Glucose homeostasis

Fasting blood glucose levels and tolerance to glucose administration were similar in WT and β_1KO animals kept on a chow diet (Fig. 3A and B). At the same time, feeding on a HFD promoted fasting hyperglycemia and glucose intolerance in both strains of animals, with much more pronounced changes observed in the β_1KO mice (Fig. 3A and B). Sensitivity to insulin was significantly decreased in B1KO mice as compared to WT animals. Feeding on an HFD only minimally affected this parameter (Fig. 3C and D).

Cholesterol and triglyceride plasma levels of β_1KO mice

Cholesterol and triglycerides plasma levels were assessed to evaluate the effect of β_1KO on lipid metabolism during high-fat feeding (Table 1). β_1KO mice did not exhibit changes in plasma cholesterol or triglyceride levels when kept on a chow diet. However, when placed on HFD, the β_1KO mice

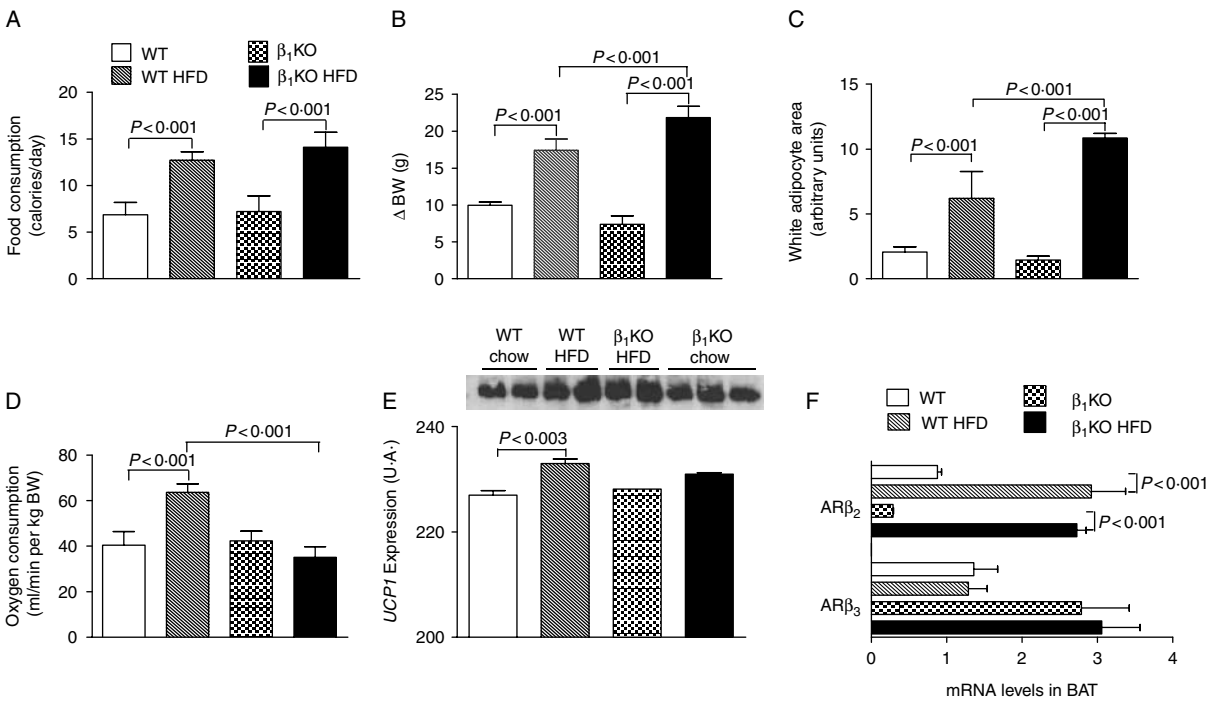

Figure 2 Lack of β_1 adrenergic receptor on diet-induced obesity of mice placed on a HFD. WT and β_1KO mice were followed for 5weeks while kept on a chow diet or on a HFD; (A) caloric intake was calculated based on daily food consumption; By (B) variances of BW (ΔBW) are shown; (C) estimated individual epididymal adipocytes area; 40 cells for each group were analyzed; (D) VO_2 as assessed during 30 min at the end of the experiment; (E) *UCP1* expression performed by western blot; (F) gene expression of β_2 and β_3 adrenergic receptors in mice kept on a chow or HFD in BAT.

