Expression of Human \( \alpha_2 \)-Adrenergic Receptors in Adipose Tissue of \( \beta_3 \)-Adrenergic Receptor-deficient Mice Promotes Diet-induced Obesity*

Catecholamines play an important role in controlling white adipose tissue function and development. \( \beta_1 \)- and \( \alpha_2 \)-adrenergic receptors (ARs) couple positively and negatively, respectively, to adenylyl cyclase and are co-expressed in human adipocytes. Previous studies have demonstrated increased adipocyte \( \alpha_2/\beta_2 \)-AR balance in obesity, and it has been proposed that increased \( \alpha_2 \)-ARs in adipose tissue with or without decreased \( \beta_2 \)-ARs may contribute mechanistically to the development of increased fat mass. To critically test this hypothesis, adipocyte \( \alpha_2/\beta_2 \)-AR balance was genetically manipulated in mice. Human \( \alpha_2 \)-ARs were transgenically expressed in the adipose tissue of mice that were homozygous (\( \alpha_2/\alpha_2 \)) or heterozygous (\( \alpha_2/\beta_2 \)) for a disrupted \( \beta_2 \)-AR allele. Mice expressing \( \alpha_2 \)-ARs in fat, in the absence of \( \beta_3 \)-ARs (\( \beta_3 \)-AR \(--/-- \) background), developed high fat diet-induced obesity. Strikingly, this effect was due entirely to adipocyte hyperplasia and required the presence of \( \alpha_2 \)-ARs, the absence of \( \beta_3 \)-ARs, and a high fat diet. Of note, obese \( \alpha_2 \)-transgenic, \( \beta_3 \)--/-- mice failed to develop insulin resistance, which may reflect the fact that expanded fat mass was due to adipocyte hyperplasia and not adipocyte hypertrophy. In summary, we have demonstrated that increased \( \alpha_2/\beta_2 \)-AR balance in adipocytes promotes obesity by stimulating adipocyte hyperplasia. This study also demonstrates one way in which two genes (\( \alpha_2 \) and \( \beta_3 \)-AR) and diet interact to influence fat mass.

The contribution of catecholamines to the control of metabolic events occurring in mature adipocytes such as lipolysis has been well documented. Human adipocytes express significant levels of \( \alpha_1 \), \( \beta_2 \), and \( \alpha_2 \)-adrenergic receptors (ARs), which couple positively (\( \alpha_1 \) and \( \beta_2 \)) and negatively (\( \alpha_2 \)) to adenylyl cyclase (1). Endogenous ligands, epinephrine and norepinephrine, activate both classes of receptors (1, 2), suggesting an important role for \( \alpha_2/\beta_2 \)-AR balance in regulating lipolysis and energy balance (1, 3, 4). Adipocytes from obese humans have increased \( \alpha_2 \)-ARs, \( \alpha_2/\beta_2 \)-AR ratios, and \( \alpha_2 \)-AR-mediated responses (3–9). In addition, longitudinal studies in animal models have shown that \( \alpha_2 \)-ARs are increased with fat cell hypertrophy and that increased \( \alpha_2/\beta_2 \)-AR balance is correlated with obesity (5, 10, 11). Thus, it has been proposed that \( \alpha_2/\beta_2 \)-AR balance affects adipose tissue development.

Murine adipocytes differ from human adipocytes in that they express many \( \beta_3 \)-ARs, in addition to \( \beta_1 \)- and \( \beta_2 \)-ARs, and very few \( \alpha_2 \)-ARs (1, 12). \( \beta_3 \)-ARs, like \( \beta_1 \)- and \( \beta_2 \)-ARs, couple positively to adenylyl cyclase. In mice, \( \beta_3 \)-ARs are expressed predominantly in white and brown adipocytes, where they are thought to play an important role in regulating lipolysis and thermogenesis (1). Surprisingly, \( \beta_3 \)-AR gene knockout mice have little or no increase in body weight and only a slight increase in body fat (13, 14). The absence of greater effects of \( \beta_3 \)-AR deficiency on fat stores could be due to the fact that murine adipocytes, unlike human adipocytes, express very few \( \alpha_2 \)-ARs (12), which if present would antagonize actions mediated by residual \( \beta_1 \)- and \( \beta_2 \)-ARs and even initiate some additional effects.

