Human $\alpha 2A$ -adrenergic receptor gene expressed in transgenic mouse adipose tissue under the control of its regulatory elements

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Abstract

Catecholamines regulate white adipose tissue function and development by acting through β - and $\alpha 2$ -adrenergic receptors (ARs). Human adipocytes express mainly $\alpha 2A$ - but few or no $\beta 3$ -ARs while the reverse is true for rodent adipocytes. Our aim was to generate a mouse model with a human-like $\alpha 2/\beta$ -adrenergic balance in adipose tissue by creating transgenic mice harbouring the human $\alpha 2A$ -AR gene under the control of its own regulatory elements in a combined mouse $\beta 3$ -AR $^{-/-}$ and human $\beta 3$ -AR $^{+/+}$ background.

Transgenic mice exhibit functional human $\alpha 2A$ -ARs only in white fat cells. Interestingly, as in humans, subcutaneous adipocytes expressed higher levels of $\alpha 2$ -AR than perigonadal fat cells, which are associated with a better antilipolytic response to epinephrine. High-fat-diet-induced obesity was observed in transgenic mice in the absence of fat cell size modifications. In addition, analysis of gene expression related to lipid metabolism in isolated adipocytes suggested reduced lipid mobilization and no changes in lipid storage capacity of transgenic mice fed a high-fat diet. Finally, the development of adipose tissue in these mice was not associated with significant modifications of glucose and insulin blood levels. Thus, these transgenic mice constitute an original model of diet-induced obesity for *in vivo* physiological and pharmacological studies with respect to the $\alpha 2/\beta$ -AR balance in adipose tissue.

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Introduction

Obesity is characterized by an excessive development of fat mass which is a consequence of increased fat cell size and/or fat cell number. Several hormones and neurotransmitters regulate adipose tissue development and metabolism. Among them, catecholamines play a major role by acting through $\alpha 2$ - and β -adrenergic receptors (ARs). They control metabolic effects such as lipolysis through regulation of adenylyl-cyclase activity and cAMP levels under the balanced control of positive $\beta 1$ -, $\beta 2$ - and $\beta 3$ -AR-dependent stimulation and $\alpha 2$ -AR-mediated inhibition (Lafontan & Berlan 1993). In humans,

differences exist in catecholamine-induced lipolysis between fat pads from various anatomical locations. Such differences are related to variations in $\alpha 2$ -AR number. Indeed, epinephrine stimulates lipolysis in internal fat depots (omental, pericolonic) whereas it inhibits lipolysis in s.c. fat pads (femoral) where $\alpha 2$ -ARs largely outnumber β -ARs (Mauriège *et al.* 1987, Castan *et al.* 1993). Moreover, it has recently been shown, using *in situ* microdialysis, that the physiological activation of $\alpha 2$ -ARs represents an important inhibitory factor for epinephrine-induced lipid mobilization during exercise (Stich *et al.* 1999). This impairment of lipolysis is enhanced in obese subjects (Stich *et al.* 2000).

Striking species-specific differences exist in fat cell $\alpha 2$ -AR expression. The highest level of $\alpha 2$ -ARs is observed in primate and human fat cells in which $\alpha 2$ -ARs largely predominate over β -ARs (Castan *et al.* 1993, Lafontan & Berlan 1995). Moreover, human white adipocytes express few (or no) $\beta 3$ -ARs (Tavernier *et al.* 1996). Conversely, most of the laboratory animal models including mice and rats exhibit a very low number of adipocyte $\alpha 2$ -ARs (Castan *et al.* 1994). In rodent adipocytes, lipolysis is mainly due to $\beta 3$ -AR activation (Lafontan 1994). Thus, human and rodent white adipocytes exhibit major differences with respect to the balance between $\alpha 2$ - and $\beta 3$ -ARs and catecholamine-dependent regulation of lipolysis.

Since no suitable animal model was available to study the involvement of $\alpha 2/\beta$ -AR balance in white adipose tissue (WAT) *in vivo*, transgenic approaches were used in mice. Recently, $\beta 3$ -AR gene disruption and human $\alpha 2A$ -AR expression in adipose tissues have been described in the Tg(ADRA2A)^{Low1} mouse (Valet *et al.* 2000). When fed a high-fat diet (HFD), these mice had increased fat mass and body weight. Adipocyte-specific expression of human $\alpha 2A$ -ARs in these mice was driven by the aP2 promoter which controls the expression of the associated gene during the early stages of adipocyte differentiation. However, fusing the human $\alpha 2A$ -AR gene with the aP2 promoter prevents any endogenous regulation of the $\alpha 2C10$ gene (encoding the $\alpha 2A$ -AR). In mammals, regulation of $\alpha 2$ -AR gene expression has been related to processes linked to the control of WAT development (Lafontan & Berlan 1995). Thus, another transgenic approach aiming at the utilization of the regulatory elements of the $\alpha 2C10$ gene and expected to preserve endogenous regulation of $\alpha 2A$ -AR appeared complementary. Ito *et al.* (1998) described a human $\beta 3$ -AR transgene containing the regulatory and coding sequences necessary for a 'human-like' pattern of expression. A genomic P1 clone containing 77 kb of human genomic DNA bearing regulatory elements of the promoter regions and the coding sequences of the human $\beta 3$ -AR were injected into mouse embryo disrupted for the murine $\beta 3$ -AR gene. In this case, $\beta 3$ -AR expression paralleled that commonly described in humans, e.g. expression in brown but not white adipocytes. A similar strategy was applied in the present study for the regulatory elements of the promoter regions and coding

sequences of the human $\alpha 2C10$ gene. A 31 kb fragment of human DNA framing the coding sequence of the human $\alpha 2C10$ gene (11 kb upstream and 18 kb downstream) was obtained, characterized and introduced transgenically into (m $\beta 3$ - / -, h $\beta 3$ +/+) mice previously described by Ito *et al.* (1998). The expression of $\alpha 2A$ -AR should then be driven by the regulatory sequences of the human $\alpha 2C10$ gene. A human-like distribution profile of ARs was expected. Therefore, we investigated the expression of human $\alpha 2A$ -AR in WAT as well as its regulation regarding fat pad location in transgenic mice. The physiological consequences of an HFD were also studied.

