Disruption of beta3 adrenergic receptor increases susceptibility to DIO in mouse

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Abstract

The brown adipose tissue (BAT) mediates adaptive changes in metabolic rate by responding to the sympathetic nervous system through β-adrenergic receptors (AR). Here, we wished to define the role played by the ARβ3 isoform in this process. This study focused on the ARβ3 knockout mice (ARβ3KO), including responsiveness to cold exposure, diet-induced obesity, intolerance to glucose, dyslipidaemia and lipolysis in white adipose tissue (WAT). ARβ3KO mice defend core temperature during cold exposure (4°C for 5 h), with faster BAT thermal response to norepinephrine (NE) infusion when compared with wild-type (WT) mice. Despite normal BAT thermogenesis, ARβ3KO mice kept on a high-fat diet (HFD; 40% fat) for 8 weeks exhibited greater susceptibility to diet-induced obesity, markedly increased epididymal adipocyte area with clear signs of inflammation. The HFD-induced glucose intolerance was similar in both groups but serum hypertriglyceridemia and hypercholesterolemia were less intense in ARβ3KO animals when compared with WT controls. Isoproterenol-induced lipolysis in isolated white adipocytes as assessed by glycerol release was significantly impaired in ARβ3KO animals despite normal expression of key proteins involved in lipid metabolism. In conclusion, ARβ3 inactivation does not affect BAT thermogenesis but increases susceptibility to diet-induced obesity by dampening WAT lipolytic response to adrenergic stimulation.

Key Words
► obesity
► lipolysis
► adaptive thermogenesis
► brown adipose tissue
► β3 adrenergic receptor

Introduction

A balance between food intake and energy expenditure is essential to preserve fuel homeostasis. Changes in caloric intake trigger physiological responses that include adaptive modifications in appetite, metabolic rate and energy expenditure in order to preserve the energy levels in the body (Weigle 1994). In small mammals of all ages and in human newborns, adaptive changes in metabolic rate triggered by cold exposure or feeding on a
high-caloric diet occur mainly in the brown adipose tissue (BAT), a specialized tissue that has the ability to dissipate energy in the form of heat (Cannon & Nedergaard 2004, Lidell & Enerback 2010). A significant portion of human adults also exhibit a functional BAT (Cinti 2006, van der Lans et al. 2014), with incidence and activity inversely correlated to ambient temperature, age and body fat content (Lidell & Enerback 2010, Kajimura & Saito 2014), indicating a possible role of this tissue in adult human energy metabolism (Lowell & Spiegelman 2000, Cypress et al. 2009).

Indeed, several studies have shown that the active BAT in healthy adult humans is influenced by cold exposure (van Marken Lichtenbelt et al. 2009, Kajimura & Saito 2014, van der Lans et al. 2014), increasing energy expenditure. These findings indicate that metabolically active BAT may participate in the control of the energy balance and adiposity in humans, as already confirmed in small rodents (Yoneshiro et al. 2011). Thereby, adult human BAT represents a promising target for anti-obesity therapeutic strategies, however, its physiological relevance remains controversial (Cinti 2006, Hibi et al. 2016).

Autonomic regulation of BAT is influenced by environmental temperature and energy levels of the body. These signals are centrally integrated and, as a result, there is autonomic discharge of norepinephrine (NE) in BAT that stimulates energy expenditure and local thermogenesis. This is the result of NE interacting with β adrenergic receptors (ARβ), triggering a sequence of events that result in cAMP accumulation, hydrolysis of triglycerides, mitochondrial uncoupling protein 1 (UCP₁) activation, all of which are necessary for BAT thermogenesis (for review see Silva (2011)). Thus, NE and other catecholamines are the main mediators of diet and cold-induced adaptive thermogenesis (Silva 2011).

