Evidence from in vitro differentiating cells that adrenoceptor agonists can increase uncoupling protein mRNA level in adipocytes of adult humans: an RT-PCR study

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Abstract In vivo data have suggested that adrenergic signals can reactivate dormant brown adipocytes in adult humans. We report here a system based on primary cultures of perirenal adipocytes from human adults and reverse transcription-PCR of uncoupling protein mRNA. Norepinephrine and compounds classified as β3-adrenoceptor agonists in rodents increased uncoupling protein mRNA level in human adipocytes (presumably brown adipocytes). Although we did not demonstrate that the observed effect was mediated by β3-adrenoceptors, it is proposed that this system could be used to appreciate the ability of β-adrenoceptor agonists to activate UCP gene transcription and help to select β3-adrenoceptor agonists and antagonists prior to in vivo trials; indeed, the difficulty in developing such drugs that are effective in humans may result from the fact that the screening of molecules has historically been made in rodents.—**Champigny, O., and D. Ricquier.** Evidence from in vitro differentiating cells that adrenoceptor agonists can increase uncoupling protein mRNA level in adipocytes of adult humans: an RT-PCR study. *J. Lipid Res.* 1996. **37:** 1907–1914.

Supplementary key words obesity • thermogenesis • polymerase chain reaction • brown adipose tissue • adrenergic agonist

The major site of thermogenesis in rodents is the brown adipose tissue (BAT), characterized by the presence in the inner mitochondrial membrane of the uncoupling protein (UCP), which uncouples respiration from phosphorylation and leads to the dissipation of energy as heat. This thermogenic function of BAT is under control of catecholamines acting through adrenoceptors (1). Though in large mammals (ovines, bovines, dogs, humans) BAT is present in neonates (2, 3) and hardly detectable in adults, the presence of large amount of UCP has been shown in perirenal adipose tissue of adult human patients with phaechromocytoma (2, 4). UCP gene expression can therefore be induced in adipose tissue of adult man in certain conditions. Moreover, studies on Finnish outdoor workers (5), and recent studies, made either on adult patients with various pathologies or alcohol consumers (6–8), indicate that, in fact, a low but significant amount of BAT does exist in human adults.

During the past 10 years, the interest of several pharmaceutical groups has focused on β-adrenoceptor agonists that mimic the action of noradrenaline and could be useful in stimulating UCP in brown adipocytes and increasing energy expenditure in obese patients. These drugs have first been tested on animal models (mainly mice and rats, more rarely dogs or lambs). Unfortunately, these drugs often proved to have undesirable side effects on the cardiovascular system and other tissues associated with agonist activity at β_1 and β_2-adrenoceptors. Recent research on β-adrenergic receptors developed these last years led to the characterization of a new adrenoceptor called β_3, essentially present in adipocytes (9–14). This research induced the development of several specific β_3 adrenoceptor agonists that proved to be efficient in selectively stimulating UCP synthesis and BAT thermogenic activity in animal models, without any side effect on heart rate (9–14).

There are discrepancies between the results obtained in animal and human studies. The D7114 β_3-adrenoceptor agonist (15) proved to be efficient in selectively stimulating BAT in rats (16) and reactivating nascent BAT in adult dogs (17), but it failed to maintain UCP expression in adipose tissue of newborn lambs (18) and

Abbreviations: UCP, uncoupling protein; BAT, brown adipose tissue; Rt-PCR, reverse-transcription polymerase chain reaction.

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to increase lipolysis in humans (19). Moreover, this compound was shown to be a β_3-adrenoceptor antagonist in rat isolated ileum (20) and its acid metabolite is a partial agonist of lipolysis mediated by the rat β_3-adrenoceptor (21). The existence of functional β_3-adrenoceptors in human fat is vigorously debated (10, 12–14, 19, 22–36). It is therefore important to test the efficiency of new drugs in humans. In vitro assays are a useful step before in vivo experiments; usually they consist in measurement of lipolysis, but this does not give any information on the ability of a compound to increase UCP mRNA level or promote UCP synthesis. In this respect, cell culture can be a useful tool. As no human preadipocyte cell line has yet been established, primary culture of preadipocytes issued from different human deposits that may contain a few brown adipocytes (perirenal or omental depot) has been used in this study. As brown adipocytes represent a very small proportion in comparison with white adipocytes in such cultures, UCP mRNA was assayed using polymerase chain reaction of mRNA previously transcribed into DNA using reverse transcriptase (RT-PCR).

