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Short communication

SR59230A blocks β_3-adrenoceptor-linked modulation of uncoupling protein-1 and leptin in rat brown adipocytes

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Abstract

Experimental evidence suggests that, by stimulating energy expenditure in brown fat, selective β_3-adrenoceptor agonists can reduce body weight in obese rodents. In order to investigate further the physiological role of β_3-adrenoceptors in brown adipocytes, we analysed the effects of selective β_3-adrenoceptor agonists and antagonists on uncoupling protein-1 and leptin gene expression in culture-differentiated brown fat cells. Our main findings were that: (i) the leptin gene is expressed in brown adipocytes; (ii) the selective β_3-adrenoceptor agonist, *N*[(2*S*)-7-carbethoxy-1,2,3,4-tetrahydronaphth-2-yl]-(2*R*)-2-hydroxy-2-(3-chlorophenil)ethanamine hydrochloride (SR58611A), inhibits leptin gene while inducing uncoupling protein-1 gene expression; (iii) these opposite effects of SR58611A are antagonized by the selective β_3-adrenoceptor antagonist, *SS*-enantiomer 3-(2-ethylphenoxy)-1-(1*S*)1,2,3,4-tetrahydronaphth-1-ylaminol]-(2*S*)-2-propanol oxalate (SR59230A), but not by the selective β_1-adrenoceptor antagonist (±)-[2-(3-carbamoyl-4-hydroxyphenoxy)-ethylamino]-3-[4(1-methyl-4-trifluoromethyl-2-imidazolyl)-phenoxy]-2 propanol (CGP20712A); and (iv) these effects are due to increased cyclic AMP levels. These results confirm by means of a different experimental approach that β_3-adrenoceptors play a central role in controlling the expression of genes that are important for brown fat function.

Keywords: Uncoupling protein-1; Leptin; Brown adipose tissue; β_3-Adrenoceptor

1. Introduction

Selective β_3-adrenoceptor agonists have been recently synthesized as potent stimulants of energy expenditure in brown fat, and therefore as useful drugs for inducing weight loss in genetically obese rodents (Ghorbani and Himms-Hagen, 1997). Of prime importance in this research field are the pharmacological criteria used to define β_3-adrenoceptor subtypes and their functional relevance. The advent of molecular biology techniques that allow the selective isolation, expression and reconstitution of receptors has simplified, if not conclusively answered these questions. One of the main problems in detecting and pharmacologically characterizing β_3-adrenoceptors has been the lack of a selective β_3-adrenoceptor antagonist, but this has been overcome by the recently described β_3-adrenoceptor antagonistic activity of a new compound, *SS*-enantiomer 3-(2-ethylphenoxy)-1-(1*S*)1,2,3,4-tetrahydronaphth-1-ylaminol]-(2*S*)-2-propanol oxalate (SR59230A) (Manara et al., 1996; Nisoli et al., 1996a), which has been shown to be active not only in rat but also in human tissues (Levasseur et al., 1995; De Ponti et al., 1996). In order to test further its usefulness as a pharmacological tool, we studied the ability of SR59230A to antagonize the β_3-adrenoceptor-linked modulation of the expression of two genes (uncoupling protein-1 (UCP1) and leptin) which are known to be involved in the thermogenic function of brown fat.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats weighing 180–200 g (Charles River, Como, Italy) were housed under standard conditions with free access to standard laboratory chow

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and water. All animal experiments were conducted in accordance with the highest standards of humane animal care.

2.2. Isolation of brown adipocytes, adipose cell culture and treatment

Brown fat precursor cells were isolated as previously described (Nisoli et al., 1996a), and 2 million cells were added to collagen-coated 50-mm diameter glass petri dishes (to avoid the wall binding of SR59230A that occurs with plastic dishes). The cells were cultivated in 2.5 ml of Dulbecco's modified Eagle's medium supplemented with 4 mM glutamine, 10% newborn calf serum, 4 nM insulin, 4 nM triiodothironine (T_3) and 10 mM HEPES with 50 IU of penicillin, 50 mg of streptomycin and 25 mg/ml of sodium ascorbate at 37°C in a water-saturated atmosphere of 6% CO_2 in air in a NAPCO 5430 incubator. The medium was exchanged with fresh prewarmed medium on day 1 (when the culture dishes were first washed), and on days 3 and 8. The confluent brown fat cells (day 8) were treated with 1 μM *N*[(2*S*)-7-carbethoxy-1,2,3,4-tetrahydronaphth-2-yl]-(2*R*)-2-hydroxy-2-(3-chlorophenil)ethanamine hydrochloride (SR58611A) alone or plus different concentrations of SR59230A for 4 h.