Norepinephrine acts primarily through three ARβ isoforms, ARβ₁, ARβ₂ and ARβ₃, the latter being the most abundant isoform expressed in mice adipose tissue. The WAT of these animals exhibits ARβ mRNA at ratios of approximately 3:1:150 for the ARβ₁, ARβ₂ and ARβ₃ isoforms, respectively (Collins et al. 1994). Notably, mice with triple knockout for ARβ₁, ARβ₂ and ARβ₃ (ARβ₁−/−,ARβ₂−/−,ARβ₃−/−KO) are obese and intolerant to cold exposure (Bachman et al. 2002, Jimenez et al. 2002), exhibiting markedly lower UCP₁ levels (Bachman et al. 2002). In addition, based largely on the phenotypic characterization of animals with single ARβ knockouts, it is well accepted that lipolysis and adaptive thermogenesis are mediated via ARβ₁ and ARβ₃, while the ARβ₂ subtype plays only a minor role in these processes (Mattsson et al. 2011, Ueta et al. 2012, Fernandes et al. 2014). For example, ARβ₃KO animals exhibit normal basal metabolic rate but are deficient in both cold- and diet-induced thermogenesis (Ueta et al. 2012). In contrast, ARβ₂KO mice have normal BAT thermogenesis but do exhibit lower body weight (Chruscinski et al. 1999) and disruption of glucose homeostasis (Santulli et al. 2012, Fernandes et al. 2014).

The relatively high level of ARβ₃ in adipose tissue supports the idea that this subtype is key for BAT growth (Nagase et al. 1994), lipolysis (Soloveva et al. 1997) and thermogenesis (Susulic et al. 1995). In fact, animals treated with ARβ₃ agonists do exhibit higher BAT activation and energy expenditure (Lidell & Enerback 2010, Xiao et al. 2015), increased lipid mobilization and reduced body fat (Arch & Wilson 1996, Atgie et al. 1997, Fisher et al. 1998, Inokuma et al. 2006, Lidell & Enerback 2010) as well as induction of UCP₁ expression (Inokuma et al. 2006). Furthermore, studies in ARβ₂KO mice indicate increased adiposity while on chow diet and an accelerated body weight gain while on a high-fat diet (HFD) (Susulic et al. 1995, Revelli et al. 1997). Notably, also in humans, some ARβ₃ mutations are associated with the development of obesity (Widen et al. 1995). However, despite inactivation of the ARβ₃, ARβ₂KO mice are not cold-sensitive and exhibit normal UCP-1 induction during cold exposure (Susulic et al. 1995, Mattsson et al. 2011). This study was undertaken to investigate the mechanisms of obesity in ARβ₃KO mice.

Materials and methods

Animals and diets
All experiments were performed following protocols approved by the Institutional Committee on Animal Research at the Center of Biological Sciences and Health, University Presbyterian Mackenzie. Eight-week-old male WT mice and mice with ARβ₃ knockout (ARβ₃KO; FVB background) were obtained from Jackson Laboratory and maintained on a 12-h light/darkness cycle, at a temperature of 24°C and given food and water ad libitum. Mice were kept on chow diet (1.8 Cal/g; 4% fat; Nuvilab CR1; Nuvital, Brazil) except for the experiments in which HFD (7.52 Cal/g; 40% fat; Rhoster, Sao Paulo, SP, Brazil) was used. In this case, before switching to HFD, all mice were fasted for 12h and then placed on HFD for 8 weeks. A total number of six mice per group were used in the experiments, unless otherwise specified. Body weight and
food intake were monitored daily. All drugs were obtained from Sigma Aldrich, unless specified.

**Intra-peritoneal glucose tolerance test**

This test was performed in the morning (around 0900 h) on the following groups: WT (control group – WT mice fed with chow diet), WT HFD (WT mice fed with HFD), ARβ3KO (ARβ3KO mice fed with chow diet) and ARβ3KO HFD (ARβ3KO mice fed with HFD). Mice were fasted for 12 h and given glucose (2 g/kg of body weight; i.p.). Blood glucose was measured using a glucometer (One Touch Ultra, Johnson & Johnson) in blood samples collected from the tail at 0 (before glucose injection), 30, 60, 90 and 120 min after glucose injection (Asensio et al. 2005).

**Cold exposure**

Conscious mice (5 WT and 7 ARβ3KO) were housed individually in cages with no bedding and exposed to cold (4°C) in a chamber with controlled temperature (Eletrolab, Sao Paulo, SP, Brazil) for 5 h. Colonic temperature was measured at hourly intervals using a high-precision thermometer connected to a rectal probe of 3 mm diameter (YSI 402, YSI, Yellow Springs, OH, USA) (Enerback et al. 1997).

