Disruption of β_3 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 $AR\beta_3$ isoform in this process. This study focused on the $AR\beta_3$ knockout mice ($AR\beta_3KO$), including responsiveness to cold exposure, diet-induced obesity, intolerance to glucose, dyslipidaemia and lipolysis in white adipose tissue (WAT). $AR\beta_3KO$ 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, $AR\beta_3KO$ 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 $AR\beta_3KO$ animals when compared with WT controls. Isoproterenol-induced lipolysis in isolated white adipocytes as assessed by glycerol release was significantly impaired in $AR\beta_3KO$ animals despite normal expression of key proteins involved in lipid metabolism. In conclusion, $AR\beta_3$ 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
- β_3 adrenergic receptor

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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, Cypess *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 (AR β), 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 AR β isoforms, AR β_1 , AR β_2 and AR β_3 , the latter being the most abundant isoform expressed in mice adipose tissue. The WAT of these animals exhibits AR β mRNA at ratios of approximately 3:1:150 for the AR β_1 , AR β_2 and AR β_3 isoforms, respectively (Collins *et al.* 1994). Notably, mice with triple knockout for AR β_1 , AR β_2 and AR β_3 (AR β_{1-3} 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 AR β knockouts, it is well accepted that lipolysis and adaptive thermogenesis are mediated via AR β_1 and AR β_3 , while the AR β_2 subtype plays only a minor role in these

processes (Mattsson *et al.* 2011, Ueta *et al.* 2012, Fernandes *et al.* 2014). For example, AR β_1 KO animals exhibit normal basal metabolic rate but are deficient in both cold- and diet-induced thermogenesis (Ueta *et al.* 2012). In contrast, AR β_2 KO 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 AR β_3 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 AR β_3 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 AR β_3 KO 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 AR β_3 mutations are associated with the development of obesity (Widen *et al.* 1995). However, despite inactivation of the AR β_3 , AR β_3 KO 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 AR β_3 KO 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 AR β_3 knockout (AR β_3 KO; 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 (1.8Cal/g; 4% fat; Nuvilab CR1; Nuvital, Brazil) except for the experiments in which HFD (7.52Cal/g; 40% fat; Rhostror, 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 0900h) on the following groups: WT (control group – WT mice fed with chow diet), WT HFD (WT mice fed with HFD), AR β_3 KO (AR β_3 KO mice fed with chow diet) and AR β_3 KO HFD (AR β_3 KO 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 AR β_3 KO) 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 AR β_3 KO 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 μ g/kg/min for 30 min.

WAT lipolysis

Samples of approximately 250 mg of epididymal WAT from WT and AR β_3 KO 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.10⁻⁶cells.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 QuantiTect 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 AR β_1 and AR β_2 were expressed as a function of the housekeeping gene β -actin for WT and AR β_3 KO mice (4–7 per group).

Western blotting

20 μ g BAT mitochondrial protein or total protein of WAT of WT and AR β_3 KO 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 AR β_3 KO mice were excised, fixed in 10% buffered formaldehyde for 24h, embedded in paraffin and sectioned.

Morphological analysis

The sequential 5 μ m 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 $\times 40$ 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 μ m²) 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 (5 μ m) 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 TNF α (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–Keuls 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 $\pm$ standard error of the mean.

Results

AR β_3 KO mice have normal adiposity and defend core temperature during acute cold exposure

When acutely exposed to cold (4°C), AR β_3 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 AR β_3 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.

AR β_3 KO iBAT responds faster to NE infusion

To assess adaptive thermogenesis and test BAT directly, AR β_3 KO animals were infused with NE and iBAT thermal response evaluated. In the WT animals, a typical iBAT

Figure 1

(A) Variation in body temperature of WT and AR β_3 KO mice fed with chow diet during cold exposure (4°C) for 5 h (*n* = 6). * vs WT with *P* < 0.01; (B) caloric intake per mouse/day of the WT and AR β_3 KO mice treated with chow diet along the weeks of treatment – values on the graph are an average of values obtained during the days of the week (*n* = 6); (C) body weight of the WT and AR β_3 KO animals during the treatment with chow diet (*n* = 6). All values are expressed as mean $\pm$ SEM and data were analyzed using Student's *t*-test.

Figure 2

(A) Variation of the iBAT temperature in WT and AR β_3 KO animals exposed to 30 min infusion of NE ($n=6$); (B) mRNA levels of AR β_1 and AR β_2 on iBAT of the WT and AR β_3 KO mice treated with chow diet ($n=6$). * vs WT with $P<0.05$. Values expressed as mean $\pm$ SEM and data were analyzed using Student's t -test.

thermal response was detected as early as 5 min of the start of the NE infusion, plateauing at about 20 min with maximum delta temperature of about 1.5°C (Fig. 2A). In contrast, the response in the AR β_3 KO animals was much faster (1 min), with a plateau that was reached by 5 min at about 2.0°C (Fig. 2A). The nonlinear regression analysis indicates that the top best-fit value for the AR β_3 KO curve is 36.36 ± 0.041 vs 35.76 ± 0.046 °C in WT control animals, with a respective LogT₅₀ of 3.5 vs 7.8 min ($P<0.009$). Notably, these differences between AR β_3 KO and WT controls were observed even as the iBAT mitochondrial UCP-1 levels remained similar in both groups of animals (Fig. 2C). This could be explained by compensatory increase in other AR β isoforms (Susulic *et al.* 1995) and indeed there was an approximately 45% increase in iBAT AR β_1 mRNA levels, whereas the AR β_2 mRNA levels remained similar to WT control animals (Fig. 2B).