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 m $\beta 3$ -AR - / -, ha2-AR+/-, h $\beta 3$ -AR+/. Targeted disruption of mouse $\beta 3$ -AR (m $\beta 3$ - / -) generated by homologous recombination (*Adrb3*^{tm1 Low1}) was described by Susulic *et al.* (1995) and the introduction of the human $\beta 3$ -AR genomic transgene was reported by Ito *et al.* (1998). Finally, the human $\alpha 2A$ -AR transgene was constructed accordingly. Briefly, human $\alpha 2$ -AR P1 bacteriophage genomic clones were isolated and screened by using specific primers: sense primer 5'-CCTCCAGGGCCCCGCAGACC-3' and anti-sense primer 5'-AGGGGAACCAGCACACCACGAACA-3'. The clones were then mapped. A selected P1 clone (DMPC-HFF#1, 69 g6) corresponding to 11 kb upstream of and 18 kb downstream from the $\alpha 2A$ -AR-coding region was injected into zygotes of (m $\beta 3$ - / -, h $\beta 3$ +/+) mice. Human $\alpha 2$ -AR genomic transgene is represented in Fig. 1. The transgenic mice called *Adrb3*^{tm1 Low1}-Tg (ADRB3-ADRA2A)^{Low1} mice according to the Jackson guidelines were identified by Southern blotting following PstI digestion of isolated genomic DNA or by PCR with selective primers. After digestion of genomic DNA by PstI enzyme and separation on an agarose gel, three bands of 9, 2.5 and 1 kb on Southern blot reveal the presence of human $\alpha 2A$ -AR and $\beta 3$ -AR. These mice will be called transgenic or (m $\beta 3$ - / -, h $\beta 3$ +/+, ha2A+/-) mice in the text. In mice

Figure 1 Schematic representation of the human $\alpha 2A$ -AR transgene. A partial restriction enzyme map of the human $\alpha 2A$ -AR is shown. The human transgene consists of the P1 clone DMPC-HFF#1, 69g6, which was linearized with NotI before microinjection. The box refers to the location of the coding sequence of the $\alpha 2C10$ gene. P1 vector refers to the P1 bacteriophage backbone.

which do not express human $\alpha 2$ -AR but human $\beta 3$ -AR, two bands (9 kb and 1 kb) were present. These mice will be called control or ($m\beta 3 - / -$, $h\beta 3 + / +$) mice in the text. Transgenic mouse lines were established by breeding transgenic founder mice with their non-transgenic littermates. Nine transgenic founders were generated and three of these were bred to establish lines P1, P3 and P6. Mice from lines P3 and P6 were maintained and analysed. All the results presented in this study were found in both P3 and P6 lines. At all times, the human $\beta 3$ -AR and $\alpha 2A$ -AR transgenes were maintained on the murine $\beta 3$ -AR gene knockout background. Transgenic mice could not be distinguished from wild type or control mice by appearance, body weight, fertility or viability.

Diets

After weaning (3 weeks old), mice (male and female) were assigned to a low-fat diet (LFD) or an HFD (UAR, Lyon, France) for 18 weeks. Calorie composition was 20% protein, 70% carbohydrate and 10% fat for the LFD, and 20% protein, 35% carbohydrate and 45% fat for the HFD. The main source of fat in the HFD was lard (20 g/100 g of food). Mice were housed conventionally in an animal room with constant temperature (20–22 °C), humidity (50–60%) and with a 12 h light/12 h darkness cycle. Body weight was determined weekly. All mice had free access to food and water throughout the experiment.

Human adipose tissue

Human adipose tissue was collected according to the guidelines of the Ethical Committee of Rangueil Hospital, Toulouse. It was obtained from healthy non-obese women (46 ± 10 years old, body mass index 24.2 ± 1.5 , $n=5$) undergoing abdominal dermolipectomy for plastic surgery. None of them had any identified metabolic disorder or was taking drugs modifying adipose tissue metabolism or catecholamine levels.

Northern blot analyses

Total RNA was extracted from mouse brain, liver, kidney, brown adipose tissue (BAT), WAT (perigonadal and s.c. fat deposits pooled) and human s.c. WAT using RNA STAT (Ams Bio-technology Ltd, Oxon, UK). Fifty microgram aliquots of total RNA were separated by electrophoresis in 1% denaturing MOPS-formaldehyde-agarose gels, transferred to nylon membranes and hybridized to a [^{32} P]dCTP-labelled specific probe derived from the $\alpha 2A$ -AR gene. However, the probe cannot distinguish between the human $\alpha 2A$ subtype and the corresponding rodent subtype formerly called $\alpha 2D$. Ethidium bromide staining of the gel was used to verify the uniformity of loading.

Real-time RT-PCR

Total RNA was extracted using RNA Easy mini (Quiagen) from isolated perigonadal adipocytes of control ($m\beta 3 - / -$, $h\beta 3 + / +$) and transgenic ($m\beta 3 - / -$, $h\beta 3 + / +$, $h\alpha 2A + / -$) mice fed an HFD. cDNA was synthesized from 2 μ g total RNA using random hexamers and murine Moloney leukaemia virus reverse transcriptase (Life Technologies, Inc.). Real time quantitative RT-PCR analyses were performed as previously described in an ABI PRISM 7700 Sequence Detection System Instrument (Taqman; Applied Biosystems, Foster City, CA, USA) (Le Lay *et al.* 2001). GAPDH expression of a control cDNA was used as an interplate calibrator, and the variability in the initial quantities of cDNA was normalized by using ribosomal 18S RNA amplifications. Results were obtained from three or four independent RNA samples from individual animals, each tested in triplicate.

