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Research Article

Leptin, but not a β_3-adrenergic agonist, upregulates muscle uncoupling protein-3 messenger RNA expression: short-term thermogenic interactions

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Abstract. The short-term effects of leptin and a β_3-adrenoceptor agonist on thermogenesis and expression of uncoupling proteins (UCPs) in brown adipose tissue (BAT) and muscle and their possible interactions were assessed. One hour after administration of the β_3-adrenoceptor agonist Trecadrine, a statistically significant increase in UCP1 messenger RNA (mRNA) expression in BAT was observed, whereas UCP2 and UCP3 in both BAT and gastrocnemius muscle were unaffected. Leptin induced an upregulation of UCP3 mRNA in muscle, with no changes in BAT UCP1 mRNA. A statistical interaction was found between leptin and Trecadrine in rectal temperature. The present study provides evidence, for the first time, of the induction of UCP3 mRNA expression in skeletal muscle by leptin in nongenetically obese animals.

Key words. Uncoupling protein; leptin; β_3-adrenergic agonist; rat.

Nonshivering thermogenesis has been thought for a long period of time to mainly occur through the uncoupling protein (UCP1) of brown adipose tissue (BAT) [1]. However, the recent discovery of two new members of this protein family, UCP2 [2, 3] and UCP3 [4, 5], in a variety of tissues has constituted a new breakthrough for understanding the neuroendocrine and molecular mechanisms involved in the regulation of thermogenesis, as well as for identifying new potential roles for leptin and β_3-adrenoceptors affecting UCPs.

Leptin—a 16-kDa secreted protein mainly produced by adipocytes [6] and apparently in a lesser extent by other tissues [7, 8]—plays a critical role in the regulation of energy balance, reducing body weight and adipose tissue mass through decreases in appetite [9–11]. An increased sympathetic outflow in response to leptin [12] may be involved in the weight-reducing effects of leptin by triggering thermogenesis [13]. Furthermore, autocrine or paracrine leptin actions on brown and white adipose tissue [14, 15] and skeletal muscle [16, 17] have been described, as well as a lipolytic activity in adipocytes induced by β_3-adrenergic agonists [18].

In this context, an interesting interplay has been published between β_3-adrenoceptor stimulation and leptin functions, suggesting that leptin could trigger the sympathetic nervous system (SNS) via the hypothalamus, thus exerting β_3-adrenoceptor-mediated effects [19]. Also, it has been reported that β_3-adrenergic receptor (β_3-AR) agonists inhibit leptin expression in vitro [20] and in vivo [21–24], but little is known about the possible physiological interactions between leptin and β_3-AR agonists on energy expenditure.

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The purpose of the current study was to assess the short-term effects of leptin, a β_3-AR agonist or a combined administration of both on thermogenesis and the potential involvement in this process of UCPs from different tissues.

Materials and methods

Animals. Five-month-old male Wistar rats weighing 423 ± 3 g were housed at 25 ± 1 °C with 12-h light cycle (8 am to 8 pm) and fed ad lib. They were fasted for 24 h before drug administration. Rats received the following intraperitoneal (i.p.) treatments: saline, leptin (Peprotech, London, UK; 0.25 mg/kg), the β_3-AR agonist Trecadrine (Wasserman-Chiesi, Barcelona/Milan; 1 mg/kg, see [25, 26] for chemical structure and pharmacological properties) or a combination of both at the same doses. After 1 h, rectal temperature and oxygen consumption were measured. Animals were killed by decapitation, and the trunk blood was collected. Gastrocnemius muscle and BAT were excised, immediately frozen in liquid nitrogen and stored at -80 °C until analysis. The gastrocnemius muscle was selected as being representative of the whole skeletal musculature for nutritional studies [27]. All experimental procedures were performed according to institutional guidelines for animal care and use at the University of Navarra.

Blood analysis. Plasma glucose levels were measured by an enzymatic assay with the Spotchem analyser (Menarini, Barcelona, Spain). Serum insulin was measured with a commercially available RIA Kit (Rat insulin [^{125}I] assay system, Amersham, Buckinghamshire, UK).

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-s sampling periods at 1-min intervals while animals were in a resting situation. The temperature of the chamber was maintained at 25 °C. Results are expressed as millilitres of oxygen consumed per gram of body weight per hour. Rectal temperature was measured before drug administration and after 1 h by a rectal probe (Yellow Springs Instruments, Yellow Springs, OH, USA) connected to a Panlab thermometer pb 0331 (Panlab, Barcelona, Spain) as described elsewhere [28].