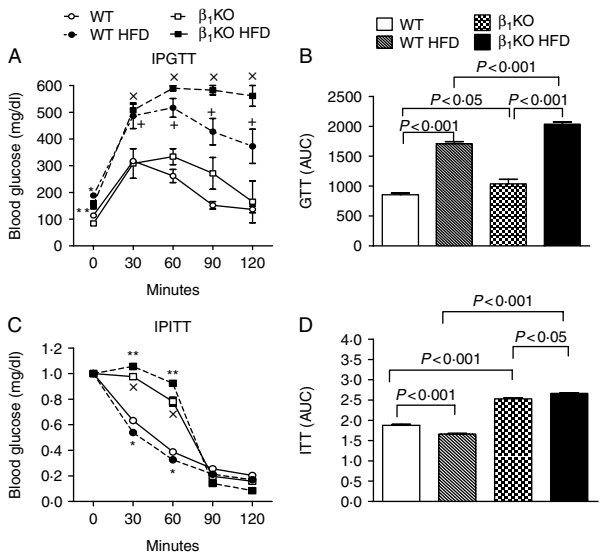

Figure 3 Lack of β_1 adrenergic receptor on glucose, insulin tolerance of mice kept on a chow diet or on a HFD. (A and B) Blood glucose levels before and after i.p. administration of 2 g/kg glucose (GTT) or (C and D) after i.p. injection of 0·5 U/kg insulin (ITT) at the indicated time points in WT and β_1KO animals kept on chow or high-fat diet; A) $^*P<0{\cdot}05$ vs WT, $^{**}P<0{\cdot}05$ vs β_1KO, $^{+}P<0{\cdot}001$ vs WT, $^{\times}P<0{\cdot}001$ vs β_1KO; C) $^*P<0{\cdot}01$ vs. WT; $^{**}P<0{\cdot}01$ vs β_1KO; $^{\times}P<0{\cdot}01$ vs WT.

exhibited a much higher elevation in plasma cholesterol and triglycerides levels compared with WT mice (Table 1). Also, high-fat feeding caused a much more dramatic increase in liver fat deposition in β_1KO when compared with WT animals, leading to the development of nonalcoholic steatohepatitis (Fig. 4).

Discussion

It is well accepted that β_3 adrenergic receptors play the lead role in thermoregulation and energy homeostasis through their mediation in sympathetic stimulation of BAT adaptive thermogenesis (Silva 2005). However, the present data challenge this paradigm by demonstrating that a major fraction of the sympathetic stimulation of BAT thermogenesis in response to cold exposure and high-fat feeding is mediated through β_1 adrenergic receptors. Targeted inactivation of the β_1 adrenergic receptor gene promoted hypothermia, limited acceleration of energy expenditure in response to cold exposure or HFD, poor thermal iBAT response to NE and DOB infusion, increased adiposity and obesity during high-fat feeding, as well as glucose intolerance, hypercholesterolemia, and hypertriglyceridemia. These findings are particularly relevant in view of the recent observation that functionally responsive BAT is present in adult individuals (Cypess *et al.* 2009, Virtanen *et al.* 2009). It is conceivable that a disruption in β_1 adrenergic receptor could play a role in thermoregulation and energy homeostasis if a similar role for the β_1 adrenergic receptor pathway is found in humans.

Our data suggest that the β_1 adrenergic receptor is fundamental for the activation of thermogenesis during cold exposure, as evidenced by the finding that β_1KO mice do not maintain their body temperature at 4 °C. In fact, the BAT of β_1KO mice is thermogenically inactive as NE or DB infusion failed to increase its temperature and histology showed a pale BAT, although the amount of BAT tissue and UCP1 levels in β_1KO are normal when compared with WT. These data confirm the previous study showing that the increase in cAMP levels is fundamental to activate BAT to produce heat, despite high levels of UCP1 (Ribeiro *et al.* 2001). Several other studies performed on mice with β_1KO have shown that compensatory mechanisms may be found in KO models (Atgie *et al.* 1997, Rohrer 1998). In fact, there was a tendency to an increase in β_3 adrenergic receptor mRNA in β_1KO BAT, although it is not sufficient to compensate the absence of β_1 as the mice developed severe hypothermia when exposed to 4 °C. This study showed that β_1 adrenergic receptor is the main isoform that mediates BAT activation during cold-induced thermogenesis.