AR β_3 KO 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 AR β_3 KO animals show greater susceptibility to HFD as reported previously (Susulic *et al.* 1995, Revelli *et al.* 1997,

Figure 3

(A) Body weight of WT and AR β_3 KO treated with chow diet or HFD for 8 weeks ($n=6$); (B) delta body weight of the WT and AR β_3 KO mice treated with chow diet or HFD for 8 weeks ($n=6$); (C) caloric intake of WT and AR β_3 KO 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 AR β_3 KO with $P<0.001$. Values expressed as mean $\pm$ SEM and data were analyzed using one-way ANOVA followed by Student–Newman–Keuls test.

Lowell 1998). Therefore, we revisited this issue by placing AR β_3 KO 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, AR β_3 KO HFD animals exhibited markedly increased epididymal adipocyte size (hypertrophy) of 24.5-fold ($P>0.01$) when compared with controls (AR β_3 KO). 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 AR β_3 KO 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 70.10³ μm^2 showing higher frequency in AR β_3 KO HFD, besides adipocytes higher than 90.10³ μm^2 were just detected in AR β_3 KO HFD.

WAT inflammation is more pronounced in AR β_3 KO 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 AR β_3 KO. Immunostaining for macrophages

Figure 4

Morphological and immune-staining characteristics of the WT and AR β_3 KO 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 mice; $n=3$ per group; scale bar: 50 μ m). (C) H&E staining; 100 $\times$; 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 TNF α . (D) qPCR analysis of isolated RNA. * vs WT with $P<0.05$; ** vs WT HFD with $P<0.05$ and # vs AR β_3 KO $P<0.01$. Values expressed as mean $\pm$ SEM. Nuclei were stained with haematoxylin (blue labelling). Bar (μ m). HE, haematoxylin and eosin staining.

(CD68), neutrophils (CD11b) and TNF α and analysis of gene expression of CD68, F4/80 and TNF α 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 AR β_3 KO HFD. Less frequently, sections for CD68 positive cells showed irregular distribution more often observed in the epididymal WAT fibrotic areas from AR β_3 KO HFD. CD68 and F4/80 mRNA expression increased in both HFD-treated groups (~5 and 16 fold, respectively). The former was higher in AR β_3 KO when compared with WT HFD (~4 fold). Increase in TNF α mRNA expression was detected only in AR β_3 KO HF (Fig. 4D). No structural differences were found in liver of the AR β_3 KO 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 AR β_3 KO (Fig. 5C, D and E). HFD-induced glucose intolerance was similar in both groups (Fig. 6) but serum triglycerides and cholesterol increased less in AR β_3 KO animals when compared with WT controls, both fed with HFD (Table 1).

WAT lipolysis is impaired in AR β_3 KO 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,

Figure 5

Liver histology of (A) WT mice fed with chow diet ($n=6$); (B) WT mice fed with HFD ($n=6$); (C) AR β_3 KO mice fed with chow diet ($n=6$); (D) AR β_3 KO mice fed with HFD for 8 weeks ($n=6$) and (E) liver triglycerides of WT and AR β_3 KO mice treated with chow diet or HFD for 8 weeks ($n=6$). [#] vs WT with $P<0.01$; ^{*} vs AR β_3 KO with $P<0.01$. Values expressed as mean $\pm$ SEM and data were analyzed using one-way ANOVA followed by Student–Newman–Keuls test.

whereas in AR β_3 KO cells this figure is only 2.3-fold, but not significantly different (Table 2). These differences in glycerol release were greatly exaggerated when WT

Figure 6

Glucose tolerance test (GTT) of the WT and AR β_3 KO mice fed with chow diet or HFD for 8 weeks ($n=6$); blood glucose at 0 (baseline), 30, 60, 90 and 120 min after glucose injection. ^{*} vs WT with $P<0.05$; [#] vs AR β_3 KO with $P<0.05$. Values expressed as mean $\pm$ SEM and data were analyzed using one-way ANOVA followed by Student–Newman–Keuls test.

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 AR β_3 KO 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 AR β_3 inactivation is supported by the observation that the expression level of key proteins involved in lipid metabolism remains unaffected in the epididymal fat of AR β_3 KO animals, including HSL and p-HSL, AMPk and p-AMPk, ATGL, perilipin and DGAT2 (Fig. 7).

