Divergent effects of an α_2-adrenergic antagonist on lipolysis and thermogenesis: Interactions with a β_3-adrenergic agonist in rats

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Abstract. This study was undertaken in order to test the hypothesis that selective β_3-AR stimulation and simultaneous blockade of α_2-AR would result in an increase of lipolysis and thermogenesis in rats. Incubation of isolated white adipocytes with the α_2-AR antagonist yohimbine produced a concentration-dependent increase in glycerol release ($P<0.001$) for all assayed concentrations (10^{-12}-10^{-6} M) and potentiated the lipolytic effect of the β_3-AR agonist Trecadrine. However, *in vivo* administration of yohimbine produced a marked decrease in body temperature (1.3-1.5°C, $P<0.001$) and blocked the thermogenic effect of Trecadrine when simultaneously administered. A similar response was observed for whole body oxygen consumption. Furthermore, yohimbine did not modify brown adipose tissue oxygen consumption, but blocked the β_3-AR-mediated increase triggered by Trecadrine. Brown adipose tissue UCP-2 and -3 mRNA expression was not changed by yohimbine. In conclusion, the present work indicates that *in vitro* α_2-AR blockade by yohimbine potentiates the β_3-AR-mediated stimulation of lipolysis. On the other hand, *in vivo* α_2-AR antagonism blocks the thermogenic effects mediated by β_3-AR stimulation, suggesting a possible interplay between the receptors.

Introduction

It is well established that catecholamines exert a dual control on white fat cell lipolysis in rats and other species: stimulation by acting on ß-adrenoceptors (ß-AR) and inhibition mediated through α_2-adrenoceptors (α_2-AR) (1). Three types of ß-AR, namely the β_1-, β_2-, and β_3-AR, have been well characterized by their distinct pharmacological properties, protein sequence and gene organization (2). Recent evidence for a fourth ß-AR called β_4-AR has been also reported (3,4). The first three ß-AR are present in white adipocytes (5). The α_2-AR is present in rat white adipocytes and it seems to belong to the α_{2A}-subtype (6), which appears to mediate inhibitory changes in the cAMP cascade. Thus, it would be reasonable to hypothesize that selective β_3-AR stimulation and simultaneous blockade of α_2-AR on white adipocytes could result in an increase of the lipolytic response.

On the other hand, the sympathetic nervous system plays an important role in energy expenditure through the regulation of thermogenesis, which constitutes a physiological defense against cold and excessive energy intake (7). Brown adipose tissue (BAT) is of special interest in the study of thermogenesis because a mitochondrial protein called uncoupling protein (UCP1) is expressed specifically in this tissue (8). UCP1 is known to uncouple oxidative phosphorylation by moving protons across the mitochondrial inner membrane toward the mitochondrial matrix (9). Recently, two further members of the UCP family have been cloned (i.e., UCP2 and UCP3). UCP3 is expressed mainly in BAT and skeletal muscle, whereas UCP2 is expressed ubiquitously (10). Thermogenesis in BAT is primarily mediated by β_3-AR, whose stimulation induce a strong induction of UCP1 expression, but have no clear effect on UCP2 and UCP3 expression in BAT and other tissues (11-13). In addition, it has been suggested that α_2-AR may participate in the regulation of thermogenesis (6). In this sense, it has been reported that administration of yohimbine to fasted dogs increases oxygen consumption and heat production (14) and that yohimbine may regulate the expression of UCP1 in BAT (15). However, the thermogenic effect of α_2-AR blockade and its consequences on UCPs expression remain unclear.

The aim of the present work was to study the *in vitro* lipolytic effect of yohimbine and its *in vivo* effect on lipolysis and thermogenesis as well as whether the α_2-AR blockade elicited by yohimbine may induce a potentiation of the lipolytic and thermogenic effects of the β_3-AR agonist Trecadrine in Wistar rats. Also, the potential involvement of BAT UCPs in some of these process was evaluated.

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Materials and methods

Animals. Five-month-old male Wistar rats weighing 420-430 g were obtained from CIFA (Centro de Investigación en Farmacobiología Aplicada, Pamplona, Spain) and housed at 25±1°C with 12 h light cycle (8 a.m. to 8 p.m.) and fed *ad libitum*. They were fasted for 24 h before *in vitro* lipolysis assays or drug administration for *in vivo* experiments.