To assess the importance of \( \alpha_2/\beta_2 \)-AR balance in adipocytes in vivo, we have combined gene targeting and transgenic approaches to create mice with increased \( \alpha_2/\beta_2 \)-AR balance in adipose tissue. Specifically, the \( \alpha_2 \)-promoter (15) was used to drive adipocyte-specific expression of \( \alpha_2 \)-ARs in mice that were either homozygous (\(--/-- \)) or heterozygous (\( +/- \)) for a disrupted \( \beta_3 \)-AR allele. Mice with genetically altered \( \alpha_2/\beta_2 \)-AR balance were then assessed for sensitivity to high fat diet-induced obesity. Of note, mice with increased \( \alpha_2/\beta_2 \)-AR balance developed diet-induced obesity secondary to adipocyte hyperplasia. These results strongly suggest that \( \alpha_2/\beta_2 \)-AR balance plays an important role in regulating fat mass.

**MATERIALS AND METHODS**

Transgenic Mice—All genetically modified animals were created and maintained on an FVB/n inbred background and were genetically identical except for the specified genetic alterations. Creation of \( \beta_3 \)--/--

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1 The abbreviations used are: AR, adrenergic receptor; kb, kilobase(s); trans, transgenic.
mice, homozygous for the Ardb3m1Lowl allele, has previously been described (13). The aP2-α2-AR transgene (see Fig. 1a) was constructed by fusing mouse aP2 fatty acid-binding protein 5'-flanking regulatory sequence (16), −5.4 kb (EcoRI) to +21 base pairs (PatI), to 1.4 kb (NcoI to HindIII) of human genomic DNA containing the human α2C10 gene (16) and the splice/polyadenylation site of SV40. Comparisons between mice with Tg(ADRA2A)Lowl and without the α2-AR transgene were all performed on littermates. Animals were group-housed at 24 °C, had free access to food and water, and were handled in accordance with the principles and guidelines established by the National Institutes of Health. Where indicated, mice were weaned at the age of 3 weeks onto low fat (D12450) or high fat (D12451) diets (Research Diets, New Brunswick, NJ). Diets were matched for protein content and had the following composition (as a % of total calories): low fat diet (10% fat, 70% carbohydrate, and 20% protein); high fat diet (45% fat, 35% carbohydrate, and 20% protein).

Radioligand Binding Assays—Specific binding of the α2-adrenergic receptor antagonist (3H)RX-821002 to fat cell membranes was determined after 30 min of incubation at 25 °C (without total binding) or with (nonspecific binding) 10 μM epinephrine (12). The maximal number of α2-AR binding sites (Bmax) and equilibrium dissociation constants (Kd) were calculated using Scatchard analysis of saturation binding data.

Lipolysis—The in vitro lipolytic response of isolated white fat cells to epinephrine without or with 10 μM selective α2-adrenergic receptor antagonist RX-821002 was measured. Adipocytes were isolated, and lipolysis was measured as described previously (12). The in vivo lipolytic response of conscious overnight-fasted mice was measured 10 min after a 0.1 mg/kg epinephrine intraperitoneal injection by non-esterified fatty acid blood levels.

mRNA Analyses—Total RNA was isolated using a Trizol homogenizer and RNA STAT-60 solution (Tel-Test “B,” Inc., Friendswood, TX). α2-AR transgene mRNA was analyzed by Northern blotting using either a specific 1.5-kb SV40 probe or 1.2-kb α2C10 probe. UCP1 mRNA levels were analyzed by Northern blotting using a specific mouse 0.3-kb UCP1 cDNA probe.

Oxygen Consumption—Oxygen consumption was measured in 10-week-old mice using the OXYMAX system 4.93 (Colombo Instruments, Columbus, OH), with a settling time of 100 s, a measuring time of 2 min, and a measured oxygen consumption of 34798.

Assessment of Fat Stores—The measurement of total body lipid content was performed as described previously (17, 18). Fat cell size and fat cell number per fat depot were determined in perigonadal fat samples using the Hirsch and Gallian method (19) of lipid extraction, osmium tetroxide fixation, and Coulter Counter analysis. Histological determinations were performed as described previously (microscopic assessment of fat cell size; 600 cells per depot quantified in paraffin-embedded, inguinal fat pad sections from female mice) (20, 21).