Radioligand binding studies

The $\alpha 2$ -ARs were identified with the selective antagonist radioligand [^3H]RX821002 on membranes prepared from isolated white adipocytes and from brain homogenates as previously described (Castan *et al.* 1993). The $\beta 1$ - and $\beta 2$ -ARs in fat cell membranes were determined by using the antagonist [^3H]CGP12177 radioligand as previously described (Carpéné *et al.* 1994). Specific binding was defined as the difference between total and non-specific binding determined in the presence of 10^{-5} M phentolamine for the $\alpha 2$ -AR and 10^{-5} M propranolol for the β -AR. The maximum number of adrenergic binding sites (B_{max}) and equilibrium dissociation constants (K_d) were calculated using Scatchard analysis of saturation binding data. Competition studies of [^3H]RX821002 binding were performed either on brain membranes from control (m $\beta 3$ - / -, h $\beta 3$ + / +) and transgenic (m $\beta 3$ - / -, h $\beta 3$ + / +, ha2A + / -) mice or on adipocyte membranes from human and transgenic mice (a pool of perigonadal and s.c. adipocytes) using 0.01 nM to 10 μM yohimbine and 2 nM of radioligand. Protein content was determined using the Lowry protein assay kit (Bio-Rad, Richmond, CA, USA).

Lipolysis

Perigonadal and s.c. (inguinal) adipose tissues from female mice fed a standard diet or 18 weeks of HFD were quickly dissected. Adipocytes were isolated using collagenase digestion as described previously (Castan *et al.* 1994). They were incubated in the presence of different agents at 37 °C and shaken gently for 90 min. Adenosine deaminase (ADA) was used to promote an increment of basal lipolysis in order to measure the antilipolytic effect of the selective $\alpha 2$ agonist, UK14304. The glycerol produced by the cells was used as an index of lipolysis. Total lipids were evaluated gravimetrically after extraction by the method of Dole and Meinertz (Castan *et al.* 1994).

Adipocyte size

Perigonadal adipocytes were isolated as described above. Fifty microlitres of adipocyte suspension were observed under a microscope connected to a camera and then analysed with Visiolab software (Biocom, Les Ulis, France). Two pictures from each

preparation were taken and the surface as well as the diameter of each adipocyte (between 30 and 80 adipocytes per photo) was measured.

DNA content of WAT

One hundred milligrams of each adipose tissue sample were minced and digested overnight by proteinase K (NaCl 100 mM, Tris 20 mM, EDTA 10 mM, SDS 1%, proteinase K 100 $\mu\text{g}/\text{ml}$). After salt precipitation, genomic DNA was isolated by ethanol precipitation and DNA content was calculated by spectrophotometry (Ultrospec 3000; Pharmacia Biotech). Linearity and reproducibility of this method was controlled using increasing amounts of adipose tissue.

Serum analyses

At the term of the experiment (21-week-old animals), blood was collected and glucose was immediately measured with a glucose meter (Glucometer 4; Bayer Diagnostics, Puteaux, France). Serum was then taken and immediately frozen. Insulin concentrations were determined with an RIA kit (Diagnostics Pasteur, Paris, France) and non-esterified fatty acids (NEFAs) were measured using a colorimetric enzymatic kit (NEFA C; Wako Pure Chemical Industries, Neuss, Germany).

Glucose tolerance tests

After 6 h fasting, mice fed an HFD for 18 weeks received an i.p. injection of glucose (1 mg/g body weight). Tail vein blood samples were collected before injection and at 10, 20, 30, 60, 120 and 180 min post-injection.

Blood pressure of conscious mice

Blood pressure was measured in control and transgenic mice (male and female) fed an HFD or LFD for 18 weeks. The mice were placed in a semicylindrical container warmed to 30 °C and measurements were made indirectly using an inflatable tail-cuff. Prior to the actual blood pressure measurements, the mice were acclimated daily for 1 week to the container. Systolic blood pressure was recorded (15 determinations in a row at three different times) by using a PowerLab/s

Figure 2 Northern blot analysis of $\alpha 2A$ -AR mRNA in various tissues. Fifty micrograms of total RNA from kidney, liver, brain, brown (BAT) and white (WAT) adipose tissue of control ($m\beta 3^{-/-}$, $h\beta 3^{+/+}$) and transgenic ($m\beta 3^{-/-}$, $h\beta 3^{+/+}$, $h\alpha 2A^{+/-}$) female mice (8 weeks old) as well as 50 μ g of total RNA from human s.c. WAT were obtained. They were subjected to Northern blot analysis. Corresponding 28S and 18S ribosomal RNA from ethidium bromide-stained gels are shown.

system connected to CHART software (AD Instruments, Milford, MA, USA).

Statistical analysis

All results are expressed as means $\pm$ S.E.M. Comparisons between groups were made by paired or unpaired Student's *t*-tests.

Results

Tissue distribution of human $\alpha 2A$ -AR mRNA levels in transgenic mice

The presence of human $\alpha 2A$ -AR mRNA was first analysed by Northern Blot analysis in different tissues of ($m\beta 3^{-/-}$, $h\beta 3^{+/+}$) control and ($m\beta 3^{-/-}$, $h\beta 3^{+/+}$, $h\alpha 2A^{+/-}$) transgenic mice (Fig. 2). Human s.c. adipocytes expressing $\alpha 2A$ -AR were used as a positive control. In control mice, $\alpha 2A$ -AR mRNA was only detected in the brain as expected. In transgenic mice, the human $\alpha 2A$ -AR is mainly expressed in WAT and to a much lower extent in BAT. In brain, $\alpha 2A$ -AR mRNA was also expressed. The transgene was not detected in kidney or in tissues known to lack $\alpha 2A$ -AR in

humans like liver. Thus, human $\alpha 2A$ -AR expression was extended to WAT in transgenic mice suggesting that the human $\alpha 2A$ -AR promoter contains the cis-elements that induce adipose-specific expression of the $\alpha 2A$ -AR gene.

