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PAPER

Effects of Trecadrine®, a β3-adrenergic agonist, on leptin secretion, glucose and lipid metabolism in isolated rat adipocytes

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OBJECTIVE: Leptin, a hormone produced in adipocytes, is a key signal in the regulation of food intake and energy expenditure. β-Adrenergic agonists have been shown to inhibit leptin gene expression and leptin secretion. The mechanisms underlying the inhibitory effects of β-adrenergic agonists have not been established. In this study, we examined the effects of Trecadrine®, a novel β3-adrenergic agonist, on basal and insulin-stimulated leptin secretion in isolated rat adipocytes. Because insulin-stimulated glucose metabolism is an important regulator of leptin expression and secretion by the adipocytes, the effects of Trecadrine on indices of adipocyte metabolism were also examined.
MEASUREMENTS: Isolated adipocytes were incubated with Trecadrine (10^{-8}–10^{-4} M) in the absence or presence of insulin (1.6 nM). Leptin secretion, glucose utilization, lactate production, glucose incorporation into CO_2 and triglyceride, as well as lipolysis (glycerol release) were determined.
RESULTS: Trecadrine induced a concentration-dependent inhibition of basal leptin secretion. Trecadrine also decreased insulin-stimulated leptin secretion; however, the effect was not as pronounced as in the absence of insulin. Treatment of adipocytes with Trecadrine increased basal glucose utilization and produced a further increase in insulin-stimulated glucose utilization. Basal lactate production was also increased by Trecadrine; however, the proportion (percentage) of glucose carbon released as lactate was unaffected. In the presence of insulin, absolute lactate production was unaffected by Trecadrine at 96 h. However, the percentage of glucose carbon released as lactate was significantly decreased by insulin treatment, and was further decreased by the co-treatment with Trecadrine. Trecadrine induced a dose-dependent increase of the absolute amount of glucose incorporated into triglyceride. However, the percentage of glucose utilized that was incorporated into triglyceride was unaffected by Trecadrine. Trecadrine did not modify the proportion of glucose utilized that was oxidized to CO_2. Trecadrine increased glycerol release after 96 h of treatment. Glycerol release was negatively correlated with leptin secretion.
CONCLUSIONS: These results suggest that alterations of glucose metabolism are not directly involved in the effects of β3-adrenergic agonists to inhibit leptin expression and secretion. The inverse relationship between leptin secretion and the increase of glycerol levels, which is an index of the activation of cAMP-dependent protein kinases, suggests that activation of the cAMP signaling pathway mediates the inhibitory effects of Trecadrine on leptin gene expression and secretion.
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Keywords: β3-adrenoceptor; leptin; insulin-mediated glucose metabolism; lactate; lipogenesis; lipolysis

Introduction

Molecules with affinity for β3-adrenergic receptors have been developed as potential drugs for the treatment of obesity and diabetes.[1,2] Trecadrine® a novel β-adrenergic agonist with a marked selectivity for β3-adrenoceptors,[3] has shown anti-diabetic properties in an alloxan-induced rat model of diabetes by decreasing hepatic glucose output and improving muscle glucose uptake.[4,5] Trecadrine has also been shown to have anti-obesity properties in a diet-induced obesity model (cafeteria-fed animals), by decreasing fat content and the weight of white adipose tissue (WAT) depots while increasing gastrocnemius muscle UCP2 mRNA, brown adipose tissue

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(BAT) UCP1 mRNA levels and WAT lipolysis and oxygen consumption.[6,7]

In vivo, Trecadrine treatment, like other β3-adrenergic agonists, causes a decrease in both leptin expression in WAT and plasma leptin levels.[7–10] The mechanisms underlying the inhibition of leptin expression and secretion by β3-adrenergic stimulation remain unclear. Previous experiments from our laboratory and others have demonstrated that insulin-mediated glucose metabolism has an important role regulating leptin expression and secretion,[11–15] Free fatty acids have been reported to inhibit leptin expression and secretion in adipocytes.[16,17] It is well known that the sympathetic nervous system regulates adipocyte glucose and fat metabolism through β-adrenergic receptors.[18]

Therefore, the aim of the present study was to determine the direct effects of Trecadine on leptin expression and secretion in isolated rat adipocytes and whether its effects were related to alterations in adipocyte glucose and/or fat metabolism. For this purpose, the effects of Trecadrine on glucose utilization, lactate production, and percentage of glucose converted to lactate, incorporated into lipid, or oxidized to CO_2 were assessed. The lipolytic effect of Trecadrine and its relationship with leptin secretion were also examined by measuring glycerol release.

Methods

Materials

Media (Dulbecco's modified Eagle's medium, DMEM), fetal bovine serum, minimal essential medium amino acids, penicillin/streptomycin and nystatin were purchased from Life Technologies (Grand Island, NY, USA). Bovine serum albumin fraction V, 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES) and insulin were purchased from Sigma Chemical Co. (St Louis, MO, USA). Collagen (Vitrogen 100) was purchased from Cohesion Technologies (Palo Alto, CA, USA). ^{14}C-glucose was obtained from NEN (Beverly, MA, USA). Trecadrine was a generous gift from Wassermann-Chiesi (Barcelona/Milano).

Animals

Male Sprague–Dawley rats (3–6 months of age) were obtained from Charles River (Wilmington, MA, USA). Animals were housed in hanging wire cages in temperature-controlled rooms (22°C) with a 12 h light–dark cycle and fed Purina chow diet (Ralston-Purina., St Louis, MO, USA) and given deionized water *ad libitum*.

