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Hypertrophy of Brown Adipocytes in Brown and White Adipose Tissues and Reversal of Diet-Induced Obesity in Rats Treated with a β_3-Adrenoceptor Agonist*

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ABSTRACT. In a previous study, we demonstrated that chronic treatment with a new β_3-adrenoceptor agonist, CL 316,243 [disodium (*R,R*)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]-amino]propyl]-1,3-benzodioxazole-2,2-dicarboxylate], promoted thermogenesis, caused the appearance of multilocular adipocytes in white adipose tissue (WAT), and retarded development of obesity in young rats eating a high-fat diet (Himms-Hagen *et al.*, *Am J Physiol* **266**: R1371–R1382, 1994). Objectives of the present study were to find out whether CL 316,243 could reverse established diet-induced obesity in rats and to identify the multilocular adipocytes that appeared in WAT. Infusion of CL 316,243 (1 mg/kg/day) reduced abdominal fat, with a decrease in enlarged adipocyte size but no loss of white adipocytes. The resting metabolic rate increased by 40–45%, but food intake was not altered. Abundant densely stained multilocular brown adipocytes expressing uncoupling protein (UCP) appeared in retroperitoneal WAT, in which a marked increase in protein content occurred. UCP content of interscapular brown adipose tissue (BAT) was also increased markedly. We suggest that the substantial increase in the resting metabolic rate induced by CL 316,243 occurs in brown adipocytes in both BAT and WAT. The origin of the brown adipocytes that appeared in WAT is uncertain. They may have been small brown preadipocytes, expressing β_3-adrenoceptors but with few mitochondria and little or no UCP, that were induced to hypertrophy by the β_3-agonist. BIOCHEM PHARMACOL **54**;1:121–131, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. thermogenesis; uncoupling protein; norepinephrine; energy balance; energy expenditure; β_3-adrenoceptors

CL 316,243¶ (or CL**) is a new, highly selective β_3-adrenergic agonist [1–3]. β_3-Adrenoceptors are present in WAT and BAT and can serve as a target for the treatment of obesity [4]. Their stimulation increases lipid mobilization in WAT and increases energy expenditure in BAT, reducing lipid stores in both tissues. In a previous study, we demonstrated that daily treatment of young growing rats with CL for 2 weeks retards the development of obesity induced by a high-fat diet [5]. This retardation was associated with an increase in metabolic rate but no change in food intake and with mitochondrial proliferation in BAT, with a large increase in the level of UCP. The DNA content of two WAT depots was lower in the CL-treated rats, but because no pretreatment group of rats was studied, it was not clear whether the treatment had simply prevented the normal increase in cellularity with age or had made cells disappear. It was also not clear whether the lower DNA level was due to fewer white adipocytes, to fewer cells other than adipocytes, or to both of these. In addition, multilocular adipocytes appeared among the unilocular white adipocytes in white adipose tissue of CL-treated rats, but the identity of these cells was not established.

The first objective of the experiment reported here was to find out whether treatment with CL could reverse DIO, which had already been established in older rats by feeding them a high-fat diet, during a period in which they continued to eat the high-fat diet. The second objective was to explore more fully the cellular changes in WAT, in particular the possibilities that the multilocular cells that appeared in WAT might be brown adipocytes expressing

* Preliminary reports of this work have been made at meetings of the North American Association for the Study of Obesity (*Obes Res* 1 (Suppl 2): 75S, 1993; *Obes Res* 3 (Suppl 3): 406S and 406S, 1995) and at the 7th International Congress of Obesity (*Int J Obes* 18 (Suppl 2): 130, 1994).

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¶ The chemical name for CL 316,243 is disodium (*R,R*)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]-amino]propyl]-1,3-benzodioxazole-2,2-dicarboxylate.

**Abbreviations: BAT, brown adipose tissue; CL, CL 316,243; DIO, diet-induced obesity; RWAT, retroperitoneal white adipose tissue; TBS, Tris-buffered saline; TCT, Tris carageenan Triton buffer; UCP, uncoupling protein; and WAT, white adipose tissue.

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UCP and that the treatment might result in the disappearance of mature white adipocytes. To distinguish between drug-induced prevention of aging-associated accretion of white adipocytes and drug-induced disappearance of mature white adipocytes, a pretreatment group of rats also was studied.

Other studies have demonstrated atypical occurrence of UCP in certain WAT depots previously thought to contain only white adipocytes and an increase in UCP content in response to cold adaptation [6–10] or to β-adrenergic stimulation [7, 8]. Based upon our previous observation of multilocular cells in WAT of CL-treated rats [5], it seemed likely that CL might likewise induce the appearance of UCP in brown adipocytes in one or more typical WAT depots. The definition of a brown adipocyte as used here is of a cell that expresses UCP [11]. We looked for brown adipocytes in WAT depots, using immunohistochemical detection of UCP. While this work was in progress, a report appeared that treatment of obese yellow KK mice with CL induced the appearance of brown adipocytes in WAT [12].

MATERIALS AND METHODS

Animals

Eighty male Sprague–Dawley rats were purchased at 13 weeks of age and housed at 24° in hanging wire mesh cages with free access to food (Agway R-M-H 4020 chow, 14.5% energy from fat) and water. After 1 week, food intake and body weights were measured weekly for the next 4 weeks. Rats were then separated into three groups of equal ranges of weights and mean weights. One group of 8 was killed at this time (week 0). The other two groups either continued to eat chow (N = 28) or were switched to a high-fat diet (Teklad 85418, 54% of energy from partially hydrogenated vegetable oil) (N = 44) for 4 weeks. Rats susceptible to DIO were selected as the 28 most rapid weight gainers from this last group. Remaining rats eating the high-fat diet (N = 16) were not studied further. Each of the diet groups was then divided into three. One group of 8 from each diet subgroup was killed at this time (4 weeks) to obtain two pretreatment groups: one with DIO, the other without. Two groups of 10 from each diet group had osmotic minipumps (Alzet 2002, Alza, Palo Alto, CA) implanted s.c. under halothane anesthesia and received 0.49 μL/hr containing either saline or CL (dose of CL was 1 $mg \cdot kg^{-1} \cdot day^{-1}$) for the next 4 weeks (the pumps were replaced after 2 weeks). Of the 10 rats in each of the four treatment groups, 8 were killed after 4 weeks (8-week saline or CL groups) and 2 were killed (for histology) a few days later. Body weights and food intake were measured weekly throughout the experimental period. During weeks 3 and 4 of treatment with saline or CL, the resting metabolic rate was measured by placing the rat in a water-jacketed chamber at 28°, volume 1 L, through which air, also at 28°, was drawn at a rate of 1 L/min (regulated with a mass flowmeter, Brooks Instrument Division, Emerson Electric Co., Hatfield, PA). Oxygen concentration in the air entering and leaving the chamber was measured with an oxygen analyzer (Beckman Industrial Oxygen Analyzer model 755) and computerized data acquisition and handling to obtain oxygen uptake by the rat as described before [5].