Drugs. Trecadrine, a diphenyl-methylene-ethylamine derived compound, whose formula and characterization as a β_3-adrenoceptor agonist have been previously published (16) was a generous gift of Wassermann-Chiesi (Barcelona/Milan). Yohimbine-hydrocloride was purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). All other chemicals and organic solvents were of reagent grade.

Isolation of white adipocytes and lipolysis measurement. Adipocytes from epididymal fat were isolated as described elsewhere (17). Briefly, white adipose tissue samples were cut into small pieces and the fragments were digested at 37°C with collagenase P (Boehringer Mannheim GmbH, Mannheim, Germany) in Krebs-Ringer bicarbonate buffer containing 3.5% bovine serum albumin (Sigma) and 6 mM glucose at pH 7.4 (KRBA). The ratio of digestion solution to adipose tissue mass was 5 ml g^{-1} and 0.75 ml of KRBA per mg of collagenase. After 90 min of incubation under continuous vigorous shaking (90 cycles min^{-1}) the fat cells were filtered through a nylon mesh and washed tree times with KRBA to eliminate the stroma-vascular fraction and collagenase. The fat cells were brought to a suitable dilution in KRBA buffer. Adipocytes were incubated in polyethylene tubes (1 ml of incubation medium) with continuous gentle shaking (30 cycles min^{-1}) in a water bath at 37°C. Drugs were added to the polyethylene tubes just prior to the beginning of the second incubation. Responses to the following drugs were evaluated: the β_3-AR agonist Trecadrine (10^{-12}-10^{-6} M), the α_2-AR antagonist yohimbine (10^{-12}-10^{-6} M) and the same range of concentrations of yohimbine in combination with the EC_{50} of Trecadrine as determined by the logit method (18). The basal lipolysis was determined in the absence of drugs. After 90 min, the incubation tubes were placed in an ice bath and 200 µl of the infranatant were removed for the enzymatic determination of glycerol released into the incubation medium, which was taken as the index of lipolytic rate. Data are expressed as percentage of increment with respect to basal after a 90-min incubation in KRBA.

Treatments for in vivo experiments. Forty rats were randomly assigned to four groups of ten animals each. Rectal temperature of the animals was measured before the administration of: saline, the β_3-adrenoceptor agonist Trecadrine (1 mg·kg^{-1} i.p), yohimbine (5 mg·kg^{-1} i.p) or a combination of both at the indicated doses. After 1 h, rectal temperature and oxygen consumption were measured as described later. Animals were killed by decapitation and trunk blood was collected. A small portion of interscapular BAT was used for *ex vivo* oxygen consumption determination. The remaining BAT was immediately frozen in liquid nitrogen and stored at -80°C until analysis. All experimental procedures were performed according to institutional guidelines for Animal Care and Use at the University of Navarra.

Oxygen consumption and rectal temperature. Oxygen consumption was assessed by means of a Jacketed Oxygen Consumption Chamber (Harvard Apparatus, Edenbridge, UK). Values represent the average volume of O_2 consumed during six consecutive 30-sec sampling periods at 1-min intervals while animals were in a resting situation. The temperature of the chamber was maintained at 25°C. Rectal temperature was measured before and 1 h after drug administration by a rectal probe (Yellow Springs Instruments, Yellow Springs, OH, USA) connected to a Panlab thermometer pb 0331 (Panlab, Barcelona, Spain) as previously described (19).

Brown adipose tissue ex vivo oxygen consumption. A fragment of BAT from each rat obtained immediately after decapitation was cut into small pieces and placed into the experimental chambers with Krebs-Ringer phosphate buffer containing 6 mM glucose at pH 7.4. Oxygen consumption was measured with a YSI Model 5300 Biological Oxygen Monitor using a Clark-type oxygen electrode as previously reported (16).

Blood analyses. Plasma glucose concentrations were determined by the glucose oxidase method (Spotchem analyzer, Menarini, Barcelona, Spain). Serum insulin levels were measured by a radioimmunoassay kit (Rat insulin [^{125}I] assay system, Amersham, Buckinghamshire, UK). Glycerol concentrations were quantified by using an enzymatic colorimetric method (Randox Laboratories, Antrim, UK). Serum leptin concentration was measured by means of a commercially available ELISA kit (Active™ Murine Leptin ELISA, Diagnostic System Laboratories, Webster, TX, USA).

