Multilocular fat cells in WAT of CL-316243-treated rats derive directly from white adipocytes

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Multilocular fat cells in WAT of CL-316243-treated rats derive directly from white adipocytes. Am J Physiol Cell Physiol 279: C670–C681, 2000.—Multilocular, mitochondria-rich adipocytes appear in white adipose tissue (WAT) of rats treated with the β3-adrenoceptor agonist, CL-316243 (CL). Objectives were to determine whether these multilocular adipocytes derived from cells that already existed in the WAT or from proliferation of precursor cells and whether new mitochondria contained in them were typical brown adipocyte mitochondria. Use of 5-bromodeoxyuridine to identify cells that had undergone mitosis during the CL treatment showed that most multilocular cells derived from cells already present in the WAT. Morphological techniques showed that at least a subpopulation of unilocular adipocytes underwent conversion to multilocular mitochondria-rich adipocytes. A small proportion of multilocular adipocytes (~8%) was positive for UCP1 by immunohistochemistry. Biochemical techniques showed that mitochondrial protein recovered from WAT increased 10-fold and protein isolated from brown adipose tissue (BAT) doubled in CL-treated rats. Stained gels showed a different protein composition of new mitochondria isolated from WAT from that of mitochondria isolated from BAT. Western blotting showed new mitochondria in WAT to contain both UCP1, but at a much lower concentration than in BAT mitochondria, and UCP3, at a higher concentration than that in BAT mitochondria. We hypothesize that multilocular adipocytes present at 7 days of CL treatment have two origins. First, most come from convertible unilocular adipocytes that become multilocular and make many mitochondria that contain UCP3. Second, some come from a cell that gives rise to more typical brown adipocytes that express UCP1. Uncoupling proteins; white adipose tissue; brown adipose tissue; thermogenesis; ultrastructure; morphology; bromodeoxyuridine; mitochondrial biogenesis; obesity; β3-adrenoceptor; endothelial cells; brown adipocytes; convertible adipocytes

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Brown adipocytes in brown adipose tissue (BAT) contain a unique protein not found in any other cell type, the uncoupling protein (UCP1). Indeed, the presence of this protein in an adipocyte usually defines that adipocyte as brown (24). Recent work has disclosed the existence of a family of related proteins, also now referred to as uncoupling proteins (3, 23, 31, 34). However, these proteins are present in tissues other than BAT, as well as in brown adipocytes, and cannot be used to define any particular cell type. BAT has traditionally been regarded as occurring in specific depots in rodents, such as interscapular, perirenal, inguinal, and axillary. The distribution differs from that of white adipose tissue (WAT) depots in these animals, which are in periovarian, retroperitoneal, inguinal, and other locations (8). However, unilocular white adipocytes that do not express UCP1 are usually present in BAT depots (5, 9). Moreover, under certain circumstances, cells with the morphological appearance of mature brown adipocytes can appear among the characteristic unilocular white adipocytes within WAT depots, a location where they are normally not seen.

Multilocular brown adipocytes, positive for UCP by immunohistochemistry, have been seen in parametrial WAT depots of cold-acclimated mice (43) and in periovarian WAT depots of cold-acclimated rats (11, 12). They also appear in WAT depots of rats (16, 17, 38) and of mice (20, 26, 32, 41) that have been treated with a selective β3-adrenoceptor (β3-AR) agonist. In mice, the β3-AR agonist-induced appearance of brown adipocytes in WAT varies considerably from one strain to another and is under complex genetic control (20, 26). We initially defined these adipocytes as brown adipocytes at a time when the existence of other UCPs was not known and on the basis of immunohistochemistry with an antisemum that the present work shows to be not entirely selective for UCP1.

It is well known that cold acclimation induces hypertrophic hyperplasia in BAT (33). Thus norepinephrine induces proliferation of precursor cells, via an action on β1-ARs, and differentiation and mitochondrial biogen-
esis in mature brown adipocytes, via an action on β3-ARs (4). Chronic stimulation by a β3-AR agonist does not induce cellular proliferation in BAT (there is no change in DNA content) but does stimulate mitochondrial biogenesis in brown adipocytes (21, 22). The result of chronic stimulation of WAT by a β3-AR agonist is more complex, and extensive remodeling of WAT occurs as its mass decreases and the unilocular white adipocytes become smaller. DNA content decreases, probably due to loss of vascular cells, since the adipocyte number remains the same (16, 17). In periovian WAT the appearance of brown adipocytes during acclimation to cold is not associated with cell proliferation (11), despite the presence of sympathetic innervation to these brown adipocytes (18).