2.3. Reverse transcriptase–polymerase chain reaction

Total RNA was isolated from treated and untreated cells using the RNAzol method (TM Cinna Scientific, Friendswood, TX). The RNAs were treated for 1 h at 37°C with six units of RNAse-free DNAse I/μg RNA in 100 mM Tris–HCl, pH 7.5, and 50 mM $MgCl_2$ in the presence of 2 units/μl of placenta RNAse inhibitor. One microgram of total RNA was reverse-transcribed with 200 units of Moloney murine leukemia virus reverse transcriptase (Promega) in 20 μl of buffer containing 0.4 mM dNTPs, 2

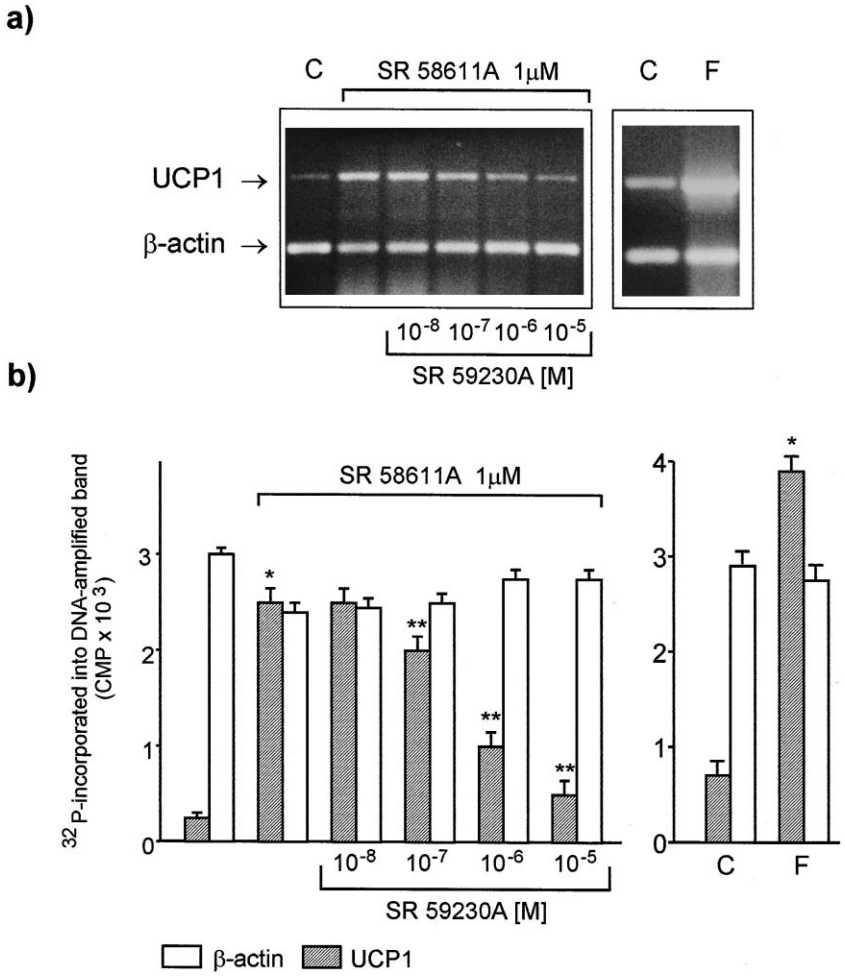

Fig. 1. Effect of SR59230A on SR58611A-stimulated UCP1 mRNA synthesis in cultured brown adipocytes. (a) Representative agarose gel showing PCR analysis of the UCP1 and β-actin mRNA from brown adipocytes treated or not with 1 μM SR58611A alone, or with 1 μM SR58611A plus different concentrations of SR59230A. In addition, the effects of 1 μM forskolin (F) on UCP1 mRNA synthesis in comparison with untreated cells (C) are shown. (b) Quantitation of the drug-induced changes in UCP1 mRNA as described in Section 2. $* P < 0.01$ vs. untreated cells; $** P < 0.01$ vs. SR58611A alone-treated cells.