Adipocyte isolation and culture

Adipocytes were isolated from epididymal fat pads of male Sprague–Dawley rats. The fat pads were minced into pieces in Krebs-Ringer HEPES buffer (pH 7.4; containing 5 mM D-glucose, 2% BSA, 135 mM NaC1, 2.2 mM $CaCl_2 \cdot 2H_2O$, 1.25 mM $MgSO_4 \cdot 7H_2O$, 0.45 mM KH_2PO_4, 2.17 mM Na_2HPO_4 and 10 mM HEPES). Adipose tissue fragments were digested in the same buffer with type I collagenase (1.25 mg/ml per 0.5 g tissue; Worthington, Lakewood, NJ, USA) at 37°C with gentle shaking at 60 cycles/min for 30 min. The resulting cell suspension was diluted in HEPES-phosphate buffer and the isolated adipocytes were then separated from the undigested tissue by filtration through a 400 μm nylon mesh and washed three times. Isolated adipocytes were then resuspended in DMEM supplemented with 1% FBS and incubated for 30 min at 37°C.

The isolated adipocytes (150 μl of 2:1 ratio of packed cells to medium) were then plated on 500 μl of a collagen matrix (Vitrogen 100, Cohesion Technologies, Palo Alto, CA, USA) in six-well culture plates. After a 45 min incubation at 37°C, the culture media containing the different treatments were added and the cells were maintained in an incubator at 37°C in 6% CO_2 for up to 96 h. Aliquots (300 μl) of the media were collected at 24, 48, 72 and 96 h, and replaced with fresh medium containing the appropriate concentration of insulin and/or Trecadrine.

Assays

Leptin concentrations in the medium were determined by a radioimmunoassay for rat leptin (Linco Research, St Charles, MO, USA), as previously described.[19] Glucose and lactate were measured with a YSI glucose analyzer (Model 2300, Yellow Springs Instruments, Yellow Springs, OH, USA).

Analysis of mRNA

Leptin mRNA level was determined by Northern blotting. The leptin cDNA probe was a 388 bp fragment of mouse leptin cDNA, which was kindly provided by Dr Charles Mobbs (Mount Sinai School of Medicine, New York, USA). The 18S ribosomal probe was obtained from Ambion (Ambion, Austin, TX, USA).

RNA was extracted according to the Gibco Life Technologies procedure using Trizol (Life Technologies Inc., Grand Island, NY, USA). The UV absorbance and integrity gels were used to estimate RNA. Leptin and 18S cDNA probes were labeled by random priming (Rediprime kit, Amersham, Buckinghamshire, UK) in the presence of ^{32}P dCTP (3000 Ci/mmol, Amersham). Unincorporated nucleotides were removed using NucTrap probe purification columns (Stratagene, La Jolla, CA, USA). For each tissue sample, 5–7 μg of total RNA were fractionated by electrophoresis on a denaturing 1% agarose gel containing 2.2 M formaldehyde and 1× MOPS running buffer. One microliter of a 50 μg/ml ethidium bromide (Gibco BRL, Gaithersburg, MD, USA) stock solution was added in order to check RNA integrity and even loading. After electrophoresis, RNA was transferred to nylon membrane (Duralon-UV, Stratagene, La Jolla, CA, USA) by overnight capillary transfer and UV cross-linked (Stratalinker 1800, Stratagene, La Jolla, CA, USA). Blots were then hybridized for 1 h at 68°C in presence of the labeled cDNA probe

(2×10^6 cpm/ml Express Hyb solution, Clontech, Palo Alto, CA, USA). After washing at high stringency, blots were exposed to X-ray films with an intensifying screen for 1 day at -80°C. To allow loading of equal mass of RNA in each well, after analysis of leptin mRNA using a single-stranded cDNA probe followed by quantification of bands from film, the blots were re-analyzed using a probe complementary to mouse 18S ribosomal RNA. Leptin mRNA was then normalized with respect to the 18S ribosomal signal.

Glucose incorporation into lipid

Glucose incorporation into lipid was measured after 96 h of culture by counting the ^{14}C radio-labeled glucose incorporated into the adipocytes.[20] Throughout the culture, the adipocytes were incubated in media containing 0.01 μCi/ml of ^{14}C glucose (NEN, Beverly, MA, USA). After 96 h the media was removed, along with any extracellular triglyceride in the wells, and 4 ml of methanol were added to each well. The adipocytes anchored into the collagen were then transferred into a 50 ml glass tube with screw cap. The well was washed again using an additional 1 ml of methanol which was also transferred to the tube. Ten milliliters of chloroform were added to each tube in order to extract the triglyceride as described by Folch.[21] Twenty-four hours later, the tube was filled with de-ionized water to separate the methanol from the chloroform containing the triglyceride. The water–methanol layer was aspirated and remaining moisture was absorbed by the adding 4 g sodium sulfate. One milliliter of the chloroform–triglyceride was pipetted into a scintillation vial and counted for radioactivity (dpms). Five milliliters of the chloroform–lipid was pipetted into pre-weighed aluminum pans. The chloroform was allowed to evaporate and the pan was weighed again to determine the retained triglyceride (mg).

Glucose oxidation

Oxidation was measured using a modification of the method of Rodbell[22] and a modification of the culture system described by Bottcher and Furst.[23] Briefly, isolated adipocytes were placed in collagen in sterile 20 ml scintillation vials. Two milliliters of treatment media containing 0.03 μCi/ml of [U-^{14}C]-glucose were added to the vials. The vials were capped with rubber stoppers fitted with a hanging center well in the presence of 6% CO_2 gas. Each well contained a 2×8 cm strip of Whatman no. 1 paper. Vials were incubated at 37°C for 48 h. After 48 h a media sample was removed from each vial using a 4 inch, 23 gauge needle. Using another syringe and 23 gauge needle, 200 μl of sodium benzethonium were placed onto the paper strip and hanging well to capture CO_2. Concentrated sulfuric acid was added to the vials in order to lyse cells and liberate all CO_2 from the collagen matrix. After 24 h, the hanging well and paper were transferred to another vial containing scintillation fluid and counted.

