Inactivation of the adrenergic receptor $\beta_2$ disrupts glucose homeostasis in mice

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Abstract

Three types of beta adrenergic receptors (AR$\beta_{1–3}$) mediate the sympathetic activation of brown adipose tissue (BAT), the key thermogenic site for mice which is also present in adult humans. In this study, we evaluated adaptive thermogenesis and metabolic profile of a mouse with AR$\beta_2$ knockout (AR$\beta_2$KO). At room temperature, AR$\beta_2$KO mice have normal core temperature and, upon acute cold exposure (4 °C for 4 h), AR$\beta_2$KO mice accelerate energy expenditure normally and attempt to maintain body temperature. AR$\beta_2$KO mice also exhibited normal interscapular BAT thermal profiles during a 30-min infusion of norepinephrine or dobutamine, possibly due to marked elevation of interscapular BAT (iBAT) and of AR$\beta_1$ and AR$\beta_3$ mRNA levels. In addition, AR$\beta_2$KO mice exhibit similar body weight, adiposity, fasting plasma glucose, cholesterol, and triglycerides when compared with WT controls, but exhibit marked fasting hyperinsulinemia and elevation in hepatic Peck (Pck1) mRNA levels. The animals were fed a high-fat diet (40% fat) for 6 weeks, AR$\beta_2$KO mice doubled their caloric intake, accelerated energy expenditure, and induced Ucp1 expression in a manner similar to WT controls, exhibiting a similar body weight gain and increase in the size of white adipocytes to the WT controls. However, AR$\beta_2$KO mice maintain fasting hyperglycemia as compared with WT controls despite very elevated insulin levels, but similar degrees of liver steatosis and hyperlipidemia. In conclusion, inactivation of the AR$\beta_2$KO pathway preserves cold- and diet-induced adaptive thermogenesis but disrupts glucose homeostasis possibly by accelerating hepatic glucose production.

Key Words
- $\beta$-adrenergic receptors
- adaptive thermogenesis
- brown adipose tissue (BAT)
- obesity

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Introduction

Brown adipose tissue (BAT) is an important site of adaptive thermogenesis, preserving thermal homeostasis in response to cold exposure in both rodents and humans (Cannon & Nedergaard 2004, Nedergaard & Cannon 2010), as well as dissipating caloric excess in rodents in response to feeding with a high caloric diet (Rothwell & Stock 1979, Vosselman et al. 2013). Thus, understanding of the mechanisms regulating BAT activity may help in development of new strategies to accelerate energy expenditure and address the current high prevalence of obesity.

In mice, BAT develops during the 3-day window E16.5–18.5 (Hall et al. 2010) and after birth can be activated by the sympathetic nervous system (SNS) through stimulation of β-adrenergic receptors (ARβ) and cAMP production (Cannon & Nedergaard 2004). In BAT, cAMP signaling not only induces the expression of genes involved in the thermogenic capacity of the tissue but also promotes lipolysis and activates the mitochondrial uncoupling protein-1 (Ucp1) that leads to heat production. The importance of this pathway is illustrated by the fact that mice in which all three ARβ isoforms have been inactivated exhibit increased susceptibility to cold exposure as well as diet-induced obesity (Bachman et al. 2002, Jimenez et al. 2002).

The ARβs are members of a family of three G-protein-coupled receptors, i.e. β1, β2, and β3, that are distributed throughout the body and play their metabolic roles by controlling glucose homeostasis, lipolysis, and insulin secretion (Gardner & Shoback 2007). All three ARβ isoforms are expressed in BAT (Collins & Survit 2001) and our recent studies have indicated that the ARβ1 is key for cold- and diet-induced BAT thermogenesis as the ARβ1KO mice develop hypothermia when exposed to cold and obesity due to being fed a high-fat diet (HFD) (Ueta et al. 2012). In contrast, the roles played by ARβ2 and ARβ3 are less clear. Inactivation of the ARβ2 gene seems to be very well tolerated in vivo given that mice with ARβ2KO exhibit only a modest increase in body fat, have normal basal metabolic rate, and respond normally to cold exposure (Susulic et al. 1995), which could be due to upregulation of ARβ1 (Atgie et al. 1997).