Rats were killed by decapitation. Interscapular and perirenal BAT depots were removed and placed in isolation medium on ice [5, 13]. They were then dissected free of adhering muscle, vasculature, and white adipose tissue, weighed, and frozen. RWAT and epididymal WAT depots were removed and weighed. Weighed portions were used for osmium fixation for counting and sizing of mature adipocytes [14]. The remainder was weighed and frozen. The tail and paws were removed from the remaining carcass which was frozen for later analysis. For carcass analysis, carcasses were autoclaved, and then homogenized in water (Waring Blendor, commercial heavy duty). Duplicate samples of homogenate were placed in extraction thimbles and allowed to drain and dry; then the fat was extracted (Soxhlet extraction) and weighed.

Assays

Frozen tissue samples were homogenized in isolation medium. Protein was measured by a modified Lowry method and UCP was measured by solid-phase radioimmunoassay in homogenates of BAT as before [13]. DNA was measured in delipidated WAT and BAT homogenates with a dye binding (Hoechst 33258) fluorometric method, and UCP in WAT was assessed by gel electrophoresis followed by western blotting, both as described previously [13].

Histology

Some samples of WAT were fixed in 10% buffered formalin solution and then embedded in paraffin. Thick (7–10 μm) sections either were stained with hematoxylin and eosin or were subjected to immunohistochemistry for UCP. For immunohistochemistry, sections were incubated with a primary antibody to UCP diluted 1:100 with TCT [Tris buffer–carageenan (0.6%)-Triton X-100 (0.3%)] overnight at 4°. The antiserum for hamster UCP had been raised in rabbits and stored in glycerol 1:1 at −20°. The next day sections were washed in TBS (0.1 M Tris and 0.14 M sodium chloride) and incubated with a secondary antibody (donkey anti-rabbit Ig, biotinylated whole antibody) diluted 1:50 in TCT buffer. After further washing in TBS and neutralization with 3% hydrogen peroxide, sections were washed again in TBS and incubated with streptavidin-horseradish peroxidase conjugate diluted with TCT at room temperature. They were washed further in TBS, and then were incubated with diaminobenzidine (200 μg/100 mL TBS plus 700 μL nickel chloride plus 10 μL hydrogen peroxide) in the dark. Sections were then dehydrated in alcohol, cleared in xylol, and mounted in Permount [15].

Some samples of WAT were fixed in buffered glutaraldehyde solution and embedded in Epon. Semi-thin sections

TABLE 1. Body weights and weights of RWAT

	Lean (chow-fed)			Obese (high fat diet)		
	Pretreatment	Saline	CL	Pretreatment	Saline	CL
			Body weights (g)			
Week 0	502 ± 7.9					
Week 4	556 ± 18.9			605 ± 7.3		
Week 8		588 ± 17.3	580 ± 17.3		623 ± 9.9	600 ± 9.1
			Weights of RWAT depots (g)			
Week 0	2.48 ± 0.28					
Week 4	3.00 ± 0.53			7.92 ± 0.57†		
Week 8		4.82 ± 0.59	2.45 ± 0.35*		9.31 ± 0.46†	4.76 ± 0.54*†‡

Rats ate chow (lean) or high-fat diet (obese) from weeks 0 to 8. Values are means ± SEM for weights for the groups of rats (N = 8 for each) killed at the times shown. Treatment with saline or CL was between weeks 4 and 8. Rats killed at weeks 0 and 4 did not receive implanted pumps.

* Significant effect of CL ($P < 0.05$) compared with saline-treated rats eating the same diet.

† Significant effect of diet ($P < 0.05$) compared with the same time and treatment groups.

‡ Significant effect of CL ($P < 0.05$) compared with the pretreated state at week 4.

(0.5 μm) were cut and stained with methylene blue and azur II [5, 16].

Statistical Analysis

Results are presented as means ± SEM. Statistical analysis used Instat software to do ANOVA followed by a Student–Newman–Keuls post hoc test. Significant differences are based on $P < 0.05$.

RESULTS

Energy Balance

Body weights of rats eating chow or a high-fat diet were not significantly different (Table 1). However, the fat content of two major abdominal depots (RWAT and epididymal WAT) (Fig. 1A) and other carcass fat (Fig. 1B) were both much greater in rats eating the high-fat diet. The large increase in abdominal fat content seen at 4 weeks was reversed to the level in chow-fed rats by 4 weeks of treatment with CL (Fig. 1A). CL-treated chow-fed rats also had less fat in their abdominal depots than did saline-treated chow-fed rats, but the difference was not as marked as that in the rats eating the high-fat diet and was due mainly to the prevention of an aging-associated increase in fat content, not to a disappearance of fat (Fig. 1A). Carcass fat other than that in the two abdominal depots noted above continued to increase through the 8-week period in the rats eating the high-fat diet; the increase ceased when CL was infused, but this fat compartment did not decrease in size with the CL treatment (Fig. 1B). Little increase in this fat compartment occurred in the chow-fed rats during the 8-week period, and CL had no effect on it. Energy intake was the same in the rats eating the high-fat diet as in rats eating chow and it was not altered by treatment with CL (Fig. 2A). The resting metabolic rate of rats treated with CL was increased substantially regardless of diet (Fig. 2B). Rectal temperatures were not altered by the CL treatment (see legend to Fig. 2).

White Adipose Tissue Depots

Both retroperitoneal and epididymal WAT depots were studied. Data are presented only for RWAT; changes in epididymal WAT were similar but usually of lesser magnitude. The elevated weight of RWAT induced by the high-fat diet was reduced by CL treatment (Table 1). CL treatment prevented the aging-associated increase in RWAT weight in the chow-fed rats but did not decrease it from that seen before the treatment (Table 1). White adipocyte size increased markedly with the development of obesity on the high-fat diet, and this increase was reversed by the treatment with CL (Fig. 3A). The number of mature white adipocytes was not altered significantly by the high-fat diet, and CL treatment did not alter this number (Fig. 3B). Despite the unchanged number of mature white adipocytes, DNA content of RWAT increased progressively with age, especially marked in the rats eating the high-fat diet (Fig. 4A); the diet-induced increase in DNA content at 4 weeks was reversed by CL treatment in rats that continued to eat the high-fat diet (Fig. 4A); in rats eating chow, the small aging-associated increase in DNA content of RWAT was prevented by CL treatment (Fig. 4A). Remarkably, protein content of RWAT was increased markedly by CL treatment, particularly in the rats eating the high-fat diet in which it increased 5-fold (Fig. 4B), although these depots weighed less and contained fewer total cells than those in the saline-treated rats eating the same diet.

Histology of WAT

Interpretation of the CL-induced changes in DNA content and weight (both decreased), mature white adipocyte number (unchanged), and protein content (increased) in both types of obese rat required histological and immunohistochemical evaluation of the tissue to assess its cellular composition.