units/ml RNAse inhibitor, 0.8 μg oligo$(dT)_{15}$ primer (Promega), and [^{32}P]dCTP. The resulting cDNA was quantified by determining the amount of radioactivity incorporated into trichloroacetic acid-precipitable nucleic acid. A control without reverse transcriptase was run for each sample to verify that the amplification was not due to residual genomic DNA. Polymerase Chain Reaction was performed using *Taq* DNA polymerase (Promega) in 25 μl of standard buffer (10 mM Tris–HCl, pH 9, 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$ and 200 μM dNTPs) containing 10 ng of cDNA from the preparations and 40 pmol of each sense- and antisense-specific oligonucleotide primer in the presence of trace amounts of radioactive dCTP. For leptin mRNA analysis, 4% deionized formamide and 4% glycerol were added to the Polymerase Chain Reaction mixture. The primer sequences were: 5′-GTGAGTTCGACAACTTCCGAAGTG-3′ and 5′-CATGAGGTCATATGTCACCAGCTC-3′ (amino acids 1–197) for UCP1 (Bouillaud et al., 1986), and 5′-CACCAAAACCCTCATCAAGAC-3′ and 5′-AGCCTGCTCAGAGCCACCACC-3′ (nucleotides 90–449) for leptin (Murakami and Shima, 1995). The primers for β-actin were as described by Gaudette and Crain (1991). Both UCP1 and leptin were amplified by using thirty cycles at 94°C for 30 s, 62°C for 30 s and 72°C for 40 s, followed by 7 min final extension at 72°C. After amplification, 10 μl of the reaction mixture was separated by electrophoresis (1.2% agarose gel in Tris–acetate–EDTA buffer) and visualized by ethidium bromide staining. β-Actin cDNA was coamplified in the same test tube with UCP1 or leptin cDNA, and the visualized bands were excluded. The radioactivity incorporated into the DNA-amplified fragments was Cerenkov counted and expressed as UCP1/β-actin or leptin/β-actin ratios.