There is great interest in the study of the ARβ2 pathway in humans. For example, activation of the ARβ2 pathway with salbutamol accelerates energy expenditure, lipolysis, and fat oxidation without affecting glucose oxidation in humans (Hoeks et al. 2003). In fact, a specific genetic variation in the ARβ2 gene is associated with blunted in vivo ARβ-mediated lipolysis and fat oxidation during ARβ stimulation (Jocken et al. 2007). In addition, polymorphisms of the ARβ2 gene are frequent in obese humans (Large et al. 1997, Takenaka et al. 2012), but studies on associations between these polymorphisms and body weight and composition, glucose tolerance, and insulin sensitivity have produced mixed results (Echwald et al. 1998, Kortner et al. 1999, Oberkofer et al. 2000, Prior et al. 2011).

Despite great interest in ARβ2 in humans, available animal models indicate only a minor metabolic role for ARβ2 (Rohrer et al. 1999). For example, it is known that ARβ2KO mice have lower body weight and smaller epididymal fat pads (Chruscinski et al. 1999), but their metabolic rate remains unaffected when compared with WT control animals (Rohrer et al. 1999). However, ARβ2KO mice have not been studied under conditions in which thermogenesis is activated. Thus, herein we looked at different metabolic parameters in ARβ2KO animals that were acutely exposed to cold or fed with a HFD. We found that ARβ2KO mice expressed increased liver phosphoenolpyruvate carboxykinase (Pepck) mRNA levels and displayed marked hyperinsulinemia, which on a HFD also results in increased fasting blood glucose. However, these animals attempt to maintain their body temperature when exposed to cold and exhibit normal catecolamine-stimulated interscapular BAT (iBAT) thermal response. When fed a HFD, ARβ2KO mice exhibited similar susceptibility to diet-induced obesity when compared with WT controls. Notably, there was a dramatic increase in the expression of both ARβ1 and ARβ3 mRNAs in the BAT of ARβ2KO mice, possibly explaining the lack of a major thermogenic phenotype.
Materials and methods

Animals
Approximately 60-day-old Friend virus B (FVB) male ARβ3-KO mice and WT FVB controls were studied, following an animal protocol approved by the Institutional Committee on Animal Research at the Center of Biological Sciences and Health-University Presbyterian Mackenzie. Each experiment was repeated two or three times on different sets of animals. As indicated, mice were kept on a chow diet (1.8 Cal/g) and water was available for drinking ad libitum in a room maintained at 25 °C. Food intake and body weight were measured daily. Acute cold exposure was performed in conscious mice housed individually in cages with no bedding in a cold room maintained at 4 °C (Eletrolab, São Paulo, SP, USA) for up to 4 h. Colonic temperature was measured temperature was measured hourly using a 1 mm wide rectal probe (Y4000, YSI, Yellow Springs, OH, USA) as previously described (de Jesus et al. 2001). Some animals were fed a HFD (5.44 Cal/g; 40% lipid; Rhoster, Sao Paulo, BR, USA) for 5 weeks.

Resting oxygen consumption (VO2)
VO2 was measured in an open-circuit respirometer system (O2-10, Sable System, Las Vegas, NV, USA) as described previously (Curcio et al. 1999). For cold exposure, mice were exposed to different temperatures (5, 15, 25, 30, 32, 34, and 36 °C) and VO2 was measured for 1 h at each temperature. The experiments were conducted between 1100 and 1900 h. Data were collected and analyzed using the Sable Systems’ software and results expressed as ml O2/min per g BW and ml O2/min as indicated. When at room temperature (25 °C), measurements were obtained over 30 min periods, in the afternoon (1400–1800 h) in animals that had access to food ad libitum.