Characterization of human $\alpha 2A$ -AR in transgenic mice

In order to quantify $\alpha 2$ -AR sites, saturation binding experiments were performed with [^{3} H]RX821002, a selective $\alpha 2$ -AR antagonist. The results are reported in Table 1. In tissues known to express endogenous $\alpha 2$ -ARs such as brain or kidney, the number of $\alpha 2$ -ARs was similar in control and transgenic mice. However, in kidney, the K_d value was higher than in other tissues, indicating that the majority of $\alpha 2$ -AR belong to another subtype, probably the B subtype as previously described (Uhlen & Wikberg 1991). In adipose tissues, $\alpha 2A$ -AR sites were only expressed in white and not in brown adipocyte membranes of transgenic mice. Nevertheless, the number of $\alpha 2A$ -AR binding sites detected in the white fat cells in transgenic mice was much lower than that commonly observed in

Table 1 Quantification of the maximal number of $\alpha 2$ -AR sites ($B_{\max}$) by [^{3} H]RX821002 radioligand binding and equilibrium dissociation constant (K_d) values in different tissues from control (m $\beta 3$ -/-, h $\beta 3$ +/+) and transgenic (m $\beta 3$ -/-, h $\beta 3$ +/+, h $\alpha 2A$ +/-) mice. Binding studies were performed as indicated in Materials and methods. $B_{\max}$ and K_d values were calculated by computer-assisted analysis of data according to a one-site model. Values are means $\pm$ S.E.M. from (n) experiments

	Control		Transgenic	
	$B_{\max}$ (fmol/mg protein)	K_d (nM)	$B_{\max}$ (fmol/mg protein)	K_d (nM)
Brain	109 $\pm$ 8 (3)	0.8 $\pm$ 0.1	97 $\pm$ 5 (4)	0.8 $\pm$ 0.1
Liver	nd (3)	—	nd (3)	—
Kidney	55 $\pm$ 1 (4)	5.9 $\pm$ 0.4	58 $\pm$ 5 (3)	4.5 $\pm$ 0.4
BAT	nd (3)	—	nd (3)	—
WAT	<15 nd (3)	—	53 $\pm$ 2 (6)	1.1 $\pm$ 0.1

nd, not detected; BAT, brown adipose tissue; WAT, white adipose tissue.

human adipocytes (Castan *et al.* 1995). As expected, in the liver, expression of $\alpha 2A$ -AR sites was not detected, in agreement with the absence of $\alpha 2A$ -AR mRNA.

In order to verify whether the $\alpha 2$ -AR receptor identified with [^{3} H]RX821002 was of human or murine origin, competition studies of [^{3} H]RX821002 binding were performed in the presence of yohimbine (an $\alpha 2$ -AR antagonist) in brain and adipocyte membranes. Indeed, it has been shown that yohimbine has a very low affinity for the rodent compared with human $\alpha 2A$ -AR (Link *et al.* 1992, Uhlen *et al.* 1998). In brain from transgenic as well as control mice, yohimbine inhibited the binding of [^{3} H]RX821002 in a concentration-dependent manner, displaying a similar population of low-affinity receptors (K_i = 135 $\pm$ 47 nM and 89 $\pm$ 38 nM respectively, n = 5). By contrast, the K_i values in adipocyte membranes from transgenic mice (K_i = 6.7 $\pm$ 1.3 nM, n = 5) revealed a population of high-affinity receptors which was similar to that obtained in humans (K_i = 4.6 $\pm$ 1.1 nM, n = 5) (Fig. 3). This result indicates that the $\alpha 2A$ -AR expressed in the brain of transgenic mice is essentially of the murine type whereas it is of the human type in WAT. Thus, the human $\alpha 2A$ -AR protein is mainly expressed in WAT and not in brain of transgenic mice.

Regulation of $\alpha 2A$ -AR expression according to the location of the fat deposit

The distribution of the $\alpha 2A$ -AR differs according to fat location in humans. Since the expression of the

$\alpha 2A$ -AR was under the control of human gene regulatory elements in transgenic mice, the level of expression of $\alpha 2A$ -ARs was investigated in two different fat deposits. Strikingly, $\alpha 2A$ -ARs were more numerous in male and female s.c. than in

Figure 3 Competition of [^{3} H]RX821002 binding to adipocyte and brain membranes by yohimbine. Experiments were performed on brain membranes from control (●) and transgenic (○) mice and on adipocyte membranes from non-obese human subjects (■) and transgenic mice (□). Each point represents the mean obtained from five separate experiments.

Figure 4 $\alpha 2A$ -AR responsiveness according to fat pad location in transgenic mice. (A) Quantification of $\alpha 2A$ -AR sites by using [^3H]RX821002 radioligand in perigonadal (empty bars) and s.c. (filled bars) adipocyte membranes from male and female transgenic mice. Results are means $\pm$ S.E.M. of $n=5-8$ independent experiments. * $P<0.05$, ** $P<0.01$, paired t -test. *In vitro* lipolytic effect of epinephrine was determined alone (empty symbols) or in the presence of 10^{-5} M RX821002 (filled symbols) in perigonadal (B) and s.c. (C) isolated fat cells from transgenic female mice. The effect of 10^{-5} M isoproterenol is represented by the histogram. Results are expressed in μ mol glycerol released/100 mg lipids during the incubation time. Each point represents the mean $\pm$ S.E.M. of $n=6$ independent experiments. * $P<0.05$ vs epinephrine alone (paired t -test). (D) Antilipolytic effect of UK14304 on ADA-stimulated (4 μ g/ml) lipolysis in perigonadal ($\square$) and s.c. ($\blacksquare$) adipocytes. Results are expressed as percent inhibition where 0% corresponds to stimulated lipolysis and 100% to basal values. * $P<0.05$ paired t -test.