Routine histology of RWAT of the saline-treated rats

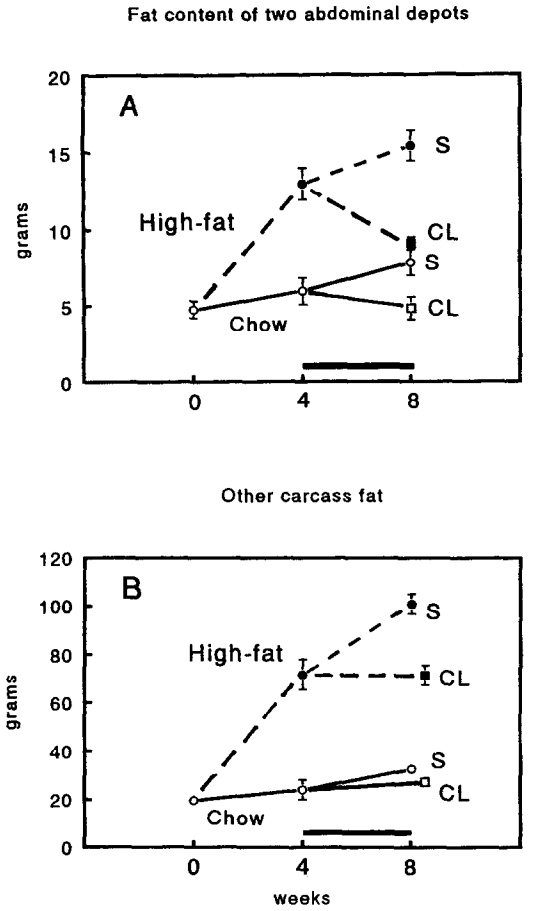

FIG. 1. Body fat in DIO and lean rats. All values are means ± SEM (N = 8). Panel A shows fat content of two major abdominal depots (epididymal and retroperitoneal). Closed symbols and dotted line are DIO rats that ate a high-fat diet from week 0 to week 8 (High-fat). Open symbols and solid line are control rats (Chow). Rats were treated with saline (S) or CL 316,243 (CL) between 4 and 8 weeks (denoted by bar along x-axis). Effect of high-fat diet at 4 weeks, $P < 0.001$. CL-DIO vs S-DIO at 8 weeks, $P < 0.001$, CL-DIO rats at 8 weeks vs DIO rats at 4 weeks, $P < 0.001$. CL vs S at 8 weeks for chow-fed rats, $P < 0.05$, CL-chow rats at 8 weeks vs chow rats at 4 weeks, not significant (NS). Panel B shows other carcass fat. Symbols, lines, and labels are the same as in Panel A. Effect of high-fat diet at 4 weeks, $P < 0.001$. CL vs S at 8 weeks, $P < 0.001$; CL-DIO rats at 8 weeks vs DIO rats at 4 weeks, NS. S-DIO rats at 8 weeks vs DIO rats at 4 weeks, $P < 0.001$.

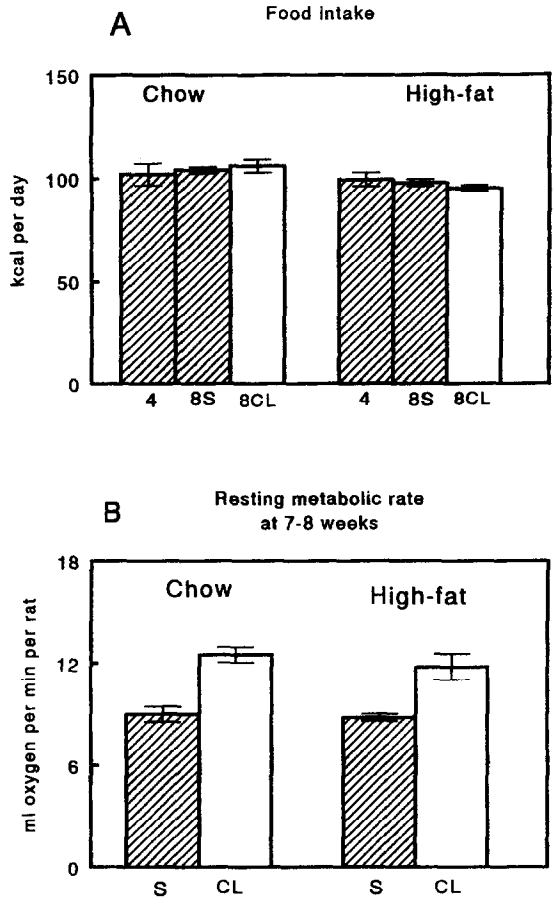

FIG. 2. Food intake and resting metabolic rates. All values are means ± SEM (N = 8). Panel A shows food intake during the first 4 weeks of diet (bar labeled 4) and during the subsequent 4 weeks of treatment with saline (bar labeled 8S) or CL (bar labeled 8CL) in chow-fed rats (Chow) and DIO rats (High-fat). There were no significant differences. Panel B shows the resting metabolic rate measured during weeks 7–8. It was significantly higher ($P < 0.001$) in CL-treated rats for both diet treatments. Rectal temperatures of the rats were 37.70 ± 0.093 (S-Chow), 38.00 ± 0.195 (CL-Chow), 37.73 ± 0.180 (S-High-fat), and 37.96 ± 0.150 (CL-High-fat). There were no significant differences in rectal temperature.

showed the typical appearance of numerous large unilocular white adipocytes, apparently the predominant cell type in the tissue; adipocytes appeared to be larger in the DIO rats (Fig. 5, C and D). Histology of RWAT of the CL-treated rats revealed unilocular white adipocytes that were smaller than those in the saline-treated rats eating the same diet and, in addition, abundant more densely stained and much smaller cells, many with a multilocular appearance (Fig. 5, A and B). Immunohistochemistry showed that these multilocular cells contained UCP (Fig. 6, A and B), not visible at all in RWAT of the saline-treated rats (Fig. 6, C and D). Higher power light microscopy of semi-thin sections showed the multilocular cells to have densely stained cytoplasm, numerous very small lipid droplets, and large pale nuclei with a prominent nucleolus, all characteristic of brown adipocytes (Fig. 7, A and B). The multilocular cells were associated with numerous capillaries and were present in clusters, between mature white adipocytes (Fig. 7A). Multilocular cells were not seen in sections of WAT of the saline-treated rats (Fig. 7, C and D). Western blotting showed that RWAT of the CL-treated rats contained a 32 kDa protein, identified as UCP (not shown).