Intraperitoneal glucose tolerance test and insulin tolerance test
Overnight fasting blood glucose was measured weekly in all animals by sampling the tail vein and using a glucose analyzer (LifeScan, Inc., Milpitas, CA, USA). For the glucose tolerance test (GTT), animals were fasted overnight and received glucose (2 g/kg) injected i.p. between 0900 and 1000 h. Blood samples were collected from the tail vein at the indicated times after the glucose load and assessed for glucose analysis. For the insulin tolerance test (ITT), food was removed 6 h before the experiment, which was carried out between 1400 and 1500 h. Blood samples were collected from the tail vein at the indicated times after injection of insulin (0.5 U/kg; i.p.) and glycemia was immediately determined using the glucose analyzer. At the end of the experimental period, blood samples were collected by cardiac puncture and assessed for insulin levels by ELISA (Marschner et al. 1974).

iBAT thermal response to norepinephrine or dobutamine infusion
This was determined under anesthesia as described previously (Ribeiro et al. 2001, Bianco et al. 2014). Briefly, mice were anesthetized with urethane (560 mg/kg, i.p.) and chloralose (38 mg/kg, i.p.) in the morning on the day of the experiment and a polyethylene (P-50) cannula was inserted into the left jugular vein. iBAT temperatures (°C) were measured using a precalibrated thermistor probe (YSI 427; Yellow Springs Instrument Co., Yellow Springs, OH, USA) surgically placed under the iBAT pad. Temperature was monitored until it reached a stable baseline (approximately 10 min), followed by infusion of norepinephrine (NE) (2 mg/ml) or the selective β1 agonist dobutamine (DB) (600 μg/ml) (Aikawa et al. 1996, Huang et al. 1998) through the left jugular vein using an infusion pump (model 2274, Harvard Apparatus, Holliston, MA, USA) at a rate of 0.643 μl/min for 30 min.

mRNA analysis
iBAT and liver were dissected and total RNA 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 RT-PCR (Promega), on a Robocycler thermocycler (Stratagene, La Jolla, CA, USA). Based on the reaction efficiency, about 120 ng of cDNA was used for amplification. Quantitative real-time PCR (RT-qPCR) was carried out using an IQ SYBR Green PCR kit (Bio-Rad) on an iCycler thermal cycler (Bio-Rad). The housekeeping gene cyclophilin A was used as an internal reference. Primer sequences were available upon request. The cycle conditions were as follows: 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 ΔΔCt method, as described previously (Christoffolete et al. 2004).
Western blot

iBAT was also processed for mitochondrial isolation. Mitochondrial proteins were then size-fractionated by 12% SDS–PAGE and probed with α-UCP1 (Santa Cruz Biotechnology).

Blood chemistry

Total serum cholesterol and triglycerides were assessed via enzymatic methods using a commercial kit (Roche Molecular Biochemicals).

Histology

After dissection, tissues were immersed in buffered formaldehyde solution and fixed for 24 h. Paraffin-embedded tissues were sectioned and processed as described for staining with hematoxylin–eosin or Masson’s trichrome (Kerr et al. 1995). The cellular area of the white adipocytes was measured by analyzing pictures of at least 40 adipocytes per animal taken at 200× magnification.

Statistical analysis

The statistical analyses were done by ANOVA followed by the Student–Newman–Keuls post-test when \( P < 0.05 \) and by analysis of covariance (ANCOVA) followed by the Bonferroni post-test as indicated.

Results

Thermogenic profile of ARβ2KO animals in response to cold or infusion with catecholamines

Mice acclimatized to room temperature were exposed to acute cold temperature at 4 °C and core body temperatures were measured hourly for up to 4 h. Both WT controls and ARβ2KO mice maintained their core temperature (Fig. 1A) by progressively accelerating resting energy expenditure (REE) (Fig. 1B). The iBAT thermal response to infusion with a defined dose of catecholamines provides information about the BAT thermogenic capacity (Ribeiro et al. 2001). In WT animals acclimatized to room temperature, infusion of NE over 30 min resulted in an increase of approximately 2.0 °C in iBAT temperature, similar to what was observed in the ARβ2KO animals (Fig. 1C).

