Modulation *in vivo* of β -adrenergic-receptor subtypes in rat brown adipose tissue by the thermogenic agonist Ro 16-8714

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The number of β_3 -adrenergic receptors (AR) in plasma membranes from interscapular brown adipose tissue (IBAT) was decreased by 62 % in lean Zucker rats treated with the thermogenic β -adrenergic agonist Ro 16-8714 as compared with controls after 72 h of treatment. The loss of β_3 -AR number was preceded by a 93 % decrease in the steady-state level of β_3 -AR mRNA at 30 h. Similar results were obtained in obese (*fa/fa*) Zucker rats. Ro 16-8714 had no effect on the number of β_1 - and β_2 -ARs in IBAT. This is the first report to demonstrate that the β_3 -AR in IBAT can be specifically down-regulated *in vivo* by exposure to a thermogenic agonist.

INTRODUCTION

Brown adipose tissue (BAT), the main effector of cold- and diet-induced thermogenesis in rodents [1,2], is under the control of the sympathetic nervous system [3], which acts through β -adrenergic receptors (β -ARs) [4]. β_1 -, β_2 - and an atypical β_3 -AR have been identified in BAT; the β_3 -AR represents the predominant β -AR subtype in this tissue [5]. As compared with β_1 - and β_2 -ARs, the β_3 -AR displays a high affinity for thermogenic agonists such as BRL 37344, and a low affinity for β -adrenergic antagonists [5,6]. Genes encoding β_2 -, β_1 - and β_3 -ARs from rats have been isolated and sequenced [7,8,5]. Recently a technique has been described which allows for quantification of the β_3 -AR relative to β_1 - and β_2 -ARs in interscapular BAT (IBAT) plasma membranes, by using [³H]CGP 12177 as a radioligand [9,10]. It is therefore now possible to measure the level of mRNA and the density of each β -AR subtype in BAT.

Desensitization of the β -AR has been the object of numerous studies. It has been shown, in particular, that long-term (several hours) exposure of various cell lines in culture to non-selective β -adrenergic agonists such as (–)-isoprenaline or to cyclic AMP analogues decreases the level of β -AR mRNA [11–14]. All data, however, are from studies on cells expressing β_2 -AR.

The possibility that an acute treatment with Ro 16-8714, which is a specific thermogenic β -adrenergic agonist [15], might desensitize rat BAT has been the object of a recent study performed on lean and obese (*fa/fa*) Zucker animals [16]. That study revealed that Ro 16-8714 has no effect in lean rats on β -AR number and on the response of adenylate cyclase to isoprenaline in the plasma membrane of IBAT. In obese animals, Ro 16-8714 had a trophic effect on IBAT, characterized by an increase in the amount of protein per tissue and an effect on the β -AR adenylate cyclase system suggestive of induction of supersensitivity [16]. In that study the effect of Ro 16-8714 treatment on the specific β -AR subtypes could not be evaluated. With the availability of DNA probes specific for three distinct β -AR subtypes, it has now become possible to study regulation of β -AR subtype expression.

The aim of the present work was to test *in vivo* the possible effect of Ro 16-8714 on β -AR subtype expression in IBAT by measuring the steady-state levels of β_1 -, β_2 - and β_3 -AR and of their corresponding mRNAs in lean Zucker rats. The effect of Ro 16-8714 treatment was also studied in obese (*fa/fa*) Zucker rats.

MATERIALS AND METHODS

Materials

All organic and inorganic chemicals were of analytical or molecular biology grade and were purchased from Merck (Darmstadt, Germany), Sigma (St. Louis, MO, U.S.A.), Fluka (Buchs, Switzerland), Gibco BRL (New York, NY, U.S.A.), Pharmacia (Uppsala, Sweden) or Boehringer (Mannheim, Germany). [³H]CGP 12177 (30 Ci/mmol) was purchased from Amersham International (Amersham, Bucks, U.K.), and [³²P]ATP (1000–3000 Ci/mmol), [α -³²P]dCTP (3000 Ci/mmol) and Gene Screen Plus membrane filters were from Du Pont–NEN (Boston, MA, U.S.A.). Ro 16-8714 was a gift from Hoffman–La Roche (Basle, Switzerland).