Extraction of total RNA and semiquantitation by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated by the Ultraspec-II RNA Isolation System (Biotecx Laboratories, Houston, TX, USA) from 100 mg of gastrocnemius muscle or BAT. After 30 min at 37 °C treatment with 10 units of RNase-free DNase I (Boehringer Mannheim, Barcelona, Spain), 1 μg of RNA was used to synthesise first-strand complementary DNA (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 dithiothreitol, 100 ng of random hexamers (Boehringer Mannheim), 1 mM each deoxynucleotide-triphosphate (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 microlitres 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 cDNA (GenBank M11814) were 5′-GTCTTAGGGACCATCACCA-3′ (sense, 351–369) and 5′-CCAGTGTAGCGGGGT-TTG-3′ (antisense, 629–646), UCP2 (GenBank AF039033) 5′-TAAAGCAGTTCTACACCAAGGG-3′ (sense, 308–329) and 5′-CGAAGGCAGAAGTGAAG-TGG-3′ (antisense, 648–667), UCP3 (GenBank U920-69) 5′-GGAACTGGAGGCGAGAGGAA-3′ (sense, 577–596) and 5′-TTTGTAGAAGGCTGTGGGGC-3′ (antisense, 926–945) and β-actin (GenBank J00691) 5′-TCTACAATGAGCTGCGTGTG-3′ (sense, 1599–1618) and 5′-GGTCAGGATCTTCATGAGGT-3′ (antisense, 2357–2376). Primers for UCPs were designed using Oligo 4.05 Primer Analysis Software (National Biosciences, Inc., Plymouth, MN, USA). cDNA was amplified for 26 (UCP2), 27 (UCP3) and 21 cycles (β-actin) in muscle, and 20 (UCP1), 32 (UCP2), 35 (UCP3) and 25 cycles (β-actin) in BAT, using the following parameters: 94 °C for 30 s, 58 °C (UCP1), 62 °C (UCP2), 60 °C (UCP3) and 59 °C (β-actin) for 30 s and 72 °C for 30 s, 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). The amplified products were resolved in a 1.5% agarose gel with ethidium bromide. Levels of messenger RNA (mRNA) were expressed as the ratio of signal intensity for each UCP relative to that for β-actin. PCR band intensities were determined by densitometric analysis with the Gel Doc 1000 ultraviolet (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 demonstrated after digestion with *Sca*I (New England Biolabs, Beverly, MA, USA) and *Bgl*II (Amersham) for UCP1, *Bam*HI (Promega) and *Sma*I (Promega) for UCP2 and *Ban*I (New England Biolabs) and *Kpn*I (Amersham) for UCP3. All restriction enzymes yielded the predicted fragments.

Statistical analysis. All results are expressed as mean ± standard error of the mean (SEM). Data were analysed using a two-way analysis of variance (ANOVA) or two-tailed unpaired *t* test when an interaction was detected. The Pearson correlation coefficient was computed to analyse correlations between two variables. The calculations were performed using the SPSS/Windows version 6.1.3 (SPSS Inc., Chicago) statistical package [29]. A *P* value lower than 0.05 was considered to be statistically significant.

Results

Trecadrine administration produced a statistically significant increase in plasma glucose levels with no changes in plasma insulin concentrations after 60 min (table 1). Plasma glucose values were slightly reduced by leptin injection, although the decrease was not statistically significant. Insulin levels were not modified by the acute leptin treatment. The simultaneous administration of leptin and the β_3-AR agonist showed no statistically significant interaction on plasma glucose or insulin concentrations. Rats treated with Trecadrine showed a marked elevation of rectal temperature (table 2). Leptin also produced an increase in body temperature. Furthermore, Trecadrine administration produced a statistically significant rise in oxygen consumption, whereas leptin only induced a small increase (7%) with no statistical significance.