RNA analysis by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated by Ultraspec™-II (Biotech Laboratories, Houston, TX, USA) from 100 mg of BAT according to the manufacturer's instructions. The yield and quality of the RNA were assessed by measuring absorbance at 260 and 280 nm and by electrophoresis on 1.5% agarose gels. After 30 min at 37°C treatment with 10 units of RNase free DNase I (Boehringer Mannheim), 1 µg of RNA was used to synthesize first-strand cDNA. The RT reaction was carried out in a volume of 20 µl containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiotreitol, 100 ng of random hexamers (Boehringer Mannheim), 1 mM each dNTP (Bioline, London, UK), 20 units of RNase inhibitor (Promega, Madison, WI, USA), 200 units of M-MLV RT (Gibco BRL, Life Technologies, Gaithersburg, MD, USA) and incubated at 37°C for 60 min. The enzyme was inactivated by heating at 95°C for 5 min. Four µl from the RT reaction were amplified in a 50 µl reaction mixture containing 40 ng of each primer, 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 2 mM $MgCl_2$, 0.1% Tween-20, 0.2 mM each dNTP and 1 unit of BioTaq™ polymerase (Bioline). Primers used to amplify UCP1, UCP2, UCP3 and ß-actin cDNA were the same as described elsewhere (13). cDNA was amplified for 20 (UCP1), 32 (UCP2), 35 (UCP3) and 25 cycles (ß-actin) using the following parameters: 94°C for 30 sec, 58°C (UCP1), 62°C

(UCP2), 60°C (UCP3) and 59°C (ß-actin) for 30 sec and 72°C for 30 sec, with a final extension step at 72°C for 7 min. Amplifications were linear under these conditions and were carried out in a GeneAmp® PCR System 2400 (Perkin Elmer, Norwalk, CT, USA). All PCR reactions for each gene were performed with the same batch of Taq polymerase in order to reduce variations in the efficiency of PCR. The amplified products were resolved in a 1.5% agarose gel with ethidium bromide. Levels of mRNA were expressed as the ratio of signal intensity for each UCP relative to that for ß-actin. PCR bands intensity were determined by densitometric analysis with the Gel Doc 1000 UV fluorescent gel documentation system and Molecular Analyst 1.4.1 software for quantitation of images (Bio-Rad, Hercules, CA, USA). The identity of PCR product amplifications was previously demonstrated (13).

Statistics. All results are expressed as mean ± SE mean. The lipolytic effect of drugs was analyzed by means of two-tailed one-sample t-tests in order to detect differences of each concentration assayed with basal values. Two-way ANOVA followed by Tukey's *post hoc* test analyzed the differences between doses. Furthermore, two-tailed unpaired t-tests were applied to compare the combined effect of yohimbine and Trecadrine with that of yohimbine alone. Data concerning *in vivo* and *ex vivo* experiments were analyzed using repeated-measure two-way ANOVA coupled to a two-tailed unpaired t-test when an interaction was detected. Pearson's correlation coefficient was computed to analyze correlations between two variables. The calculations were performed using the SPSS/Windows version 7.5.2S (SPSS, Chicago, IL, USA). A P-value lower than 0.05 was considered statistically significant.

Results

Lipolysis studies. The incubation of isolated white adipocytes with yohimbine produced a statistically significant (P<0.001) concentration-dependent increase in glycerol release for all concentrations tested. The average percent change increase from baseline lipolytic activity ranged from 16±2% for the lowest concentration (10^{-12} M) to 111±6% for the highest one (10^{-6} M). Trecadrine induced a pronounced stimulation of lipolysis (P<0.001) producing a 175±8% increase with respect to baseline at the 10^{-6} M concentration. When adipocytes were incubated simultaneously in the presence of Trecadrine [at a concentration of $3.13x10^{-10}$ M (EC_{50})] and yohimbine (10^{-12}-10^{-6} M) the lipolytic effect was enhanced (Fig. 1). In order to test for potential interactions, the effect of Trecadrine (EC_{50}) plus yohimbine referred to that of Trecadrine (EC_{50}) was compared with the effect of yohimbine referred to baseline. The analysis showed the existence of a significantly positive interaction (P<0.001) between the lipolytic effects of Trecadrine and yohimbine on white fat cells at all concentrations of yohimbine assayed.