Animals

Female lean (*Fa/Fa*) and obese (*fa/fa*) Zucker rats (9 weeks old) were kept at room temperature (about 21 °C) with 12 h of illumination per day and fed *ad libitum* with Provimi Lacta chow (Cossonay, Switzerland). The animals were dosed orally with either Ro 16-8714 (2 μ mol = 0.87 mg/kg) in 5 % (w/v) gum arabic (5 ml/kg) or with the vehicle alone at time 0, 24 and 48 h, and killed at the indicated times. IBAT was rapidly excised and plasma membranes were prepared from the pooled IBAT of three rats as previously described [17], except that for obese-rat IBAT the fat was re-homogenized twice in 4 ml of homogenizing medium at room temperature to improve the recovery of adsorbed subcellular membranes. Protein concentrations were determined by the method of Lowry *et al.* [18]. The 5'-nucleotidase activity was measured as described previously [17]. In all the groups (lean or obese rats treated with Ro 16-8714 or with vehicle alone) the specific activity of the plasma-membrane enzyme 5'-nucleotidase, expressed per mg of plasma-membrane protein, was similar, indicating that the degree of purification of the IBAT plasma membranes was independent of the experimental conditions [16]. The tissues to be used for RNA isolation were rapidly dipped into liquid nitrogen.

Binding studies

In radioligand-binding studies, membranes were incubated for 30 min at 37 °C in 50 mM-Tris/HCl (pH 7.4)/10 mM-MgCl₂ (total volume 0.5 ml) containing GTP (50 μ M) and the indicated amount(s) of [³H]CGP 12177. The binding of the radioligand to the membranes was determined by filtration using a Brandel

Abbreviations used: BAT, brown adipose tissue; IBAT, interscapular BAT; AR, adrenergic receptor; poly(A)⁺ RNA, poladenylated RNA.

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M-24 R apparatus. The technique to measure the binding of [3 H]CGP to β_3 -AR and β_1/β_2 -AR is as follows. A concentration of radiolabelled ligand close to the K_d of the β_3 -AR is used in the absence or presence of 10 μ M BRL 37344, which displaces only the binding to β_3 -AR. Specific binding to β_3 -AR is then defined as the difference between total binding, obtained in the absence of competing ligand, and the binding obtained in the presence of BRL 37344. Specific binding to β_1/β_2 -AR subtypes in IBAT can be quantified as the difference between [3 H]CGP 12177 binding in the presence of 10 μ M BRL 37344 and in the presence of 100 μ M(-)-propanolol, a concentration sufficient to displace [3 H]CGP 12177 binding to all β -AR subtypes in IBAT membranes [10].

RNA isolation

Total IBAT RNA was isolated by the Cs^+ /trifluoroacetic acid gradient method of Okayama *et al.* [19]. Briefly, the frozen tissues were pulverized in liquid nitrogen, transferred directly to 19 ml of 5.5 M-guanidium isothiocyanate/25 mM-sodium citrate/0.5% lauryl sarcosine/200 mM- β -mercaptoethanol, pH 7.0, and homogenized with a Polytron homogenizer (setting 5) for 20 s. The homogenate was centrifuged at 350 g for 5 min and the supernatant was layered over 17 ml of Cs^+ /trifluoroacetic acid at a density of 1.5 g/ml containing 0.1 M-EDTA at pH 7.4, and centrifuged at 110000 g for 24 h. The poly(A) $^+$ RNA was isolated from the total RNA by the method of Aviv & Leder [20], using prepacked oligo(dT)-cellulose columns from Pharmacia. The yields of poly(A) $^+$ RNA varied between 2 and 4%.

RNA blot hybridization

Poly(A) $^+$ RNA (15 μ g) was electrophoresed in an agarose gel containing formaldehyde as described by Lehrach *et al.* [21] and transferred to Gene Screen Plus membranes by capillary blotting. A rat heart β_1 -AR cDNA (J. C. Venter, D. A. Robinson & C. M. Fraser, unpublished work), a rat heart β_2 -AR cDNA [7] and a rat IBAT β_3 -AR cDNA [5] were used. β_1 -AR and β_2 -AR cDNA are gifts from Dr. J. Craig Venter, Section of Receptor Biochemistry and Molecular Biology, NIH, Bethesda, MD, U.S.A. The probes were labelled by random priming with [α - 32 P]dCTP to a specific radioactivity of approx. 1×10^9 d.p.m./ μ g of DNA. The (dT) $_{12-18}$ probe obtained from Pharmacia was labelled by T4 polynucleotide kinase with [γ - 32 P]ATP. RNA blots were hybridized overnight at 60 $^{\circ}\text{C}$ in $4 \times \text{SSC}$ (0.6 M-NaCl/0.06 M-sodium citrate/100 mM- NaH_2PO_4 (pH 6.0)/0.25% SDS/5 $\times$ Denhardt's solution/10% dextran sulphate plus 100 μ g of denaturated salmon sperm DNA/ml, and then washed successively in a solution of $0.1 \times \text{SSC}$ /0.1% SDS at 55 $^{\circ}\text{C}$ and 68 $^{\circ}\text{C}$ for 15 min and exposed to Kodak X-AR films at -70 $^{\circ}\text{C}$. Size estimates of the RNA species were established by comparison with an RNA ladder. Densitometric analysis of autoradiograms was performed with a high-resolution laser-beam densitometer. Student's unpaired *t* test was used to determine statistical significance.

