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Metabolic response to various β-adrenoceptor agonists in β_3-adrenoceptor knockout mice: Evidence for a new β-adrenergic receptor in brown adipose tissue

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1 The β_3-adrenoceptor plays an important role in the adrenergic response of brown and white adipose tissues (BAT and WAT). In this study, *in vitro* metabolic responses to β-adrenoceptor stimulation were compared in adipose tissues of β_3-adrenoceptor knockout and wild type mice. The measured parameters were BAT fragment oxygen uptake (MO_2) and isolated white adipocyte lipolysis.

2 In BAT of wild type mice (−)-norepinephrine maximally stimulated MO_2 4.1 ± 0.8 fold. Similar maximal stimulations were obtained with β_1-,β_2- or β_3-adrenoceptor selective agonists (dobutamine 5.1 ± 0.3, terbutaline 5.3 ± 0.3 and CL 316,243 4.8 ± 0.9 fold, respectively); in BAT of β_3-adrenoceptor knockout mice, the β_1- and β_2-responses were fully conserved.

3 In BAT of wild type mice, the β_1/β_2-antagonist and β_3-partial agonist CGP 12177 elicited a maximal MO_2 response (4.7 ± 0.4 fold). In β_3-adrenoceptor knockout BAT, this response was fully conserved despite an absence of response to CL 316,243. This unexpected result suggests that an atypical β-adrenoceptor, distinct from the β_1-, β_2- and β_3-subtypes and referred to as a putative β_4-adrenoceptor is present in BAT and that it can mediate *in vitro* a maximal MO_2 stimulation.

4 In isolated white adipocytes of wild type mice, (−)-epinephrine maximally stimulated lipolysis 12.1 ± 2.6 fold. Similar maximal stimulations were obtained with β_1-, β_2- or β_3-adrenoceptor selective agonists (TO509 12 ± 2, procaterol 11 ± 3, CL 316,243 11 ± 3 fold, respectively) or with CGP 12177 (7.1 ± 1.5 fold). In isolated white adipocytes of β_3-adrenoceptor knockout mice, the lipolytic responses to (−)epinephrine, to the β_1-, β_2-, β_3-adrenoceptor selective agonists and to CGP 12177 were almost or totally depressed, whereas those to ACTH, forskolin and dibutyryl cyclic AMP were conserved.

Keywords: β-adrenoceptor agonists; CGP 12177; β_3-adrenoceptor; knockout; respiratory rate; lipolysis; brown adipose tissue; white adipocytes

Introduction

The β_3-adrenoceptor, first isolated from a human genomic library (Emorine *et al*., 1989) and then from rat brown adipose tissue (BAT) cDNA libraries (Muzzin *et al*., 1991; Granneman *et al*., 1991) is expressed in rodent BAT and white adipose tissue (WAT) (Giacobino, 1995). In these tissues, the three β-adrenoceptor subtypes (β_1-, β_2- and β_3-) co-exist, the β_3-adrenoceptor being the predominant subtype (Giacobino, 1995). This coexistence raises the question of the respective biological significance of the various β-adrenoceptor subtypes.

A decrease in the expression of the β_3-adrenoceptor has been consistently reported in the BAT and WAT of genetically obese rodents (Muzzin *et al*., 1991; Giacobino, 1995). This observation led to the suggestion that a defect in the β_3-adrenoceptor might be involved in the pathogenesis of obesity and diabetes.