perigonadal adipocytes (Fig. 4A) from transgenic mice. Expression of $\beta 1$ - and $\beta 2$ -ARs was also determined in isolated adipocytes from control and transgenic mice (female) by using [^3H]CGP12177, a $\beta 1$ -, $\beta 2$ -AR antagonist. The maximum number of sites for both $\beta 1$ - and $\beta 2$ -ARs was similar between control and transgenic mice without differences according to fat pad location (18 ± 1 fmol/mg

protein, $K_d = 0.21 \pm 0.04$ nM in perigonadal adipocyte membranes, vs 17 ± 1 fmol/mg protein, $K_d = 0.29 \pm 0.06$ nM in s.c. adipocyte membranes in control mice, $n=3$ and 19 ± 1 fmol/mg protein, $K_d = 0.31 \pm 0.04$ nM vs 23 ± 2 fmol/mg protein, $K_d = 0.26 \pm 0.02$, $n=3$ in transgenic mice). So, as in humans, the $\alpha 2/\beta$ -AR ratio in WAT is in favour of the $\alpha 2A$ -AR, particularly in s.c. adipocytes.

Regulation of lipolysis in transgenic mice expressing human $\alpha 2A$ -AR

It was expected that a higher expression of $\alpha 2A$ -AR in s.c. adipocytes should be related to a lower lipolytic response to catecholamines. In order to assess the efficiency of the $\alpha 2$ -adrenergic responsiveness in the different fat pads, isolated adipocytes from transgenic female mice were stimulated with different concentrations of the endogenous amine epinephrine alone or in the presence of 10^{-5} M RX821002, a selective $\alpha 2$ antagonist. Isoproterenol at 10^{-5} M was also tested as a positive control for the maximal lipolysis induced by $\beta 1$ - and $\beta 2$ -ARs stimulation. Epinephrine promoted glycerol release with a significant effect at 10^{-6} M and 10^{-5} M in both perigonadal and s.c. adipocytes. When RX821002 was added to the incubation medium, a significant increment in the lipolytic effect of 10^{-5} M epinephrine was obtained. It was significantly stronger in s.c. than in perigonadal adipocytes (Fig. 4B and C). The maximum effect at 10^{-6} M epinephrine plus 10^{-5} M RX821002 was similar to that of isoproterenol in both fat deposits. In addition, 10^{-6} M selective $\alpha 2$ agonist UK14304 induced a significantly stronger antilipolytic effect in s.c. than in perigonadal fat cells ($75.7 \pm 7.4\%$ inhibition vs $52.5 \pm 4.1\%$) with similar efficacy ($EC_{50} = 112 \pm 25$ nM in perigonadal and 68 ± 26 nM in s.c. fat cells), confirming the higher responsiveness to $\alpha 2$ -adrenergic stimulation in s.c. adipocytes from transgenic mice (Fig. 4D).

Effects of an HFD; physiological consequences in transgenic mice

Since $\alpha 2A$ -AR expression has been described to increase with obesity (Lafontan & Berlan 1995), control and transgenic mice were fed either an LFD or an HFD from age 3 to 21 weeks. The consequences of these diets were measured on body weight, adipocyte size, adipocyte gene expression of lipid metabolism, $\alpha 2$ -adrenergic responsiveness, plasma parameters and blood pressure.

Body weight

No difference was observed between control and transgenic mice fed an LFD (Fig. 5A). However, when fed an HFD, the body weight gain of transgenic mice was significantly elevated in

comparison with control mice (Fig. 5B). This difference became significant after 3 weeks of HFD (6-week-old animals) for the females and after 4 weeks of HFD (7-week-old animals) for the males. At the end of the diet period, the final weight gain of transgenic mice exceeded that of controls by 22% in males and 21% in females. This increased body weight was related to a rise of fat pad weight; the mass of perigonadal and s.c. fat deposits was higher in transgenic compared with control mice fed an HFD (Table 2). These results indicate that $\alpha 2A$ -AR expression in WAT enhances the fattening effect of HFD.

Adipocyte size

To assess the contribution of fat cell size and/or adipocyte number in the expansion of adipose tissue, fat cell size and DNA content were measured. This was done in adipose tissue from female transgenic mice since they had a higher fat pad weight gain than males. In addition, the measurements were made in female perigonadal adipose tissue in which the largest increase of mass was observed. The diameter of perigonadal adipocytes from female transgenic mice fed an HFD was not significantly different from that of control mice fed an HFD or transgenic mice fed an LFD (Fig. 6). The DNA content of perigonadal adipose tissue was twice as high in adipose tissue of transgenic females fed an HFD as in control (220.0 ± 32.8 μ g/fat pad, $n=5$, vs 111.6 ± 15.0 μ g/fat pad, $n=4$, respectively, $P<0.05$). So, the development of WAT in 21-week-old transgenic mice fed an HFD is not associated with hypertrophy but rather with hyperplasia.

Adipocyte gene expression

mRNA levels were determined in isolated perigonadal adipocytes and the expression of various genes involved in lipid metabolism was evaluated.

Values were normalized to ribosomal RNA levels. mRNA levels of genes involved in lipid storage and mobilization such as lipoprotein lipase (LPL) and hormone-sensitive lipase (HSL), respectively, were studied. LPL mRNA levels were not modified whereas HSL mRNA levels were significantly lower in perigonadal adipocytes from HFD-fed transgenic compared with control mice (Table 3). Recently, the cholesterol balance within

Figure 5 Time course of body weight increase in male and female mice fed either a low-fat (LFD) or a high-fat (HFD) diet. After weaning, control (○) and transgenic (■) mice were subjected to either an LFD (A) or an HFD (B) for 18 weeks. Results are means $\pm$ S.E.M. of 9–13 mice. * $P < 0.05$ vs control, unpaired t -test.