Lipolysis

Glycerol released into the media at 96 h was assayed to evaluate lipolysis. Glycerol was determined using Sigma Diagnostics Triglyceride (GPO-Trinder) reagent following the procedure indicated by the manufacturer (Sigma Diagnostics, Inc, St Louis, MO, USA).

Data analysis

Glucose utilization was assessed by measuring the concentration of glucose in the media in each well before and at 24, 48, 72 and 96 h with corrections for the amount of glucose that was removed and added during media sampling and replacement. Lactate production was calculated as the increase of media lactate at 24, 48, 72 and 96 h with corrections for the amount of lactate removed and added with media replacement. The amount of carbon released as lactate per amount of carbon taken up as glucose over 96 h was calculated as Δ[lactate]/Δ[glucose], where Δ is the change and expressed as a percentage. The amount of glucose incorporated into CO_2 was calculated as (dpms collected on Whatman 1 strip) (total glucose)/total dpms and expressed as a percentage of total glucose utilized. The amount of glucose incorporated into lipid was calculated as ((dpms extracted in 1 ml chloroform)(total glucose)/total dpms)$\times$10 ml chloroform. This value was normalized over the amount of lipid recovered from the well and expressed as a percentage of total glucose utilized. The experimental results from each adipocyte suspension prepared from a single animal were analyzed in relation to a control well from the same suspension. The means were compared by ANOVA followed by a Bonferroni's *post-hoc* test (GraphPad Prism, GraphPad Software Inc, San Diego, CA, USA).

Results

Effect of Trecadrine on leptin expression and secretion

Trecadrine (10^{-8}–10^{-4} M) induced a concentration-dependent inhibition of basal leptin secretion from the first 24 h of treatment (Figure 1A). Over 96 h of treatment, the inhibitory effect of Trecadrine on leptin secretion was significant ($P<0.001$) at concentrations of 10^{-5} and 10^{-4} M (46.5 ± 7.2 and 39.6 ± 8.5% of control). Insulin at 1.6 nM induced a significant increase on leptin secretion over the 96 h (220 ± 18.2% of control, $P<0.001$). Co-treatment with Trecadrine in the presence of insulin decreased insulin-stimulated leptin secretion (Figure 1B). However, the effect of Trecadrine to inhibit leptin secretion was not as potent as in the absence of insulin (80.2 ± 5.1 and 84.1 ± 6.1% for 10^{-5} and 10^{-4} M respectively).

The pattern of Trecadrine's effects on leptin expression at 48 h of treatment mimics those observed for leptin secretion (Figure 2). Trecadrine induced a decrease in basal leptin mRNA levels (40.8 ± 5.2 and 35.1 ± 7.8% of control for 10^{-6} and 10^{-4} M). As expected, insulin had a potent effect to increase leptin mRNA levels (251.3 ± 9.2% of control) and

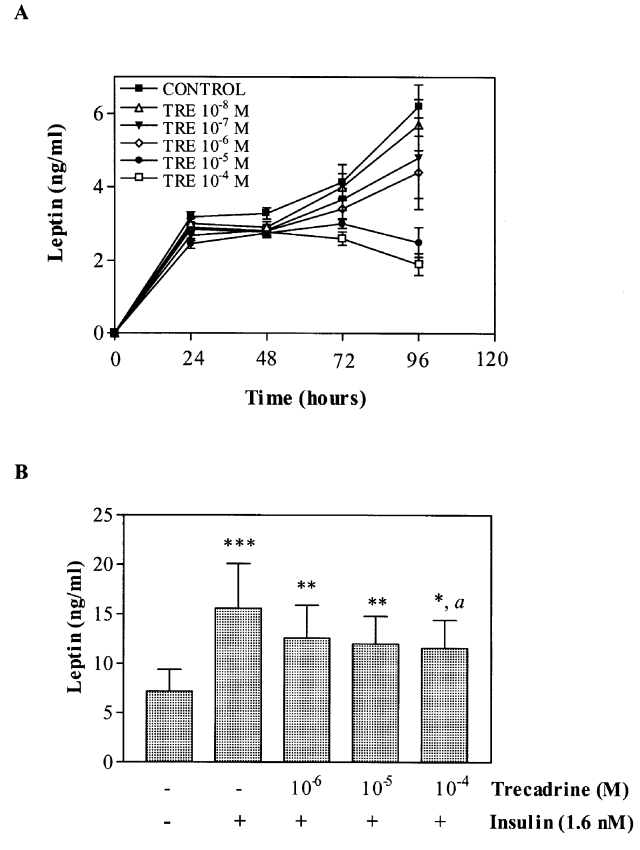

Figure 1 Effects of Trecadrine on basal (A) and insulin-stimulated (B) leptin secretion by isolated rat adipocytes over 96 h in culture. Results (mean ± s.e.) are representative of six independent experiments. $^{*}P<0.05$; $^{**}P<0.001$; and $^{***}P<0.001$ *vs* corresponding control. $^{a}P<0.05$ *vs* insulin-treated cells.

Trecadrine modestly reduced the effect of insulin to increase leptin mRNA levels (78.5 ± 0.4 and 77.3 ± 0.3% of insulin for 10^{-6} and 10^{-4} M Trecadrine).