**Interscapular BAT (iBAT) thermal response to the NE infusion**

WT and ARβ3KO mice that had been kept at room temperature (6 and 3, respectively) were anaesthetized with urethane (560 mg/kg; i.p.) and chloralose (38 mg/kg; i.p.) and had one jugular vein cannulated with a polyethylene (P-50) cannula. iBAT temperature (°C) was monitored using a precalibrated thermistor probe (YSI 427 Skin, Yellow Springs, OH, USA) surgically placed under the iBAT pad (Ribeiro et al. 2001, Bianco et al. 2014). Throughout the experiment, the animals were maintained on a warm pad to stabilize body temperature. After a stable baseline was reached (approximately 5 min), NE infusion (2 mg/mL) was started using an infusion pump (model 2274, Harvard Apparatus, Holliston, MA, USA) at a rate of 8 μg/kg/min for 30 min.

**WAT lipolysis**

Samples of approximately 250 mg of epididymal WAT from WT and ARβ3KO mice fed with chow diet or HFD (5–6 per group) were excised, minced and digested with collagenase as described previously in the presence of adenosine deaminase (ADA, Sigma, 0.2 U/mL, pH 7.45) (Rodbell 1964, Susulic et al. 1995). Cells were then exposed to ascorbic acid or isoproterenol (Sigma Chemical). The rate of lipolysis was estimated by measuring the release of glycerol (Sigma Chemical) and expressed as nmol.10⁻⁶ cells.h⁻¹. Cells were also kept on 10% formaldehyde for subsequent measurement adipocyte diameter. Total adipocyte volume used in each reaction was estimated with a haematocrit card. Adipocyte diameter and total volume were used as internal references.

**Blood chemistry**

Serum cholesterol and triglycerides were measured using clinical kits (Cholesterol Liquiform, Labtest, Brazil; Triglycerides Liquiform, Labtest).

**mRNA analysis by real-time PCR (RT-qPCR)**

Total BAT and WAT RNA were extracted using TRIzol (Invitrogen) and used to synthesize cDNA with the SuperScript First-Strand Synthesis System Kit (Invitrogen) in a Mastercycler thermocycler (Eppendorf, Hamburg, Germany). About 120 ng of cDNA were used as template in the Real-Time PCR reaction, using the kit Quant iTect SYBR Green PCR (Qiagen). Each cycle included 2 min at 50°C, 15 min at 95°C, 15 s at 94°C, 30 s at 60°C, 30 s at 72°C, 15 s at 95°C, 15 s at 55°C and 15 s at 95°C for 50 cycles. The relative expressions of ARβ1 and ARβ2 were expressed as a function of the housekeeping gene β-actin for WT and ARβ3KO mice (4–7 per group).

**Western blotting**

20 μg BAT mitochondrial protein or total protein of WAT of WT and ARβ3KO mice fed with chow diet or HFD (3–5 per group) were size fractionated using 12% SDS-PAGE, transferred to a nitrocellulose membrane and probed with UCP1 (1:5000 dilution), HSL, p-HSL, AMPK, p-AMPK, ATGL, perilipin and DGAT2 goat-polyclonal IgG primary antibody solution (1:1000 dilution) (Santa Cruz Biotechnology), and β-actin (1:2000 dilution) (ABCAM) was used as a loading control. The secondary antibody solution used was donkey anti-goat IgG conjugate HRP (Santa Cruz Biotechnology), in a 1:2000 dilution.
Histology
Samples of WAT and liver of all the four groups of WT and ARβ3 KO mice were excised, fixed in 10% buffered formaldehyde for 24h, embedded in paraffin and sectioned.

Morphological analysis
The sequential 5μm sections obtained were first stained with haematoxylin-eosin (H&E) and then, with Picrosirius red. The sections were analyzed with a Leica microscope (DM 750), equipped with filters to provide circularly polarized light. Picrosirius red staining sections allowed collagen fibres detection with different colours (Batista et al. 2016). Type I collagen fibres appear orange to red, whereas the thinner type III collagen fibres were stained with a yellow to a green hue. Tissue images were obtained with an x 40 objective lens, recorded on a digital camera (DFC 295, Leica), displayed on a high-resolution monitor (LG, Flatron, E1941) and analyzed with SigmaScan Pro image analysis (Chicago, IL, USA). Measurement of adipocyte size (as the area in μm2) was acquired by Imagen Pro-Plus 6.0 (100 adipocytes per stained section) and performed with a custom-designed software programme written using MATLAB (v. R2010b; The MathWorks, Natick, MA, USA).