RESULTS

By using [3 H]CGP 12177 as a radioligand, it is possible to demonstrate the existence of two populations of binding sites in IBAT plasma membranes with K_d values of 0.64 and 31 nM [10]. It was previously shown that the low-affinity binding site represents the β_3 -AR subtype, whereas the high-affinity binding site represents the β_1 - and β_2 -AR subtypes. As shown in Fig. 1, there is a time-dependent decrease in the density of the β_3 -AR in IBAT plasma membranes after daily administration of a single dose of Ro 16-8714. At 72 h after beginning the treatment, the level of β_3 -AR in rats given Ro 16-8714 is 38% of that measured

in animals dosed with vehicle alone. In contrast, the density of the β_1/β_2 -ARs in animals given Ro 16-8714 is unchanged, or perhaps slightly increased at 30 h after beginning the treatment, as compared with controls.

Because previous studies have demonstrated that β -AR down-regulation may be associated with changes in mRNA levels [11-14], we also examined in parallel the effect of Ro 16-8714 administration on the levels of β -AR subtype-specific mRNA in IBAT in treated versus control animals. The poly(A) $^+$ blots were hybridized with the β_1 -, β_2 - and β_3 -AR and the ^{32}P -labelled (dT) $_{12-18}$ probes. As described previously [22], the densitometric-analysis values for each β -AR subtype were normalized for the actual amount of poly(A) $^+$ RNA on the membrane, which was determined by using the corresponding ^{32}P -labelled oligo(dT) signal. It was thus possible to calculate the changes in the amount of each β -AR subtype in a known amount of poly(A) $^+$ in the obese and/or treated as compared with control groups. Fig. 2 is a representative autoradiogram. It illustrates the previously described observation of a decrease in the β_3 -AR mRNA level in obese compared with lean rats [5]. The apparent increase in the β_1 -AR mRNA signal observed in obese as compared with lean rats was not reproducible. In fact, there is no significant change in the mean level of the β_1 -AR mRNA in obese as compared with lean rats [5]. Fig. 2 also shows that, 30 h after the beginning of Ro 16-8714 treatment, the β_3 -AR mRNA signal has practically disappeared and the β_2 -AR mRNA signal is decreased in both lean and obese rats. Table 1 shows the results of densitometric analysis of five or six autoradiograms. At 30 h after beginning treatment, the level of β_3 -AR mRNA is decreased by 93%, the level of β_1 -AR mRNA is unchanged and the level of β_2 -AR mRNA is decreased by 48% as compared with control animals. The amounts of plasma membrane and of total mRNA isolated from IBAT were not affected in lean rats by Ro 16-8714 treatment (results not shown).

Fig. 1. Effect of Ro 16-8714 on the density of high- (○) and low- (●) affinity [3 H]CGP 12177 binding sites (β_1/β_2 -AR and β_3 -AR respectively) in IBAT from lean Zucker rats 30 and 72 h after beginning treatment

The binding values were calculated in fmol of [3 H]CGP 12177 bound/mg of plasma membranes. The results are means $\pm$ S.E.M. of 3-4 experiments. The values obtained in Ro 16-8714-treated rats were compared with those obtained in corresponding sham-treated controls, and the results are expressed as percentages of control values: **P* < 0.05 versus respective controls.

Fig. 2. Steady-state level of β_3 -, β_1 - and β_2 -AR subtype mRNA in lean (L) or obese (*fa/fa*) (O) Zucker-rat IBAT treated for 30 h with Ro 16-8714 (+) or with vehicle alone (-)

Poly(A)⁺ RNA (15 μ g) was electrophoresed, transferred to membrane filters and hybridized with ³²P-labelled β_3 -, β_1 - and β_2 -AR rat cDNA and oligo(dT) probes. The figure shows a representative autoradiogram. The positions of marker RNAs are shown in kilobases (kb).