The aim of the present study was to obtain a good differentiation of human preadipocytes in primary culture, and to check this system as a tool to analyze the response of the human UCP gene to adrenoceptor agonists.

MATERIALS AND METHODS

Cell isolation and culture

Adipose tissue samples were obtained from different adipose depots of human adult patients undergoing abdominal surgery in agreement with National Ethics Committee regulation. These samples were kept at room temperature in phosphate-buffered saline (PBS) containing 20 mg/ml of bovine serum albumin (BSA) until transfer to the laboratory. They were then repeatedly rinsed in PBS in order to remove blood, and carefully dissected to discard fibrous material and blood vessels. Samples from a same depot were then put into a dish containing culture medium and weighed.

The tissue was then cut into small pieces and digested in Krebs-Ringer buffered with 100 mM HEPES and containing glucose 0.9 mg/ml, BSA 15 mg/ml, and collagenase 1.5 mg/ml. The ratio of digestion solution to adipose tissue mass was usually around 5 ml/1 g. Digestion was carried out for 30 to 45 min at 37°C. The digested tissue was filtered onto a 250-nm nylon filter to discard nondigested pieces. The suspension was allowed to stand for 10 min at room temperature for decantation of mature adipocytes, which were then discarded by aspiration. The remaining cell suspension was filtered onto a 60-nm nylon filter and cells were pelleted by centrifugation, 10 min at 800 *g*. The numerous red blood cells were eliminated by incubation with erythrocyte lysing buffer for 10 min at room temperature, as described by Hauner et al. (37). Stromal vascular cells were then pelleted, washed twice with culture medium, and counted in a Malassez cell.

Cells were inoculated into sterile dishes at a density of 10,000 to 15,000 cells/cm^2. Culture medium was a mixture of Dulbecco modified Eagle's medium (DMEM)/Ham's F12 nutritive medium (1:1; v/v) supplemented with biotin 0.016 mM, pantothenic acid 0.018 mM, glutamine 5 mM, glucose 16 mM, HEPES 15 mM, penicillin 100 U/ml, streptomycin 50 mg/ml, and ascorbate 100 mM. Fetal calf serum (FCS 10%) was added to this medium on day 0 of culture. On day 1 the dishes were washed twice with PBS and filled with the above medium supplemented with insulin 510 nM, transferrin 120 μM, and triiodothyronine 0.2 nM. This serum-free chemically defined medium was referred to as ITT medium, according to Deslex, Négrel, and Ailhaud (38). Dexamethasone 100 nM and isobutylmethylxanthine (IBMX) 500 mM were added to the medium to enhance adipose differentiation. On day 4 cells were refed with ITT medium supplemented with dexamethasone alone. On day 6 cells began to differentiate, as judged by the appearance of lipid droplets. There was no other change of medium. Differentiation proceeded gradually until day 11, when cells were usually collected. The degree of differentiation, estimated through counting of cells containing lipid droplets, varied between 40 and 80%.

Cells were usually treated on day 11 or 12 and collected 4 h later. Different β_3-agonists were tested, in comparison with dibutyryl cyclic AMP 1 mM or norepinephrine 10 μM. The β_3-adrenoceptor agonists used here were D2079 and D7114 (Zeneca Pharmaceuticals), CGP 12177 (Ciba-Geigy), BRL 37344 (SmithKline Beecham), and CL 316,243 (Wyeth Ayerst-American Cyanamid). Cells were washed twice with PBS and either frozen at -20°C or harvested directly in the lysis buffer.