Immunohistochemistry
Immunohistochemistry of epididymal WAT samples were carried out with sections fixed in buffered formalin and embedded in paraffin. Deparaffinized sections (5μm) were stained with H&E. After quenching of endogenous peroxidase activity with 0.3% H2O2 in methanol and blocking of free protein-binding sites with 5% normal goat serum, sections were immune stained for immune cells: macrophages with CD68 anti-mouse KP-1 monoclonal antibody (Abcam, ab955), neutrophils with CD11b (Abcam, ab75476) and TNFα (Abcam, ab6671) antibodies. Specific secondary antibodies were peroxidase (horseradish peroxidase) conjugated. Histochemical reactions were performed using Vecta stain ABC Kit.

Statistical analysis
Student’s t-test or one-way ANOVA followed by Student–Newman–Keul’s test were used throughout as indicated (GraphPad Software). For brown fat thermogenic response, we used linear regression analysis. For lipolysis assay, two-way ANOVA was used followed by Student–Newman–Keul’s test. P<0.05 was used to reject the null hypothesis. All results were expressed as mean± standard error of the mean.

Results
ARβ3 KO mice have normal adiposity and defend core temperature during acute cold exposure
When acutely exposed to cold (4°C), ARβ3 KO mice maintain core temperature for up to 5 h (Fig. 1A), reflecting their ability to maintain thermal homeostasis. This is not explained by better insulation (given their propensity to obesity (Susulic et al. 1995, Revelli et al. 1997, Lowell 1998)), that is, we found ARβ3 KO mice consume similar amounts of calories (Fig. 1B) and grow normally, reaching adulthood with similar body weight (Fig. 1C) and similar adiposity as assessed by the area of the epididymal adipocytes to WT controls.

ARβ3 KO iBAT responds faster to NE infusion
To assess adaptive thermogenesis and test BAT directly, ARβ3 KO animals were infused with NE and iBAT thermal response evaluated. In the WT animals, a typical iBAT
ARβ3KO mice exhibit greater susceptibility to diet-induced obesity

BAT thermogenesis can also be triggered by HFD and, given that NE-induced BAT thermogenesis is better than normal, here we wanted to test whether the ARβ3KO animals show greater susceptibility to HFD as reported previously (Susulic et al. 1995, Revelli et al. 1997, Lowell 1998). Therefore, we revisited this issue by placing ARβ3KO animals on a HFD for 8 weeks, and found that they gained significantly more body weight (Fig. 3A and B) despite ingesting the same amount of calories (Fig. 3C). Additionally, ARβ3KO HFD animals exhibited markedly increased epididymal adipocyte size (hypertrophy) of 24.5-fold (P<0.01) when compared with controls (ARβ3KO). HFD also induced adipocyte hypertrophy in WT (6.9-fold, P>0.01), compared with WT controls. The adipocyte hypertrophy was 1.6-fold higher in ARβ3KO group when compared with WT, both fed with HFD (Fig. 4A). To determine the adipocyte size distribution, we measured the two-dimensional area of 100 individual adipocytes from epididymal WAT (Fig. 4B). Consistent with results for average adipocyte size, adipocytes sized between 10 and 70.104μm showing higher frequency in ARβ3KO HFD, besides adipocytes higher than 90.104μm were just detected in ARβ3KO HFD.