To test the role played *in vivo* by the β_3-adrenoceptor, mouse models with a targeted disruption of their β_3-adrenoceptor were developed (Susulic *et al*., 1995; Revelli *et al*., 1997). Mice homozygous for the disrupted allele (−/−), referred to as β_3-adrenoceptor knockout (β_3KO) mice, responded normally to chronic cold exposure (Susulic *et al*., 1995 and our unpublished data) and, although they did not become overtly obese, showed higher body fat (Susulic *et al*., 1995; Revelli *et al*., 1997) and lower body protein (Revelli *et al*., 1997) contents as well as increased food intake (Revelli *et al*., 1997) when compared to wild type (WT) controls. *In vivo*, the stimulatory effects of (−)-isoproterenol on the metabolic rate and on the level of circulating fatty acids were reported to be similar in β_3KO and WT mice (Susulic *et al*., 1995). Conversely, *in vitro* (−)-isoproterenol stimulation of adenylyl cyclase was strongly decreased in BAT and WAT cell membranes of β_3KO as compared to WT mice while that of lipolysis was either absent or decreased by 30% in isolated white adipocytes of β_3KO as compared to WT mice depending on the conditions of incubation of the cells (Susulic *et al*., 1995). The phenotype of the β_3-adrenoceptor targeted disruption was therefore surprisingly close to normality despite the observed alterations of the *in vitro* metabolic responses to nonselective β-adrenoceptor agonists in BAT and WAT. These findings suggested that *in vivo*, some alternative pathways might compensate for the decreased *in vitro* responses to catecholamines in β_3KO mice.

Pharmacological studies suggest the existence of a new, yet unidentified, β-adrenoceptor subtype in various tissues (Mohell and Dicker, 1989; Molenaar *et al*., 1991; Deng *et al*., 1996; Galitzky *et al*., 1997; Kaumann, 1997; Kaumann and Lynham, 1997; Malinowska and Schlicker, 1997). Some of these studies, using the nonconventional partial β-adrenocep-

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tor agonist CGP 12177 (Mohell and Dicker, 1989) support the existence of an atypical β-adrenoceptor in WAT (Deng *et al*., 1996; Galitzky *et al*., 1997) and cardiac muscle (Kaumann, 1997; Kaumann and Lynham, 1997; Malinowska and Schlicker, 1997). Altogether, these results suggested that CGP 12177 is not only an agonist for the β_3-adrenoceptor but might also be a tool of choice for the study of atypical β-adrenoceptors in tissues devoid of β_3-adrenoceptors.

The aim of the present work was to study *in vitro* the adrenergic response in BAT and WAT of β_3KO mice. The parameters measured were the oxygen uptake (MO_2) of BAT fragments and the lipolysis of isolated white adipocytes in response to specific β_1-, β_2- and β_3-adrenoceptor agonists and to CGP 12177.

Methods

All organic and inorganic chemicals were of analytical or molecular biology grade. They were purchased from Merck (Darmstadt, Germany), Boehringer Mannheim (Mannheim, Germany), Sigma Chemical Co. (St. Louis, Missouri, U.S.A.) and Fluka (Buchs, Switzerland). The β_3-adrenoceptor agonist CL 316,243 was a generous gift from Wyeth-Ayerst (Princeton, New Jersey, U.S.A.), the β_1, β_2-adrenoceptor antagonist and β_3-adrenoceptor agonist ($\pm$)-4-(3-t-butylamino-2-hydroxypropoxy)benzimidazol-2-one (CGP 12177) from Ciba-Geigy (Basel, Switzerland) and the β_1-adrenoceptor agonist dobutamine was purchased from Research Biomedicals International (Natick, Massachusetts, U.S.A.). The selective β_1-agonist TO 509 and β_2-agonist procaterol were generous gifts by Tanabe Seiyaku (Osaka, Japan) and by Otsuka Pharmaceutical (Tokashima, Japan), respectively.

Targeted disruption of the β_3-adrenoceptor

The targeted disruption, i.e. the cloning of the 129 Sv mouse β_3-adrenoceptor gene, the construction of the targeting plasmid, the electroporation and the screening of the ES cells, the injection of the ES cell line carrying the disrupted β_3-adrenoceptor into C57BL/6J blastocysts and the screening of the progeny have been described in detail elsewhere (Revelli *et al*., 1997). Colonies of WT (+/+) and β_3KO (−/−) mice were established starting from offspring of heterozygous (+/−) parent mice. The identification of the WT or β_3KO mice was performed by Southern blot analysis of mouse tail genomic DNA as previously described (Revelli *et al*., 1997).