RNA analysis and RT-PCR analysis

Total RNA was extracted by the hot phenol method. For reverse-transcription polymerase chain reaction (RT-PCR), 10 to 400 ng total RNA was treated with 200 units of Maloney murine leukemia virus reverse transcriptase (Gibco BRL) in 20 μl of PCR buffer (Tris-HCl 20 mM, pH 8.4; 50 mM KCl; 2.5 mM $MgCl_2$; 0.1 mg/ml bovine serum albumin) containing 0.4 mM each dNTP; 50 mM dithiothreitol, 1 mg oligo-dT (25, 39). A control without reverse transcriptase was done. After synthesis of cDNAs (42°C for 50 min), human UCP cDNA was amplified by 30 cycles of temperature (95°C 30 sec, 76°C 10 sec, 58°C 30 sec, 62°C 10 sec, 68°C 10 sec, 72°C 30

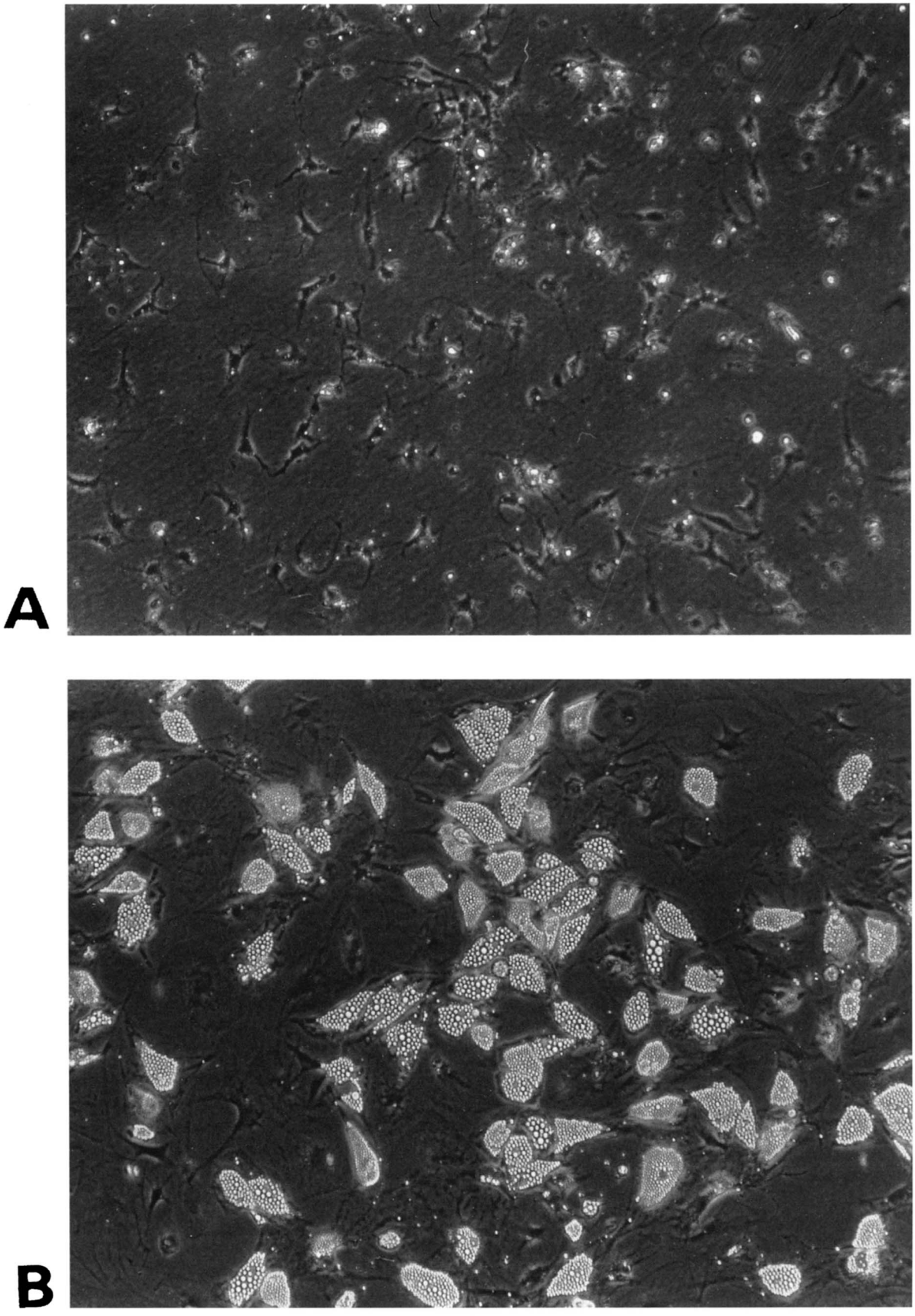

Fig. 1. Morphology of in vitro differentiating human adipocytes cultured in serum-free medium. A: Precursor cells of fibroblastic type at day 4 of culture. B: Differentiated adipocytes at day 11 of culture (magnification: ×700).

sec) followed by 5 min extension at 72°C in a temperature cycler (Hybaid) in 100 µl of prewarmed PCR buffer containing 2.5 unit of *Thermophylus aquaticus* polymerase (Perkin Elmer-Cetus), 140 nM each sense and anti-sense oligonucleotide primers, and 400 µM each dNTP.