WAT inflammation is more pronounced in ARβ3KO mice after diet-induced obesity

Considering the scope of data regarding WAT remodelling induced by HFD, notably throughout adipocyte hypertrophy and the modification in total collagen density (fibrosis) (Sun et al. 2011), we also examined the presence of inflammation and infiltrated cells in epididymal WAT from ARβ3KO. Immunostaining for macrophages

Figure 3
(A) Body weight of WT and ARβ3KO treated with chow diet or HFD for 8 weeks (n=6); (B) delta body weight of the WT and ARβ3KO mice treated with chow diet or HFD for 8 weeks (n=6); (C) caloric intake of WT and ARβ3KO treated with chow diet or HFD for 8 weeks (n=6). * vs WT with P<0.05; ** vs WT HFD with P<0.01; * vs ARβ3KO with P<0.001. Values expressed as mean±SEM and data were analyzed using one-way ANOVA followed by Student–Newman–Keuls test.

Lowell 1998). Therefore, we revisited this issue by placing ARβ3KO animals on a HFD for 8 weeks, and found that they gained significantly more body weight (Fig. 3A and B) despite ingesting the same amount of calories (Fig. 3C). Additionally, ARβ3KO HFD animals exhibited markedly increased epididymal adipocyte size (hypertrophy) of 24.5-fold (P<0.01) when compared with controls (ARβ3KO). HFD also induced adipocyte hypertrophy in WT (6.9-fold, P>0.01), compared with WT controls. The adipocyte hypertrophy was 1.6-fold higher in ARβ3KO group when compared with WT, both fed with HFD (Fig. 4A). To determine the adipocyte size distribution, we measured the two-dimensional area of 100 individual adipocytes from epididymal WAT (Fig. 4B). Consistent with results for average adipocyte size, adipocytes sized between 10 and 70.104μm showing higher frequency in ARβ3KO HFD, besides adipocytes higher than 90.104μm were just detected in ARβ3KO HFD.
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Figure 4
Morphological and immune-staining characteristics of the WT and ARβ3KO mice fed with chow diet or HFD for 8 weeks; photomicrographs illustrate the most representative images considering data related to morphometric analysis of sectional area (A) and (B) of adipocytes size distribution (three sections per mouse; n=3 per group; scale bar: 50 mm). (C) H&E staining, 100×; Picrosirius red staining. Collagen fibres are presented in different colours. Type I collagen fibres are orange to red; identification of different immune cell types were stained with markers of macrophages (CD68), neutrophils (CD11b) and for TNFα. (D) qPCR analysis of isolated RNA. * vs WT with P<0.05; ** vs WT HFD with P<0.05 and # vs ARβ3KO P<0.01. Values expressed as mean±SEM. Nuclei were stained with haematoxylin (blue labelling). Bar (μm). HE, haematoxylin and eosin staining.

(CD68), neutrophils (CD11b) and TNFα and analysis of gene expression of CD68, F4/80 and TNFα were carried out (Fig. 4C). Additionally, in tissue sections stained for CD11b positive cells, we found the higher intensity that was more pronounced in the surrounding of adipocytes in ARβ3KO HFD. Less frequently, sections for CD68 positive cells showed irregular distribution more often observed in the epididymal WAT fibrotic areas from ARβ3KO HFD. CD68 and F4/80 mRNA expression increased in both HFD-treated groups (~5 and 16 fold, respectively). The former was higher in ARβ3KO when compared with WT HFD (~4 fold). Increase in TNFα mRNA expression was detected only in ARβ3KO HFD (Fig. 4D). No structural differences were found in liver of the ARβ3KO animals while on a chow diet (Fig. 5A and B) and feeding on a HFD resulted in a severe macrovesicular liver steatosis, with triglyceride accumulation in large hepatocyte vacuoles but similar in WT and ARβ3KO (Fig. 5C, D and E). HFD-induced glucose intolerance was similar in both groups (Fig. 6) but serum triglycerides and cholesterol increased less in ARβ3KO animals when compared with WT controls, both fed with HFD (Table 1).

WAT lipolysis is impaired in ARβ3KO adipocytes

Next, we studied basal and stimulated lipolysis in isolated white adipocytes of all animals. In WT control cells obtained from animals kept on a chow diet, exposure to the non-selective β-agonist isoproterenol leads to an approximately 3.6-fold stimulation in glycerol release,
control cells obtained from animals kept on HFD were tested. In this case, the isoproterenol-stimulated glycerol release reached more than twice the levels seen in animals kept on a chow diet (Table 2). In contrast, there was no greater lipolytic response to isoproterenol in the cells obtained from ARβ3KO mice kept on a HFD, with levels remaining at less than half of those observed in WT control cells (Table 2). That these differences are the result of ARβ3 inactivation is supported by the observation that the expression level of key proteins involved in lipid metabolism remains unaffected in the epididymal fat of ARβ3KO animals, including HSL and p-HSL, AMPk and p-AMPk, ATGL, perilipin and DGAT2 (Fig. 7).