Tissue respiratory rate measurements

Seven to nine week-old WT and β_3KO male mice were killed by cervical dislocation followed by decapitation. Interscapular BAT was dissected and two fragments 18–20 mm long, 1 mm thick and 10–14 mg wet weight were placed into the experimental chambers and perifused with Krebs Ringer bicarbonate buffer (KRB) containing 5 mM glucose. The medium was gassed with a mixture of 95% O_2 and 5% CO_2 and was maintained at 30 ± 0.2°C.

The respiratory rate of interscapular BAT fragments was measured by indirect calorimetry as described in detail by Barde *et al*. (1975) under basal conditions and in response to increasing concentrations of various β-adrenoceptor agonists. All values for O_2 uptake rate (MO_2) were measured during steady state respiration.

Lipolysis in isolated white adipocytes

In order to get an amount of white adipose tissue sufficient for subsequent adipose isolation, 8-months-old WT and β_3KO female mice were used. The periovarian fat was excised and isolated adipocytes were obtained as previously described (Tavernier *et al*., 1996) by collagenase digestion of WAT fragments in KRB containing HEPES (10 mM), albumin (2 g/100 ml) (KRBHA) and glucose (6 mM) at pH 7.4 and 37°C under gentle shaking at around 60 cycles/min. At the end of the incubation, fat cells were filtered through a silk screen and washed three times with KRBHA buffer to eliminate collagenase. Packed cells were brought to a suitable dilution in KRBHA buffer for lipolysis. β-adrenoceptor agonists at suitable dilutions were added to the cell suspension (2000–3000 cells/assay) just before the beginning of the assay in a final volume of 100 μl. After 90 min of incubation, the tubes were placed in an ice bath and 20–50 μl aliquots of the infranatant were taken for enzymatic determination of the glycerol (Bradley and Kaslow, 1989) released in the incubation medium which was used as the index of fat cell lipolysis.

Results

BAT respiratory rate

The mean basal MO_2 values were not significantly different in β_3KO as compared to WT BAT fragments (50 ± 2 *vs* 46 ± 3 nmol of $O_2 \times h^{-1} \times$ (mg tissue wet weight^{-1}), respectively) (Figure 1). Dose-response curves were carried out with each agonist in order to determine the concentrations at which maximal stimulation occurred (data not shown). Those concentrations were 1 μM for (−)-norepinephrine, 5 μM for the β_1-adrenoceptor agonist dobutamine, 1 μM for the β_2-adrenoceptor agonist terbutaline, 0.01 μM for the β_3-adrenoceptor agonist CL 316,243 and 0.5 μM for the nonconventional β_3-adrenoceptor agonist, CGP 12177. The results illustrated in Figures 1 and 2 were obtained using these respective concentrations.

In WT mouse BAT fragments the maximal level of MO_2 obtained upon stimulation by the natural ligand (−)-norepinephrine was 186 ± 43 nmol of O_2. $\times h^{-1} \times$ (mg tissue)$^{-1}$.

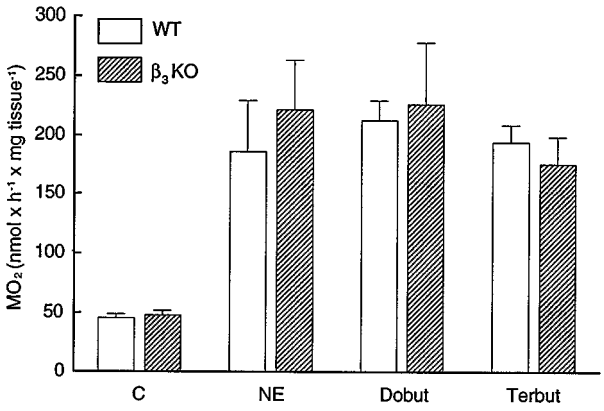

Figure 1 MO_2 in brown adipose tissue fragments of WT (open bars) and β_3KO (hatched bars) mice. Basal (C) and maximal MO_2 values obtained upon stimulation by (−)-norepinephrine 1 μM (NE), the β_1-adrenoceptor agonist dobutamine 5 μM (Dobut) and the β_2-adrenoceptor agonist terbutaline 1 μM (Terbut) are shown. They are the mean $\pm$ s.e.m. of five to fifteen experiments and expressed as nmol of $O_2 \times h^{-1} \times$ (mg of tissue wet weight)$^{-1}$.