Effect of Trecadrine on glucose utilization

Trecadrine induced a concentration-dependent increase in basal glucose utilization over 96 h (Figure 3A), which was significant at 10^{-6}, 10^{-5} and 10^{-4} M (123 ± 9.9, $P<0.05$; 139.1 ± 16.3 and 172.9 ± 24.6%, $P<0.01$ of control, respectively). This increase in basal glucose uptake was observed from the first 24 h of treatment (data not shown). As expected, insulin (1.6 nM) induced a significant increase in glucose uptake (170.5 ± 24.0% of control, $P<0.01$). When adipocytes were co-treated with Trecadrine in the presence of insulin, a concentration-dependent increase in glucose uptake was also observed. This stimulatory effect of Trecadrine on insulin-stimulated glucose uptake was, however, less potent (125.8 ± 7.0% for the 10^{-4} M concentration; $P<0.05$) than the effects of Trecadrine on basal glucose uptake (Figure 3B).

Effect of Trecadrine on lactate production

As shown in Table 1, Trecadrine increased absolute lactate production at 10^{-5} and 10^{-4} M (127.6 ± 9.0 and 144.6 ± 10.3%). However, the proportion of carbon released as lactate per amount of carbon taken up as glucose over 96 h was unaffected by Trecadrine treatment (Table 1). Lactate production at 96 h was similar in adipocytes treated with insulin alone and those co-treated with Trecadrine (Table 2). As we have previously reported, the percentage of glucose carbon released as lactate was significantly decreased by insulin treatment (41.7 ± 7.8 *vs* 25.5 ± 5.8, $P<0.001$), and co-treatment with 10^{-4} M Trecadrine decreased this percentage (25.5 ± 5.8 *vs* 20.0 ± 4.6, $P<0.05$; Table 2).

Effect of Trecadrine on glucose incorporation into triglyceride

The amount of glucose incorporated into triglyceride was increased by Trecadrine in a dose-dependent manner (162.6 ± 29.3 and 168.8 ± 21.5% of control for 10^{-5} and 10^{-4} M, respectively). However, the proportion (percentage) of glucose incorporated into triglyceride of the total 96 h glucose utilization was not significantly modified by Trecadrine treatment at any concentration (Table 1).

As expected, insulin caused a significant increase in glucose incorporation into triglyceride (254.7 ± 98.8%); however insulin did not significantly increase the percentage of glucose incorporation into triglyceride. Co-treatment with Trecadrine at concentrations 10^{-5} and 10^{-4} M further increased the amount of glucose incorporated into triglyceride (125.5 ± 9.7 and 139.8 ± 11.4% of insulin-treated adipocytes). However, the percentage of glucose incorporated into triglyceride of the total 96 h glucose utilization was not significantly modified in cells co-treated with insulin and Trecadrine in comparison to those treated with insulin alone (Table 2).

Effect of Trecadrine on glucose oxidation

Trecadrine (10^{-6} and 10^{-4} M) did not modify the percentage of glucose oxidized to CO_2 (Table 1). Insulin, however, caused a significant increase ($P<0.01$) in the oxidation of glucose into CO_2, which was not affected by the co-treatment with Trecadrine (Table 2).

Effect of Trecadrine on lipolysis

The effects of Trecadrine on lypolysis were evaluated by determining the amount of glycerol released into the media over 96 h of culture. Trecadrine induced a concentration-dependent increase (Figure 4) in glycerol release, which was significant at concentrations of 10^{-6}, 10^{-5} and 10^{-4} M

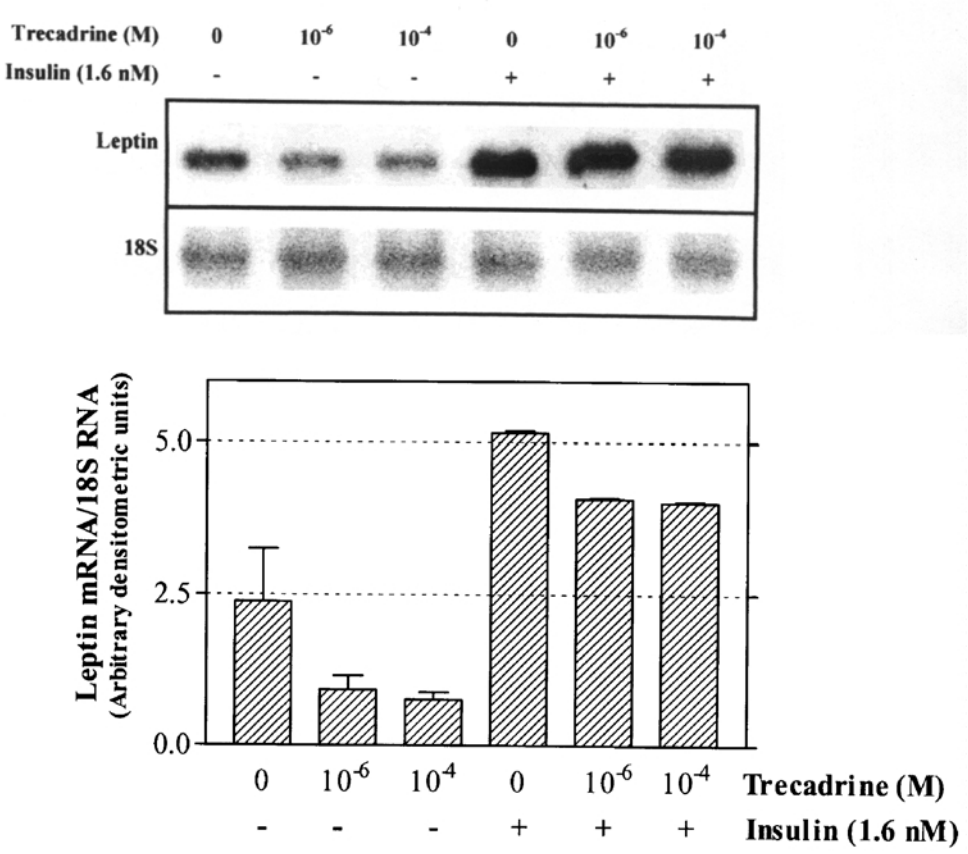

Figure 2 Effects of Trecadrine on basal and insulin-stimulated leptin mRNA expression, as assessed by Northern blots. The expression level of 18S ribosomal RNA was determined and used as an internal control to correct for minor variation in total RNA amount. Densitometric scanning was used to determine the relative amount of leptin mRNA and 18S RNA. Values are mean ± s.e. from two experiments.