Table 1. Effect of Ro 16-8714 on the steady-state levels of β_1 -, β_2 - and β_3 -AR mRNA in IBAT of lean Zucker rats

Poly(A)⁺ RNA (15 μ g) was electrophoresed, transferred to membrane filters and hybridized with ³²P-labelled rat β_1 -, β_2 - and β_3 -AR cDNA and oligo(dT) probes. The rats had been treated for 30 h with Ro 16-8714 or with vehicle alone. The results are means $\pm$ S.E.M. of the numbers of experiments in parentheses. The values obtained in Ro 16-8714-treated rats were compared with those obtained in corresponding vehicle-treated controls, and the results are expressed as percentages of control values: ***P* < 0.01, ****P* < 0.001 versus respective controls.

[³² P]cDNA	Value (% of control)
β_1 -AR	116 $\pm$ 16 (6)
β_2 -AR	52 $\pm$ 9 (5)**
β_3 -AR	7.0 $\pm$ 5.0 (5)***

In obese *fa/fa* Zucker rats the effects of Ro 16-8714 treatment on the β_3 -AR were comparable with those observed in lean rats. There was a 89% (*P* < 0.001; *n* = 4) decrease in the density of β_3 -AR at 72 h that was preceded by a 98% (*P* < 0.001; *n* = 5) decrease in the level of the β_3 -AR mRNA at 30 h. There was, as in the lean rat, no significant change in the density of β_1/β_2 -AR, which was observed up to 72 h after beginning treatment. A 37% decrease (*P* < 0.05; *n* = 5) in the level of β_1 -AR mRNA and a 76% decrease (*P* < 0.005; *n* = 4) in the level of β_2 -AR mRNA were observed at 30 h.

DISCUSSION

Exposure of cultured cells to β -adrenergic agonists has been found to modulate the mRNA level of the β_2 -AR subtype [11,12]. This modulation occurs in two steps: a short-term exposure increases the level of receptor-specific mRNA, whereas continued exposure results in a gradual decline in this level [14]. The results of the present study demonstrate that administration of the thermogenic β -adrenergic agonist Ro 16-8714 to lean and obese Zucker rats induces a marked down-regulation of the β_3 -AR of the IBAT over a time course of 72 h. The loss of membrane β_3 -AR in IBAT is preceded by a comparable decrease in the steady-state level of β_3 -AR specific mRNA in this tissue. Administration of Ro 16-8714 has essentially no effect on the level of β_1/β_2 -AR in IBAT, consistent with its known selectivity for the atypical β_3 -AR which is presumed to mediate thermogenesis in this tissue.

It was observed that Ro 16-8714 administration was associated with a decrease in the β_2 -AR mRNA in lean rats. The effect of β_2 -AR mRNA may reflect some interaction between Ro 16-8714 and the β_2 -AR under the conditions of treatment *in vivo*. Alternatively, it is possible that the interaction of Ro 16-8714 with β_3 -AR may down-regulate the β_2 -AR mRNA via production of an intracellular messenger. There was no detectable decrease in the density of β_1/β_2 -AR in IBAT plasma membranes of treated animals. However, the β_1 -ARs are about 2.5-fold more abundant than the β_2 -ARs in IBAT plasma membranes [22,23]. Thus a small change in the level of β_2 -AR would be difficult to quantify.

It was recently shown that the steady-state level of β_3 -AR is decreased in obese Zucker (*fa/fa*) rats as compared with lean controls, whereas the level of β_1 - and β_2 -ARs is unchanged [5]. In the present study, the effect of Ro 16-8714 treatment was demonstrated to be comparable in lean and obese rats.

The present study represents the first report on the effect of a selective β_3 -adrenergic agonist administered *in vivo* on the expression of β -AR subtypes in IBAT. Exposure to the thermogenic agonist Ro 16-8714 selectively down-regulates the β_3 -AR. The change in β_3 -AR mRNA that precedes the loss of receptor number suggests that chronic administration of Ro 16-8714 may decrease the rate of β_3 -AR gene transcription and/or decrease the stability of β_3 -AR mRNA. Elucidation of the molecular mechanism involved in the down-regulation and the biological consequences of the decrease in the density of β_3 -ARs in IBAT require further investigation.

Analysis of the results of our previous study on the effect of Ro 16-8714 treatment on IBAT [16] and of those of the present work leads to the hypothesis that administration of the thermogenic agonist induces a transitory state of supersensitivity, at least in obese rats, followed by a specific down-regulation of β_3 -AR in both lean and obese animals. Chronic treatment with Ro 16-8714 does not seem to produce a desensitization in obese (*fa/fa*) Zucker rats, as assessed by the observation of sustained stimulation of thermogenesis [15]. It might be that the down-regulation of β_3 -AR observed in this study 72 h after beginning

treatment with Ro 16-8714 is compensated in the whole animal by the trophic effect of the drug on the BAT [16].

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