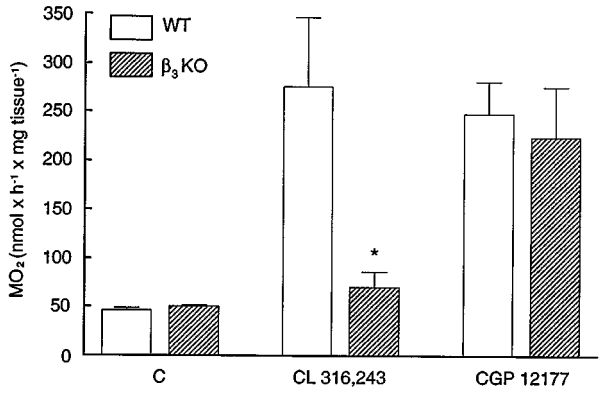

Figure 2 MO_2 in brown adipose tissue fragments of WT (open bars) and β_3KO (hatched bars) mice. Basal (C) and maximal MO_2 values obtained upon stimulation by the β_3-adrenoceptor agonist CL 316,243 0.01 μM and CGP 12177 0.5 μM are shown. They are the mean $\pm$ s.e.m. of four to six experiments and expressed as nmol of $O_2 \times h^{-1} \times$ (mg of tissue wet weight)$^{-1}$. *$P<0.05$.

The maximal levels of MO_2 obtained in the presence of dobutamine, terbutaline, CL 316,243 and CGP 12177 were not different from those obtained with (−)-norepinephrine. Expressed as fold-increases, the stimulations of MO_2 were 4.1 ± 0.8 for (−)-norepinephrine, 5.1 ± 0.3 for dobutamine, 5.3 ± 0.3 for terbutaline, 4.8 ± 0.9 for CL 316,243 and 4.7 ± 0.4 for CGP 12177 (Figures 1 and 2).

In β_3KO BAT fragments, (−)-norepinephrine, dobutamine and terbutaline induced MO_2 responses similar to those obtained in WT BAT (Figure 1). Surprisingly, CGP 12177 also induced similar responses in β_3KO as compared to WT BAT in terms of maximal stimulation (and of affinity, data not shown) whereas CL 316,243 had no effect in β_3KO BAT (Figure 2).

Isolated white adipocyte lipolysis

Basal lipolysis was not significantly different in β_3KO as compared to WT adipocytes (180 ± 40 *vs* 120 ± 20 nmol of glycerol released $\times 90$ min$^{-1} \times$ (100 mg of lipids)$^{-1}$, respectively). Dose-response curves carried out for each agonist are shown in Figure 3a–c. The concentrations needed for maximal stimulation were 10 μM for (−)-epinephrine, 1 μM for the β_1-adrenoceptor agonist TO509, 100 μM for the β_2-adrenoceptor agonist procaterol, 1 μM for the β_3-adrenoceptor agonist CL 316,243 and 10 μM for the nonconventional agonist CGP 12177.

In WT mouse white adipocytes the maximal level of lipolysis obtained upon stimulation by the natural ligand (−)-epinephrine was 1360 ± 290 nmol $\times 90$ min$^{-1} \times$ (100 mg of lipids)$^{-1}$ (12.1 ± 2.6 fold increase) (Figure 3a). The maximal levels of lipolysis obtained in the presence of the β_1-, β_2- and β_3-adrenoceptor selective agonists were not different from those obtained in the presence of (−)-epinephrine (Figure 3b and c). Expressed as fold-increases, stimulations of lipolysis were 11.6 ± 2.4 for TO509, 10.9 ± 2.5 for procaterol, 11.4 ± 2.6 for CL 316,243 and 7.1 ± 1.5 for CGP 12177, respectively.