(123.5 ± 9.3, 136.9 ± 7.1 and 162.6 ± 9.8% of control, $P < 0.05$, $P < 0.001$ and $P < 0.001$, respectively). The increase in glycerol levels was negatively correlated ($r = -0.503$, $P < 0.0001$) to the inhibition of leptin secretion in Trecadrine treated adipocytes.

Discussion

β3-Adrenergic agonists have been reported to inhibit leptin expression and secretion in rodents primary adipocytes in culture.[24,25] An inhibition of leptin secretion was also observed after administration of Trecadrine or other β3-adrenergic agonist drugs to rats and mice.[7–10] The effect of insulin to stimulate leptin expression and secretion by primary rat adipocytes is well established.[11,24,26–28] In the present study, an inhibitory effect of Trecadrine was also observed on insulin-stimulated leptin secretion. However, the inhibitory effect was less potent than in the absence of insulin, suggesting that insulin was able to partially reverse the inhibitory effects of Trecadrine on leptin expression and secretion. Gettys *et al*[24] reported a potent inhibition of insulin-stimulated leptin secretion from isolated adipocytes using the β3-AR selective agonist CL316,243. This large inhibitory effect may be due to the differences in the treatment conditions (eg short-term *vs* longer culture periods or studying adipocytes in suspension *vs* adipocytes anchored to a matrix) or to the potency of the β3-agonist employed. A recent study using cultured human adipose tissue reported that the inhibition of leptin secretion induced by isoproterenol, a nonselective β-adrenergic agonist, was reversed in the presence of insulin.[29]

Previous data from our laboratory indicate that glucose metabolism has an important role in the regulation of leptin expression and secretion in isolated cultured rat adipocytes and the transcriptional activity of the leptin promoter in 3T3-Ll cells.[11,30,31] Inhibition of glucose uptake with 2-deoxy-D-glucose, phloretin or cytochalasin B, or inhibition of glycolysis, with NaF or iodoacetate, decreases insulin-stimulated leptin gene expression and leptin secretion.[11] Several studies have previously reported that β3-adrenergic agonists can increase glucose uptake by adipose tissue and skeletal muscle *in vivo* and *in vitro*.[32–35]

Trecadrine has been shown to alter hexose uptake in several tissues. Administration of 1 mg/kg of Trecadrine for 4 days caused an increase of 2 deoxy-D-glucose uptake in

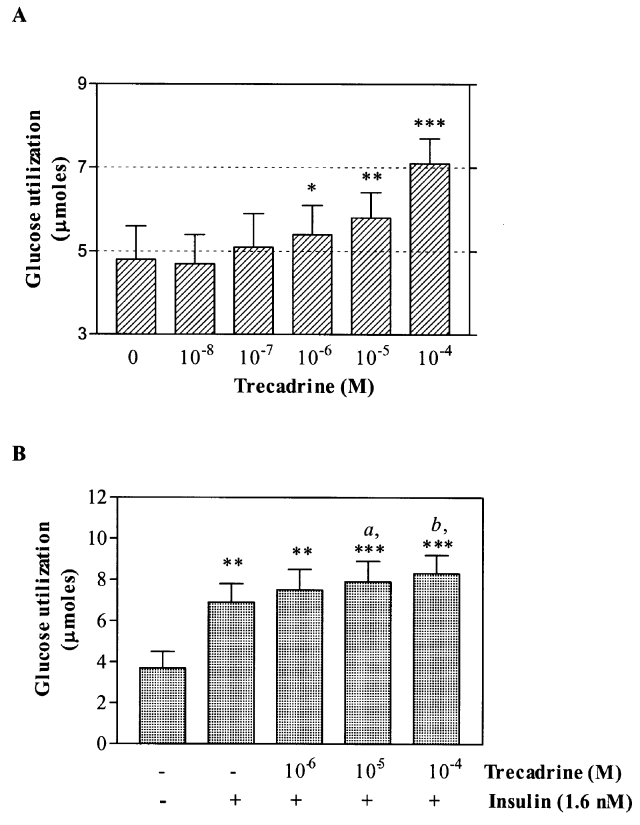

Figure 3 Effects of Trecadrine on basal (A) and insulin-stimulated (B) glucose utilization by isolated rat adipocytes over 96 h in culture. Data are mean ± s.e. of six independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$. $^{a}P < 0.05$; $^{b}P < 0.01$ *vs* insulin-treated cells.

extensor digitorium longus and in gastronemius muscles, while no changes were found in soleus muscle and WAT.[5] It is unclear, however, if the ability of Trecadrine and other β3-adrenergic agonists to promote glucose uptake is mediated by increasing the non-insulin dependent glucose utilization or by promoting the recruitment of insulin-regulated glucose transporters.[32,33] In the present study, Trecadrine induced a concentration-dependent stimulation of both basal and insulin-stimulated glucose uptake by isolated adipocytes, which is consistent with the previously reported anti-diabetic properties of Trecadrine.[4,5]

Previously published studies from our laboratory suggest that the metabolism of glucose beyond pyruvate, and not glucose utilization *per se*, is involved in the action of glucose and insulin to stimulate leptin secretion. For example, leptin secretion is inversely related to the proportion of glucose that is metabolized to lactate.[11] Furthermore, metformin, an antidiabetic drug that stimulates adipocyte glucose uptake, appears to inhibit leptin secretion by increasing the anaerobic metabolism of glucose to lactate.[30] Our present data shows that Trecadrine does not alter the percentage of glucose carbon released as lactate, suggesting that Trecadrine-induced inhibition of leptin production is not mediated by increasing anaerobic glucose metabolism.