Several hypotheses can be advanced for the origin of the multilocular adipocytes that appear in WAT under the circumstances outlined above. First, it is possible that they arise from brown adipocyte precursors already present in the WAT. It is known that 10–15% of precursors isolated from WAT become brown adipocytes rather than white adipocytes when cultured (25), and precursors to cells of the brown adipocyte lineage are present in human WAT depots (14). Second, it has been hypothesized that they arise by direct conversion of white adipocytes present in the tissue (27, 28) and that they do not necessarily express UCP1 (29, 30). Third, it has been suggested that some unilocular white adipocytes are in reality “masked” brown adipocytes that revert to the brown adipocyte phenotype in response to stimulation (6).

The present research started with two principal questions. First, are the multilocular adipocytes that appear in WAT depots in response to stimulation by a β3-AR agonist derived from cells that already exist in the tissue or has cell proliferation occurred in a subpopulation of precursor cells? We used 5-bromo-2′-deoxyuridine (BrdU) to label cells that had undergone mitosis in the tissue during the treatment with the β3-AR agonist CL-316243 (CL) and identified the cells that were labeled. Second, are the multilocular adipocytes that appear in WAT the same as the multilocular brown adipocytes expressing UCP1 that are present in BAT or are they another cell type masquerading as brown adipocytes? We quantitated the changes in mitochondrial protein content of interscapular BAT (IBAT) and retroperitoneal WAT (RWAT) and compared the proteins, including UCP1 and UCP3, in the mitochondria isolated from these tissues using gel electrophoresis and Western blotting. We also used electron microscopy, to compare the ultrastructure of the multilocular adipocytes and their mitochondria with that of brown adipocytes in BAT, and immunohistochemistry, using a UCP1 selective antiserum, to assess the presence of UCP1 in individual multilocular adipocytes.

**MATERIALS AND METHODS**

**Animals**

Two sets of rats were studied, one in Ottawa, Canada, and the other in Ancona, Italy. In Ottawa, 10 male Sprague-Dawley rats were purchased (Charles River, St. Constant, Quebec, Canada) at 12 wk of age and housed individually at 24°C in wire mesh cages with free access to food (Agway R-M-H 4020 chow, 14.5% energy from fat) and water until they were 20 wk old. They were then separated into two groups of five rats of equivalent weights (561.4 ± 14.7 g for those to be treated with saline and 564 ± 5.7 g for those to be treated with CL). Both groups had osmotic minipumps (Alzet 2001) implanted subcutaneously under halothane anesthesia and received a volume of 0.49 μl/h. One group received saline, the other group received CL (dose was 1 mg·kg⁻¹·day⁻¹) for 1 wk.

In Ancona, 20 male Sprague-Dawley rats were purchased from Harlan (Correzzana, Milan, Italy) at 20 wk of age and housed in individual cages at 20–24°C, with free access to chow and water. After 1 wk, 12 rats, under anesthesia, had mini-osmotic pumps (Alzet 2001) implanted subcutaneously at a median dorso-thoracic level and received 1 μl/h of a saline solution of CL 314, 243 (dose was 1 mg·kg⁻¹·day⁻¹) for 7 days. The control group of eight animals received only saline. The rats receiving CL treatment were divided in two groups: seven rats were injected daily with BrdU in saline (Sigma, St. Louis, MO), at a dose of 50 mg/kg for the first 3 days of CL treatment (CL 1–3 animals), and another five rats were injected with BrdU during the last 4 days of the CL treatment (CL 4–7). In the control group, four rats received BrdU on days 1–3 (SAL 1–3) and four on days 4–7 (SAL 4–7).

