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Stimulation of uncoupling protein synthesis in white adipose tissue of mice treated with the β3-adrenergic agonist CGP-12177

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Abstract. The effects of chronic treatment with the β3-adrenergic receptor agonist CGP-12177 on uncoupling protein (UCP) synthesis in interscapular brown adipose tissue (IBAT), various white fat depots and skeletal muscle have been examined in the mouse (daily injection for 15 days at a dose of 0.5 mg/kg). The treatment increased the IBAT UCP content and led to the expression of UCP in inguinal white adipose tissue. The increase in IBAT UCP content took place in the absence of tissue hypertrophy, and despite the increase in total body UCP content, no changes in body weight were observed after the treatment. The results confirm that ectopic expression of UCP in non-BAT tissues can be induced after chronic adrenergic stimulation.

Key words. UCP; thermogenesis; CGP-12177; β3-adrenergic agonist; mice.

Mammalian brown adipose tissue (BAT) specifically functions to produce heat (thermogenesis) in order to facilitate regulation of body temperature and also to expend excess energy, hence participating in body weight regulation. A key molecule for metabolic thermogenesis is the thermogenin or uncoupling protein (UCP), a 33-kDa inner-mitochondrial membrane protein that is usually expressed only in BAT in mammals [1, 2]. UCP functions as a proton transporter, allowing the dissipation as heat of the proton gradient generated by the respiratory chain and thereby uncoupling oxidative phosphorilation [3, 4].

In response to an augmented demand on BAT for heat production (as during acclimation to the cold or to high-calorie diets), the tissue undergoes a recruitment process in which a series of events are induced, all intended to increase its total thermogenic potential, including the stimulation of UCP synthesis, mitochondriogenesis and brown adipocyte cell proliferation [2, 5]. The recruitment process in BAT is under adrenergic control, with norepinephrine released by sympathetic nerves distributed to the tissue and acting mainly through β-adrenergic receptors (β-AR) coupled to adenylyl cyclase as the main physiological regulator [5, 6]. In adipocytes, three isoforms of β-ARs are known – β1-, β2- and β3-ARs [7]. From results obtained in brown adipocytes differentiating in culture, it has been proposed that β1-AR mediates the norepinephrine-induced proliferation of preadipocytes [8], while β3-AR mediates the acute stimulatory effects of norepinephrine on UCP synthesis and thermogenic activity in mature cells [9, 10]. β3-AR is expressed predominantly, but not exclusively, on brown and white adipocytes [11]. Given the involvement of β3-ARs in lipolysis and facultative thermogenesis, and hence in energy expenditure, a considerable interest has focused on β3-selective agonists as promising antiobesity drugs.

In spite of the well-accepted view of exclusive expression of UCP in BAT, recently Nagase et al. [12] re-

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ported the induction of UCP expression in white fat pads and even in skeletal muscle of yellow KK obese mice after chronic treatment with CL316,243, a potent β3-AR agonist. The feasibility of a pharmacologically induced extension of UCP expression to non-BAT tissues raises new expectations.

Here, we selected an absolute β3-AR agonist, CGP-12177, to study its effect on UCP synthesis in interscapular BAT (IBAT) and certain non-BAT tissues of nonobese mice. CGP-12177 was originally developed as a $\beta1/\beta2$-antagonist; it was later shown to act as a β3-agonist on isolated brown fat cells and on brown fat membrane preparations [13]. CGP-12177 can therefore be considered as an absolute β3-agonist in the sense that, irrespective of the dose used, it does not activate β1- or β2-ARs, unlike other β3-agonists, which at high dose also transactivate β1- and β2-ARs. We found that mice chronically treated with CGP-12177 had increased UCP levels in IBAT, although the tissue did not undergo a complete recruitment process. The treatment also stimulated the ectopic appearance of UCP in inguinal white adipose tissue (WAT), and occasionally in the quadriceps muscle, but had no effect on body weight.

Materials and methods

Materials. ($\pm$)-CGP-12177 was obtained from RBI (Natick, MA, USA). Other reagents were supplied by Sigma, and routine chemicals used were from Merck and Panreac.

Animals. Male NMRI mice (4 weeks old, 20.6 ± 0.3 g, obtained from CRIFFA, Barcelona, Spain) were acclimated to 22 °C, with a 12 h light/12 h dark cycle and free access to a standard chow diet (Panlab, Barcelona, Spain). They were randomly assigned to two experimental groups: control animals and CGP-12177-treated animals, with four and five animals per group, respectively. The latter were given a daily subcutaneous injection of CGP-12177 (0.5 mg/kg) in 100 µl of saline; control animals were injected with saline only. After 15 days of treatment, the mice were killed with CO_2, followed by cervical dislocation. IBAT, inguinal (subcutaneous), epididymal, mesenteric and retroperitoneal WAT depots, and quadriceps and gastrocnemius muscles were rapidly removed in their entirety, weighed, frozen in liquid nitrogen and stored at -70 °C. The dose of CGP-12177 was chosen on the basis of previous reports indicating that a similar dose (0.75–1 mg/kg) was capable of eliciting a thermogenic response when acutely injected in rodents, including elevated oxygen consumption [13, 14] and increased UCP expression in IBAT [15].