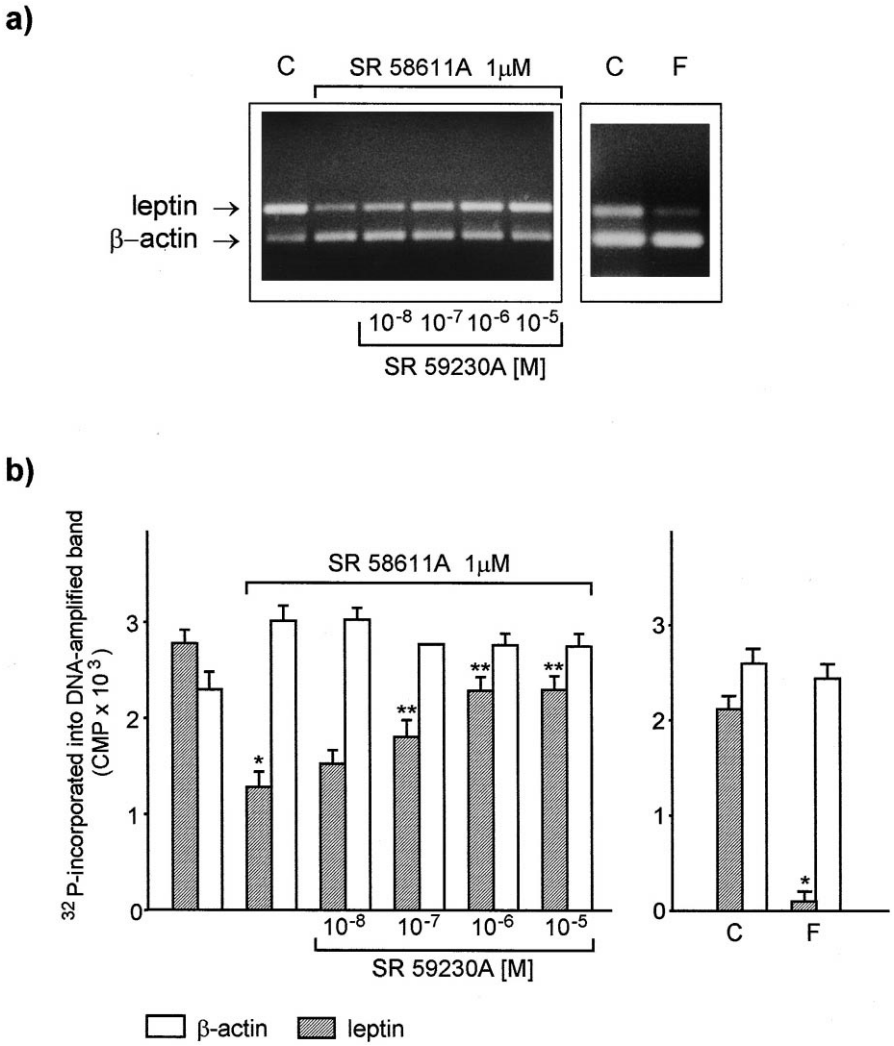

Fig. 2. Effect of SR59230A on SR58611A-inhibited leptin mRNA synthesis in cultured brown adipocytes. (a) Representative agarose gel showing PCR analysis of the leptin and β-actin mRNA from brown adipocytes treated or not with 1 μM SR58611A alone, or with 1 μM SR58611A plus different concentrations of SR59230A. In addition, the effects of 1 μM forskolin (F) on leptin mRNA synthesis in comparison with untreated cells (C) are shown. (b) Quantitation of the drug-induced changes in leptin mRNA as described in Section 2. $* P < 0.01$ vs. untreated cells; $** P < 0.01$ vs. SR58661A alone-treated cells.

3. Results

The addition of 1 μM SR58611A to culture-differentiated brown adipocytes induced an intense UCP1 gene expression that peaked when the cells were nearly confluent. Exposure of confluent cells to SR59230A for 4 h concentration dependently antagonized this SR58611A-induced increase in UCP1 mRNA levels (Fig. 1A). In contrast, 100 nM (±)-[2-(3-carbamoyl-4-hydroxyphenoxy)-thylamino]-3-[4(1-methyl-4-trifluoromethyl-2-imidazolyl)-henoxy]-2 propanol (CGP20712A), the selective β_1-adrenoceptor antagonist, did not modify the effect of SR58611A (^{32}P-incorporated cpm into UCP1-amplified bands were: untreated cells, 100 ± 12, 1 μM SR58611A-treated cells, 2800 ± 28, and 1 μM SR58611A plus 100 nM CGP20712A-treated cells, 2750 ± 31). A primer pair based on the published β-actin sequence was added to each Polymerase Chain Reaction tube to allow structural protein mRNA co-amplification, which generated a 241-bp fragment. β-actin mRNA was measured as an internal control of the amount of starting cDNA template because some authors have reported that, under selected experimental conditions, the yield of the reverse-transcribed Polymerase Chain Reaction-amplified DNA fragment linearly correlates with the amount of the original RNA template (Srivastava et al., 1992). The radioactivity of the different DNA fragments, obtained after Polymerase Chain Reaction amplification of cDNA with primers selective for each target gene can therefore be taken as an index of steady-state mRNA levels (see Fig. 1B).

Fig. 2A shows that the exposure of confluent brown fat cells to 1 μM SR58611A for 4 h induced a marked decrease in leptin mRNA levels, whereas SR59230A concentration dependently antagonized this effect (see Fig. 2B). CGP20712A at 100 nM did not modify this inhibitory effect of the β_3-adrenoceptor agonist (^{32}P-incorporated c.p.m. into leptin-amplified bands were: untreated cells, 2900 ± 24, 1 μM SR58611A-treated cells, 1200 ± 30, and 1 μM SR58611A plus 100 nM CGP20712A-treated cells, 1250 ± 19). Although Bianchetti and Manara (1990) have reported that SR58611A at micromolar concentrations can stimulate β_2-adrenoceptors in rat colon preparations, we decided to perform our Reverse Transcriptase–Polymerase Chain Reaction experiments by using 1 μM SR58611A to obtain clear results on the two studied mRNA species. Indeed, it is well known that β_2-adrenoceptors are the least expressed β-adrenoceptor subtype in brown adipocytes (Collins et al., 1994) and a vast literature demonstrates that UCP1 and leptin gene expression are modulated by noradrenaline mainly through by β_3- but not β_2-adrenoceptors (Nisoli et al., 1996a; Moinat et al., 1995).