For the biochemical studies, rats were killed by decapitation, and IBAT and RWAT were removed and placed in ice-cold isolation medium (13). They were then cleaned of adherent connective tissue and muscle and, in the case of BAT, of visible WAT, and weighed. The remaining tissue was weighed and homogenized in isolation medium, and mitochondria were isolated as described previously (13). Samples of homogenates and of mitochondria were frozen for later analyses. RWAT, rather than epididymal WAT, was chosen because in previous studies we found change in epididymal WAT to be less marked than in RWAT (16, 17).

For morphological studies, animals were weighed, anesthetized with xylazine-ketamine (0.4%–0.15% 500 g body wt), and perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Tissues dissected were IBAT and RWAT. For control tests of BrdU incorporation, small intestine and esophagus were also sampled. Samples for light microscopy and immunohistochemistry were further fixed by immersion in the same fixative overnight at 4°C, and then tissues were embedded in paraffin blocks. For electron microscopy, small fragments of IBAT and RWAT were fixed in 2% glutaraldehyde-2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 4 h, postfixed in 1% osmium tetroxide, and embedded in an Epon-Araldite mixture. Semithin sections (2 μm) were stained with toluidine blue, and thin sections were obtained with an MT-X ultratome (RMC, Tucson, Arizona), stained with lead citrate, and examined with a CM10 transmission electron microscope (Philips, Eindhoven, Netherlands).

**Immunohistochemistry**

BrdU. Incorporated BrdU was detected with a mouse monoclonal antibody (Sigma, clone BU-33; St. Louis, MO) and visualized by the avidin and biotinylated horseradish peroxidase macromolecular complex (ABC) method. Three-micrometer sections of tissue were attached on clean glass slides treated with poly-L-lysine solution (Sigma) and left to dry at 40°C for 2 days. After deparaffinization and rehydration with distilled water, sections were first treated with 3% hydrogen peroxide in distilled water for 5 min to inactivate endogenous peroxidase. To
detect incorporated BrdU, the DNA must be denatured to allow antibody access. We used microwave antigen retrieval (36) with the following schedule: double irradiation in a 650-W microwave oven (Whirlpool, MWO 104) for 5 min in 0.01 M citrate buffer, pH 6.0, with 5 min cooling between the first and second irradiation, after which the slides were left in hot citrate buffer for an additional 15 min and finally rinsed in phosphate-buffered saline (PBS) for 30 min. The sections were incubated successively in: 1) primary antibody against BrdU, diluted 1:100 in PBS containing 1% normal horse serum, to block nonspecific staining, for 1 h at 40°C, in a humid chamber; 2) PBS, twice for 15 min; 3) biotinylated secondary antibody, horse anti-mouse IgG (Vector Labs, Burlingame, CA) 1:200, 30 min at room temperature; 4) PBS, twice for 15 min; 5) ABC complex (Vector Labs) for 1 h; 6) PBS twice for 15 min; 7) enzymatic development of peroxidase with 0.05% diaminobenzidine hydrochloride (Sigma, St. Louis, MO) and 0.02% hydrogen peroxide in 0.05 Tris, pH 7.6, for 4 min. Tests of the specificity of reaction were performed for the immunological sequence and the BrdU incorporation. For the immunological sequence, negative control slides were prepared by substitution of mouse IgG for the primary antibody. For the BrdU incorporation in replicating cells, tissues of the same animals, with known turnover time of cells, were studied (small intestine in which labeled cells broaden and pass up the crypt, nearing the villus base by 14 h and the villus tip by 48 h and esophagus whose turnover time is ~8 days) (40). In animals treated with BrdU in the first 3 days (CL and SAL 1–3 groups), the interstitial villi were negative and the superficial layer of the epithelium of the esophagus mucosa showed BrdU-positive nuclei as expected. In animals treated with BrdU in the last 4 days (CL and SAL 4–7 group), the intestinal villi (base and apex) and the basal layer of epithelium of the esophagus mucosa showed BrdU-positive nuclei as expected (Fig. 1C).

UCP1. Polyclonal sheep antibodies raised against rat UCP1 were used as previously described (5) for UCP1 immunohistochemistry.

**Morphometric Analysis**

One midline sagittal section of the entire depot on one side of each animal was used to quantify the occurrence of different cell types. Ten different fields, viewed at ×400 magnification, were used to count ~800 adipocytes per CL-treated animal in superior, middle, and inferior regions of the depot. More fields were needed in saline-treated animals because the adipocytes were larger. Distribution of multilocular adipocytes in CL-treated rats was mostly diffuse, but a few patches composed mainly of multilocular cells, were observed.