Amplification of cDNA was linear up to 600 ng initial RNA for UCP. In parallel to UCP cDNA amplification, β-actin cDNA was also amplified. Sequences of the sense and antisense oligonucleotides were respectively: 5′-TAGGTATAAAGGTGTCCTGG-3′ and 5′-CACTTTTGTACTGTCCTGGTGG-3′ for UCP (amino acid 53 to 249), 5′-CGACGAGGCCCAGAGCAAGC-3′ and 5′-CCAGGGCGACGTAGCACAGC-3′ for actin (amino acid 55 to 221). Using these primers, the length of the fragments, calculated from the structure of the corresponding genes, were 590 and 500 base pairs for UCP (40) and actin, respectively. PCR products were directly sequenced using sense and antisense oligonucleotides.

PCR products (10 µl aliquots) were visualized by electrophoresis through 1.5% agarose gels. Gels were blotted to nylon membranes that were hybridized to a human UCP probe (41). Filters were washed 2 × 15 min in 2 × SSC (1 × SSC is 150 mM NaCl and 15 mM sodium citrate), 0.1% SDS at 65°C and 2 × 10 min in 0.1 SSC, 0.1% SDS. Membranes were then exposed for 2 to 16 h to NIF RX-100 (Fuji) films with intensifying screens.

RESULTS

Most of the experiments were carried out with precursor cells isolated from perirenal adipose tissue of patients with Conn disease (primary hyperaldosteronism), or kidney cancer or phaeochromocytoma. Cells from other depots (omental, perivesical, epiploic, and even subcutaneous) were also successfully cultivated. The efficiency of differentiation, as judged by the presence of lipid droplets in the cells, varied between 40 and 80%, depending on the experiment (**Fig. 1**).

RNA was prepared from in vitro differentiated adipocytes treated with different adrenoceptor agonists. To check the linearity of the detection of UCP mRNA by RT-PCR, an increasing amount of RNA was used. A good linearity was obtained using 20 to 600 ng RNA. Therefore, in all cultures, RT-PCR was carried out using 50, 100, and 200 ng adipocytes RNA (**Fig. 2**). Using these conditions, a 590 bp band corresponding to the expected size (not observed in absence of reverse transcriptase) was obtained (Fig. 2). An induction of UCP mRNA by β_3-adrenoceptor agonists was observed (Fig. 2, **Fig. 3,** and **Fig. 4**) whereas actin mRNA was not induced (Fig. 4). Actually, the effect of D 2079 compound (41) on UCP mRNA level was only observed at high doses (10^{-4} and 10^{-5} M, Fig. 2) and this compound was almost ineffective at 10^{-6} or 10^{-7} M, whereas a higher induction was observed with low dosage (10^{-7} M) of CGP 12177 and BRL 37344 (Fig. 3 and **Table 1**). Remarkedly, when using 20 ng or 50 ng RNA prepared from 1-month-old baby BAT (Figs. 3 and 4), a strong signal was obtained that was several fold stronger than the signal obtained with 200 ng RNA from in vitro differentiated and stimulated adipocytes. We also tested CL 316,243, which is a new and highly specific β_3-adrenoreceptor agonist in rodents (11). This compound was shown to be a strong activator