Table 2 Fat pad weight of control ($m\beta 3^{-/-}$, $h\beta 3^{+/+}$) and transgenic ($m\beta 3^{-/-}$, $h\beta 3^{+/+}$, $h\alpha 2A^{+/-}$) mice fed a high-fat (HFD) or low-fat (LFD) diet for 18 weeks. Mice fed an LFD or HFD were killed at the age of 21 weeks and perigonadal (PG) as well as subcutaneous (SC) fat pads were dissected out and weighed (g). Data are means $\pm$ S.E.M. from 9–13 mice

	LFD		HFD	
	PG	SC	PG	SC
Male				
Control	0.99 $\pm$ 0.11	0.67 $\pm$ 0.08	1.19 $\pm$ 0.12	1.33 $\pm$ 0.24°
Transgenic	0.90 $\pm$ 0.10	0.60 $\pm$ 0.06	1.37 $\pm$ 0.14°	2.01 $\pm$ 0.19°°,*
Female				
Control	1.19 $\pm$ 0.09	0.64 $\pm$ 0.06	1.84 $\pm$ 0.28°	1.02 $\pm$ 0.17°
Transgenic	1.02 $\pm$ 0.15	0.57 $\pm$ 0.08	2.64 $\pm$ 0.19°°,*	1.42 $\pm$ 0.14°°

* $P < 0.05$ for control vs transgenic, ° $P < 0.05$ and °° $P < 0.01$ for LFD vs HFD.

Figure 6 Effect of diet on perigonadal adipocyte size. Size of isolated white adipocytes from perigonadal fat deposits of female control (empty bars) and transgenic mice (filled bars) fed either an LFD or an HFD for 18 weeks were measured as described in Materials and methods. Values are means±S.E.M. of *n*=5–7 separate determinations in each group performed in duplicate.

the adipocytes has been shown to be altered as a result of fat cell hypertrophy (Le Lay *et al.* 2001). So, we compared the expression of several genes involved in cholesterol metabolism in control and transgenic mice fed an HFD. The sterol regulatory element binding protein-2 (SREBP2) gene and its targets like the low-density lipoprotein receptor

Table 3 mRNA levels in isolated adipocytes of control (mβ3–/–, hβ3+/+) and transgenic (mβ3–/–, hβ3+/+, hα2A+/–) mice fed an HFD for 18 weeks. Mice fed an HFD were killed at the age of 21 weeks and perigonadal adipocytes were prepared as described in Materials and methods. Values are means±S.E.M. from three or four independent determinations and normalized to 18S RNA

Gene	Normalized expression relative to 18S (×10 ^{–3})	
	Control	Transgenic
SREBP2	0.011±0.004	0.007±0.003
LDLR	0.012±0.003	0.008±0.003
SR-B1	0.300±0.170	0.220±0.110
Caveolin-2	0.299±0.193	0.209±0.085
LPL	5.427±1.841	3.907±0.978
HSL	0.041±0.001	0.029±0.006*

*P<0.05, *t*-test.

Table 4 Quantification of the maximal number of α2-AR sites (*B*_{max}) by using [³H]RX821002 radioligand and equilibrium dissociation constant (*K*_d) values in different fat pads of transgenic (mβ3–/–, hβ3+/+, hα2A+/–) mice fed an HFD for 18 weeks. Binding studies were performed on fat cell membranes as indicated in Materials and methods. Quantification was done on both perigonadal (PG) and subcutaneous (SC) fat cells from the same animal. *B*_{max} and *K*_d values were calculated by computer-assisted analysis of data according to a one-site model. Values are means±S.E.M. from four to seven mice

	<i>B</i> _{max} (fmol/mg protein)		<i>K</i> _d (nM)	
	PG	SC	PG	SC
Gender				
Male	35±6	54±8*	1.2±0.2	1.2±0.3
Female	45±10	58±8*	1.05±0.16	1.18±0.19

*P<0.05, paired *t*-test.

(LDLR) (that controls the rate limiting steps for cholesterol uptake), the scavenger receptor type B1 (SR-B1), the ATP binding cassette that controls cholesterol efflux from cells, and the caveolin 2 genes were not differentially expressed in adipocytes of transgenic vs control mice fed an HFD. All together, these results on gene expression confirm the absence of marked fat cell hypertrophy and suggest a reduced ability to mobilize fat in WAT of transgenic mice fed an HFD.

α2-adrenergic responsiveness

After an 18 week period of HFD associated with a rise in fat pad mass and body weight, the maximal antilipolytic effect of 10^{–6} M UK14304 in adipocytes of transgenic mice (52.3 ± 7.5% inhibition vs 78.2 ± 2.4%, *n*=3 in perigonadal and s.c. fat cells respectively) was not modified. The maximum number of α2A-AR sites was unchanged also (Table 4).

Serum parameters and glucose tolerance tests

Obesity is often associated with glucose intolerance and the development of type II diabetes. In order to know whether the obese transgenic mice had any metabolic disturbances, blood glucose, serum insulin and NEFA levels were measured. No significant differences were observed in glucose or insulin levels between transgenic and control mice (male and female) at the end of the HFD period

Table 5 Blood glucose, serum insulin and non-esterified fatty acids (NEFA) levels in control ($m\beta 3^{-/-}$, $h\beta 3^{+/+}$) and transgenic ($m\beta 3^{-/-}$, $h\beta 3^{+/+}$, $ha2A^{+/-}$) mice fed either an LFD or HFD. Values are means $\pm$ S.E.M. from 5–10 mice. After weaning, mice were fed an LFD or HFD for 18 weeks