The following morphometric quantitations were performed at the light microscopic level on RWAT sections of CL-treated and control animals after 7 days of treatment. The percentages of multilocular adipocytes in all adipocytes (~800 adipocytes/animal), of UCP1-positive multilocular adipocytes in the total multilocular adipocytes, of BrdU-positive cells in all the cell types present, of BrdU-positive endothelial cells in the total BrdU-positive cells, of BrdU-positive endothelial cells in the total endothelial cells, and of BrdU-positive multilocular adipocytes in the total multilocular adipocytes were counted.

**Assays**

Protein was measured by a modified Lowry method, and cytochrome oxidase was measured in tissue homogenates as described before (13, 21).

**Gel Electrophoresis and Western Blotting**

Samples of mitochondria from all five animals in each group were assessed. Figures show one representative blot or stained gel for each antiserum used. Mitochondrial proteins were separated using SDS-PAGE with 16% acrylamide gels. Standard molecular mass markers, detectable by enhanced chemiluminescence, were from Santa Cruz Biotechnology.

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**Fig. 1.** 5-Bromodeoxyuridine (BrdU) immunohistochemistry. A: negative multilocular cell (M) close to positive endothelial cells (E) is visible in retroperitoneal white adipose tissue (RWAT) of a CL-316243 (CL)-treated rat (×1,000 magnification). B: multilocular positive cell (same depot as in A; ×1,500 magnification). C: positive control shows villi of the intestinal mucosa of a CL-treated rat (×100 magnification).
(132, 90, 55, 43, 34, and 23 kDa). Proteins were transferred to nitrocellulose membranes that were then stored at −20°C. Membranes were blocked in 5% skim milk in 0.1% Tween 20 in PBS for 1 h at room temperature. Primary antibody incubation was in 3% bovine serum albumin for 1 h at room temperature. Primary antibodies used were rabbit anti-hamster purified UCP antiserum at a dilution of 1:16,000 to detect UCP1 and anti-human UCP3 peptide antiserum at a dilution of 1:100 (Chemicon AB-3046) to detect UCP3. The rabbit anti-hamster UCP antiserum was the one used in previous studies (16, 17). Standard proteins included purified rat UCP (for UCP1) and BAT mitochondria from wild-type mice and from transgenic mice with deficiency of UCP3 (to identify which band was UCP3) as well as recombinant UCP3 (Stratagene; the mouse BAT mitochondria were provided by Dr. Marc Reitman of the National Institutes of Health). The secondary antibody was donkey anti-rabbit IgG (Santa Cruz) diluted in 5% skim milk in PBS and incubated for 1 h at room temperature. Detection used enhanced chemiluminescence (Amersham kit), and the image was captured on Polaroid film using a minicamera (Bio-Rad). Some gels were not transferred and were stained with Coomassie blue for 1 h at room temperature followed by overnight destaining.

RESULTS

Origin of Multilocular Adipocytes

After 7 days of treatment with CL, an average of 17 ± 3.6% of the RWAT adipocytes had a multilocular appearance, whereas 0.3 ± 0.22% were multilocular in saline-treated animals (P < 0.001). To find out whether the multilocular cells derived from proliferation of precursors, we treated the animals with BrdU, a substance that is incorporated into the DNA of replicating cells and that subsequently persists in the nuclei of the cells deriving from that replication and can be revealed by immunohistochemistry. BrdU can be toxic if administered for long periods; therefore, we used two groups of animals: the first received BrdU during the first 3 days (group 1–3) and the second during the last 4 days (group 4–7) of treatment with CL. An average of 2.9 ± 0.65% in group 1–3 and 17.8 ± 1.5% in group 4–7 of all the RWAT cells in CL-treated animals had BrdU-positive nuclei (vs. 0.8 ± 0.25% and 3.3 ± 0.31%, respectively, in the two saline-treated groups). Most of the BrdU-positive cells were endothelial cells: 97.3 ± 1.08% (group 1–3) and 98.6 ± 0.4% (group 4–7; Fig. 1). In fact, 8.7 ± 1.51% (group 1–3) and 27.1 ± 7% (group 4–7) of all endothelial cells were labeled by BrdU in CL-treated animals. In contrast, of all the multilocular cells, 94.1 ± 1% in group 1–3 and 93.6 ± 2.8% in group 4–7 cells were negative for BrdU, strongly suggesting that the multilocular cells do not derive from a mitotic proliferation of precursors (Fig. 1).