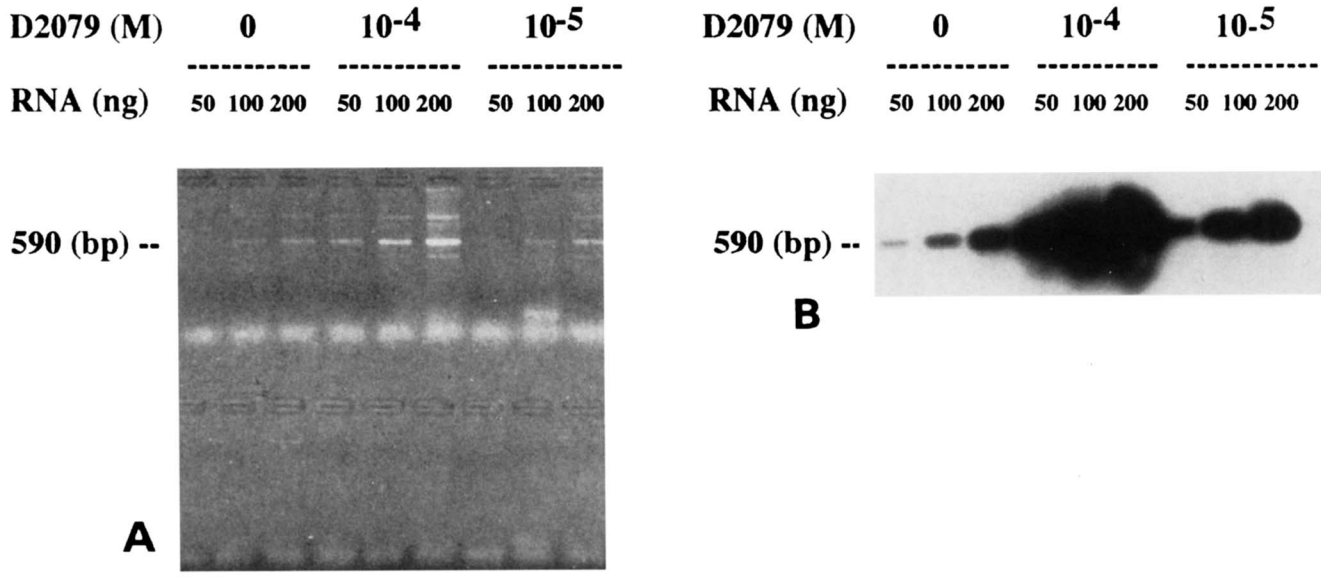

Fig. 2. Stimulation by D 2079 of UCP mRNA expression in human adipocytes differentiated in vitro. In vitro differentiated adipocytes were untreated or treated with 10^{-4} or 10^{-5} M D 2079 for 4 h. RNA (50, 100 or 200 ng) was then analyzed by RT-PCR. A: panel displays ethidium bromide staining of agarose gel electrophoresis of PCR products; RNA was treated (upper part) or non-treated (lower part) with reverse transcriptase. B: panel displays corresponding Southern blot hybridized to human UCP probe.

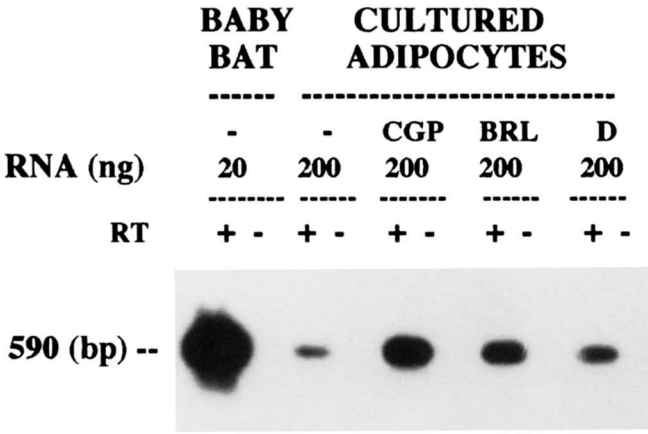

Fig. 3. Effects of different β_3-adrenoceptor agonists on UCP mRNA expression in human adipocytes differentiated in vitro. PCR samples (590 bp) obtained from RNA treated (RT+) or untreated (RT-) with reverse transcriptase were transferred onto nylon membrane and hybridized to human UCP probe. Before amplification by PCR, human UCP cDNA fragments were synthesized from 20 ng RNA of baby brown adipose tissue (BABY BAT) or 200 ng RNA of in vitro differentiated adipocytes (CULTURED ADIPOCYTES). Before RNA extraction, adipocytes were untreated or treated for 4 h with 10^{-7} M CGP 12177 (CGP), 10^{-7} M BRL 37344 (BRL), or 10^{-5} M D 2079 (D).

and inducer of mouse BAT and UCP (11). At the concentration of 10^{-6} M, CL 316,243 increased UCP mRNA but was less active than BRL 37344 (Fig. 4).