Brown Adipose Tissue

Both interscapular and perirenal BAT depots were studied. Data are presented only for interscapular BAT (IBAT); changes in perirenal BAT were similar. Total protein content and UCP content were increased markedly by CL

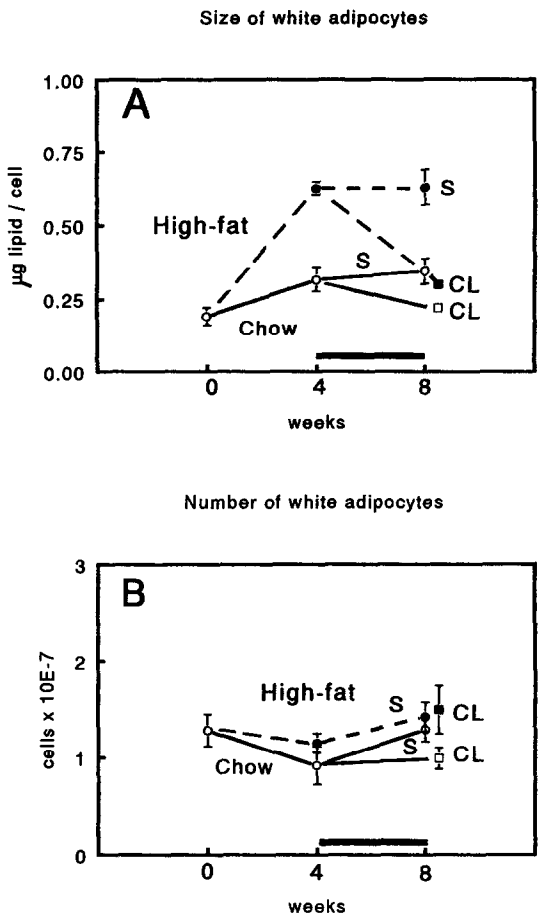

FIG. 3. Size and number of white adipocytes in RWAT as assessed by the osmium fixation method [14]. All values are means ± SEM (N = 8). Symbols, lines, and labels are the same as in Fig. 1A. Panel A shows adipocyte size. DIO rats at 4 weeks vs chow rats at 4 weeks, $P < 0.001$. CL-DIO rats at 8 weeks vs saline-DIO (S-DIO) rats at 8 weeks, $P < 0.001$, and vs DIO rats at 4 weeks, $P < 0.001$. S-DIO rats at 8 weeks vs DIO rats at 4 weeks, NS. CL-chow rats at 8 weeks vs S-chow rats at 8 weeks, $P < 0.05$, and vs chow-rats at 4 weeks, NS. S-chow rats at 8 weeks vs chow rats at 4 weeks, NS. Panel B shows total adipocyte number. The y-axis shows total number of cells multiplied by 10^{-7}. There were no significant differences.

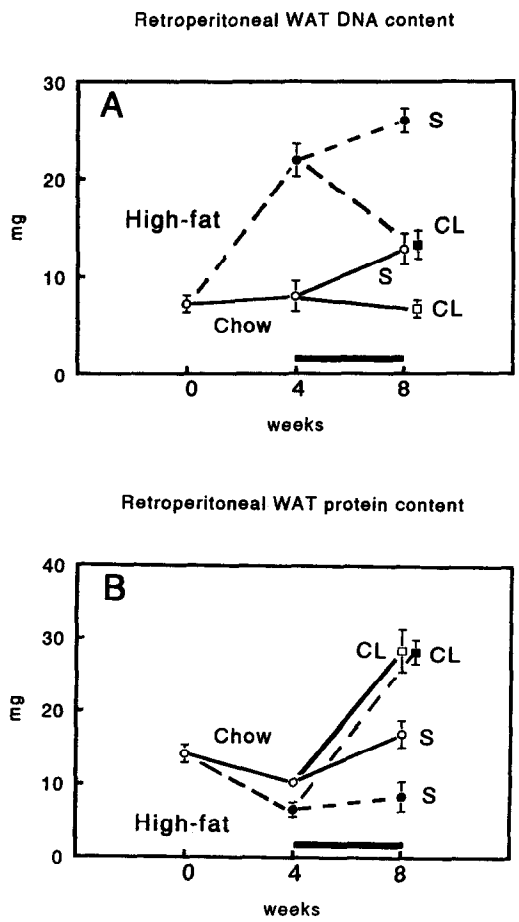

FIG. 4. DNA and protein contents of RWAT. All values are means ± SEM (N = 8). Symbols, lines, and labels are the same as in Fig. 1A. Panel A shows DNA content. DIO at 4 weeks vs chow at 4 weeks, $P < 0.001$. CL-DIO at 8 weeks vs saline-DIO (S-DIO) at 8 weeks, $P < 0.001$, and vs DIO at 4 weeks, $P < 0.001$. S-DIO at 8 weeks vs DIO at 4 weeks, NS. CL-chow at 8 weeks vs S-chow at 8 weeks, $P < 0.05$, and vs chow at 4 weeks, NS. Panel B shows total protein content. CL-DIO at 8 weeks vs S-DIO at 8 weeks, $P < 0.001$, and vs DIO at 4 weeks, $P < 0.001$. CL-chow at 8 weeks vs S-chow at 8 weeks, $P < 0.001$, and vs chow at 4 weeks, $P < 0.001$.

treatment. The increases were similar in rats eating chow and in rats eating the high-fat diet (Fig. 8, A and B). As in our previous study [5], DNA content of IBAT was not altered by CL treatment (not shown).

DISCUSSION

CL treatment is shown here to reverse established DIO in rats that continued to eat a high-fat diet during the treatment. The DIO had developed without any increase in energy intake; thus, it was a manifestation of the high metabolic efficiency with which a high-fat diet is processed. The negative energy balance associated with CL-induced reversal of DIO was brought about by an increase in energy expenditure, without any change in energy intake. The loss of fat was primarily from the intraabdominal WAT depots. Other carcass fat, while ceasing to accrue, was not reduced by the CL treatment. The obesity induced by diet was hypertrophic (increased size of white adipocytes) rather than hyperplastic (increased number of white adipocytes), and the enlarged size of the adipocytes was returned to normal by the drug treatment. The drug had less effect on the smaller cells in the chow-fed control rats, and it did not alter the number of white adipocytes in either the DIO or the chow-fed rats.

An increase in energy expenditure induced by a β_3-adrenoceptor agonist is likely to occur in brown adipocytes. The substantial increase in energy expenditure induced by the CL treatment was associated with alterations in two distinct populations of brown adipocytes. First, there was an expansion of the mitochondrial compartment in brown adipocytes in BAT. In our previous study, we showed that there was no change in the relative proportions of different