In β_3KO white adipocytes, the lipolytic responses to (−)-epinephrine, TO509, procaterol and CGP 12177 were depressed by 80% ($P<0.02$), 78% ($P<0.05$), 89% ($P<0.025$) and 100% ($P<0.05$) respectively, compared to WT adipocytes. CL 316,243 had no effect on β_3KO adipocyte lipolysis up to a concentration of 10 nM which induced a near maximal lipolytic response in WT adipocytes. At higher concentrations a small but significant ($P<0.01$) effect was observed in β_3KO white adipocytes (Figure 3a–c). As shown in Figure 4, the lipolytic effects of ACTH (1 μM), forskolin (100 μM) and dbcAMP (1 mM) were not different in β_3KO as compared to WT adipocytes.

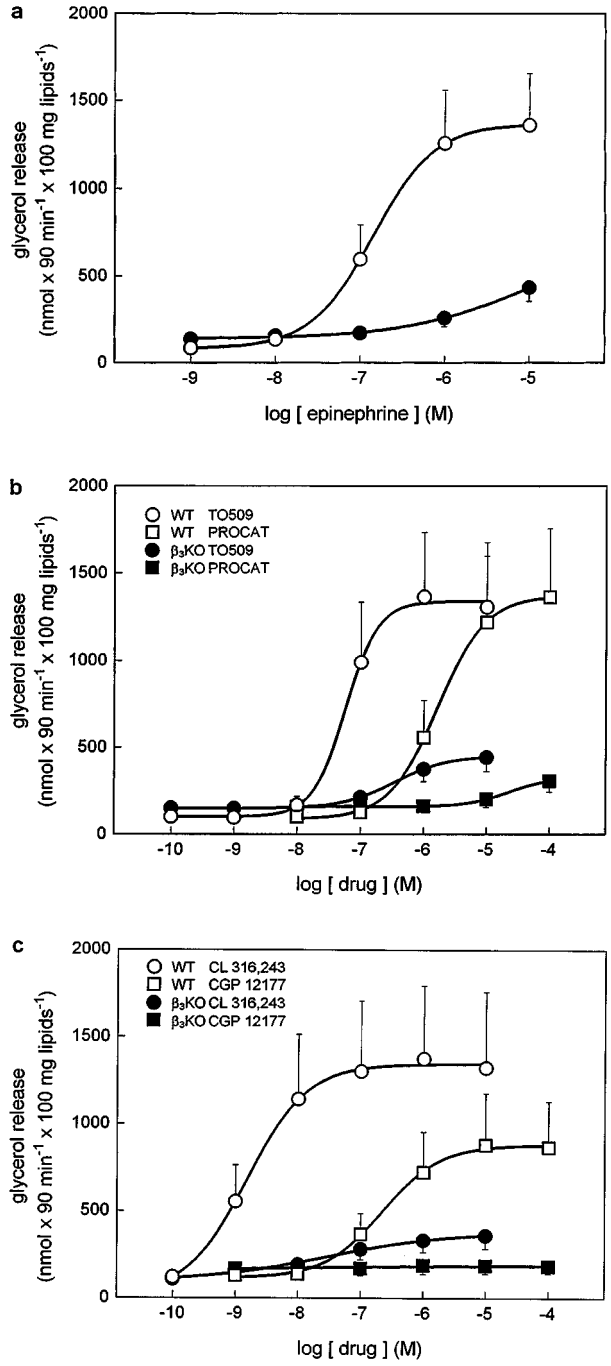

Figure 3 Lipolysis in isolated white adipose cells of WT (open symbols) and β_3KO (closed symbols) mice, as a function of increasing concentrations of (a) (−)-epinephrine (b), the β_1-adrenoceptor agonist TO509 and the β_2 adrenoceptor agonist procaterol and (c) CL 316,243 and CGP 12177. The values are the mean $\pm$ s.e.m. of six experiments and expressed as nmol of glycerol released $\times 90$ min$^{-1} \times$ (100 mg of lipids)$^{-1}$.