	Mice	Gender	Diet	
			LFD	HFD
Glucose (mg/dl)	Control	Male	184 $\pm$ 11	175 $\pm$ 14
	Transgenic	Male	167 $\pm$ 9	191 $\pm$ 14
	Control	Female	160 $\pm$ 8	162 $\pm$ 12
	Transgenic	Female	168 $\pm$ 13	180 $\pm$ 7
Insulin (μU/ml)	Control	Male	39.5 $\pm$ 7.9	52.3 $\pm$ 7.8
	Transgenic	Male	51.4 $\pm$ 8.1	54.3 $\pm$ 8.7
	Control	Female	26.5 $\pm$ 3.9	21.4 $\pm$ 3.6
	Transgenic	Female	20.9 $\pm$ 4.5	24.1 $\pm$ 5.1
FFA (mM)	Control	Male	2.38 $\pm$ 0.37	2.23 $\pm$ 0.32
	Transgenic	Male	1.82 $\pm$ 0.24	2.01 $\pm$ 0.09
	Control	Female	2.51 $\pm$ 0.20	2.31 $\pm$ 0.15
	Transgenic	Female	1.89 $\pm$ 0.25	2.57 $\pm$ 0.12 ^a

^a $P < 0.05$, t -test between LFD and HFD transgenic females. FFA, free fatty acids.

(Table 5). Glucose tolerance tests (Fig. 7) revealed an apparent but not significant rightward shift of the curve following i.p. glucose injection associated with a slight alteration in insulin sensitivity (not shown) between control and transgenic mice fed an

Figure 7 Glucose tolerance tests in control and transgenic mice fed an HFD. Glucose was determined on blood samples from the tail vein of control (■) and transgenic mice (□) fed an HFD for 18 weeks. Data are means $\pm$ S.E.M. for $n=6$ mice in each group.

HFD. NEFA in serum were only significantly different between female control and transgenic mice fed an HFD (Table 5).

Mean blood pressure

Hypertension, another disease commonly associated with obesity, was also evaluated. Mean blood pressure was compared between control and transgenic mice fed an HFD or an LFD. No significant difference was observed (Table 6), indicating that this model of obese transgenic mice does not develop hypertension.

Table 6 Mean blood pressure (mmHg) in control ($m\beta 3^{-/-}$, $h\beta 3^{+/+}$) and transgenic ($m\beta 3^{-/-}$, $h\beta 3^{+/+}$, $ha2A^{+/-}$) mice fed either an LFD or HFD. Blood pressure was recorded as described in Materials and methods. Values represent means $\pm$ S.E.M. from three mice in each group

	Mice	LFD	HFD
Gender			
	Male		
	Control	144 $\pm$ 2	148 $\pm$ 4
	Transgenic	140 $\pm$ 1	140 $\pm$ 7
Female	Control	141 $\pm$ 1	139 $\pm$ 2
	Transgenic	142 $\pm$ 2	138 $\pm$ 3

Discussion

In this study, genetic engineering was used in the mouse to promote the expression of human $\alpha 2A$ -ARs under the control of human $\alpha 2A$ -AR gene regulatory elements. The human transgene was expressed in mouse WAT and the physiological significance of the $\alpha 2A$ -AR was investigated. The $\alpha 2/\beta$ -AR functional balance mimics that previously described in human WAT. Interestingly, like in humans, a regulation of $\alpha 2A$ -AR expression, according to the fat pad location, was also found. Stimulation of $\alpha 2A$ -AR induced resistance to catecholamine-induced lipolysis mainly in s.c. adipose tissue. Only transgenic mice fed an HFD become obese without modification of fat cell size. Analysis of adipocyte gene expression related to lipid metabolism in transgenic mice fed an HFD suggested reduced lipid mobilization and no changes in lipid storage capacity.

The distribution of $\alpha 2$ -ARs is known to be widespread in both humans and animals. Tissue specificities have been described concerning the distribution of different $\alpha 2$ -AR mRNA subtypes: $\alpha 2C2$, $\alpha 2C4$ and $\alpha 2C10$ genes encoding $\alpha 2B$ -, $\alpha 2C$ - and $\alpha 2A$ -AR respectively (Handy *et al.* 1993, Berkowitz *et al.* 1994). Although most of the tissues usually express more than one $\alpha 2$ -AR subtype, white adipocytes only express the $\alpha 2A$ -AR subtype in humans (Castan *et al.* 1995). This receptor is not expressed in mouse adipocytes but is weakly expressed in rats adipocytes (Castan *et al.* 1994). Moreover, in rodents, $\alpha 2A$ -AR, formerly called $\alpha 2D$ -AR, exhibits pharmacological differences when compared with human $\alpha 2A$ -AR (Cheung *et al.* 1982). Thus, we transgenically expressed the human $\alpha 2C10$ gene encoding the $\alpha 2A$ subtype in mice under its regulatory elements. Surprisingly, in tissues already known to express $\alpha 2A$ -AR such as brain, transgenic mice did not overexpress human $\alpha 2A$ -ARs mRNA or protein (identified by binding studies). By contrast, overexpression of $\alpha 2A$ -AR mRNA and protein was observed in transgenic mouse white adipocytes leading to a functional antilipolytic response with an $\alpha 2$ agonist. According to these results, we hypothesize that the human DNA fragment used (e.g. 5'-, 3'-flanking and coding sequences of the human $\alpha 2C10$ gene) and the endogenous promoter of the murine $\alpha 2C10$ could compete differently for murine brain or WAT-specific transcription factors leading to

differences in human receptor expression in these tissues. Indeed, human $\alpha 2A$ -AR was not detected in brain while it was expressed in adipocytes of transgenic mice. Moreover, human and rodent $\alpha 2A$ -ARs can be pharmacologically distinguished by the use of the $\alpha 2$ antagonist yohimbine (Link *et al.* 1992); rodent $\alpha 2A$ -AR exhibits a lower affinity for this compound than human $\alpha 2A$ -AR. In wild type and transgenic mice brains, competition studies performed with yohimbine revealed the predominance of the murine over the human $\alpha 2A$ -AR. Nevertheless, we cannot totally exclude the presence of some human $\alpha 2A$ -AR undetected by the binding assay. Analysis of $\alpha 2A$ -AR mRNA revealed the absence or a very weak signal in BAT (probably due to some white adipocyte contamination), but no receptor protein was detected. In kidney, high K_d values for [^3H]RX821002 (typical of other $\alpha 2$ -AR subtypes such as $\alpha 2C$ and $\alpha 2B$) stress the fact that the human $\alpha 2A$ -AR is poorly expressed in this tissue by comparison with other subtypes. No signal was found in tissues known not to express $\alpha 2A$ -AR such as liver.