Circulating Blood Metabolites and Hormones—Whole blood was collected and analyzed for blood glucose levels (One Touch blood glucose meter, LifeScan Inc., Milpitas, CA). Serum was isolated and assayed for non-esterified fatty acids (NEFA C kit, Wako Pure Chemical Industries, Ltd.), insulin, and leptin (mouse insulin or leptin kit, Linco Research Inc., St. Louis, MO).

RESULTS

To evaluate the physiologic significance of adipocyte α2-ARs, we had previously generated and studied transgenic mice, on a wild-type β3-AR (+/+ background, which express human α2A-ARs in white and brown fat (22) (transgene shown in Fig. 1a). Despite the presence of abundant α2-AR binding sites, transgenic mice had normal body weight and fat content (data not shown). We hypothesized that the absence of an effect of α2-ARs on fat stores was due to the presence of abundant β3-ARs, which along with β1- and β2-ARs override the inhibitory action of transgenically expressed α2-ARs.

In the present study, we assessed the importance of α2β-AR balance in adipocytes by creating α2-AR transgenic mice (Tg(ADRA2A)Lowl) on a β3-AR −/− background (mice −/− or +/− for the Ardb3m1Lowl allele) (13). The transgene employed was the α2P-promoter/human α2A-AR construct mentioned above (shown in Fig. 1a). As expected, mRNA encoding human α2A-AR was expressed in white and brown adipocytes, but not in liver, kidney, skeletal muscle, brain, intestine, heart, or non-adipocyte cells resident within adipose tissue (stroma-vascular fraction) (Fig. 1b). Using the α2-AR-selective radioligand, (3H)RX-821002, few α2-AR binding sites were found in membranes isolated from white adipocytes and brown adipose tissue of β3 −/− mice, confirming that murine adipocytes express very few α2-ARs (Fig. 1c).

Epinephrine is a agonist for both β- and α2-ARs, stimulates lipolysis in white adipocytes by increasing cAMP levels (1). As predicted, the human-like α2β-AR balance obtained in α2-trans, β3 −/− mice shifted the epinephrine concentration-response curve for stimulation of lipolysis to the right (Fig. 2a, left panel). This effect was lost when the α2-AR-selective antagonist, RX-821002, was present (Fig. 2a, right panel). In
addition, the α2-AR agonist, UK14304, inhibited lipolysis in a concentration-dependent fashion (data not shown). Finally, displacement of [3H]RX-821002 binding by epinephrine in α2-trans, β3−/− fat cell membranes (data not shown) gave the expected shallow competition curve with high and low affinity components (KᵢH, 0.81 nM; KᵢL, 30 nM) as classically described in human fat cells (7). These results demonstrate that α2-ARs in transgenic adipocytes are coupled to Gi protein. As expected, the in vivo circulating free fatty acid response to a single injection of epinephrine was blunted in α2-trans, β3−/− mice (Fig. 2b). These in vitro and in vivo studies demonstrate that α2-ARs in white adipocytes of α2-trans, β3−/− mice functionally antagonize epinephrine-induced stimulation of lipolysis (similar to what has been observed using isolated human white adipocytes) (6).

The effects of α2-AR expression on brown fat function were assessed. Cold exposure induces sympathetic nervous stimulation of UCP1 gene expression and thermogenesis in brown adipocytes, and this response plays an important role in maintaining the body temperature of mice (25–27). Compared with wild-type mice, β3−/− mice had impaired induction of UCP1 mRNA and decreased body temperature following acute cold exposure (Fig. 2c). These responses were not inhibited further by expression of α2-ARs in brown fat (α2-trans, β3−/− mice) (Fig. 2c). In addition, a single injection of epinephrine stimulated energy expenditure to a similar degree in β3−/− mice and α2-trans, β3−/− (Fig. 2d). These studies suggest that brown adipocyte function, in contrast to white adipocyte function, is not impaired by transgenic expression of α2-ARs.