In order to determine whether the observed effects were due to increased cAMP levels, the confluent cells were exposed for 4 h to 1 μM forskolin, the ubiquitous cAMP stimulator. Fig. 1 and Fig. 2 show that forskolin induced an increase in UCP1 and a decrease in leptin mRNA levels, thus suggesting that cAMP plays a significant role in modulating the expression of these genes. As expected SR59230A, which acts at receptor level, did not antagonize the postreceptor effects of forskolin (data not shown).

4. Discussion

The β_3-adrenoceptor is mainly expressed in brown and white adipose tissues; its stimulation in brown fat leads to increased adenylyl cyclase activity, and thus to cAMP accumulation, protein kinase A activation, lipid breakdown and UCP1 expression and activity (Nisoli et al., 1996a). Although β_3-adrenoceptor expression and thermogenic brown fat function are markedly impaired in obese rodents (Collins et al., 1994), it is well known that prolonged injection of selective β_3-adrenoceptor agonists can stimulate energy expenditure in their brown adipocytes. The β_3-adrenoceptor is thus a valuable pharmacological target for the treatment of obesity (Strosberg and Pietri-Rouxel, 1996).

One of the main problems in detecting and pharmacologically characterizing the β_3-adrenoceptor has been the lack of a selective β_3-adrenoceptor antagonist (Nisoli and Carruba, 1997), but this has been overcome by the advent of a new compound, SR59230A (Nisoli et al., 1996a). In order to investigate the selective properties of this drug, we used it to challenge the β_3-adrenoceptor-mediated effects on UCP1 and leptin gene expression in brown fat cells.

The expression of leptin is apparently controversial, but there is no doubt that brown fat and cultured brown adipocytes synthesize and release the hormone (Moinat et al., 1995; Nisoli et al., 1996b). However, Cinti et al. (1997) immunohistochemically showed that leptin is produced only by the adipocytes that are morphologically reminiscent of their mature white counterparts (i.e., with the appearance of only monolocular lipid droplets). Our findings confirm that leptin mRNA is expressed in cultured brown adipocytes and that this expression is decreased by treatment with SR58611A. They also show that SR59230A antagonizes the decrease of SR58611A-induced leptin mRNA. Moreover, SR58611A (as well as noradrenaline) can induce UCP1 gene expression, and this effect is also completely antagonized by SR59230A.

Since forskolin mimics the effects of β_3-adrenoceptor stimulation on UCP1 and leptin gene expression, it is clear that these events are mediated by cAMP accumulation in brown adipocytes.

Leptin may have autocrine or paracrine functions in brown fat, as well as food-intake modulating functions in the central nervous system (Levin et al., 1996). Indeed it has been recently demonstrated that in *ob/ob* mice brown fat the expression of uncoupling protein-3 and UCP1 is low and increases with leptin treatment (Gong et al., 1997).

Cohen et al. (1996) have suggested that leptin secretion may be involved in obesity-linked insulin resistance, because it antagonizes the insulin signaling pathway by decreasing the insulin-induced tyrosine phosphorylation of insulin receptor substrate-1. The observation that selective β_3-adrenoceptor stimulation decreases leptin gene expression may therefore help to explain the improvements in insulin resistance obtained with β_3-adrenoceptor agonists, regardless of any weight reduction. Furthermore, Gettys et al. (1996) have reported that β_3-adrenoceptor agonists can antagonize insulin-stimulated leptin release, suggesting that insulin and β_3-adrenoceptors may represent opposing arms of a regulatory system controlling leptin production in rat adipocytes.

Since SR59230A also acts in human tissues and cells, our results suggest that it may be a useful tool for studying the pharmacological profile of thermogenic compounds that are active in humans.

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