Our previous results suggest that glucose utilization stimulates leptin production by directing the metabolism of glucose to a fate other than anaerobic lactate production, possibly oxidation or lipogenesis.[11,30,36] Trecadrine did not, however, significantly alter the percentage of glucose metabolized to triglyceride or to CO_2, either in the absence or presence of insulin. In this study, insulin substantially increased the percentage of glucose oxidized to CO_2, while it did not significantly affect the percentage of glucose incorporated into triglyceride. This suggests that the effect of glucose utilization to stimulate leptin production involves the oxidative metabolism of glucose to CO_2. Therefore, a mechanism independent of altering the pathways of glucose metabolism is likely be involved in the effect of Trecadrine to inhibit leptin expression and secretion.

Binding of agonists to β-ARs activates the adenylate cyclase causing an increase of intracellular c-AMP.[18] Interventions that elevate c-AMP, such as incubation with forskolin or isoproterenol, inhibit leptin release by adipose tissue.[37] The mechanisms underlying this inhibition are not known. Activation of β3-adrenoceptors stimulates lipolysis in adipose tissue.[24,38] An increase in the activity of hormone sensitive lipase, the enzyme that catalyzes the hydrolysis of triglyceride to glycerol and fatty acids,[39] has

Table 1 Effects of Trecadrine on glucose metabolism. Lactate production, the percentage of glucose carbon released as lactate, and lipogenesis as assessed by the amount and the percentage of glucose carbon incorporated into triglyceride (TG) were determined over 96 h in culture. The effects of Trecadrine on the percentage of glucose oxidized to CO_2 were analysed over 48 h in culture. Data are mean ± s.e.m. of six independent experiments

		Trecadrine (M)			
	Control	*10^{-7}*	*10^{-6}*	*10^{-5}*	*10^{-4}*
Lactate production (μmol)	4.1 ± 0.7	4.4 ± 0.8	4.6 ± 0.8	4.9 ± 0.6**	5.6 ± 0.7**
Glucose to lactate (%)	50.7 ± 10.6	42.6 ± 6.0	54.0 ± 12.3	44.2 ± 5.9	40.3 ± 4.5
Lipogenesis (nmol glu/mg TG)	1.9 ± 0.4	2.3 ± 0.4	2.4 ± 0.4	2.8 ± 0.4**	2.9 ± 0.4**
Glucose to TG (%)	27.2 ± 4.3	26.9 ± 6.6	30.9 ± 6.6	25.3 ± 3.9	24.8 ± 3.8
Glucose to CO_2[a] (%)	20.2 ± 1.7	ND	21.0 ± 1.5	ND	18.6 ± 1.1

$^{**}P < 0.01$; ND, non determined.
[a]Determined in cultured cells obtained from different animals than the other parameters.

Table 2 Effects of Trecadrine in the presence of 1.6 nM insulin on glucose metabolism. Lactate production, the percentage of glucose carbon released as lactate, and lipogenesis as assessed by the amount and the percentage of glucose carbon incorporated into triglyceride (TG) were determined over 96 h in culture. The effects of Trecadrine on the percentage of glucose oxidized to CO_2 were analysed over 48 h in culture. Data are mean ± s.e.m. of six independent experiments

		Insulin (1.6 nM)			
		Trecadrine (M)			
	Control	*0*	*10^{-6}*	*10^{-5}*	*10^{-4}*
Lactate production (μmol)	3.4 ± 0.6	2.8 ± 0.6	2.9 ± 0.6	2.9 ± 0.6	2.8 ± 0.5
Glucose to lactate (%)	41.7 ± 7.8	25.5 ± 5.8***	23.5 ± 5.4***	23.5 ± 6.2***	20.0 ± 4.6***†
Lipogenesis (nmol glu/mg TG)	1.9 ± 0.2	3.7 ± 1.4*	4.2 ± 1.5*	4.3 ± 1.4**‡	4.8 ± 1.5***‡
Glucose to TG (%)	29.2 ± 4.7	32.0 ± 5.3	37.2 ± 5.5	32.6 ± 5.0	38.1 ± 3.9
Glucose to CO_2[a] (%)	20.2 ± 1.7	23.6 ± 1.8**	22.9 ± 2.2	ND	22.7 ± 1.7

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ *vs* corresponding control. $^{\dagger}P < 0.05$; $^{\ddagger}P < 0.01$ *vs* insulin treated cells. ND, non determined.
[a]Determined in cultured cells obtained from different animals than the other parameters.

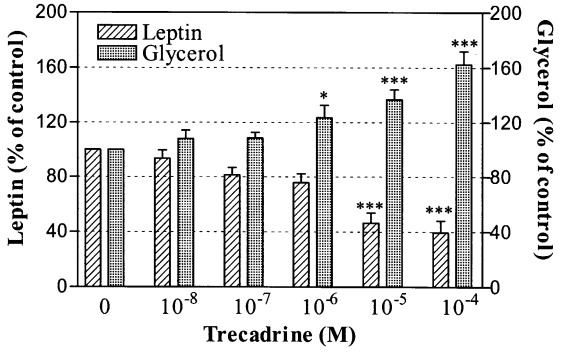

Figure 4 Effects of Trecadrine on glycerol and leptin production by isolated rat adipocytes over 96 h in culture. Results are given as percentage of control. Data are mean ± s.e. of six independent experiments. *$P < 0.05$; ***$P < 0.001$.