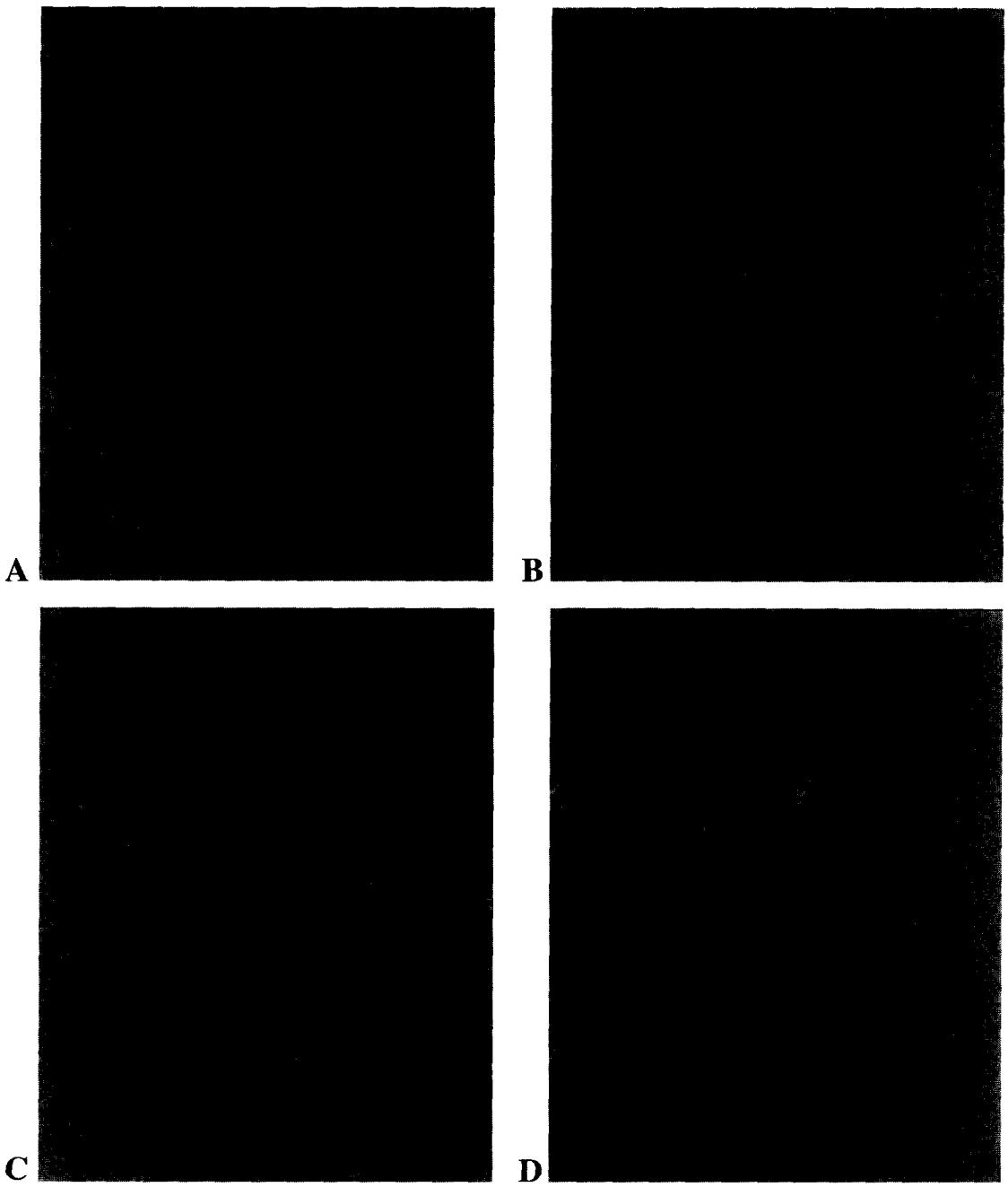

FIG. 5. Histology of RWAT. Hematoxylin and eosin-stained 10-μm sections of RWAT of lean, CL-treated (A), DIO, CL-treated (B), lean, saline-treated (C) and DIO, saline-treated (D) rats. Magnification: 400×. Note the large number of very small cells, many of them multilocular, in the CL-treated rats (A and B) and the apparent preponderance of unilocular white adipocytes in saline-treated rats (C and D).

cell types in BAT of CL-treated rats [5]; hence we conclude in the present study that the 10-fold increase in UCP content occurred in brown adipocytes already present. Second, abundant multilocular brown adipocytes that expressed UCP appeared in what is traditionally regarded as white adipose tissue, the RWAT. Their appearance was accompanied by a large increase in total protein content of the WAT, presumably present mainly in mitochondria in these brown adipocytes and occurring despite a diminution in the total number of cells (as assessed by the DNA content), a decrease in size but not in number of mature white adipocytes, and a corresponding decrease in tissue weight. We suggest that the overall increase in energy expenditure induced by CL occurred in two distinct populations of thermogenically competent brown adipocytes: those in BAT and those induced to appear in WAT by the drug treatment. (The origin of the latter population is discussed in more detail below.)

The increase in energy expenditure was not compensated for by any increase in energy intake. It is known that treatment with a β_3-adrenoceptor agonist suppresses leptin expression in WAT and decreases the concentration of