Effects of drug administration on rectal temperature and in vivo oxygen consumption. Acute i.p. administration of Trecadrine to fasted Wistar rats induced a significant rise (P<0.001) in rectal temperature. On the contrary, yohimbine administration produced a marked decrease (P<0.001) of body temperature (1.3-1.5°C), which could not be counteracted by Trecadrine administration. Furthermore, a statistically significant interaction (P=0.044) between them was found (Fig. 2a).

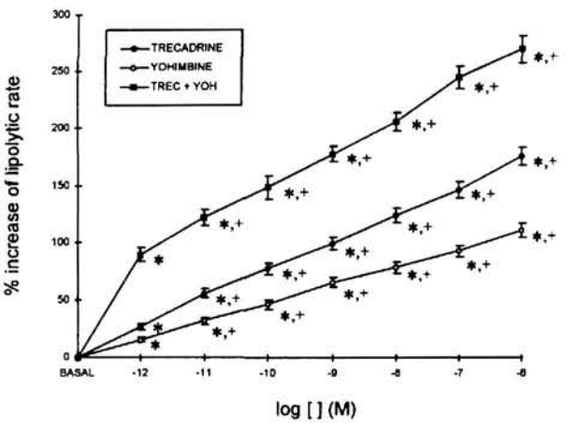

Figure 1. Concentration-response curves for stimulation of glycerol release from white adipocytes elicited by the $ß_3$-AR agonist Trecadrine, the α_2-AR antagonist yohimbine and a combination of both. For the combined study, adipocytes were incubated with concentrations of yohimbine ranging from 10^{-12} to 10^{-6} M in presence of the EC_{50} of Trecadrine ($3.13x10^{-10}$ M). Each value is the mean ± SEM (vertical bars, Trecadrine or yohimbine, n=16; Trecadrine plus yohimbine, n=8). *P<0.001 when compared to the basal value (by two-tailed one-sample t-test), +P<0.001 when compared with previous lower concentration (by two-way ANOVA followed by *Tukey's* test).

The thermogenic effect of Trecadrine was confirmed by the statistically significant rise (P<0.001) on whole body oxygen consumption observed after its administration. Yohimbine reduced significantly oxygen consumption and inhibited the Trecadrine-induced increase, following a similar pattern to that observed for rectal temperature, finding again a statistically significant (P=0.027) negative interaction (Fig. 2b).

The parallelism observed between rectal temperature and oxygen consumption was reinforced by the statistically significant positive correlations observed between whole body oxygen consumption and change in body temperature (r=0.656, P<0.00001; Fig. 2c) and final rectal temperature (r=0.579, P<0.0001).

Effects on ex vivo oxygen consumption of brown adipose tissue fragments. Fragments of BAT obtained from rats, which had received Trecadrine only, showed a 20% increase in oxygen consumption with respect to control animals (P=0.034). This effect was not observed when animals received simultaneously yohimbine. In this context, a statistically significant (P=0.027) negative interaction between Trecadrine and yohimbine was found, while yohimbine administered alone had no effect (Fig. 3a). BAT oxygen consumption showed positive correlations with whole body oxygen consumption (r=0.468, P=0.009; Fig. 3b) and rectal temperature gradient (r=0.511, P=0.004; Fig. 3c).

Effects on blood biochemistry. Acute Trecadrine administration produced a slight but statistically significant (P=0.026) elevation of serum glucose concentrations. Yohimbine *per se* had no effect, but blunted the effect of Trecadrine when both