The transgenic mice used in the present study express both the $\alpha 2A$ - and $\beta 3$ -AR human transgenes and are lacking murine $\beta 3$ -AR. No significant modification of body weight or fat mass has been observed in mice overexpressing human $\beta 3$ -AR in BAT ($m\beta 3 - / -$, $h\beta 3 + / +$ mice). $\beta 3$ -AR defects are usually associated with the development of obesity (Collins *et al.* 1994). In mice lacking murine $\beta 3$ -AR, no significant increase of body weight has been found while the total body fat stores were significantly higher (Susulic *et al.* 1995, Revelli *et al.* 1997). So, it is not excluded that disruption of the $\beta 3$ -AR gene leads to a predisposition to the development of obesity or to compensatory adaptations of endogenous $\beta 1/\beta 2$ -AR that will limit the development of obesity. Slight variations of $\beta 1$ -AR and $\beta 2$ -AR mRNA levels have been reported in $\beta 3$ -AR null mice vs wild type mice (Susulic *et al.* 1995). In the present study, no modification was observed in the amount of $\beta 1/\beta 2$ -AR protein of WAT from transgenic mice. These amounts were similar to values reported by Commins *et al.* (2000) in $\beta 3$ -AR-deficient mice. $\alpha 2A$ -AR expression in WAT of transgenic mice did not change $\beta 1/\beta 2$ -AR number either. However, the presence of human $\alpha 2A$ -AR and the absence of murine $\beta 3$ -AR in adipose tissue combined with HFD feeding caused a 20% increase in body weight. These results agree with those

obtained in Tg(ADRA2A)^{Lowl} mice (Valet *et al.* 2000). So, whatever the transgene used (either aP2-promoter driving adipocyte-specific expression or human $\alpha 2$ -AR gene and its own regulatory elements) the same phenotype (increased body and fat weight with HFD feeding) was observed, underlying the importance of the $\alpha 2/\beta$ -AR balance in adipocytes *in vivo*.

Divergences but also similarities characterize the two transgenic mouse models expressing $\alpha 2A$ -ARs in WAT. The originality of the transgenic mice studied here is characterized by preservation of the human $\alpha 2A$ -AR gene regulations, which is not the case for Tg(ADRA2A)^{Lowl} mice. The present study focuses on the regulation of $\alpha 2A$ -AR expression according to the anatomical location of fat. As in humans, $\alpha 2A$ -ARs numerically predominate over β -ARs in s.c. fat cells of transgenic mice. Epinephrine is the preferential amine for the $\alpha 2A$ -AR and plays a specific role in the control of lipolysis in s.c. fat cells *in vitro* (Mauriège *et al.* 1987) as well as *in vivo* (Sitch *et al.* 1999). The lipolytic action of epinephrine was enhanced after $\alpha 2A$ -AR blockade in both models. Interestingly, regardless of the weak expression of $\alpha 2A$ -ARs in white adipocytes of (m $\beta 3$ – / – , h $\beta 3$ + / + , ha2A + / –) transgenic mice, their stimulation is related to a strong and almost full inhibition of lipolysis probably due to a better coupling of $\alpha 2A$ -ARs to intracellular transduction pathways compared with Tg(ADRA2A)^{Lowl} or even human adipocytes.

The major converging point between the two transgenic models is the enhanced development of adipose tissue following 18 weeks of HFD in the absence of marked adipocyte hypertrophy. In the present study, transgenic mice fed an HFD have no larger perigonadal adipocytes and a higher DNA content in this fat pad than control mice. Analysing the expression of different adipocyte genes involved in lipid metabolism does not support a change in lipid storage capacity per cell. Thus, recruitment of new adipocytes (hyperplasia) rather than hypertrophy is associated with increased fat deposition. These results were obtained at a time when the obesity is already established and a different pattern of fat cell size and cell number could be observed during the dynamic phase of obesity development. In humans, the hypertrophy observed in s.c. compared with internal adipocytes could be associated with greater expression of $\alpha 2A$ -ARs in this depot. In transgenic mice fed an HFD, neither

adipocyte size nor $\alpha 2$ -adrenergic responsiveness was modified, whatever the fat location considered. Such a result suggests that the pattern of $\alpha 2A$ -AR expression in humans is not primarily due to the hypertrophic status of the adipocytes but seems directly linked to specific tissue factors. However, we can hypothesize that a higher amount of $\alpha 2A$ -AR associated with a stronger antilipolytic activity later leads to s.c. adipocyte enlargement in humans.

Finally, both transgenic mouse models become obese without significant modifications of blood glucose or insulin levels, whereas in most obese models these levels are high with an HFD (Cooney *et al.* 1989, West *et al.* 1992). However, a slightly decreased insulin sensitivity suggested by an apparent but not significant rightward shift of the glucose tolerance test curve led us to hypothesize potential modifications of glucose disposal rates in (m $\beta 3$ – / – , h $\beta 3$ + / + , ha2A + / –) transgenic mice.

In conclusion, the transgenic mice developed in the present work provide a unique model to further study the incidence of nutritional or hormonal factors on the regulation of the $\alpha 2C10$ gene. This model could also be used to assess the efficiency of novel adrenergic anti-obesity pharmacological drugs. Finally, such a model will be useful to better understand the interaction between HFD and $\alpha 2A$ -AR involvement in the development of WAT.

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