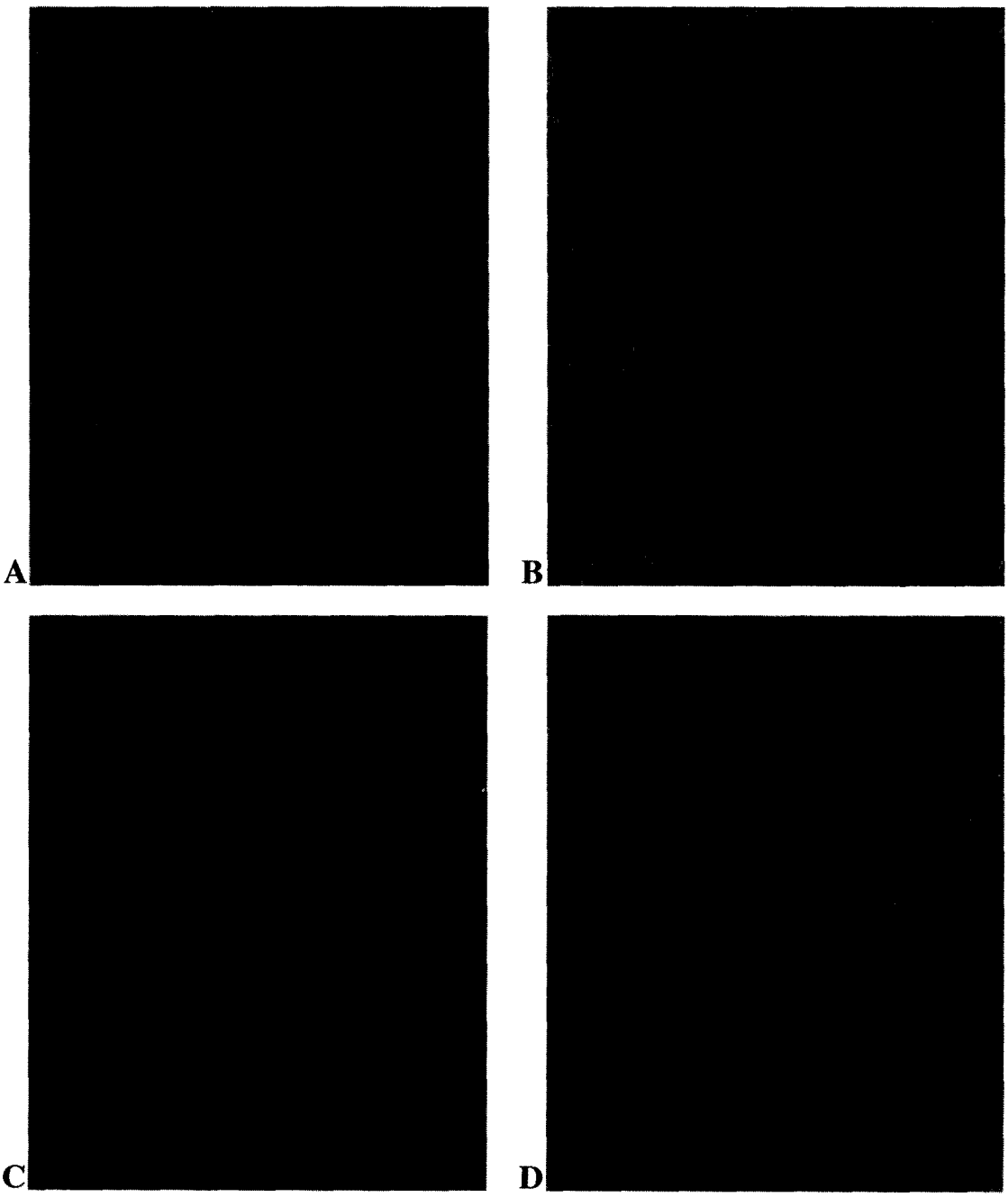

FIG. 6. Immunohistochemical detection of UCP in RWAT. UCP in 10-μm sections of RWAT of lean, CL-treated (A), DIO, CL-treated (B), lean, saline-treated (C) and DIO, saline-treated (D) rats. Magnification: 400×. Multilocular cells in CL-treated rats contain UCP (black stain); hence, they are defined as brown adipocytes.

leptin in blood [17, 18]. The failure of energy intake to rise to meet expenditure must therefore be independent of any decrease in leptin concentration [17], which would itself be expected to increase intake. It seems likely that the extra heat production induced by CL was responsible for suppressing energy intake [19].

The DNA content of the BAT was not changed by CL treatment, as also seen in our previous shorter study [5], presumably because the precursor interstitial cells possess only β_1-adrenergic receptors [20] so that the mitogenic effect of norepinephrine, seen, for example, during cold adaptation [16], does not occur with selective β_3-adrenergic stimulation. One report of an increase in DNA content of BAT in response to β_3-adrenoceptor stimulation [21] used a different and less selective β_3-adrenoceptor agonist, BRL 35135, acknowledged to possess both β_1- and β_3-adrenergic agonist properties [21].

Although DIO did not increase the number of mature white adipocytes in WAT, it was associated with an increase in total cell number, as assessed from DNA content, and this increase in non-adipocyte cells was reversed by the CL treatment. The cells that appeared then

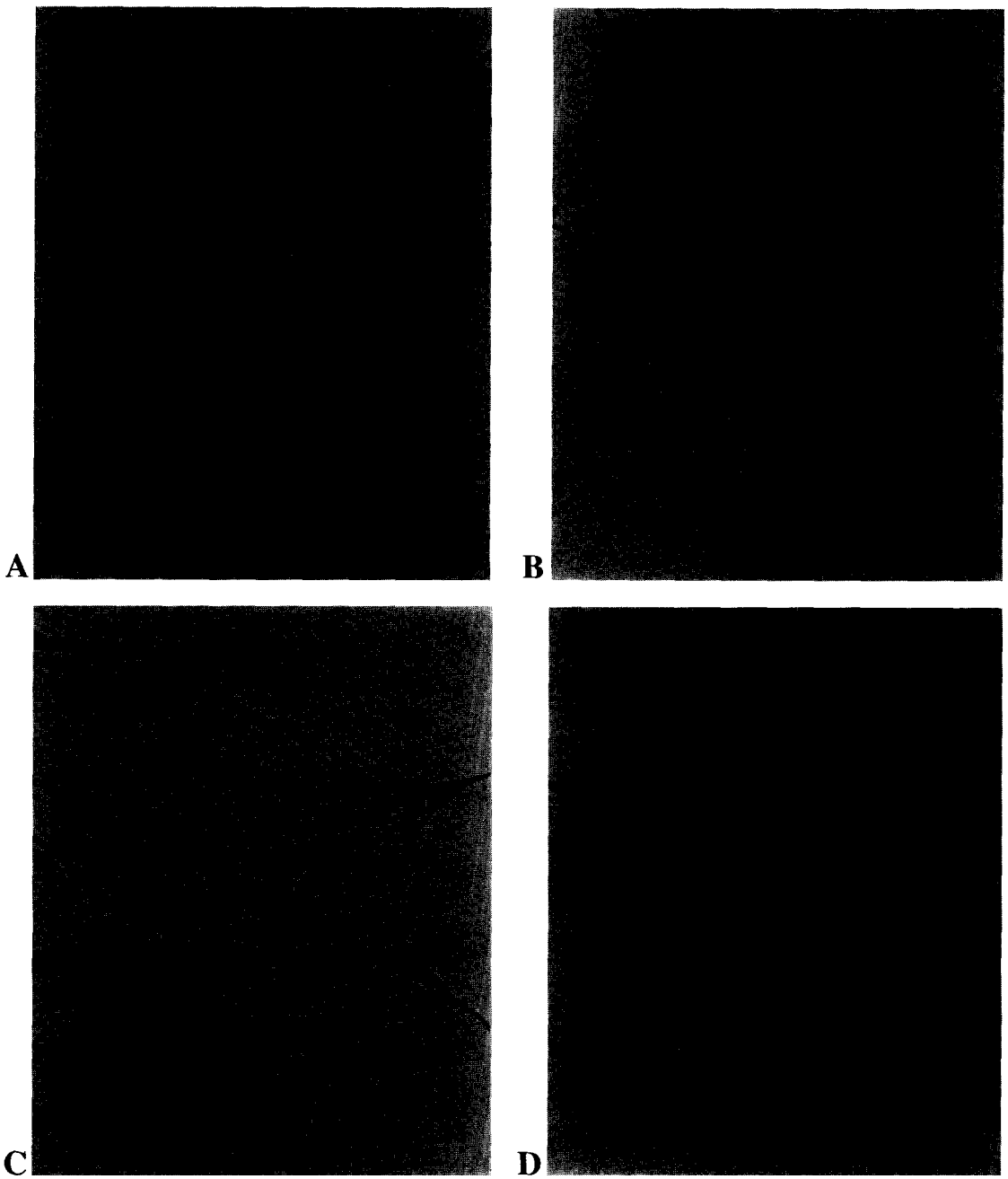

FIG. 7. Semi-thin sections of retroperitoneal WAT of CL-treated rats. Methylene blue and azur blue II-stained semi-thin (0.5 μm) sections of RWAT of lean, CL-treated (A), DIO, CL-treated (B), lean, saline-treated (C) and DIO, saline-treated (D) rats. Magnification: 2000×. In the CL-treated lean rat, the multilocular nature and the large palely stained nuclei with a dense nucleolus characterize these cells further as typical brown adipocytes. They are associated with numerous capillaries (A). Also visible are triangular-shaped interstitial cells. In the DIO rat (B), multilocular cells tended to cluster around unilocular white adipocytes; they had larger lipid droplets than the brown adipocytes in the lean rats (A).