been reported in adipose tissue of animals treated with Trecadrine.[40] In the present study, Trecadrine induced a concentration-dependent increase of lipolysis as assessed by glycerol release. Furthermore, an inverse relationship between lipolysis and the inhibition of leptin secretion was observed. The increase in glycerol suggests a parallel increase in FFA resulted from the lipolytic effect of Trecadrine, as has been previously reported with norepinephrine.[41] It has been shown that FFAs can inhibit leptin expression and secretion by adipocytes.[17] A decrease in the insulin-stimulated activity of the leptin promoter in primary rat adipocytes has also been reported after treatment with FFA.[42] This suggests that the effect of Trecadrine to suppress leptin expression and secretion may be a consequence of increased fatty acid release. Another mechanism potentially involved in the inhibition of leptin production by β3-adrenergic agonists is activation of the ERK1/2 MAP kinase pathway which is known to be induced by cAMP.[43]

In conclusion, Trecadrine inhibited leptin expression and secretion by rat adipocytes. Trecadrine also increased glucose utilization, but did not alter the anaerobic metabolism of glucose to lactate or the incorporation of glucose into lipid or glucose oxidation to CO_2. These results suggest that the inhibition of leptin expression and secretion induced by Trecadrine is not mediated by alterations of the pathways of adipocyte glucose metabolism. The inverse correlation between leptin secretion and lipolysis, an index of the activation of cAMP-dependent protein kinases, suggests that activation of cAMP-dependent pathways mediates the effects of β3-adrenergic agonists such as Trecadrine to inhibit leptin expression and secretion.

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References
1 Weise M, Frishman WH, Michaelson MD, Abdeen MA. The pharmacological approach to the treatment of obesity. *J Clin Pharmacol* 1997; **37**: 453–473.
2 Weyer C, Gautier JF, Danforth E. Development of beta3-adrenoceptor agonists for the treatment of obesity and diabetes-an update. *Diabetes Metab* 1999; **25**: 11–21.
3 Barrionuevo M, Milagro FI, Cenarruzabeitia E, Martínez JA. Potential anti-diabetic applications of a new molecule with affinity for β3-adrenoceptors. *Life Sci* 1996; **59**: PL141–146.