Acute treatment with Trecadrine increased the expression of UCP1 mRNA in BAT (fig. 1). The expression of UCP2 and UCP3 was unaffected by β_3-AR agonist administration in both BAT and gastrocnemius muscle. Treatment with leptin had no effect on UCP1, UCP2 and UCP3 mRNA in BAT, but produced a rise of UCP3 mRNA in gastrocnemius muscle (fig. 2). No changes in UCP2 mRNA expression in muscle were observed. The simultaneous administration of Trecadrine and leptin had no synergistic effect on BAT and muscle expression of UCPs. A statistical interaction between the β_3-AR agonist and leptin was found concerning rectal temperature when administered together, which apparently indicates that the maximum value achieved after β_3-AR agonist administration is not increased by the combined effect of both agents. A marginal statistical association ($r = 0.534$, $P < 0.08$) between rectal temperature increment and muscle UCP3 mRNA was found when control and leptin-treated animals were analysed together.

Discussion

The dose of Trecadrine chosen was assessed in our laboratory in a long-term treatment of diet-induced obese rats [28] and diabetic rats [25]. In addition, the assayed dose of leptin has been reported to have both antiobesity and antidiabetic effects in many studies [9–11]. The short-term effect on glycemia induced by Trecadrine may be explained by the stimulation of hepatic gluconeogenesis and/or glycogenolysis, which has been reported after administration of different β-adrenergic agonists [30, 31]. The duration (acute stimu-

Table 1. Plasma glucose and insulin 1 h after a single intraperitoneal injection of the β_3-AR agonist Trecadrine (1 mg/kg), leptin (0.25 mg/kg), their combination or saline in 24-h fasted Wistar rats.

					Statistical analysis		
	Control	β_3-AR agonist	Leptin	Lep + β_3-AR	*P* β_3-AR	*P* Lep	*P* interaction
Plasma glucose (mmol/l)	7.9 ± 0.2	8.6 ± 0.2**	7.3 ± 0.2	8.4 ± 0.3**	0.0018	0.0993	0.4714
Plasma insulin (pmol/l)	328 ± 53	271 ± 22	341 ± 31	329 ± 57	0.4534	0.4213	0.6181

Statistical significance of the differences between treatments was evaluated using a two-way ANOVA: $^{**}P<0.01$ main effect of Trecadrine. Values are mean ± SEM, $n = 10$ per group.

Table 2. Rectal temperature gradient (Δ) and oxygen consumption 1 h after a single intraperitoneal injection of the β_3-AR agonist Trecadrine (1 mg/kg), leptin (0.25 mg/kg), their combination or saline in 24-h fasted Wistar rats.

					Statistical analysis		
	Control	β_3-AR agonist	Leptin	Lep + β_3-AR	*P* β_3-AR	*P* Lep	*P* interaction
Δ Rectal temperature (°C)[1]	0.04 ± 0.08	0.79 ± 0.16^{++}	0.53 ± 0.10^{++}	0.71 ± 0.16^{++}	0.0009	0.1187	0.0327
Oxygen consumption (ml O_2/g/h)	0.75 ± 0.03	1.04 ± 0.03***	0.80 ± 0.05	1.01 ± 0.05***	0.0001	0.7819	0.3095

[1]Rectal temperature was measured before and 1 h after administration. Statistical significance of the differences between treatments were evaluated using a two-way ANOVA: $^{***}P<0.001$ main effect of Trecadrine. When an interaction was detected, two-tailed unpaired *t* test was used: $^{++}P<0.01$ versus control group, $^{+++}P<0.001$ versus control group. Values are mean ± SEM, $n = 10$ per group.