disappeared were not mature white adipocytes, judging from the unchanged number of these cells. The reduction occurred at a time when brown adipocytes were actually appearing in the tissue and white adipocytes remaining unchanged in number. A decrease in DNA content of WAT has also been seen in long-term food-deprived rats [22] and in streptozotocin-diabetic rats [23]. In food-deprived rats, as in the present experiment, the decrease in DNA content was not accompanied by any loss of white adipocytes [22]. Although some adipocytes disappeared in the streptozotocin-diabetic rats [23], it seems likely that the adipocytes that remained had so little lipid as to be indistinguishable from interstitial cells by light microscopy, and hence were not counted in the histological procedure used. We suggest that the cells that disappeared from WAT of the obese rats were vascular cells, recruited to support increased circulation of blood in the 3-fold expanded mass of the WAT and lost when the mass decreased under the

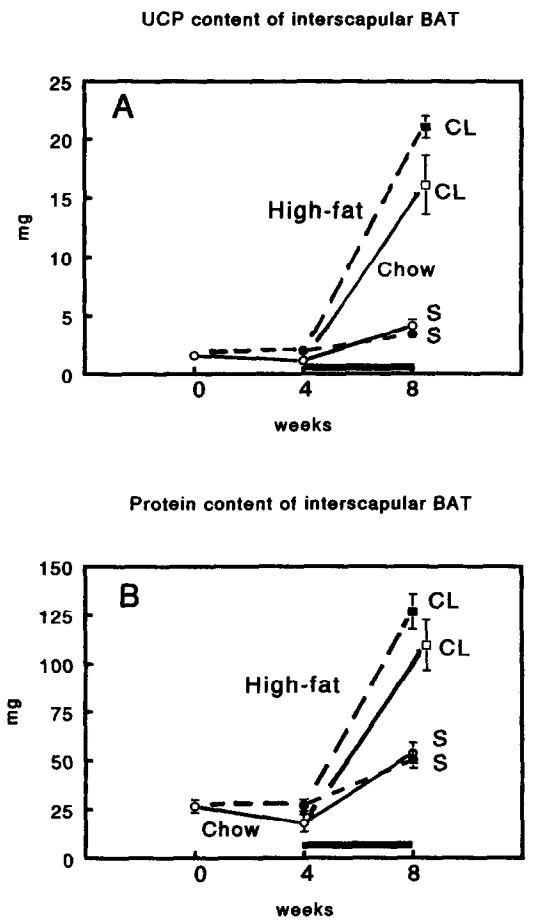

FIG. 8. UCP and protein content of interscapular BAT. Values are means ± SEM (N = 8). Symbols, lines, and labels are the same as in Fig. 1A. Panel A shows UCP content. CL at 8 weeks vs saline (S) at 8 weeks or vs 4 weeks, $P < 0.001$ for both diet treatments. Panel B shows total protein content. CL at 8 weeks vs S at 8 weeks or vs 4 weeks, $P < 0.001$ for both diet treatments.

influence of the drug. There is some information about the role of angiogenesis in altered blood flow in WAT in obesity [24], but the mechanisms involved in the disappearance of the expanded vasculature of WAT when its fat content has been reduced appear to have been neglected.

The origin of the multilocular, UCP-expressing cells that appeared in RWAT of CL-treated rats remains an enigma. Their expression of UCP defines them as brown adipocytes [11]. Since the DNA content of the tissue actually decreased at the time these cells appeared, it seems improbable that brown adipocyte precursor cells had proliferated in response to the stimulus, although we cannot exclude the possibility that proliferation of one cell type was more than balanced by disappearance of another cell type. We suggest therefore that very small precursors to brown adipocytes, perhaps brown preadipocytes or protoadipocytes [25], are present in WAT depots and are induced to differentiate into mature brown adipocytes by sustained β_3-adrenergic stimulation, undergoing both marked mitochondrial proliferation and lipogenesis, thereby acquiring triacylglycerol stores. It seems unlikely that CL has promoted proliferation and differentiation of precursor interstitial cells in WAT since it does not have this effect on precursor interstitial cells in BAT, where these cells possess β_1- rather than β_3-adrenoceptors [20]. We suggest that these precursors to brown adipocytes are always present in WAT, but are very small, with a low content of mitochondria, UCP, and lipogenic enzymes, and are not readily seen in routine histological sections. Perhaps they lack the rich sympathetic and sensory innervation characteristic of brown adipocytes in BAT depots. We suggest that these precursor cells are already committed to the brown adipocyte lineage and that they possess β_3-adrenoceptors but are in an inactive state because of lack of suitable stimulation. Thus, they are induced to differentiate and hypertrophy only by high concentrations of a β_3-adrenoceptor agonist. Others have described dormant or masked brown adipocytes in certain WAT depots [6–10]. Moreover, precursors of brown adipocytes are present in the stromal-vascular fraction of WAT (at least in the Siberian hamster) and mature into brown adipocytes during culture, when they constitute 10–15% of the total adipocytes formed [26]. Our hypothesis differs from the three hypotheses advanced by Casteilla and colleagues [6] to take into account the apparent presence of β_3-adrenoceptors on the cells (brown adipocyte precursor interstitial cells in BAT do not possess these receptors) and the lack of measurable change in the number of white adipocytes (the brown adipocytes that appeared could not have been derived from cells with the characteristics of white adipocytes).

Another possible explanation for the origin of the brown adipocytes in WAT is that uncommitted precursor cells are available that are stimulated to differentiate and become committed to the brown adipocyte lineage by a product released locally from the intensely stimulated white adipocytes surrounding them. A vascular origin for these uncommitted cells is possible [27], and it is noteworthy that the clusters of brown adipocytes that appeared in RWAT of the CL-treated rats were associated with the vasculature. Activation by free fatty acids (FFA) (or a specific FFA or product thereof such as a prostaglandin) of a member of the PPARγ family of nuclear hormone receptors and, as a consequence, promotion of adipocyte differentiation is a possibility [28, 29]. PPARγ is expressed strongly in adipose tissues [30]. Forced expression of PPARγ can induce adipose differentiation in cell lines that normally would not follow this route [30]. Moreover, thiazolidinediones, known to be ligands for the PPARγ receptor [31], enhance expression of UCP in brown adipocytes in which this expression is stimulated by norepinephrine [32]. However, it is not known whether this receptor could play a role in determining whether a cell will develop into a brown or white adipocyte.

The level of expression of the β_3-adrenoceptor in adult human adipose tissues is controversial [4], although there is no doubt that it is expressed in newborn human BAT [33]. However, precursor cells from adult human adipose tissues

can differentiate in culture and can be induced to express UCP by β_3-adrenoceptor agonists, including CL, that is, they differentiate into brown adipocytes [34]. Thus, it remains possible that use of β_3-adrenoceptor agonists in adult obese humans might, by inducing the appearance of brown adipocytes in adipose tissues, raise energy expenditure sufficiently to be of help in the reversal of obesity.