Table 1 summarizes the different attempts to increase UCP mRNA level in in vitro differentiated adipocytes cultured from human fat from adults. Obvious UCP mRNA induction was systematically observed when cells were treated either with dibutyryl cAMP or norepinephrine. In all cases BRL 37344 was active, excepted in one experiment in which it was used at 10^{-8} M. In 16 experiments out of 19, D 2079 (at high concentration) was able to increase UCP mRNA level. Only two experiments were carried out using CGP 12177 or CL 316,243, respectively, but they were all positive. It was observed that CGP 12177 was the more efficient for increasing UCP mRNA level.

DISCUSSION AND CONCLUSION

We found no difference in response between tissue from phaeochromocytoma patients and those with kidney cancer or Conn disease, except that the UCP mRNA level was higher in cells cultured from patients with phaeochromocytoma. In one case, we could detect the UCP mRNA using Northern blotting analysis of RNA of cells from a patient operated for a phaeochromocytoma. In fact, in most experiments, the requirement of RT-PCR was imposed by the low level of UCP mRNA in in vitro differentiated adipocytes from adult humans. As the UCP gene is believed to be uniquely transcribed in brown adipocytes, the very weak level of UCP mRNA in cultured adipocytes, which are essentially white adipocytes, can be explained by a very small number of brown adipocytes present in culture dishes. We tried, but failed, to detect the UCP using immunohistochemistry, to see whether, as expected, UCP expression was at reasonable levels in a small number of cells or was at a very low level in many cells. In comparison with infant brown adipose tissue, the cultured cells expressed 100- to 500-fold less UCP mRNA. There is presently no other system to assay UCP induction in human brown adipocytes, as primary culture from infant brown adipose tissue is not feasable and no human cell line is available. Nevertheless, the semi-quantitative RT-PCR method used here seems to be valuable.

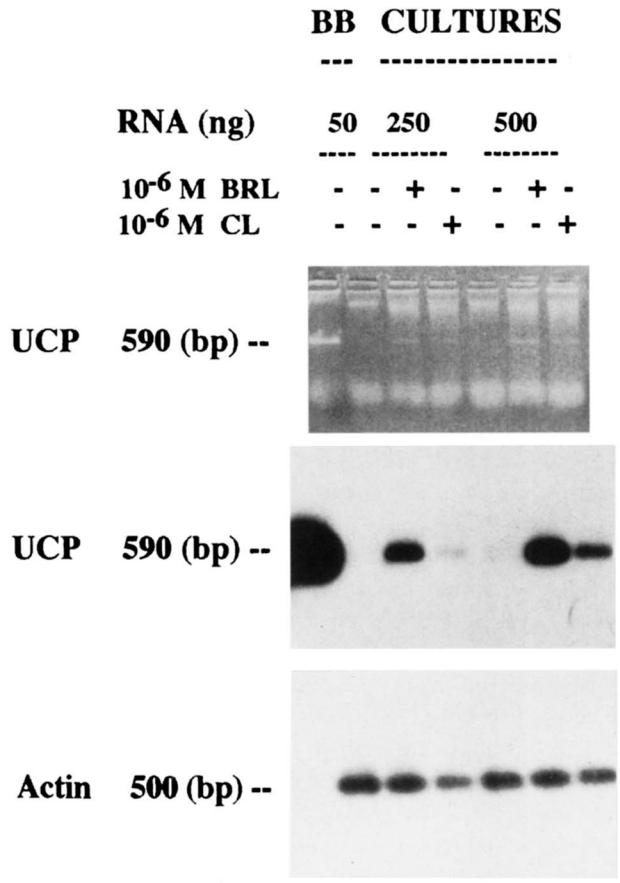

Fig. 4. Stimulation by BRL 37344 or CL 316,243 of UCP mRNA expression in human adipocytes differentiated in vitro. Adipocytes (CULTURES) were untreated or treated with 10^{-6} M agonist for 4 h and both UCP mRNA and actin mRNA levels were measured by RT-PCR analysis of 250 or 500 ng RNA. Upper part of the figure shows ethidium bromide staining of 590 bp UCP mRNA-PCR product; central part of the figure corresponds to Southern analysis of PCR product using human UCP DNA as a probe; the lower part of the figure is the Southern analysis of the 500 bp actin mRNA-PCR product from culture cells. The UCP PCR product from 50 ng baby brown fat (BB) is shown; actin cDNA from baby brown fat was not amplified.