Immunoblotting for UCP. Each tissue was homogenized in 8 to 10 volumes of PBS (phosphate-buffered saline: 137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer, pH 7.4) in a Teflon/glass homogenizer (10 strokes). The homogenate was centrifuged at 1500*g* for 5 min at 4 °C, and the fat-free infranant was used for protein and UCP determination. Total protein content was measured by the method of Bradford [16]. UCP was determined by immunoblotting as previously described [10]. Briefly, 30–100 µg of total protein was fractionated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE; 10% polyacrylamide) according to Laemmli [17], and electrotransferred onto a nitrocellulose membrane. Blocking and development of the immunoblot were performed using an ECL (enhanced chemiluminescence) western blotting analysis system (Amersham, Buckinghamshire, UK), following the manufacturer's instructions; rabbit polyclonal antiserum against purified rat UCP obtained in our laboratory [18] was used as primary antibody. For quantitative analysis, the bands were scanned with a BioImage computing densitometer (Millipore, Bedford, MA, USA).

Results and discussion

Intact mice were treated for 15 days with a daily dose of CGP-12177 (0.5 mg/kg). The treatment did not significantly affect food intake or body weight, which at the end of the experiment was 30.4 ± 0.7 g for treated animals and 28.4 ± 0.4 g for control animals. Thus, under the conditions used, the effects of CGP-12177 were apparently restricted to UCP (see below).

Table 1 shows total tissue weight, protein concentration and total protein content of IBAT, and the different WAT depots (inguinal, epididymal, mesenteric and retroperitoneal) and skeletal muscles (quadriceps and gastrocnemius) examined. The weight of the adipose pads was not significantly affected by the treatment, although we detected a slight decrease of IBAT and epididymal WAT weight together with a slight increase in the protein concentration in these tissues and also in the inguinal and mesenteric WAT depots of treated animals, which would suggest a relative loss of tissue lipids as an effect of the CGP-12177 treatment. The total protein content of the IBAT and WAT depots analysed, on the other hand, remained unchanged after the treatment. The lack of effect of CGP-12177 on IBAT weight and total protein content differs from the widely described hypertrophic effect of chronic cold exposition [6], norepinephrine treatment [19, 20] and treatment with other β3-AR agonists, such as CL316,243 [12].

Skeletal muscles were also examined after CGP-12177 treatment. As shown in table 1, a significant increase in the quadriceps muscle weight of treated animals was observed, with an even more marked increase in the

Table 1. Effect of CGP-12177 treatment on total tissue weight, protein concentration, total protein content and specific UCP content of mouse IBAT, WAT depots and skeletal muscles.

Tissue	Tissue weight (mg)	Protein concentration (μg/mg of tissue)	Total protein (mg/tissue)	Specific UCP
Interscapular BAT				
control	134 ± 10	46.0 ± 5.1	6.24 ± 1.00	100
CGP-treated	118 ± 5	52.8 ± 3.3	6.22 ± 0.31	287 ± 43*
Inguinal WAT				
control	253 ± 13	18.3 ± 1.3	4.64 ± 0.40	−
CGP-treated	268 ± 32	21.6 ± 0.9	5.70 ± 0.53	+
Epididymal WAT				
control	342 ± 40	9.77 ± 1.24	3.26 ± 0.36	−
CGP-treated	290 ± 20	12.6 ± 0.6	3.66 ± 0.32	−
Mesenteric WAT				
control	114 ± 31	27.2 ± 4.1	3.26 ± 1.08	−
CGP-treated	128 ± 23	29.9 ± 3.6	3.58 ± 0.36	−
Retroperitoneal WAT				
control	96.5 ± 9.3	17.7 ± 1.8	1.69 ± 0.19	−
CGP-treated	95.4 ± 24.0	17.2 ± 1.7	1.49 ± 0.18	−
Quadriceps muscle				
control	664 ± 32	40.7 ± 0.9	26.9 ± 0.8	−
CGP-treated	851 ± 25*	47.5 ± 1.8*	40.4 ± 2.2*	−/+
Gastrocnemius muscle				
control	366 ± 41	44.0 ± 1.5	16.0 ± 1.9	−
CGP-treated	408 ± 24	45.8 ± 1.6	18.7 ± 1.2	−

CGP-treated mice received a daily subcutaneous injection of CGP-12177 (0.5 mg/kg) in 100 μl of saline for 15 days ($n = 5$); control animals received saline only ($n = 4$). Animal samples were processed as described in 'Materials and methods'. Results represent means ± SEM.
*Statistically significant effects of the CGP treatment vs. control ($P < 0.05$, Student's t-test). The mean specific UCP content in IBAT in the control group was set to 100%, and the value of treated animals was expressed relative to this. For the rest of tissues, the (+) symbol indicates UCP appearance and the (−) symbol indicates that UCP was not detected in the immunoblots.

tissue total protein content. The same tendency was seen for the gastrocnemius muscle, although to a much lesser extent. The increase in muscle mass is intriguing, because muscle hypertrophy is usually attributed to a $\beta 2$-agonist effect [21], and CGP-12177 displays $\beta 1/\beta 2$-AR antagonist properties [13]. Since there is some evidence that CGP-12177 also binds to an atypical (non-$\beta 1$, non-$\beta 2$, non-$\beta 3$) β-AR in tissues from rodents and humans [22, 23], one possibility is that the observed effect could be mediated through this putative new β-AR. In any case, further investigation is needed to explain the positive effect of chronic CGP-12177 treatment on muscle mass.