Discussion

This study of the AR β_3 KO mouse revealed two novel findings: first, there is a faster and more intense thermal response of AR β_3 KO BAT during infusion with NE, likely reflecting increased expression of AR β_1 . This suggests that the increase in AR β_1 , which was also seen by others (Susulic *et al.* 1995), overcompensates for the AR β_3 inactivation and is able to mediate cAMP production within brown adipocytes. These results explain why the AR β_3 KO 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 AR β_3 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 AR β_3 KO 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 AR β_3 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 AR β_3 KO BAT (Fig. 2C) and that the thermal response to catecholamine infusion is better than expected (Fig. 2A) explains the AR β_3 KO mouse's thermoregulation ability during acute cold exposure. In fact, these findings are reminiscent of the previous observations that the AR β_3 KO mouse exhibits normal isoproterenol-induced acceleration of oxygen consumption (Susulic *et al.* 1995).

While these observations downplay the role played by AR β_3 in adaptive thermogenesis, these findings highlight a degree of functional redundancy shared by the three AR β subtypes, which mitigates the phenotype caused by

Table 1 Serum cholesterol and triglycerides levels (mg/dL) of the WT and AR β_3 KO mice after treatment with chow diet or HFD for 8 weeks.

	WT	WT HFD	AR β_3 KO	AR β_3 KO HFD
Cholesterol	128.44 $\pm$ 3.26	170.31 $\pm$ 7.48*	115.36 $\pm$ 5.75	134.64 $\pm$ 4.98 [#] ,**
Triglycerides	127.81 $\pm$ 3.47	167.81 $\pm$ 6.82*	111.98 $\pm$ 8.6	135.42 $\pm$ 4.77 [#] ,**

* vs WT with $P < 0.01$; [#] vs AR β_3 KO with $P < 0.05$; ** vs WT HFD with $P < 0.01$. Values are expressed as mean $\pm$ SEM of 6 animals per group.

the AR β_3 inactivation (Susulic *et al.* 1995, Rohrer 1998, Jimenez *et al.* 2002). Indeed, the AR β_3 KO BAT exhibits increased expression of AR β_1 but not AR β_2 (Fig. 2B) (Susulic *et al.* 1995). Given the importance of AR β_1 in BAT thermogenesis (Ueta *et al.* 2012), the overexpression of this AR isoform is likely to explain the normal AR β_3 KO BAT phenotype.

Despite better than normal BAT thermogenesis, AR β_3 KO animals kept on HFD developed more severe obesity, indicating greater susceptibility to diet-induced obesity. In fact, AR β_3 KO 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 AR β_3 KO. 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 AR β_3 KO mouse did not exhibit exaggerated intolerance to glucose (Fig. 6), suggesting that AR β_3 could play a role modulating the decrease in insulin sensitivity during high-fat feeding. Also notable was the fact that AR β_3 KO 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 AR β_3 KO 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 AR β_3 deletion in this condition needs further investigation.

Previous studies indicated that the isoproterenol-induced adenylate cyclase activity and lipolysis are diminished in AR β_3 KO-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 AR β_3 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, AR β_3 KO 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, AR β_3 KO 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 AR β_3 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.10⁻⁶cells.h⁻¹) released by isolated white adipocytes of WT and AR β_3 KO mice after treatment with chow diet or HFD for 8 weeks.

	WT	WT HFD	AR β_3 KO	AR β_3 KO HFD
Basal levels	3257 $\pm$ 1056	5221 $\pm$ 990	3194 $\pm$ 692	4812 $\pm$ 973
Isoproterenol	8909 $\pm$ 963	20242 $\pm$ 1768*	6003 $\pm$ 809	9583 $\pm$ 1103**

* vs WT with $P < 0.001$; ** vs WT HFD with $P < 0.001$. Values are expressed as mean $\pm$ SEM of 6 animals per group.

Figure 7

Expression of lipolytic and lipogenic enzymes in epididymal white fat of WT and $\text{AR}\beta_3\text{KO}$ fed with chow or HFD for 8 weeks ($n=6$). (A) Hormone-sensitive lipase (HSL); (B) phosphorylated hormone-sensitive lipase (p-HSL); (C) ratio between HSL and p-HSL; (D) AMP-activated protein kinase (AMPK); (E) phosphorylated AMP-activated protein kinase (p-AMPK); (F) ratio between AMPK and p-AMPK; (G) adipose triglyceride lipase (ATGL); (H) perilipin and (I) diacylglycerol acyltransferase-2 (DGAT2). * vs WT with $P<0.05$; # vs $\text{AR}\beta_3\text{KO}$ with $P<0.05$. Values expressed as mean $\pm$ SEM and data were analyzed using one-way ANOVA followed by Student–Newman–Keuls test.

In conclusion, this study indicates that the $\text{AR}\beta_3$ 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 $\text{AR}\beta_3\text{KO}$ 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.

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