4 Milagro FI, Gómez-Ambrosi J, Martínez-Ansó E, Martínez JA. Efffect of a β3-agonist on liver glucokinase gene expression in alloxan-diabetic rats. *J Physiol Biochem* 1999; **55**: 25–32.
5 Milagro FI, Gómez-Ambrosi J, Forga L, Martínez JA. A β3-adrenergic agonist increases muscle GLUT1/GLUT4 ratio, and regulates liver glucose utilization in diabetic rats. *Diabetes, Obes Metab* 1999; **1**: 97–104.
6 Berraondo B, Bonafonte A, Fernández-Otero MP, Martinez JA. Effects on energy utilization of a β3-adrenergic agonist in rats fed on a cafeteria diet. *Eating Weight Disord* 1997; **2**: 130–137.
7 Berraondo B, Marti A, Duncan JS, Trayhurn P, Martínez JA. Up-regulation of muscle UCP2 gene expression by a new beta-3 adrenoceptor agonist, tracadrine, in obese (cafeteria) rodents, but down-regulation in lean animals. *Int J Obes Relat Metab Disord* 2000; **24**: 156–163.
8 Mantzoros CS, Qu D, Friederich RC, Susulic VS, Lowell BB, Maratos-Flier E, Flier JS. Activation of beta(3) adrenergic receptors suppresses leptin expression and mediates a leptin-independent inhibition of food intake in mice. *Diabetes* 1996; **45**: 909–914.
9 Trayhurn P, Duncan JS, Rayner DV, Hardie LJ. Rapid inhibition of ob gene expression and circulating leptin levels in lean mice by the β3-adrenoceptor agonist BRL 35135A and ZD2079. *Biochem Biophys Res Commun* 1996; **228**: 605–610.
10 Li H, Matheny M, Scarpace PJ. β3-Adrenergic-mediated suppression of leptin gene expression in rats. *Am J Physiol* 1997; **272**: E1031–E1036.
11 Mueller WM, Gregoire FM, Stanhope KL, Mobbs CV, Mizuno TM, Warden CH, Stern JS, Havel PJ. Evidence that glucose metabolism regulates leptin secretion from cultured rat adipocytes. *Endocrinology* 1998; **139**: 551–558.
12 Wellhoener P, Fruehwald-Schultes B, Kern W, Dantz D, Kerner W, Born J, Fehm HL, Peters A. Glucose metabolism rather than insulin is a main determinant of leptin secretion in humans. *J Clin Endocrinol Metab* 2000; **85**: 1267–1271.
13 Havel PJ. Role of adipose tissue in body-weight regulation: mechanisms regarding leptin production and energy balance. *Proc Nutr Soc* 2000; **59**: 359–371.
14 Havel PJ. Peripheral signals conveying metabolic information to the brain: short-term and long-term regulation of food intake and energy homeostasis. *Exp Biol Med* 2001; **226**: 963–977.
15 Havel PJ. Control of energy homeostasis and insulin action by adipocyte hormones: leptin, acylation stimulation protein, and adiponectin. *Curr Opin Lipidol* 2002; **13**: 51–59.
16 Rentsch J, Chiesi M. Regulation of ob gene mRNA levels in cultured adipocytes. *FEBS Lett* 1996; **379**: 55–59.
17 Shintani M, Nishimura H, Yonemitsu S, Masuzaki H, Ogawa Y, Hosoda K, Inoue G, Yoshimasa Y, Nakao K. Downregulation of leptin by free fatty acids in isolated rat adipocytes: effects of triacsin C, palmitate and bromopalmitate. *Metab Clin Exp* 2000; **49**: 326–330.
18 Nonogaki K. New insights into sympathetic regulation of glucose and fat metabolism. *Diabetologia* 2000; **43**: 533–549.
19 Landt M, Gingerich RL, Havel PJ, Mueller WM, Schoner B, Hale JE, Heiman ML. Radioimmunoassay of rat leptin: sexual dimorphism reversed from humans. *Clin Chem* 1998; **44**: 565–570.
20 Stanhope KL, Moreno-Aliaga MJ, Kras KM, Havel PJ. Assessment of lipogenesis in long-term cultured rat adipocytes, [Abstract.] *Obes Res* 1999; **7**: 74S.
21 Folch I, Lees M, Sloane-Stanley GH. A simple method for the isolation and purification of total lipids from animal tissue. *J Biol Chem* 1957; **193**: 497–509.
22 Rodbell M. Metabolism of isolated fat cells. Effects of hormones on glucose metabolism and lipolysis. *J Biol Chem* 1964; **239**: 375–380.
23 Bottcher H, Furst P. Microcalorimetric and biochemical investigations of thermogenesis and metabolic pathways in human white adipocytes. *Int J Obes Relat Metab Disord* 1996; **20**: 874–881.
24 Gettys TW, Harness PJ, Watson PM. The β3-adrenergic receptor inhibits insulin-stimulated leptin secretion from isolated rat adipocytes. *Endocrinology* 1996; **137**: 4054–4057.
25 Slieker LJ, Sloop KW, Surface PL, Kriaucinas A, LaQuier F, Manetta J, Bue-Valleskey J, Stephens TW. Regulation of expression of mRNA and protein by glucocorticoids and cAMP. *J Biol Chem* 1996; **271**: 5301–5304.
26 Hardie LJ, Guilhot N, Trayhurn P. Regulation of leptin production in cultured mature white adipocytes. *Horm Metab Res* 1996; **28**: 685–689.
27 Yoshida T, Hayashi M, Monkawa T, Saruta T. Regulation of obese mRNA expression by hormonal factors in primary cultures of rat adipocytes. *Eur J Endocrinol* 1996; **135**: 619–625.
28 Bradley R, Cheatham B. Regulation of ob gene expression and leptin secretion by insulin and dexamethasone in rat adipocytes. *Diabetes* 1999; **48**: 272–278.
29 Fain JN, Cowan GS, Buffington C, Li J, Pouncey L, Bahouth SW. Syngergism between insulin and low concentrations of isoproterenol in the stimulation of leptin release by cultured human adipose tissue. *Metab Clin Exp* 2000; **49**: 804–809.
30 Mueller WM, Stanhope KL, Gregoire F, Evans JL, Havel PJ. Effects of metformin and vanadium on leptin secretion from cultured rat adipocytes. *Obes Res* 2000; **8**: 530–539.
31 Moreno-Aliaga MJ, Stanhope KL, Havel PJ. Transcriptional regulation of the leptin promoter by insulin-stimulated glucose metabolism in 3T3-L1 adipocytes. *Biochem Biophys Res Commun* 2001; **283**: 544–548.
32 De Souza CJ, Hirshman MF, Horton ES. CL-316,243, a β3-specific adrenoceptor agonist, enhances insulin-stimulated glucose disposal in nonobese rats. *Diabetes* 1997; **46**: 1257–1263.
33 Shimizu Y, Kielar D, Minokoshi Y, Shimazu T. Noradrenaline increases glucose transport into brown adipocytes in culture by a mechanisms different from that of insulin. *Biochem J* 1996; **314**: 485–490.
34 Abe H, Minokoshi Y, Shimazu T. Effect of a β3-adrenergic agonist, BRL35135A, on glucose uptake in rat skeletal muscle in vivo and in vitro. *J Endocrinol* 1993; **139**: 479–486.
35 Liu YL, Cawthorne MA, Stock MJ. Biophagic effects of the β-adrenoceptor agonist, BRL 37344, on glucose utilization in rat isolated skeletal muscle. *Br J Pharmac* 1996; **117**: 1355–1361.
36 Havel PJ, Stanhope KL, Moreno-Aliaga MJ, Kras KM, Evans J. Potential role for mitochondrial metabolism in regulating leptin production by cultured rat adipocytes. [Abstract.] *Obes Res* 1999; **7**: 63S.
37 Fain JN, Bahouth SW. Regulation of leptin release by mammalian adipose tissue. *Biochem Biophys Res Commun* 2000; **274**: 571–575.
38 Lipworth BJ. Clinical pharmacology of β3-adrenoceptors. *J Clin Pharmacol* 1996; **42**: 219–230.
39 Langin D, Holm C, Lafontan M. Adipocyte hormone-sensitive lipase: a major regulator of lipid metabolism. *Proc Nutr Soc* 1996; **55**: 93–109.
40 Berraondo B, Martínez JA. Free fatty acids are involved in the inverse relationship between hormone-sensitive lipase (HSL) activity and expression in adipose tissue after high-fat feeding or β3-adrenergic stimulation. *Obes Res* 2000; **8**: 255–261.
41 Wang MY, Lee Y, Unger RH. Novel form of lipolysis induced by leptin. *J Biol Chem* 1999; **274**: 17541–17544.
42 Fukuda H, Iritani N. Transcriptional regulation of leptin gene promoter in rats. *FEBS Lett* 1999; **455**: 165–169.
43 Soeder KJ, Snedden SK, Cao W, Della Rocca GJ, Daniel KW, Luttrell LM, Collins S. The β3-adrenergic receptor activates mitogen-activated protein kinase in adipocytes through Gi-dependent mechanism. *J Biol Chem* 1999; **274**: 12017–12022.