The possibility that ARβ2KO animals compensate for the disruption of the ARβ2 pathway by activating other β-adrenergic pathways was tested by measuring mRNA levels for Arβ1 and Arβ3 in the BAT of ARβ2KO animals. Indeed, mRNA levels for both ARs were greatly elevated in the ARβ2KO BAT (Fig. 1D). However, the iBAT thermal response to the selective β1 agonist DB was similar in WT and in β2KO animals (Fig. 1E), indicating that the ARβ2KO BAT maintains its thermogenic capacity, which can be maximally stimulated via the ARβ1 pathway.

Susceptibility of ARβ2KO mice to diet-induced obesity

Adult ARβ2KO mice exhibited similar caloric intake (Fig. 2A) and body weight (Fig. 2B). Mice were then fed a HFD and monitored for 8 weeks, these animals exhibited increased caloric intake with no differences between ARβ2KO and WT controls (Fig. 2A). At the same time, all animals gained weight and those on the HFD gained approximately twice as much, again with no differences between ARβ2KO and WT controls (Fig. 2B and C). REE as expressed in relation to BW (VO2 ml/min/g BW) was not different in ARβ2KO when compared with WT controls (Fig. 2D) even when data were plotted as a function of BW (Supplementary Figure 1A, see section on supplementary data given at the end of this article) as suggested previously (Tschop et al. 2012). At the

Figure 1

Effects of the lack of β2 adrenergic receptor on cold-induced thermogenesis. (A) Central body temperature during cold exposure (4 °C) for 3 h of WT and ARKOβ2; (B) oxygen consumption during exposure of WT and ARKOβ2 mice to decreasing temperatures; (C) brown adipose tissue thermogenic response to norepinephrine (NE) infusion of WT and ARKOβ2 mice; (D) gene expression of β1 and β3 adrenergic receptors in mice kept on a chow or HFD in BAT (cross hatched (black and white), WT; white, ARβ3KO); (E) brown adipose tissue thermogenic response during infusion of DB of WT and ARKOβ2 mice. Entries are mean ± S.E.M. of five animals per group.
same time, REE was accelerated by 8 weeks of HFD in both WT controls and ARβ2KO animals, albeit less so in the latter group (Fig. 2D). These differences between the two groups of animals remained even when data were plotted as a function of BW (Supplementary Figure 1B, C and D). When the same data are expressed in terms of absolute values (VO2 ml/min) and statistically controlled for the effects of body weight (covariant) then the HFD-induced acceleration in REE was only observed in the WT controls (Fig. 2E). The epididymal white adipocyte area (Fig. 2F) and Ucp1 levels also behaved similarly in both ARβ2KO and WT controls (Fig. 2G), except that ARβ2KO mice on a chow diet exhibited significantly higher Ucp1 levels when compared with WT controls.

Glucose and insulin tolerance in ARβ2KO mice

Fasting blood glucose levels were monitored weekly during the 7-week experimental period (Fig. 3A) and no differences were observed until the end of the first week of HFD. From that point on, all animals on the HFD developed fasting hyperglycemia that was transiently more intense in the WT controls but that during weeks 5–7 became more pronounced in the ARβ2KO animals (Fig. 3A). Upon further investigation during week 8 of the experiment, all animals fed a HFD exhibited an apparent greater intolerance to glucose as assessed by the area under the curve of the GTT (Fig. 3B and C). However, when blood glucose was expressed at each time-point as a percentage of basal values, only the WT controls fed with the HFD exhibited mild glucose intolerance, whereas no differences were found in the ARβ2KO animals (Fig. 3D and E).