The increase in oxygen consumption induced by lower temperatures is severely impaired in β_1KO mice but not entirely abolished. The small increase in oxygen consumption observed in cold-exposed β_1KO mice may be due to shivering thermogenesis, the first line of defense against cold environments in mammals. Other mechanisms of BAT-independent cold-induced thermogenesis should be considered for the oxygen consumption increase observed in β_1KO. UCP1-ablated mice clearly present a UCP1-independent thermogenesis when stimulated by NE, a situation similar to cold stimulation (Enerback *et al.* 1997, Feldmann *et al.* 2009). Nonetheless, these mechanisms are not sufficient to compensate for the lack of BAT thermogenesis in

Table 1 Lack of β_1 adrenergic receptor on lipid metabolism of mice placed on chow diet or high-fat diet. Values are mean±S.E.M. of six animals per group

	WT	WT HFD	β_1KO	β_1KO HFD
Cholesterol	91·5±9·8	158·9±37·8[a]	82·3±15·7	185·7±48·9[b]
Triglycerides	100·1±8·1	163·7±5·1[a]	107·2±9·1	203·9±12·1[b,c]

[a]$P<0{\cdot}001$ vs WT.
[b]$P<0{\cdot}01$ vs β_1KO.
[c]$P<0{\cdot}001$ vs WT HF.

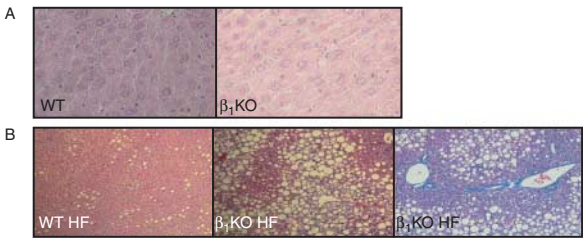

Figure 4 Liver histology. Sections of liver stained by H&E (A) or Masson's trichrome (B); magnification is 180×.

order to maintain body temperature, as β_1KO mice maintained at 4 °C have a significant drop in body temperature and die if not warmed. These data are consistent with the concept that BAT is the main site mediating body temperature regulation by the SNS in mice and also that this physiological response is mediated by the β_1 adrenergic receptor.

Given that BAT is the main site for cold-induced thermogenesis and that it is logical to assume that as the β_1KO mice are cold sensitive, they should be more prone to develop obesity when fed with a HFD. In agreement with our data, a previous study showed that transgenic mice over-expressing the β1 adrenergic receptor in adipose tissue are resistant to obesity (Soloveva *et al.* 1997).

The present data showed that the absence of the β1 adrenergic receptor leads to a more severe metabolic syndrome induced by HFD. The animals showed significant alterations of metabolic parameters such as hyperglycemia, hypertriglyceridemia, and hypercholesterolemia. Also, β_1KO mice presented an increase in liver fat deposition and the development of nonalcoholic steatohepatitis.

Although hyperglycemia is commonly related to obesity, it is possible that the hyperglycemia observed in β1KO mice may be due to a direct effect of the absence of the β_1 adrenergic receptor. Previous work with NE-induced activation of glucose uptake in brown adipocytes has implicated the β_1 and β_3 adrenergic receptors in SNS-mediated glucose uptake in brown adipocytes, allowing for synergy between SNS and insulin signaling (Nikami *et al.* 1996, Chernogubova *et al.* 2004). In conclusion, our data give strong support to the idea that β_1 adrenergic receptors are the main mediators of the facultative thermogenesis activated by SNS and have a fundamental role in regulating energy expenditure.

Declaration of interest

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

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