Morphology and Immunohistochemistry

Immunohistochemistry at the light microscopy level showed a markedly heterogeneous appearance of the adipocytes in CL-treated rats (Fig. 2). An average of only 8.4% of the multilocular cells expressed UCP1 by immunohistochemistry, with a maximum of 33.3% and a minimum of 0% in the different animals. Electron microscopy showed that very marked mitochondrial biogenesis must have occurred in many of the adipocytes (see Figs. 3–6). Many of the multilocular cells had the typical ultrastructure of brown adipocytes with numerous small lipid droplets and mitochondria (Fig. 3), but only a minority of them had the complete differentiation of the mitochondria characteristic of brown adipocytes. A close juxtaposition of mitochondria and lipid droplets was often observed (Fig. 3). Some of the multilocular cells showed villous extrofilaments of the cytoplasm typical for white adipocytes in late stages of the delipidation process (Fig. 4) and never described for brown adipocytes during active thermogenesis (7, 8). No attempt was made to quantitate the proportion of multilocular cells showing this morphology. It was evident mainly in the cells with little remaining lipid. Electron microscopy also showed an aspect of some unilocular cells not found in controls: a thickened peripheral cytoplasmic rim rich in mitochondria (Fig. 5), suggesting that mitochondrial biogenesis had been stimulated in these cells also.

IBAT morphology after 7 days of CL treatment differed from that of saline-treated animals, in that the brown adipocytes of CL-treated rats showed smaller cytoplasmic lipid droplets and numerous mitochondria with abundant cristae (Fig. 6). Immunohistochemistry showed that brown adipocytes of CL-treated rats were more intensely stained than controls by the UCP1 antiserum (not shown). In a previous study, a greater density of immunogold staining was seen in BAT mitochondria of CL-treated rats than in BAT mitochondria of saline-treated rats (21).

Biochemical Indications of Mitochondrial Biogenesis

In both IBAT and RWAT, CL treatment increased protein content, total cytochrome oxidase activity, and the amount of protein recovered in the isolated mitochondria (Fig. 7, A–C). At the same time, the wet weight of IBAT did not change (0.688 ± 0.0624 g in CL-treated rats vs. 0.736 ± 0.085 g in saline-treated rats; not significant), whereas the wet weight of RWAT, a much larger tissue, decreased by ~50% (4.28 ± 0.642 g in CL-treated rats vs. 8.25 ± 0.454 g in saline-treated rats; P = 0.001). In absolute terms, the changes in protein, cytochrome oxidase, and mitochondrial protein recovered were similar for the two tissues. Protein content increased by 58 mg in RWAT and by 32 mg in IBAT. Cytochrome oxidase content increased by 110 μmol/min in RWAT and by 114 μmol/min in IBAT. Mitochondrial protein recovered increased by 2.0 mg in RWAT and by 1.9 mg in IBAT. Results are consistent with a similar stimulation of total mitochondrial biogenesis in the two tissues above a baseline content of mitochondria that was much higher in the IBAT than in the RWAT. The very low amount of mitochondrial protein recovered from RWAT of saline-treated rats
and the low level of cytochrome oxidase in this tissue are consistent with the sparse and small mitochondria usually present in unilocular white adipocytes. Note that there was a 10-fold increase in mitochondrial protein recovered from RWAT in the CL-treated rats compared with the saline-treated rats, but only a 1-fold increase in the mitochondrial protein recovered from IBAT. Thus; 90% of the mitochondrial protein in RWAT of CL-treated rats was newly made under the influence of CL stimulation, whereas; 50% of the mitochondrial protein in IBAT of CL-treated rats was newly made.