Sensitivity to insulin was similar in ARβ2KO and WT controls, with both groups exhibiting a slight but significant decrease in the AUC when kept on a HFD (Fig. 3F and G). As expected, during week 7, insulin levels after overnight fasting were elevated in WT controls placed on the HFD (Fig. 3H). At the same time, insulin levels were dramatically elevated in the ARβ2KO animals regardless of the type of diet (Fig. 3H). The elevated fasting glucose – not associated with decreased insulin sensitivity or glucose...
were observed between ARβ2 and WT mice fed a HFD. No differences in serum cholesterol and triglycerides after they were fed a HFD. Parameters increased similarly in both groups of animals, i.e. ARβ2KO and WT animals (Fig. 4). Liver steatosis in ARβ2KO mice Feeding on a HFD caused liver steatosis indistinctly in both groups of animals, i.e. ARβ2KO and WT animals (Fig. 4). Cholesterol and triglyceride plasma levels in ARβ2KO mice No differences in serum cholesterol and triglycerides were observed between ARβ2KO and WT animals. Both parameters increased similarly in both groups of animals after they were fed a HFD.

Discussion

It is well recognized that ARβs play a significant role in metabolic control and energy homeostasis, including adaptive thermogenesis (Bachman et al. 2002). The results of the present studies indicate that inactivation of the ARβ2 pathway interferes with glucose homeostasis, most probably by accelerating hepatic neoglucogenesis and glucose production, which is largely neutralized by a marked elevation in insulin levels. However, feeding the animals with a HFD stresses the systems further, beyond what can be compensated for by an elevation of insulin secretion, resulting in fasting hyperglycemia and relative glucose intolerance.

Although data obtained in the ARβ1KO mouse indicated that the ARβ1 pathway is important for BAT thermogenesis (Ueta et al. 2012), the present studies indicate that the role played by ARβ2 in BAT thermogenesis is much less significant and possibly redundant. Animals with inactivation of the ARβ2 pathway exhibit a normal baseline metabolic profile and respond indistinguishably from WT controls when exposed to cold, or treated with infusions of adrenergic agonists, but exhibit lesser acceleration of REE when fed a HFD. Alternatively, it is conceivable that the ARβ2 pathway plays an important role in BAT thermogenesis and the apparent lack of a
thermogenic phenotype is explained by the overexpression of ARβ1 and ARβ3 in BAT. Thus, ARβ2KO mice provide a suitable model for studying the interrelationships between the ARβ2 pathway and glucose homeostasis, which is also observed in humans. However, the minor role played by the ARβ2 pathway in BAT thermogenesis is in contrast to the wealth of literature supporting a role for ARβ2 in energy homeostasis and adaptive thermogenesis in humans (Hoeks et al. 2003). If confirmed, this could indicate important differences in the mechanisms involved in energy homeostasis between the two species.

ARβ2 activation promotes dilation of smooth muscle in different organs and structures but also plays an important role in energy homeostasis by slowing peristaltic movements and gastrointestinal secretion, as well as promoting lipolysis, insulin secretion, gluconeogenesis, and glycogenolysis, thus leading to overall lower levels of blood glucose (Gardner & Shoback 2007). Therefore, it is notable that ARβ2KO animals exhibit a marked elevation in serum insulin when on a chow diet and, when on a HFD, they exhibit greater disruption in glucose homeostasis, with fasting hyperglycemia and relative glucose intolerance (Ukkola & Bouchard 2001). Furthermore, in obese postmenopausal women, different ARβ2 haplotypes were associated with glucose intolerance, and thus may mediate insulin action, glucose tolerance, and potentially risk for type 2 diabetes mellitus (Prior et al. 2011).

Previous studies with β agonists-induced activation of glucose uptake in astrocytes and skeletal muscle cells indicated a key role of β2 in SNS-mediated glucose uptake, allowing for synergy between SNS and insulin signaling (Nevzorova et al. 2006, Catus et al. 2011). This is in contrast to the present observation that overall sensitivity to insulin is not modified in the ARβ2KO mice (Fig. 3F and G). At the same time, ARβ1 and ARβ3 have been recognized as key mediators in glucose uptake (Nikami et al. 1996, Chernogubova et al. 2004) and our data supports these findings. In addition, while metoprolol, a β1 antagonist, worsens insulin resistance, carvedilol, a β2 and α1 antagonist, decreases insulin resistance in type 2 diabetes patients (Phillips et al. 2008).