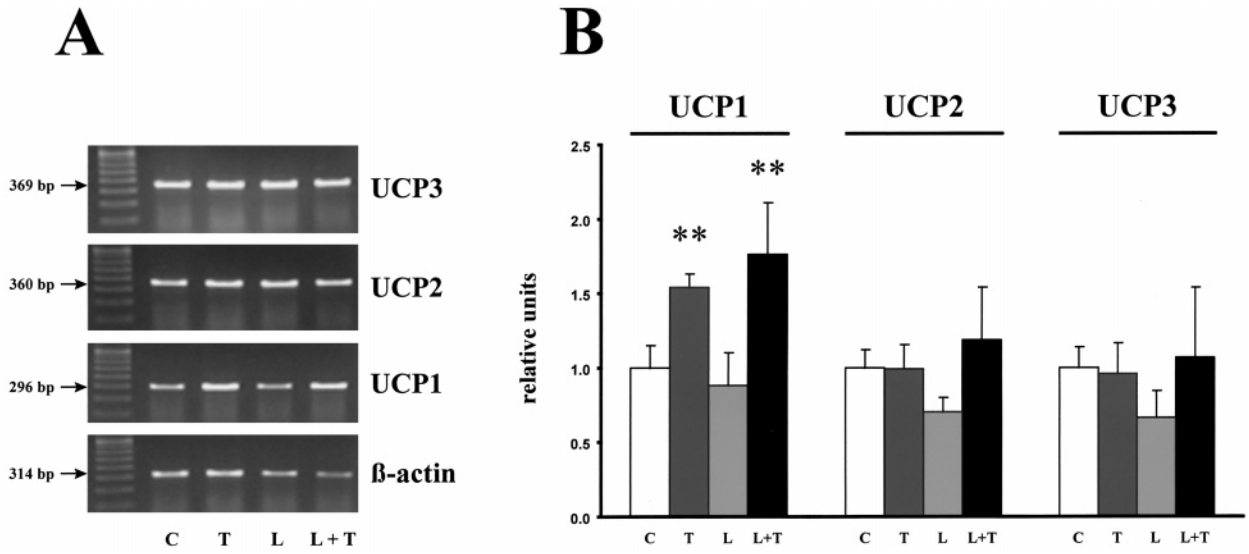

Figure 1. Uncoupling proteins mRNA in BAT 1 h after a single intraperitoneal dose of saline, Trecadrine (1 mg/kg), leptin (0.25 mg/kg) or both. (*A*) A representative RT-PCR of UCPs and β-actin. Lane 1, 100-bp ladder; lane 2, control (C); lane 3, Trecadrine (T); lane 4, leptin (L); lane 5, leptin plus Trecadrine (L + T). (*B*) Densitometric quantitation of UCP's expression in control (C, white bar), Trecadrine (T, dark-grey bar), leptin (L, light-grey bar) and leptin plus Trecadrine (L + T, black bar) treated rats. Data represent the mean ± SEM of the ratio between each UCP to β-actin. The expression of each UCP in control rats was assumed to be 1 ($n = 6$ in each group). $^{**}P < 0.01$ main effect of Trecadrine (two-way ANOVA).

lation) and the assayed dose may be responsible of the lack of effect on plasma insulin concentrations.

Trecadrine administration produced a marked increment in rectal temperature and oxygen consumption as expected when a β_3-AR agonist is administered [18, 32]. In contrast, leptin—at the assayed dose—only increased rectal temperature. In this sense, a thermogenic effect following the administration of fragment peptides, designed after the OB protein, were previously observed in our laboratory [33]. Interestingly, a statistical interaction among the effects of leptin and Trecadrine on rectal temperature was observed. The nature of this interaction did not follow an additive or synergistic pattern, which may suggest a shared action pathway, although this situation was not further confirmed by the results concerning expression of UCPs, and therefore, a common thermogenic mechanism other than UCPs might be involved.

The present study shows that the expression of UCP2 and UCP3 mRNA in two major thermogenic tissues (i.e. BAT and skeletal muscle) is not subject to short-term regulation by β_3-AR agonists despite the existence of these receptors in those tissues [34], which is in agreement with a previous report [35]. Recently, upregulation of UCP3 mRNA in BAT by the β_3-AR agonist BRL 35135 was described [36]. However, these results were obtained after a 3-week treatment, thus showing that β_3-AR agonists may have a long-term effect on BAT UCP3 mRNA expression. The lack of effect on muscle UCP2 and UCP3 after Trecadrine treatment complements data obtained by other researchers after short-term treatment with other β_3-AR agonists [35].

The thermogenic action of leptin may be attributed to upregulation in muscle UCP3 mRNA expression. In this context, the existence of functional leptin receptors (OB-Rb) has been reported in skeletal muscle [16, 37], suggesting that this tissue is a target organ for leptin action. This observation is reinforced by the fact that a marginal statistical association between rectal temperature increase and muscle UCP3 mRNA was found. This effect is in accordance with recent findings in leptin-treated *ob*/*ob* mice [35, 38]. In contrast, no leptin-mediated change in BAT UCPs was observed, which contrasts with a reported increase of UCP1 in BAT in rats after leptin treatment. However, the increase observed was only evident after 5 days of daily administration [39], suggesting that UCP1 may be a long-term mediator of the thermogenic effect elicited by leptin in rats. Additionally, some researchers [40] have found higher levels of BAT UCP1 in mice after daily very high dose (20 mg/kg) leptin treatment in a longer experiment. Others obtained an upregulating effect after 7 days of leptin infusion in *ob*/*ob* mice, but not in lean mice [41]. All these results suggest that in genetically obese animals leptin would have a normalising effect in BAT, which is further supported by the reduction in