To assess effects of α2β3-AR balance on body weight and total body lipid content, β3−/− mice and α2-trans, β3−/− mice were fed high fat and low fat diets from age 3 weeks to 20 weeks. When fed a low fat diet, body weights were similar in β3−/− mice and α2-trans, β3−/− mice (Fig. 3a). In contrast, when fed a high fat diet, body weights were markedly greater in α2-trans, β3−/− mice compared with β3−/− mice (Fig. 3b). Of interest, the effect of α2-AR expression on body weight was greater in female mice. A second line of α2-AR transgenic mice was created which, compared with the first line, expressed 50% of wild-type β3-AR expression with α2-trans, β3−/− mice (Fig. 3c). UCP1 mRNA levels were analyzed by Northern blotting using a specific mouse 0.3-kb UCP1 cDNA probe (6). All results were expressed as the mean ± S.E.

To assess the contribution of β3-AR deficiency in mediating the positive effect of α2-AR expression on high fat diet-induced obesity, α2-trans, β3−/− mice (line 1) were crossed with wild-type mice (+/+ for the β2-AR allele). All offspring were +/− for the β3-AR allele, whereas approximately 50% of offspring were positive for the α2-AR transgene. As above, mice were fed a high fat diet from age 3 weeks to 20 weeks. In contrast to studies performed using β3−/− mice, α2-AR expression failed to promote high fat diet-induced obesity in β3−/− mice (Fig. 3d). Thus, development of high fat diet-induced obesity required both the presence of α2-ARs in fat and the absence of β3-ARs.
perigonadal depots of high fat diet-fed mice. Fat cell size was decreased in α2-trans, β3−/− mice by 25% in females (not statistically significant) and by 32% in males. Fat cell number, on the other hand, was markedly increased in α2-trans, β3−/− mice 3.5-fold in females and 1.7-fold in males. These findings indicate that expansion of adipose tissue mass in 20-week-old high fat diet-fed α2-trans, β3−/− mice is due to adipocyte hyperplasia and not to an increase in fat cell size. This observation was confirmed using an alternative method of fat cell size determination (20, 21) (microscopic assessment of fat cell size; 600 cells per depot quantified in paraffin-embedded, inguinal fat pad sections from female mice) (data not shown).

Obesity is usually associated with elevated blood levels of glucose, insulin, free fatty acids, and leptin. It has been proposed that these features of obesity are due to the presence of enlarged adipocytes (28, 29). However, as shown in Fig. 4c, obese α2-trans, β3−/− mice, have normal blood glucose and insulin levels and reduced fatty acid levels, which is in agreement with hyperplasia without changes in adipocyte size observed in these mice. The weak but significant rise in blood leptin levels is not associated with increased leptin mRNA expression in adipose tissue (data not shown) but probably with the higher number of adipocytes.

DISCUSSION

In the present study we have used genetic engineering in mice to test the hypothesis that α2/β-AR balance in adipocytes is an important determinant of total body fat stores. By creating mice that have a “human-like” pattern of AR expression in fat (predominance of α2- over β1- and β2-ARs and absence of β3-ARs), we have demonstrated that increased α2/β-AR balance promotes high fat diet-induced obesity in mice. Notably, the development of obesity requires the presence of α2-ARs on adipocytes, the absence of β3-ARs, and a high fat diet, suggesting an important interaction between two genes (α2 and β3-AR) and diet on the regulation of total body fat stores.

The present study clearly indicates that increased α2/β-AR balance in adipocytes promotes high fat diet-induced obesity. However, the mechanism for this effect has yet to be established. Three possibilities are worthy of further discussion. Firstly, impaired sympathetic activation of lipolysis in white adipocytes could lead to increased accumulation of triglyceride. Secondly, impaired sympathetic activation of thermogenesis in brown adipose tissue could cause decreased energy expenditure and, consequently, positive energy balance. Thirdly, impaired sympathetic activation of white adipocytes could cause, via mechanisms to be discussed below, hyperplasia of white adipose tissue. Detailed analysis of α2-trans, β3−/− mice indicates that the first and second possibilities are less likely to be true. Obesity due to either impaired lipolysis or decreased energy expenditure would be expected to cause adipocyte enlargement, a feature common to nearly all models of obesity (30, 31). In the case of α2-trans, β3−/− mice, obesity was due entirely to adipocyte hyperplasia. In addition, brown fat function appeared not to be impaired in α2-trans, β3−/− mice. Thus, the fact that enlarged fat mass in α2-trans, β3−/− mice is due entirely to the proliferation of small adipocytes strongly suggests that high α2/β-AR balance promotes adipocyte hyperplasia.