Because it was clear that more mitochondria had been generated in both WAT and BAT in response to CL, the increase being 10 times more marked in the WAT because of the low amount present without treatment, the next question was whether these two tissues were making the same kind of mitochondria. Gel electrophoresis of identical amounts of mitochondrial protein followed by Coomassie blue staining revealed several differences between the banding pattern for RWAT mitochondria of CL-treated rats and that of IBAT mitochondria of the same animals (Fig. 8A), indicating that the new mitochondria in WAT were not typical BAT mitochondria. The banding pattern was similar for BAT mitochondria from saline and CL-treated rats, indicating that the new mitochondria made in BAT in response to CL closely resemble those already present in the BAT. Gel electrophoresis of identical amounts of IBAT and RWAT

![Fig. 2. Immunohistochemistry for uncoupling protein (UCP1) of 2 different areas (A and B) of RWAT of a CL-treated rat, using a selective sheep anti-UCP antiserum (origin, Dr. D. Ricquier; Ref. 5; ×400 magnification). Insets: enlargements (×1,000) of the areas enclosed by squares. Note the heterogeneity of morphology of the adipocytes, ranging from large unilocular to small multilocular adipocytes. Only a few of the latter are positive for UCP1 (brown stain).](image)
mitochondrial proteins followed by Western blotting using an antiserum to human UCP3 peptide showed the presence of UCP3 in BAT mitochondria of saline-treated rats and a selective increase in UCP3 in BAT mitochondria of CL-treated rats (Fig. 8B). (Because the antiserum is not totally selective for UCP3 and UCP3 standard protein was not available, we used also BAT mitochondria from transgenic mice with deficiency of UCP3 as well as mitochondria from wild-type mice to identify which band was UCP3.) UCP3 was not detected in RWAT mitochondria of saline-treated rats but was present in RWAT mitochondria of CL-treated rats. The increase was more marked in the RWAT mitochondria than in the IBAT mitochondria. Gel electrophoresis followed by Western blotting using an antiserum raised in rabbits to purified UCP1 from hamster BAT showed a selective increase in UCP1 in BAT mitochondria of CL-treated rats (Fig. 9). No UCP1 was detected in RWAT mitochondria from saline-treated rats, whereas UCP1 did appear in RWAT mitochondria from CL-treated rats. However, the level of UCP1 detected in RWAT mitochondria (10 μg protein on gel in Fig. 9) was less than in IBAT mitochondria (1 μg on the gel), indicating again that the new mitochondria in RWAT either were not typical BAT mitochondria or that they were a mixture of a few typical BAT mitochondria with many mitochondria of another kind.

Because neither antiserum used was totally selective, numerous other bands appeared on the blots. The anti-UCP3 peptide antiserum recognized more and different lower molecular mass proteins in RWAT mitochondria than in the same amount of IBAT mitochondria (Fig. 8B). Conversely, the rabbit anti-purified hamster UCP antiserum recognized more higher molecular mass proteins in the small amount of BAT mitochondria than in the larger amount of WAT mitochondria (Fig. 9). It is not possible to identify at present these many other proteins that differ in IBAT and RWAT mitochondria of CL-treated rats. However, the lack of selectivity of the antisera allows also the conclusion that there are...
differences in protein composition between the mitochondria from these two sources.

DISCUSSION

There are two principal findings in this study of remodeling of RWAT induced by chronic stimulation with the β3-AR agonist CL and associated with a marked reduction in wet weight of the RWAT. First, the mitochondria-rich multilocular adipocytes that appear among the unilocular white adipocytes in brown adipose tissue (BAT). Mitochondria are numerous but their morphology differs from that in a typical brown adipocyte (compare with Fig. 3). Second, the mitochondria-rich multilocular adipocytes that appear mostly from direct conversion of a subpopulation of preexisting unilocular white adipocytes. Second, the

Fig. 4. A: electron microscopy of a multilocular adipocyte in RWAT of a CL-treated rat (×6,300 magnification). Note the villous extrusions not normally seen in brown adipocytes in brown adipose tissue (BAT). Mitochondria are numerous but their morphology differs from that in a typical brown adipocyte (compare with Fig. 3). B: enlargement of the framed area in A (×17,000 magnification). v, Villous extrusions.