As expected from its $\beta 3$-agonist condition, CGP-12177 stimulated UCP synthesis in IBAT, with treated mice having almost threefold the UCP levels of control mice (table 1 and fig. 1). Moreover, UCP was clearly detected in inguinal WAT of all CGP-injected animals, but not in that of any saline-treated animals (see the representative immunoblot in fig. 1). In addition, we detected a small amount of UCP (a weak band) in the quadriceps muscle of three out of the five animals treated with CGP-12177, but not in that of any control animals (results not shown). No signals for UCP were detected in the remainder of tissues analysed, neither in CGP-injected animals nor in control animals. The appearance of UCP in non-BAT tissues has been recently described in obese yellow mice submitted to chronic treatment with the $\beta 3$-AR agonist CL316,243 [12]. In particular, these authors found expression of UCP in different white fat pads and in skeletal muscles. They also found that nonobese animals were much less sensitive to adrenergic stimulation, with ectopic UCP expression

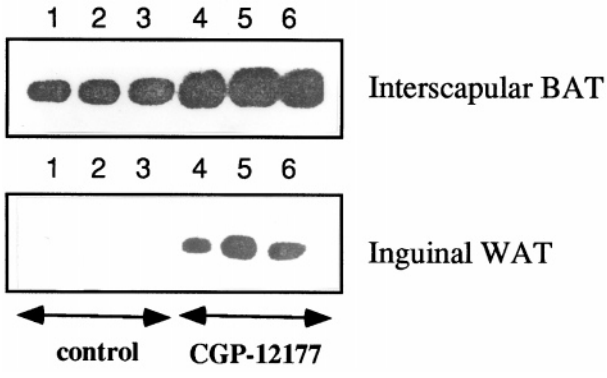

Figure 1. Immunoblot for UCP in IBAT and inguinal WAT of control and CGP-12177-treated animals. Mice were treated as indicated in table 1, and fat-free extracts containing 30 μg of protein for IBAT and 100 μg of protein for inguinal WAT were used for western blot analysis. Lanes 1–3, control animals; lanes 4–6, CGP-12177-treated animals.

restricted to inguinal WAT. Our results confirm that UCP synthesis can be induced in non-BAT tissues of non-obese animals, particularly in inguinal WAT, and show that the increased UCP levels do not necessarily correlate with IBAT hypertrophy and body weight loss.

Evidence from brown fat cell culture systems indicates that the recruitment process of BAT, which implies both proliferation and differentiation (with stimulation of UCP synthesis) of cells, involves different ARs [5, 8, 9, 10, 24]. The increase in UCP appearance after treatment of intact animals with CGP-12177 obtained here provides in vivo evidence for a major role of β3-AR in mediating norepinephrine-induced UCP synthesis, while its lack of effect on other indicators of BAT recruitment is in agreement with the involvement of other adrenoceptors in brown adipocyte proliferation. Thus, CGP-12177 was not able to induce a full trophic response in BAT, probably because this agent is devoid of $\beta1/\beta2$-agonist properties, exhibiting on the contrary $\beta1/\beta2$-antagonist properties [13]. CGP-12177 also lacks the α1-AR stimulatory properties of norepinephrine, which have been proposed to affect the expression of transcription factors and through this to influence BAT growth and cell proliferation [5].

Chronic treatment with CGP-12177 did not decrease the body weight of our lean NMRI mice. Since food intake was unaffected, the lack of body weight loss implies that, over all, no increase in metabolic rate was elicited by the treatment. At first glance, this could seem contradictory with the increased UCP content found in the CGP-12177-treated animals. It must be taken into account, however, that the metabolic rate depends on other factors, in addition to facultative thermogenesis, and that even facultative thermogenesis itself depends on other factors, in addition to the UCP content per se. In particular, when dealing with CGP-12177, the possibility exists that this agent, due to its β1-antagonist effects throughout the body, may alter oxygen supply to thermogenic tissues in such a way that it becomes limiting for full thermogenesis, especially considering that the $\beta1/\beta2$-antagonist effect of CGP-12177 is observed with very low doses compared with those needed to evoke its β3-agonist effect [14]. In fact, it has been reported that, after a single CGP-12177 injection, lean intact animals increase their oxygen consumption but lose the capability to respond to a second injection of norepinephrine [14]. The failure of our repeated CGP-12177 treatment to increase metabolic rate may be related to this refractoriness phenomenon.

In conclusion, chronic stimulation of mice with an absolute β3-AR agonist, CGP-12177, led to an increase of IBAT UCP content and to ectopic expression of UCP in inguinal WAT, without producing noticeable IBAT hypertrophy or body weight loss.

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