TABLE 1. Summary of experiments

Compound	Number of Experiments	Number of Experiments Showing UCP mRNA Level Increase
Dibutyryl cAMP, 10^{-3} M	5	5
Norepinephrine, 10^{-5} M	4	4
BRL 37344		
10^{-5} M	1	1
10^{-6} M	5	5
10^{-7} M	5	5
10^{-8} M	2	1
D7114, 10^{-6} M	1	1
D2079		
10^{-4} M	8	8
10^{-5} M	7	6
10^{-6} M	3	2
10^{-7} M	1	0
CGP 12177, 10^{-7} M	2	2
CL 316,243, 10^{-6} M	2	2

Human adipocytes differentiated in vitro were incubated with dibutyryl cAMP or norepinephrine, or the respective β_3-adrenoceptor agonists for 4 h before RNA extraction and UCP mRNA analysis.

The objective of the present work was neither to make a detailed pharmacological analysis of β-adrenoceptors present at the surface of human adipocytes nor to really compare the different β_3-adrenoceptor-agonists presently available. A complete functional characterization of β_3-adrenoceptor subtype involved in thermogenesis in human adipocytes remains to be done. Actually, the goal of this study was to test the capacity of available β_3-adrenoceptor-agonists to increase UCP mRNA level in human adipocytes, similar to what is known in rodent brown adipocytes. Clearly, the data presented here demonstrate that all tested β_3-adrenoceptor-agonists can increase UCP mRNA level. However, some agonists were active at low concentration whereas other agonists required high concentrations. Therefore, it is concluded that compounds classified as β_3-adrenoceptor-agonis ts, on the basis of their effects on rodent cells, increase UCP mRNA in human brown adipocytes as they do in animals, although this first study did not demonstrate which subtype(s) of adrenoceptor was recruited by the agonists used. The positive effects of adrenoceptor agonists observed here on the level of UCP mRNA indicate that UCP gene transcription and brown adipocytes can be reactivated in humans. However, they do not mean that such drugs can really activate thermogenesis in normal or obese humans. For instance, it has been reported that D2079, which was active at high concentration in our system, failed to activate thermogenesis in obese humans treated for 2 weeks (41); similarly, D7114 had no significant effect on energy expenditure in lean men over 14 days (42). In other respects, the positive effects of BRL 37344 and CGP 12177 at low concentration on UCP mRNA level noticed here are in agreement with their stimulatory action on lipolysis in human adipocytes or energy expenditure of non-human subjects (10, 19, 24, 29, 30, 36). CL 316,243, active here, is a potent activator of rodent brown adipose tissue thermogenesis (11, 43) and a selective partial agonist of lipolysis in human adipocytes (19). The weak development of β_3-adrenoceptor agonists active in humans results, at least in part, from the fact that drugs were selected on the basis of their capacity to trigger thermogenesis in rodents. In other respects, it was recently reported that no β_3-adrenoceptor agonist was able to stimulate lipolysis in white or brown adipose tissue from young baboons (44). Interestingly, Wheeldon, McDevitt, and Lipworth (45) and Liu et al. (46) have recently obtained data that support a significant contribution of β_3-adrenoceptor activation to isoprenaline- or ephedrine-induced thermogenesis in humans.

We conclude that adrenergic agonists can directly induce UCP mRNA in adipocytes of adult humans and also propose that primary cultures of human adipocytes can be used to assay the ability of new adrenoceptor-agonists to increase UCP mRNA in humans, prior to in vivo trials.

We express our gratitude to Dr. Jean-Marc Duclos (Hôpital Saint-Joseph, Paris) for his constant help in providing us with adipose tissue biopsies. We also thank Dr. Brian Holloway for the gifts of D7114 and D2079, Drs. Cawthorne and Arch for the gift of BRL 37344, and Drs. Claus and Danforth for the gift of CL 316,243. Our research was supported by the Centre National de la Recherche Scientifique, Direction des Recherches, Etudes et Techniques, Association de Recherche sur le Cancer and by Zeneca-Imperial Chemical Industry.

Manuscript received 5 March 1996 and in revised form 29 May 1996.

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