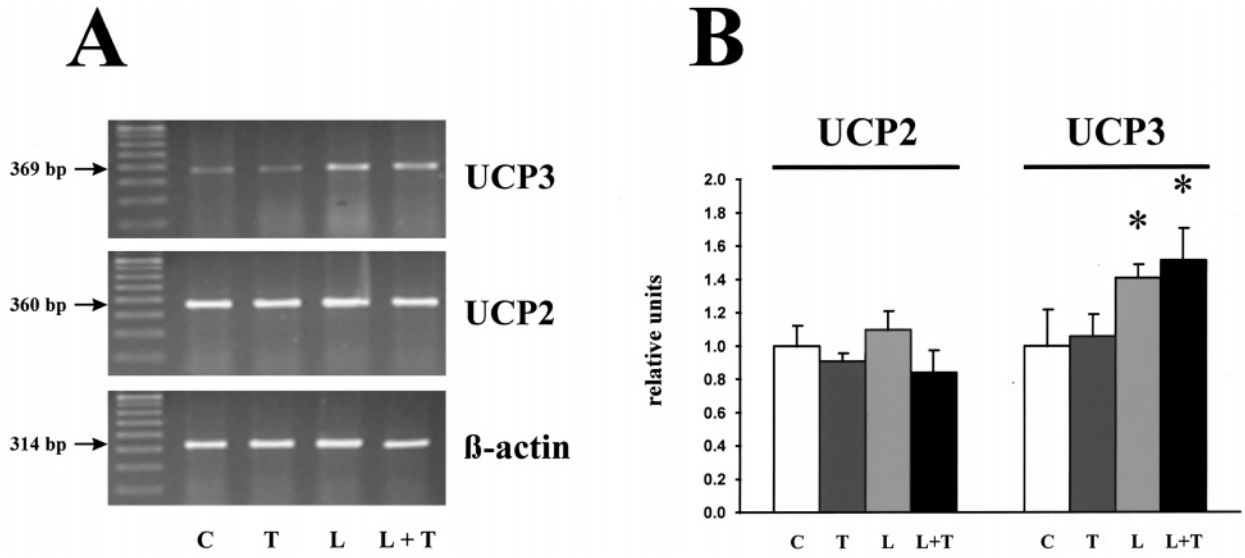

Figure 2. Uncoupling protein-2 and -3 mRNA in gastrocnemius muscle 1 h after a single intraperitoneal dose of saline, Trecadrine (1 mg/kg), leptin (0.25 mg/kg) or both. (*A*) A representative RT-PCR of UCP2, UCP3 and β-actin. Lane 1, 100-bp ladder; lane 2, control (C); lane 3, Trecadrine (T); lane 4, leptin (L); lane 5, leptin plus Trecadrine (L + T). (*B*) Densitometric quantitation of UCP2 and UCP3 expression in control (C, white bar), Trecadrine (T, dark-grey bar), leptin (L, light-grey bar) and leptin plus Trecadrine (L + T, black bar) treated rats. Data represent the mean ± SEM of the ratio between each UCP to β-actin. The expression of each UCP in control rats was assumed to be 1 ($n = 6$ in each group). *$P < 0.05$ main effect of leptin (two-way ANOVA).

BAT UCP2 and the increase in BAT UCP3 observed after leptin treatment in *ob*/*ob* mice, but not in normal animals [35].

The lack of interaction at the level of UCPs between Trecadrine and leptin supports the hypothesis that their individual effects may take place via different mechanisms and that the increase in gastrocnemius muscle UCP3 mRNA expression by leptin occurs by a direct SNS-independent effect.

In summary, short-term leptin thermogenic effects may be mediated by an increase in skeletal muscle UCP3 mRNA expression rather than by an elevation in the expression of UCP1 in BAT. In addition, we provide some evidence that short-term leptin thermogenic effects occur by ways other than those of β_3-AR agonists, although a statistical interaction between both treatments has been obtained for rectal temperature measurements.

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