The role of the ARβ2 pathway in adaptive thermogenesis was tested during cold exposure or feeding on a HFD, two processes that involve multiple organs and tissues, e.g. hypothalamus, skeletal muscle, liver, and white adipose tissue and BAT. The latter is where most heat is generated in response to SNS stimulation (Enerback et al. 1997). Thus, the present data do not support a major role for ARβ2 in cold- or diet-induced thermogenesis.
First, the ARβ2KO animals maintained their core temperature and increased VO₂ during acute cold exposure (4 h), indicating normal BAT function. That this is a process primarily dependent on BAT is illustrated by the dramatic hypothermia developed during a similar time frame of cold exposure in UCP-1KO mice (Enerback et al. 1997). That the BAT thermogenesis is largely preserved in the ARβ2KO animals is further verified by the observation that their iBAT responds normally to NE infusion. Notably, the only biochemical difference observed in the ARβ2KO BAT was a slightly elevated baseline mitochondrial UCP1 content, a finding of undetermined significance. Second, when fed a HFD the overall thermogenic response seems to be preserved in the ARβ2KO animals given that caloric intake, weight gained, and the size of white fat depots behaved similarly to those of WT controls. This occurred despite lesser acceleration of the REE, at least as assessed through a limited 1 h-diurnal VO₂ study.

ARβ2 is present (Sell et al. 2004) and fully functional (Atgie et al. 1997) in the murine BAT. Although its inactivation would be expected to result in a detectable phenotype, previous studies have indicated that over-expression of other Arβ isoforms could provide a compensatory mechanism. For example, increased signaling via the ARβ1 or ARβ2 pathways was thought to explain the resistance to diet-induced obesity of ARβ1KO mice (Atgie et al. 1997). In fact, there is marked overexpression of Arβ1 and Arβ2 mRNA in the BAT of ARβ2KO animals. However, when treated with infusions of the ARβ1-selective agonist, DB, ARβ2KO animals exhibited a similar iBAT thermal response, indicating that the ARβ1 is not hyper-responsive to stimulation. Although this could reflect limitations in other steps of the BAT thermogenic pathway, at face value these data strengthen the argument that ARβ2 plays only a minor role if any in BAT thermogenesis.

The present data indicate that ARβ2 does not play a significant role in lipid metabolism, given that ARβ2KO mice exhibit similar hyperlipidemia when fed a HFD. In addition, ARβ2KO animals fed a HFD developed liver steatosis indistinguishable from WT controls. These data are relevant considering that ARβ blockers have been implicated in the increase in triglycerides and cholesterol plasma levels of hypertensive patients (Sarafidis & Bakris 2006, Bell et al. 2009). Also, patients with the ARβ2-Glu27 variant have hypertriglyceridemia with elevated cholesterol levels (Iaccarino et al. 2005). However, long-term treatment with oral broxaterol, a selective ARβ2 agonist, has minimal metabolic effects on lipid metabolism (Petraglia et al. 1990). Thus, it is not clear that ARβ2KO mice would be useful for modelling the role played by the ARβ2 pathway in lipid metabolism in humans.

In conclusion, the ARβ2KO pathway does play a significant role in glucose homeostasis, probably by accelerating hepatic glucose production that is largely neutralized by increased insulin secretion. Fasting hyperglycemia only occurs if the system is further stressed, for example by feeding a HFD. The study of the ARβ2KO mouse also indicated that the ARβ2 pathway plays a less significant role in the acute BAT thermogenic response and overall diet-induced thermogenesis.

**Supplementary data**
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-13-0526.

**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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