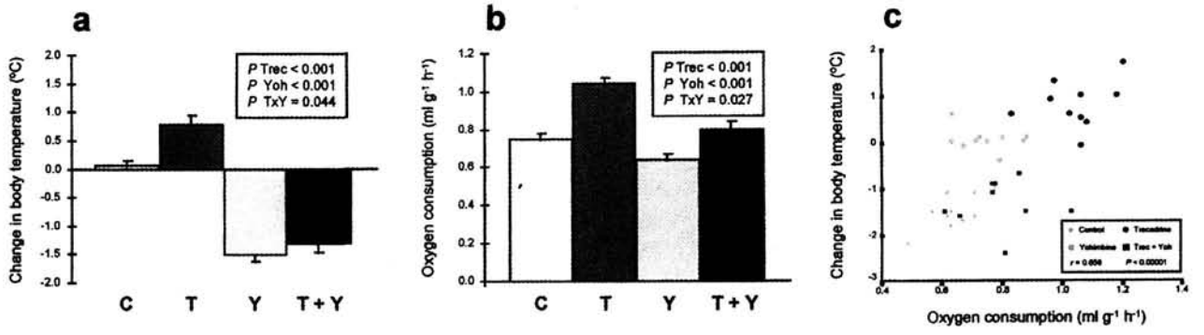

Figure 2. Effect of acute (1 h) i.p. administration of Trecadrine (1 mg·kg^{-1}), yohimbine (5 mg·kg^{-1}), both drugs combined at the same doses or vehicle on rectal temperature (a) and whole body oxygen consumption (b) of Wistar rats. Values are mean ± SEM, n=10 per group. C, control; T, Trecadrine; Y, yohimbine; T + Y, Trecadrine plus yohimbine. Correlation analysis of both variables is shown in (c). Statistical analysis by two-way ANOVA (a) and (b) or Pearson's (c) is shown in the figure.

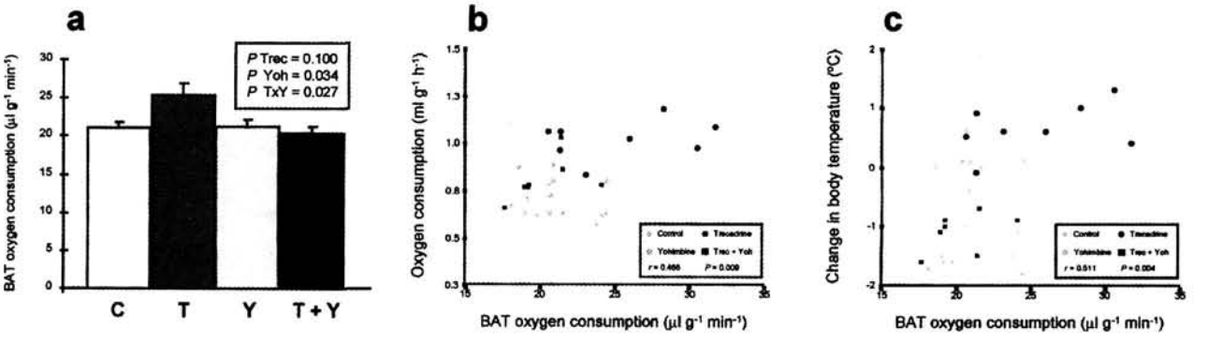

Figure 3. Effect of acute (1 h) i.p. administration of Trecadrine (1 mg·kg^{-1}), yohimbine (5 mg·kg^{-1}), both drugs combined at the same doses or vehicle on oxygen consumption of fragments of BAT of Wistar rats determined *ex vivo* (a). Values are mean ± SEM, n=7-8 per group. C, control; T, Trecadrine; Y, yohimbine; T + Y, Trecadrine plus yohimbine. Correlation analysis of BAT oxygen consumption with whole body oxygen consumption (b) and change in rectal temperature (c). Statistical analysis by two-way ANOVA (a) or Pearson's (b) and (c) is shown in the figure.

Table I. Acute effects on blood biochemistry 1 h after a single intraperitoneal injection of the β_3-AR agonist Trecadrine (1 mg·kg^{-1}), yohimbine (5 mg·kg^{-1}), their combination or saline in 24-h fasted Wistar rats.

					ANOVA-2		
	Control	Trecadrine	Yohimbine	T + Y	P Trec	P Yoh	P TxY
Glucose (mmol·l^{-1})	7.9±0.2	8.6±0.2[a]	8.1±0.2	7.7±0.2[b]	0.483	0.034	0.009
Insulin (ng·ml^{-1})	1.91±0.31	1.57±0.13	1.62±0.28	1.62±0.20	0.500	0.642	0.502
Glycerol (mmol·l^{-1})	0.30±0.02	0.62±0.04	0.36±0.03	0.71±0.04	<0.001	0.023	0.576
Leptin (ng·ml^{-1})	0.89±0.28	0.83±0.20	1.06±0.20	1.26±0.28	0.752	0.233	0.585

Data are mean ± SEM, n=10 per group. Statistical significance of the differences between treatments were evaluated using repeated-measure two-way ANOVA coupled to a two-tailed unpaired t-test when an interaction was detected: [a]P<0.05 vs control group. [b]P<0.01 vs Trecadrine treated group. T + Y, Trecadrine plus yohimbine.

drugs were administered simultaneously. Thus, a statistically significant (P=0.009) negative interaction between them was observed. Neither Trecadrine nor yohimbine modified plasma *insulin* concentrations 1 h after administration. Serum glycerol concentrations were increased by acute administration of both Trecadrine (P<0.001) and yohimbine (P=0.023). Serum leptin concentrations were not modified by any treatment (Table I).