Discussion

This study of the ARβ3KO mouse revealed two novel findings: first, there is a faster and more intense thermal response of ARβ3KO BAT during infusion with NE, likely reflecting increased expression of ARβ1. This suggests that the increase in ARβ1, which was also seen by others (Susulic et al. 1995), overcompensates for the ARβ3 inactivation and is able to mediate cAMP production within brown adipocytes. These results explain why the ARβ3KO mouse defends its core temperature when acutely exposed to cold. Secondly, that the greater susceptibility to diet-induced obesity exhibited by these animals is specifically due to ARβ3 inactivation in white (epididymal) adipocytes, given that the key enzymes involved in lipolysis are normally expressed in these animals (Fig. 7).

It is unexpected that the ARβ3KO mouse is capable of thermoregulation during acute cold exposure (Fig. 1A) (Susulic et al. 1995, Mattsson et al. 2011) in light of the previous studies indicating an important role played by ARβ3 in adaptive thermogenesis (Carpene et al. 1993, Widen et al. 1995, Atgie et al. 1997, Lafontan et al. 1997, Lowell 1998, Inokuma et al. 2006). The finding that UCP1 levels are normal in ARβ3KO BAT (Fig. 2C) and that the thermal response to catecholamine infusion is better than expected (Fig. 2A) explains the ARβ3KO mouse’s thermoregulation ability during acute cold exposure. In fact, these findings are reminiscent of the previous observations that the ARβ3KO mouse exhibits normal isoproterenol-induced acceleration of oxygen consumption (Susulic et al. 1995).

While these observations downplay the role played by ARβ3 in adaptive thermogenesis, these findings highlight a degree of functional redundancy shared by the three ARβ subtypes, which mitigates the phenotype caused by
Table 1  Serum cholesterol and triglycerides levels (mg/dL) of the WT and ARβ3KO mice after treatment with chow diet or HFD for 8 weeks.

<table>
<thead>
<tr>
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<th>WT</th>
<th>WT HFD</th>
<th>ARβ3KO</th>
<th>ARβ3KO HFD</th>
</tr>
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<tbody>
<tr>
<td>Cholesterol</td>
<td>128.44 ± 3.26</td>
<td>170.31 ± 7.48*</td>
<td>115.36 ± 5.75</td>
<td>134.64 ± 4.98**</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>127.81 ± 3.47</td>
<td>167.81 ± 6.82*</td>
<td>111.98 ± 8.6</td>
<td>135.42 ± 4.77**</td>
</tr>
</tbody>
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* vs WT with P < 0.01; * vs ARβ3KO with P < 0.05; ** vs WT HFD with P < 0.01. Values are expressed as mean ± SEM of 6 animals per group.

the ARβ3 inactivation (Susulic et al. 1995, Rohrer 1998, Jimenez et al. 2002). Indeed, the ARβ3KO BAT exhibits increased expression of ARβ1 but not ARβ2 (Fig. 2B) (Susulic et al. 1995). Given the importance of ARβ1 in BAT thermogenesis (Ueta et al. 2012), the overexpression of this AR isoform is likely to explain the normal ARβ3KO BAT phenotype.