Discussion

The β_3-adrenoceptor is the major mediator of the lipolytic and thermogenic effects of high catecholamine concentrations in

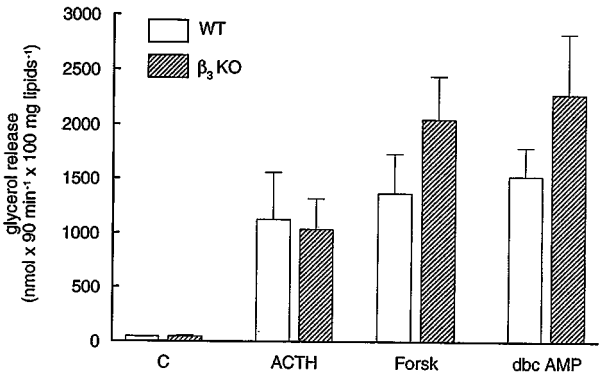

Figure 4 Lipolysis in isolated white adipose cells of WT (open bars) and β_3KO (hatched bars) mice. Basal (C) and maximal lipolysis values obtained upon stimulation by ACTH 1 μM, forskolin 100 μM (Forsk) and dibutyryl cyclic AMP 1000 μM (dbcAMP) are shown. The values are the mean $\pm$ s.e.m. of six experiments and expressed as nmol of glycerol released $\times 90\ \text{min}^{-1} \times$ (100 mg of lipids)$^{-1}$.

BAT and WAT (Giacobino, 1995; Granneman, 1990; Zhao *et al*., 1994). The observation that mice with a targeted disruption of their β_3-adrenoceptor were cold-resistant and did not become overtly obese (Susulic *et al*., 1995; Revelli *et al*., 1997) suggested that they developed compensatory pathways. In this study the thermogenic and lipolytic responses to β-adrenoceptor agonists were measured in BAT and WAT of β_3KO and WT mice.

Respiration of BAT fragments

In β_3KO BAT fragments, a lack of response to CL316,243 validates *in vitro* our knockout model and confirms, once again, that CL316,243 is a highly specific β_3-adrenoceptor agonist (Susulic *et al*., 1995; Revelli *et al*., 1997; Grujic *et al*., 1997). The findings that the thermogenic responses to (−)-norepinephrine and to the β_1-adrenoceptor agonist dobutamine were not decreased in β_3KO as compared to WT BAT fragments, and that the β_2-adrenoceptor stimulation was able to induce maximal MO_2 responses in WT and β_3KO BAT fragments, are surprising. Indeed, it has been shown that the relative amounts of β_1-, β_2- and β_3-adrenoceptors represent 30 and 70%, respectively, of the total β-adrenoceptor population in rat BAT (Muzzin *et al*., 1992). In the BAT of β_3KO mice, due to the absence of β_3-adrenoceptor and to the observed 3 fold decrease in the amount of β_1-adrenoceptor mRNA (Revelli *et al*., 1997), the total β-adrenoceptor population should be decreased by 80–90%. Altogether, our results show that in both WT and β_3KO mice only a fraction of the β-adrenoceptors can mediate a full metabolic response, supporting the hypothesis of a transduction reserve between AC and lipolytic activities in rodent adipocytes (Hollenga *et al*., 1991). This might also be due to a preferential coupling between the receptor and AC as was shown for the β_2-adrenoceptor in heart (Kaumann, 1997).

The CGP 12177-mediated stimulation of lipolysis (Van Liefde *et al*., 1993) and thermogenesis (Zhao *et al*., 1994) has been previously attributed to the activation of β_3-adrenoceptors. Surprisingly, we found that the thermogenic response was conserved in β_3KO BAT fragments, indicating that the CGP 12177-induced increase in MO_2 in this tissue was mediated by a receptor subtype distinct from β_1-, β_2- and β_3-adrenoceptors, namely by an atypical β-adrenoceptor. These data suggest that this atypical β-adrenoceptor, like the β_1-, β_2- and β_3-adrenoceptors, has the capacity to mediate by itself the full BAT thermogenic response. Pak and Fishman (1996) have shown that CGP 12177 can act as a β_1-adrenoceptor agonist when β_1-adrenoceptors are overexpressed. In our β_3KO mice the β_1-adrenoceptor mRNA is in fact decreased (Revelli *et al*., 1997). It is therefore highly unlikely that in our study CGP 12177 act *via* β_1-adrenoceptors. It is not excluded, however, that it might act *via* receptors unrelated to the β-adrenoceptors.