The form of obesity observed in high fat diet-fed α2-trans, β3−/− mice is atypical because it is due entirely to adipocyte hyperplasia. In this regard, these animals do not represent murine models of “typical” human obesity (31). Obesity in humans as well as rodents is nearly always associated with adipocyte hypertrophy and hyperplasia. Typically, adipocyte hypertrophy occurs early during the development of obesity. It has been speculated that adipocytes, upon reaching a “critical

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upon this similarity, it is possible that increased proliferation-activated receptor- (PPAR-γ) in mice with (α2-trans, β3−/−) or without (β3−/−) the α2-AR transgene (n = 8–11; mean ± S.E.; *, p < 0.05; **, p < 0.01; unpaired, 2-tailed t test). c, blood parameters (whole blood glucose, serum free fatty acids (FFAs), insulin, and leptin) in mice with (α2-trans, β3−/−) or without (β3−/−) the α2-AR transgene (n = 8–11; mean ± S.E.; *, p < 0.05; **, p < 0.01; unpaired, 2-tailed t test).}

FIG. 4. Assessment of fat stores and blood parameters in 20-week-old high fat diet-fed mice. α and β, total body lipid, fat pad weights, and fat cell size and number in female (α) and male (β) mice with (α2-trans, β3−/−) or without (β3−/−) the α2-AR transgene (n = 8–11; mean ± S.E.; *, p < 0.05; **, p < 0.01; unpaired, 2-tailed t test). In this regard, α2-trans, β3−/− mice resemble rodents treated with thiazolidinediones (32, 33), agonists of peroxisome proliferator-activated receptor-γ. Based upon this similarity, it is possible that increased α2/β-AR balance in adipocytes somehow leads to activation of peroxisome proliferator-activated receptor-γ, possibly through generation of PPAR-γ ligands.

High fat diet-fed α2-trans, β3−/− mice develop an obesity that is characterized by an increase in both adipocyte number and lipid storage without any increase in fat cell size. The findings suggest that, when fed a high fat diet, α2-trans, β3−/− mice develop obesity through two mechanisms: (i) an increase in fat cell number due to increased preadipocyte recruitment and (ii) an increase in the ability to store lipids due to impaired epinephrine-stimulated lipolytic activity. If increased lipid storage was not present, then average adipocyte size would have been decreased by an amount reciprocal to the increase in fat cell number. Because this was not the case, it must be assumed that lipid storage was also increased, an effect presumably mediated by α2-AR-induced antilipolytic activity, potentiated by the absence of β3-ARs. Thus, the increased fat mass in α2-trans, β3−/− mice appears to be due to both preadipocyte recruitment and increased lipid storage in the newly recruited adipocytes.

Brown adipocyte function appears not to have been impaired by transgenic expression of α2-ARs in β3−/− mice. This assessment is based upon the observation that cold exposure-induced changes in UCP1 mRNA in brown fat and body temperature as well as epinephrine-induced effects on whole body oxygen consumption were not impaired in α2-trans, β3−/− mice compared with β3−/−control mice. This raises the possibility that α2-ARs in brown adipocytes were not negatively coupled to adenylate cyclase. The reason for such failure of coupling in brown adipocytes, but not white adipocytes, is presently unknown.

In summary, the present study clearly demonstrates that increased α2/β-AR balance in adipose tissue promotes diet-induced obesity. These findings suggest that increased α2/β-AR balance, which is frequently observed in human obesity (3–9), has physiologic significance in the generation of adipocyte hyperplasia and the obese state. Identification of the biochemical mechanism by which α2/β-AR balance and high fat diet promote adipocyte hyperplasia will focus on the possible roles of lysophosphatidic acid and peroxisome proliferator-activated receptor-γ, α2-trans, β3−/− mice should provide a unique opportunity to explore the mechanisms by which expansion of adipose tissue mass is regulated.
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