Despite better than normal BAT thermogenesis, ARβ3KO animals kept on HFD developed more severe obesity, indicating greater susceptibility to diet-induced obesity. In fact, ARβ3KO animals showed higher levels of adipocyte hypertrophy, followed by severe infiltration of macrophages and increased levels of extracellular matrix components, in particular type I collagen. This scenario suggests that WAT remodelling induced by HFD is a more severe process in ARβ3KO. Some studies have demonstrated the relationship regarding the contribution of adipocyte hypertrophy to WAT expansion, in both humans and animal models of obesity (Lee et al. 2014, Rutkowski et al. 2015). Such conditions can lead to a countless of effects, including hypoxia, adipocyte cell death, increased chemokine secretion and dysregulation in fatty acid fluxes (Sun et al. 2011, 2013). Consequently, enhanced macrophage infiltration is required to create an environment for the remodelling process. The end point of WAT remodelling induced by obesity is associated with severe insulin resistance and lipotoxic side effects (Sun et al. 2013). Despite severe WAT remodelling, the ARβ3KO mouse did not exhibit exaggerated intolerance to glucose (Fig. 6), suggesting that ARβ3 could play a role modulating the decrease in insulin sensitivity during high-fat feeding. Also notable was the fact that ARβ3KO animals kept on a HFD exhibited less severe hypercholesterolaemia and hypertriglyceridaemia when compared with WT control animals also kept on HFD (Table 1). This could reflect the greater lipid deposit observed in the ARβ3KO WAT and liver. In fact, same obese individuals can preserve systemic insulin sensitivity, as well as obesity-associated lipotoxic side effects, as aforementioned. Such condition is evident even in the presence of WAT expansion, a condition proposed as ‘metabolically healthy obese’ state (Ruderman et al. 1981). However, the possible role of ARβ3 depletion in this condition needs further investigation.

Previous studies indicated that the isoproterenol-induced adenylyl cyclase activity and lipolysis are diminished in ARβ3KO-isolated adipocytes only when adenosine deaminase and the stable adenosine receptor agonist N6-phenylisopropyladenosine (PIA) were used (Susulic et al. 1995). However, the physiological significance of these findings is not clear given that the in vivo lipolytic response to isoproterenol was not affected by the ARβ3 inactivation (Susulic et al. 1995). These observations led us to hypothesize that a lipolysis defect in WAT could be exacerbated during HFD and thus explain the greater susceptibility of these animals to diet-induced obesity. In fact, ARβ3KO isolated adipocytes obtained from HFD animals exhibited less than half of the lipolytic response to isoproterenol as compared with HFD WT controls (Table 2), even though they have normal levels of the key enzymes involved in lipolysis. In contrast, ARβ3KO isolated adipocytes obtained from animals kept on chow diet behaved similarly to WT controls.

Therefore, the observations from this study point to a great physiological relevance for the ARβ3 in the epididymal WAT lipolysis, particularly when the white adipocytes are filled with triglycerides as a result of feeding on a HFD.

Table 2  Differences between basal levels and after stimulation with isoproterenol of glycerol (nmol.10^-6 cells.h^-1) released by isolated white adipocytes of WT and ARβ3KO mice after treatment with chow diet or HFD for 8 weeks.

<table>
<thead>
<tr>
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<th>WT</th>
<th>WT HFD</th>
<th>ARβ3KO</th>
<th>ARβ3KO HFD</th>
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<tbody>
<tr>
<td>Basal levels</td>
<td>3257 ± 1056</td>
<td>5221 ± 990</td>
<td>3194 ± 692</td>
<td>4812 ± 973</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>8909 ± 963</td>
<td>20242 ± 1768*</td>
<td>6003 ± 809</td>
<td>9583 ± 1103**</td>
</tr>
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</table>

* vs WT with P < 0.001; ** vs WT HFD with P < 0.001. Values are expressed as mean ± SEM of 6 animals per group.
In conclusion, this study indicates that the ARβ3 plays a fundamental role in the regulation of body weight by mediating adrenergic stimulation of WAT lipolysis, particularly when the adipocytes are loaded with triglycerides. Given the blunted WAT lipolytic response to isoproterenol, the ARβ3 KO develops much greater susceptibility to diet-induced obesity with WAT inflammation and more severe hepatic steatosis.

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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Author contribution statement
N Z P, B P P N, F S H, C R M, A L V A and T S H conceived and carried out experiments; F S E, C L L, M L B Jr, A C B and M O R conceived experiments and analyzed data. All authors were involved in writing the paper and had final approval of the submitted and published versions.

References


Socoloveva V, Graves RA, Rasenick MM, Spiegelman BM & Ross SR 1997 Transgenic mice overexpressing the beta 1-adrenergic receptor in adipose tissue are resistant to obesity. *Molecular Endocrinology* **11** 27–38. (doi:10.1210/me.111.1.27)


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