The presence of an atypical β-adrenoceptor named β_4-adrenoceptor has previously been evoked in several tissues on pharmacological basis. This is the case of human WAT in which the β_3-adrenoceptor mRNA is either practically absent (Deng *et al*., 1996) or represents at most 20% of the total amount of β-adrenoceptor transcripts (Tavernier *et al*., 1996) but in which a large population of low affinity [^{3}H]-CGP 12177 binding sites (Deng *et al*., 1996) and a lipolytic effect of CGP 12177 (Lönnqvist *et al*., 1995; Enocksson *et al*., 1995) have been demonstrated. Recently, the use of a new, selective β_3-adrenoceptor antagonist, SR59, 230A, allowed to attribute the lipolytic and cardiostimulant effects of CGP 12177 in rat WAT and heart, respectively, to the β_4-adrenoceptor (Galitzky *et al*., 1997; Kaumann, 1997; Malinowska and Schlicker, 1997). The present study, for the first time, indicates in BAT the presence of an atypical β-adrenoceptor which mediates the thermogenic effect of CGP 12177. Furthermore, the stimulatory effect of CGP 12177 was identical in WT and β_3KO BAT suggesting that β_3- and putative β_4-adrenoceptors were stimulated simultaneously in a non additive way by CGP 12177. Our results raise the question of the redundancy of the various β-adrenoceptor subtypes in mediating the effects of catecholamines on BAT. In this context, the particular role for the putative β_4-adrenoceptor in BAT is an open question.

Lipolysis in isolated white adipocytes

In WAT, MO_2 being too low to allow for accurate measurements, lipolysis in isolated adipocytes was measured. It should be noted that, on the other hand, the recovery of dispersed brown adipocytes from BAT is too low to allow for lipolysis studies in this tissue.

Our metabolic study on isolated white adipocytes of β_3KO mice reveals a phenotype which contrasts with that observed in BAT. Indeed, our results show an unexpected fall in β_1-, β_2- and CGP 12177 adrenergic responsiveness in β_3KO white adipocytes despite a functional non-adrenergic AC pathway and a normally responsive AC-lipolysis axis. This lack of response to catecholamines is not due to predominant α_2-adrenoceptor activity mediating inhibition of AC activity, since addition of the α_2-adrenoceptor antagonist RX821002 (10 μM) did not induce a response to (−)-epinephrine (10 μM) in β_3KO isolated adipocytes (data not shown). The observed divergence between BAT and WAT responses to adrenergic stimulation in β_3KO mice might be due to differences in the transduction reserve (Hollenga *et al*., 1991) of both tissues. It might also be due to a defect in the signalling in β_3KO isolated adipocytes upstream of AC, possibly at the level of G_s proteins. Studies on β_3KO WAT are currently under way to test this possibility. This particular WAT phenotype does not allow the identification of a putative β_4-adrenoceptor in this tissue.

Our results underline the marked different consequences of β_3-adrenoceptor deficiency on the *in vitro* metabolism of BAT and WAT. In BAT, the β_3-adrenoceptor is not essential for a normal thermogenic response to adrenergic stimulation. Indeed, the β_1-, β_2-adrenoceptors, as well as the putative β_4-adrenoceptor could mediate maximal thermogenic responses. Conversely, in white adipocytes, the

presence of the β_3-adrenoceptor is essential for normal β-adrenergic-mediated lipolytic function. The absence of overt obesity in β_3KO mice suggests that *in vivo* alternative pathways may be able to maintain most of the lipolytic activity in WAT.

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