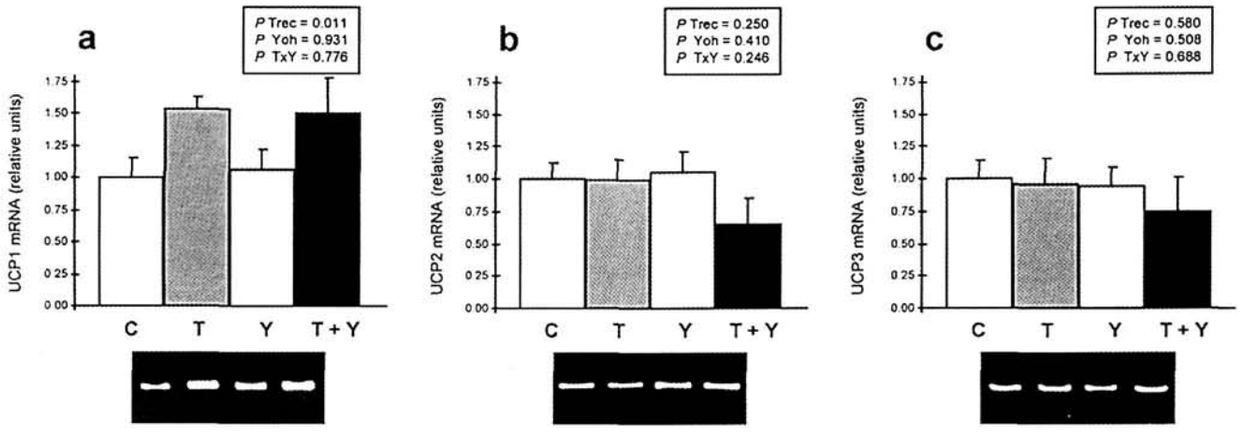

Figure 4. Effect of acute (1 h) i.p. administration of Trecadrine (1 mg·kg^{-1}), yohimbine (5 mg·kg^{-1}), both drugs combined at the same doses or vehicle on UCP1 (a), UCP2 (b) and UCP3 (c) mRNA expression in BAT measured by RT-PCR. Data represent the mean ± SEM of the ratio between each UCP to ß-actin. The expression of each UCP in control rats was assumed to be 1 (n=6 in each group). Statistical analysis by two-way ANOVA is shown on top. Representative photographs of RT-PCR products are shown on bottom. C, control; T, Trecadrine; Y, yohimbine; T + Y, Trecadrine plus yohimbine.

Effects on UCP mRNA expression in BAT. UCP1 mRNA expression in BAT was significantly (P=0.011) upregulated 1 h after Trecadrine administration, while UCP2 and UCP3 were unaffected by this $ß_3$-AR agonist. Yohimbine administration did not modified the expression of any UCP studied (Fig. 4).

Discussion

Catecholamines exert a dual control on white fat cell lipolysis in rats and other species by stimulating ß-AR and inhibiting α_2-AR (1). Initially, it was believed that rat adipocytes did not have α_2-AR-mediated inhibition of lipolysis (20). This assumption, based mainly on the lack of effect of clonidine, was reassessed by using the selective α_2-AR agonist UK 14304 (21). Incubation of white adipocytes in the presence of yohimbine produced a concentration-dependent increase in the glycerol released to the medium. These results, are in agreement with previous findings reported by Muhlbachova *et al* (22) and indicate that the α_2-AR antagonist yohimbine has a lipolytic effect *per se*. It is difficult to explain how yohimbine can exert a direct effect on white adipocytes given the fact that an antagonist, by definition, has no biological effect. Therefore, we hypothesized that yohimbine could have an inverse agonist activity at the α_{2A}-AR as has been suggested for other α_{2A}-AR antagonist (23). On the other hand, the $ß_3$-AR agonist, Trecadrine induced a marked *in vitro* lipolytic effect on white adipocytes. This effect is in accordance with previous *in vitro* studies of Trecadrine (24) and other $ß_3$-AR agonists (3,25,26). A potentiation of the lipolytic effect of Trecadrine was achieved by co-incubation with yohimbine.