Fig. 5. Electron microscopy of a unilocular white adipocyte in RWAT of a CL-treated rat. The thick peripheral rim contains numerous mitochondria similar to those in the multilocular cell in Fig. 4 (×20,000 magnification). Note that, together with the prevailing large "unilocular" lipid droplet (L), numerous small lipid droplets are present among the mitochondria. Inset: light microscopy of the same resin-embedded tissue (×400 magnification). The framed area corresponds to the electron micrograph.
protein composition of the abundant new mitochondria in the multilocular adipocytes in RWAT is not the same as that of the mitochondria in multilocular adipocytes in IBAT in the same animals. Many of the multilocular adipocytes in RWAT of the CL-treated rats have the morphological appearance of brown adipocytes, with numerous mitochondria and lipid droplets, but only a few of them have a complete differentiation of the

Fig. 6. Electron microscopy of a brown adipocyte in BAT of a CL-treated rat. Note that the typical mitochondria are similar to those shown in Fig. 3 (×14,000 magnification).

Fig. 7. Biochemical analyses of interscapular BAT (IBAT) and RWAT of saline (SAL)- or CL-treated rats. A: total protein content (effect of CL treatment \( P = 0.0006 \) for IBAT, \( P = 0.0028 \) for RWAT). B: protein recovered in isolated mitochondria (effect of CL treatment \( P = 0.0041 \) for IBAT, \( P = 0.0091 \) for RWAT). C: total cytochrome oxidase activity, as a measure of mitochondrial mass (effect of CL treatment \( P = 0.0013 \) for IBAT, \( P = 0.0049 \) for RWAT). The rats weighed 576 ± 14.2 g (SAL) and 575 ± 10.3 g (CL) at the end of the treatment [not significant (NS)], and weight gains during the 1 wk were 15.3 ± 3.91 g (SAL) and 11.2 ± 6.68 g (NS).
mitochondria to that characteristic of stimulated brown adipocytes, and only a few are positive for UCP1 by immunohistochemistry. Moreover, some of the multilocular adipocytes in RWAT showed villous extroflexions of the cytoplasm, typical of white adipocytes during the late stages of loss of lipid (7, 8) but never described for brown adipocytes during active thermogenesis. Differences in composition between RWAT mitochondria of CL-treated rats and IBAT mitochondria of CL-treated rats are indicated by the banding pattern of stained gels and the pattern of proteins detected by immunoblotting, using nonselective antisera to either UCP purified from hamster BAT or to a peptide of human UCP3. An increase in both UCP1 and UCP3 concentration was detected in IBAT mitochondria. These two uncoupling proteins were present in mitochondria isolated from RWAT of CL-treated rats, but UCP1 was present at a much lower level than in BAT mitochondria of the same animals, whereas UCP3 was more abundant in RWAT mitochondria than in IBAT mitochondria. Neither UCP1 nor UCP3 was detectable in RWAT mitochondria of saline-treated animals.

It seems likely, therefore, that most of the multilocular, mitochondria-rich adipocytes present in RWAT of CL-treated rats differ from the typical brown adipocytes present in IBAT of the same animals. We previously defined them as brown adipocytes (16, 17) before the existence of other uncoupling proteins was known and on the basis of reactivity with the nonselective antiserum shown now in this study to react with other mitochondrial proteins in CL-treated rats. These adipocytes should perhaps not be named “brown” if that term defines an adipocyte that expresses UCP1 but is rather convertible, meaning derived from unilocular adipocytes that do not express UCP1, following the nomenclature of Loncar (27–30). We cannot exclude the existence of a small proportion of more typical brown adipocytes, those that were immunopositive for UCP1, that may have arisen from brown preadipocytes already present in the WAT, and the expression of abundant UCP1 and also UCP3 in the mitochondria of these cells. We also cannot exclude the eventual differentiation, under continuous stimulation by CL, of the convertible adipocytes into brown adipocytes, i.e., containing UCP1. Because the isolated mitochondria were...
proteins detected in BAT; those referred
clusively in BAT and muscle in rats. However, at
UCP3 was initially reported to be expressed ex-
tREATED rats and in leptin-treated rats
lanes 2 contain 10 times more protein than
lane 5 (of RWAT mitochondria) or CL-treated
lanes 4 and 5, 10 μg of RWAT mito-
contain 10 times more protein than lanes 2 and 3 (IBAT mitochondria), so that the same exposure could be used for both. Only 3 proteins detected in BAT (~38, ~34, and ~30 kDa) appear in RWAT mitochondria of the CL-treated rat but are not detected in the BAT mitochondria.

