Multilocular fat cells in WAT of CL-316243-treated rats derive directly from white 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, and axillary. The distribution differs from that of white adipose tissue (WAT) depots in these animals, which are in perivarian, 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 peri-ovarian WAT 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 antisem that the present work shows to be not entirely selective for UCP1.

It is well known that cold acclimation induces hyperplastic hypertrophy in BAT (33). Thus norepinephrine induces proliferation of precursor cells, via an action on β1-ARs, and differentiation and mitochondrial biogen-
Orchis in mature brown adipocytes, via an action on 
\( \beta_3 \)-ARs (4). Chronic stimulation by a \( \beta_3 \)-AR agonist 
does not induce cellular proliferation in BAT (there is 
no change in DNA content) but does stimulate mito-
chondrial biogenesis in brown adipocytes (21, 22). The 
result of chronic stimulation of WAT by a \( \beta_3 \)-AR ago-
nist 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 adipocy-
type number remains the same (16, 17). In periovian 
WAT the appearance of brown adipocytes during accli-
ma tion 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 al-
ready present in the WAT. It is known that 10–15% of 
precursors isolated from WAT become brown adipo-
cytes 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 adipocy-
tes 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 
\( \beta_3 \)-AR agonist derived from cells that already exist 
in the tissue or has cell proliferation occurred in a sub-
population 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 
\( \beta_3 \)-AR agonist CL-316243 (CL) and identified the cells 
that were labeled. Second, are the multilocular adipocy-
tes 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 mi-
tochondrial protein content of interscapular BAT 
(IBAT) and retroperitoneal WAT (RWAT) and com-
pared the proteins, including UCP1 and UCP3, in the 
mitochondria isolated from these tissues using gel elec-
trophoresis and Western blotting. We also used elec-
tron microscopy, to compare the ultrastructure of the 
multilocular adipocytes and their mitochondria with 
that of brown adipocytes in BAT, and immunohisto-
chemistry, 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 
2002; Alza, Palo Alto, CA) implanted subcutaneously under 
halothane anesthesia and received a volume of 0.49 \( \mu \)l/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 \( \mu \)l/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 decapita-
tion, 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 mito-
chondria 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, anes-
thetized with xylazine-ketamine (0.4%-0.1%/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 
in 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 
\( \mu \)m) 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 trans-
mision electron microscope (Philips, Eindhoven, Netherlands).

Immunohistochemistry

BrdU. Incorporated BrdU was detected with a mouse mono-
clonal antibody (Sigma, clone BU-33; St. Louis, MO) and visual-
ized by the avidin and biotinylated horseradish peroxidase 
macromolecular complex (ABC) method. Three-micrometer sec-
tions 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 dist-
tilled 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 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 quantitate 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.

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).

UCP1 ORIGINS OF MULTILOCULAR ADIPOCYTES IN WHITE ADIPOSE TISSUE

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(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. Mary-Ellen Harper of the University of Ottawa as part of a collaborative study with 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 extrusions 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 ± 0.545 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 144 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 the 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 un-coupling 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).
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 adipocytes in RWAT arise mostly from direct conversion of a subpopulation of preexisting unilocular white adipocytes. Second, the

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**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

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**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 continued stimulation by CL, of the convertible adipocytes into brown adipocytes, i.e., containing UCP1. Because the isolated mitochondria were

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**Fig. 8. Mitochondrial proteins in IBAT and RWAT.**

A: Coomassie blue stained gel. B: Western blot of an identical gel probed with an anti-UCP3 peptide antiserum (Chemicon). Lanes 1 and 9 are molecular mass markers (Cruz Marker, Santa Cruz Biotechnology) and lane 8 has 5 ng of recombinant UCP3 (Stratagene). Note that the marker proteins and the UCP3 standard protein are barely or not at all visible in the Coomassie blue-stained gel because the amount of protein is so small. Lanes 4 and 5 have 10 μg of IBAT mitochondrial protein (lane 4, SAL-treated rat; lane 5, CL-treated rat). Lanes 6 and 7 have 10 μg of RWAT mitochondrial protein from the same rats (lane 6, SAL-treated rat; lane 7, CL-treated rat). Lanes 2 and 3 have 10 μg of mouse IBAT mitochondrial protein from a wild-type mouse (lane 2) and a UCP3-deficient transgenic mouse (lane 3), included for the purpose of identifying the location of UCP3 on the gel. Note that the standard UCP3 protein (lane 8) is a fusion protein with a higher molecular mass (41 kDa) than the UCP3 identified by its absence in the knockout mouse (compare lanes 2 and 3, band at ~36 kDa). Note that the RWAT mitochondria of the SAL-treated rat (lane 6) have one major protein and many other, barely visible bands. Proportions differ in RWAT mitochondria of the CL-treated rat (lane 7, ~90% newly made mitochondria), with many bands appearing. The banding pattern for RWAT mitochondria (lane 7) is different from that for BAT mitochondria from the same CL-treated rat (lane 5). Note that an equal amount of mitochondrial protein (10 μg) is in each of the lanes containing rat mitochondrial proteins. The antiserum reacts with a low molecular mass (~23 kDa) protein in rat BAT that is not detected in mouse BAT, and this protein, absent from rat BAT, is increased in amount in RWAT of the CL-treated rat. The antiserum also reacts with numerous other proteins that are present in RWAT of the CL-treated rat that are not detected in BAT of the same rat.
proteins detected in BAT (dria), so that the same exposure could be used for both. Only 3
clusively in BAT and muscle in rats (39). However, at least a subpopulation of WAT cells, those referred
contain 10 times more protein than
contain 10 times more protein than lanes 4 and 5 (IBAT 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
proteins detected in BAT [−38, −34, and −82 kDa (the last in only some rats)] are also detected in WAT of the CL-treated rat (but absent from WAT of the SAL-treated rat). Several 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 (7, 8, 24).

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
tohere 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 (16, 17, 21)? 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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