The results obtained confirm our hypothesis and demonstrate that $ß_3$-AR-mediated stimulation of lipolysis is more effective after α_2-AR blockade. In the *in vivo* experiments, 1 h after drug administration, serum concentrations of glycerol were taken as an index of lipolytic activity. Both Trecadrine and yohimbine produced a statistically significant increase of glycerol levels. The increase in glycerol concentrations triggered by yohimbine administration has been reported to take place in rabbits (27), dogs (28), and humans (29). However, to our knowledge there is no published evidence of this effect in rats and our findings support either an *in vivo* stimulation of lipolysis or an inhibition of antilipolytic process by yohimbine in rats. The simultaneous administration of Trecadrine and yohimbine did not potentiate the *in vivo* lipolytic response as opposed to the *in vitro* effect. Perhaps, the occurrence of physiological catecholamines or the existence of a contraregulatory mechanism avoiding an excessive lipolytic response by adipocytes does not allow to obtain *in vivo* the potentiation observed *in vitro*.

Trecadrine administration produced a marked thermogenic response as evidenced by the increase in rectal temperature and whole body oxygen consumption. Similar results have been obtained with other $ß_3$-AR agonists (30,31). By contrast, yohimbine produced a marked reduction in rectal temperature and oxygen consumption. Reported evidence concerning α_2-AR regulation of body temperature in rodents is inconsistent. Thus, hypothermic responses after both i.p. α_2-AR agonist (dexmedetomidine) administration (32) and s.c. α_2-AR antagonist (delequamine) administration (33) have been described. Yohimbine produces a hypothermic effect in rats (34,35), but also attenuates/prevents the hypothermia produced by clonidine (36,37). Our data show that yohimbine clearly reduces body temperature and oxygen consumption and that these effects are predominant over the thermogenic effect of Trecadrine as supported by the detected interactions. Moreover, both effects are strongly associated as shown by the statistical correlation between them. The reduction in energy expenditure reported herein is in disagreement with the thermogenic effect described by Galitzky *et al* (14) in dogs, which may be explained by differences between species, probably depending on the different patterns of distribution of α_2-AR in brain.

Trecadrine administration increased BAT oxygen consumption as described for other $ß_3$-AR agonists (4,38). Yohimbine had no effect on BAT oxygen consumption *per se*. However, when yohimbine and Trecadrine were administered

simultaneously the effect of Trecadrine was prevented. This observation may suggest the existence of a cross-talk between α_2-AR and β_3-AR in this tissue.

In order to explore the possible mechanisms involved in the changes concerning thermogenesis, the expression of UCPs in BAT was analyzed. As expected, UCP1 expression in BAT was upregulated by acute Trecadrine administration (13). On the other hand, it has been previously demonstrated that yohimbine potentiates the noradrenaline-mediated increase in UCP1 expression in cultured brown adipocytes (15). However, in this study yohimbine had no *in vivo* effect and did not block the effect of Trecadrine when simultaneously administered. This finding contrast with the results obtained *ex vivo* with BAT fragments and could be explained by specific effects of yohimbine on the activity of UCP1 or other proteins involved in thermogenesis, but not on UCP1 expression. The lack of effect of yohimbine on UCP1 expression suggests that the *in vivo* lipolytic effect of yohimbine is not a consequence of an increase in synaptic noradrenaline elicited by α_2-AR blockade as proposed (6), but seems to suggest a direct effect. BAT UCP2 and UCP3 mRNA were not modified by Trecadrine or yohimbine administration. Similar results have been obtained by other investigators in acute treatments with different β_3-AR agonists (39,40) and to our knowledge this is the first study concerning α_2-AR regulation of BAT UCP2 and UCP3 expression. Therefore, it can be concluded that BAT UCP2 and UCP3 seem not contribute to the acute thermogenic changes observed after β_3-AR agonism or α_2-AR antagonism.

In summary, *in vitro* α_2-AR blockade by yohimbine present a marked lipolytic effect on rat white adipocytes and potentiates the β_3-AR-mediated stimulation of lipolysis induced by Trecadrine. Acute yohimbine administration stimulates glycerol release, but does not potentiate the effect of Trecadrine, and induces changes in fuel utilization, interfering with the effect of Trecadrine on serum glucose concentrations. Yohimbine produces a marked decrease in rectal temperature and whole body oxygen consumption blocking the thermogenic effect of Trecadrine. These effects are not fully explained by changes in BAT UCPs and seem to be centrally mediated.

Acknowledgements

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