Fig. 9. Western blot of isolated mitochondria from IBAT and RWAT probed with an antiserum to purified hamster UCP1. Lanes 1 and 7, molecular mass markers (Cruz Marker, Santa Cruz Biotechnology). Lane 6, 10 ng purified rat BAT UCP. Lanes 2 and 3, 1 μg of IBAT mitochondrial protein from a SAL-treated (lane 2) or CL-treated (lane 3) rat. Lanes 4 and 5, 10 μg of RWAT mitochondrial protein from the same SAL-treated (lane 4) or CL-treated (lane 5) rat. Note that lanes 4 and 5 (RWAT mitochondria) contain 10 times more protein than lanes 2 and 3 (IBAT mitochondria), so that the same exposure could be used for both. Only 3 lower molecular mass bands (between ~20 and ~30 kDa) appear in RWAT mitochondria of the CL-treated rat but are not detected in the BAT mitochondria.

derived from a mixed population of adipocytes, both convertible and more typical brown as well as more typical unilocular white adipocytes, we are not able to conclude whether convertible adipocytes express UCP1 at a very low level or whether at least some do not express this protein at all. We are able to conclude only that the level of UCP1 in mixed mitochondria isolated from RWAT at 7 days of treatment was much lower than that in isolated IBAT mitochondria. White adipose tissue of saline-treated rats had very few mitochondria, in keeping with the sparse, slender mitochondria usually seen in the white adipocytes in this tissue.

The finding of endothelial cell proliferation in response to the CL treatment is probably secondary to the release of growth factors from neighboring adipocytes stimulated by the CL. It is known that vascular endothelial growth factor is secreted by both brown (1, 2, 37) and white (10, 37, 44) adipocytes and is a potent mitogen for endothelial cells.

Our finding of an increase in UCP3 protein in mitochondria isolated from RWAT of rats after chronic CL treatment would be in agreement with reports of an increase in mRNA for UCP3 in WAT of β3-AR agonist-treated rats (15, 19, 23, 42) and in leptin-treated rats (35). UCP3 was initially reported to be expressed exclusively in BAT and muscle in rats (39). However, at least a subpopulation of WAT cells, those referred to here as convertible adipocytes, has the ability to make this protein when stimulated.

A fundamental part of the long-term thermogenic response of brown adipocytes to β3-AR agonists is stimulation of mitochondrial biogenesis. This can apparently occur not only in brown adipocytes in BAT but also in a subpopulation of white adipocytes in WAT. Still unresolved are the nature and regulation of the gene(s) involved in the coordinated synthesis of the multitude of proteins needed for mitochondrial biogenesis, which necessarily accompanies the proliferation of mitochondria as well as the selective increase in synthesis of UCP-1 and of UCP-3 induced by norepinephrine.

If the multilocular adipocytes in RWAT of CL-treated rats are not typical brown adipocytes, can we be sure that they have an adaptive thermogenic function? Are they, as has been suggested, the location of part of the whole body thermogenic response that occurs during CL-treatment of rats? The answer to this question cannot be ascertained at present and will require the isolation of these cells and their mitochondria and the study of their metabolic properties. Also, elucidating the many protein differences between BAT and WAT mitochondria in CL-treated rats will clearly take much further investigation.

Perspective

β3-AR agonists have been and are still of great interest as drugs that might increase energy expenditure and hence be useful in the treatment of obesity in humans (22). A drawback to this approach often mentioned is that adult humans have relatively little BAT. It is, therefore, necessary to understand more about how β3-AR agonists evoke such a remarkable transformation of unilocular adipocytes to mitochondria-rich multilocular adipocytes in WAT depots of rodents. In particular, we need to know whether these convertible adipocytes have a thermogenic capacity when they are in their multilocular mitochondria-rich state and whether similar convertible adipocytes are present in WAT depots in humans and can be provoked into a thermogenic function by treatment with β3-AR agonists.

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