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References

1. Bloom JD, Dutia MD, Johnson BD, Wissner A, Burns MG, Largis EE, Dolan JA and Claus TH, Disodium (*R,R*)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]-amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate (CL 316,243). A potent β-adrenergic agonist virtually specific for β_3 receptors. A promising antidiabetic and antiobesity agent. *J Med Chem* **35:** 3081–3084, 1992.
2. Dolan JA, Muenkel HA, Burns MG, Pellegrino SM, Fraser CM, Pietri F, Strosberg AD, Largis EE, Dutia MD, Bloom JD, Bass AS, Tanikella TK, Cobuzzi A, Lai FM and Claus TH, *Beta*-3 adrenoceptor selectivity of the dioxolane dicarboxylate phenethanolamines. *J Pharmacol Exp Ther* **269:** 1000–1006, 1994.
3. Largis EE, Burns MG, Muenkel HA, Dolan JA and Claus TH. Antidiabetic and antiobesity effects of a highly selective β_3-adrenoceptor agonist (CL 316,243). *Drug Dev Res* **32:** 69–76, 1994.
4. Himms-Hagen J and Danforth E Jr., The potential role of beta-3 adrenoceptor agonists in the treatment of obesity and diabetes. *Curr Opin Endocrinol Diabetes* **3:** 59–65, 1996.
5. Himms-Hagen J, Cui J, Danforth E Jr, Taatjes DJ, Lang SS, Waters BL and Claus TH, Effect of CL-316,243, a thermogenic β_3-agonist, on energy balance and brown and white adipose tissues in rats. *Am J Physiol* **266:** R1371–R1382, 1994.
6. Casteilla L, Cousin B, Viguerie-Bascands N, Larrouy D and Pénicaud L, Hétérogénéité et plasticité cellulaires des tissus adipeux. *Médecine/Sciences* **10:** 1099–1106, 1994.
7. Cousin B, Casteilla L, Dani C, Muzzin P, Revelli JP and Pénicaud L, Adipose tissues from various anatomical sites are characterized by different patterns of gene expression and regulation. *Biochem J* **292:** 873–876, 1993.
8. Cousin B, Cinti S, Morroni M, Raimbault S, Ricquier D, Pénicaud L and Casteilla L, Occurrence of brown adipocytes in rat white adipose tissue: Molecular and morphological characterization. *J Cell Sci* **103:** 931–942, 1992.
9. Loncar D, Convertible adipose tissue in mice. *Cell Tissue Res* **266:** 149–161, 1991.
10. Young P, Arch JRS and Ashwell M, Brown adipose tissue in the parametrial fat pad of the mouse. *FEBS Lett* **167:** 10–14, 1984.
11. Himms-Hagen J and Ricquier D, Brown adipose tissue. In: *Handbook of Obesity* (Eds. Bray GA, Bouchard C and James WPT), in press. Marcel Dekker, New York; 1997.
12. Nagase I, Yoshida T, Kumamoto K, Umekawa T, Sakane N, Nikami H, Kawada T and Saito M, Expression of uncoupling protein in skeletal muscle and white fat of obese mice treated with thermogenic β_3-adrenergic agonist. *J Clin Invest* **97:** 2898–2904, 1996.
13. Cui J and Himms-Hagen J, Rapid but transient atrophy of brown adipose tissue in capsaicin-desensitized rats. *Am J Physiol* **262:** R562–R567, 1992.
14. Hirsch J and Gallian E, Methods for the determination of adipose cell size in man and animals. *J Lipid Res* **9:** 110–119, 1968.
15. Brandtzaeg P, Tissue preparation for immunocytochemistry. In: *Techniques in Immunocytochemistry I* (Eds. Bullock GR and Petrusz PP), pp. 2–75. Academic Press, London; 1982.
16. Géloën A, Collet AJ and Bukowiecki LJ, Role of sympathetic innervation in brown adipocyte proliferation. *Am J Physiol* **263:** R1176–R1181, 1992.
17. Mantzoros CS, Qu D, Frederich RC, Susulic VS, Lowell BB, Maratos-Flier E and Flier JS, Activation of β_3 adrenergic receptors suppresses leptin expression and mediates a leptin-independent inhibition of food intake in mice. *Diabetes* **45:** 909–914, 1996.
18. Collins S and Surwit RS, Pharmacologic manipulation of *ob* expression in a dietary model of obesity. *J Biol Chem* **271:** 9437–9440, 1996.
19. Himms-Hagen J, Role of brown adipose tissue thermogenesis in control of thermoregulatory feeding in rats: A new hypothesis that links thermostatic and glucostatic hypotheses for control of food intake. *Proc Soc Exp Biol Med* **208:** 159–169, 1995.
20. Bronnikov G, Houstek J and Nedergaard J, β-Adrenergic, cAMP-mediated stimulation of proliferation of brown fat cells in primary culture. Mediation via β_1 but not via β_3 adrenoceptors. *J Biol Chem* **267:** 2006–2013, 1992.
21. Arbeeny CM, Meyers DS, Hillyer DE and Bergquist KE, Metabolic alterations associated with the antidiabetic effect of β_3-adrenergic receptor agonists in obese mice. *Am J Physiol* **268:** E678–E684, 1995.
22. Miller WH Jr, Faust IM, Goldberger SC and Hirsch J, Effects of severe long-term food deprivation and refeeding on adipose tissue cells in the rat. *Am J Physiol* **245:** E74–E80, 1983.
23. Géloën A, Roy PE and Bukowiecki LJ, Regression of white adipose tissue in diabetic rats. *Am J Physiol* **257:** E547–E553, 1989.
24. Crandall DL, Herzlinger HE, Saunders BD and Kral JG, Developmental aspects of the adipose tissue renin-angiotensin system: Therapeutic implications. *Drug Dev Res* **32:** 117–125, 1994.
25. Géloën A, Collet AJ, Guay G and Bukowiecki LJ, *In vivo* differentiation of brown adipocytes in adult mice: An electron microscopic study. *Am J Anat* **188:** 366–372, 1990.
26. Klaus S, Ely M, Encke D and Heldmaier G, Functional assessment of white and brown adipocyte development and energy metabolism in cell culture. *J Cell Sci* **108:** 3171–3180, 1995.
27. Cinti S and Morroni M, Brown adipocyte precursor cells: A morphological study. *Ital J Anat Embryol* **100** (Suppl 1), 75–81, 1995.
28. Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM and Evans RM, 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 is a ligand for the adipocyte determination factor PPARγ. *Cell* **83:** 803–812, 1995.
29. Kliewer SA, Lenhard JM, Wilson TM, Patel I, Morris DC and Lehmann JM, A prostaglandin J_2 metabolite binds peroxisome proliferator-activated receptor γ and promotes adipocyte differentiation. *Cell* **83:** 813–819, 1995.
30. Tontonoz P, Hu E and Spiegelman BM, Regulation of adipocyte gene expression and differentiation by peroxisome proliferator activated receptor γ. *Curr Opin Genet Dev* **5:** 571–576, 1995.
31. Lehmann JM, Moore LB, Smith-Oliver T, Wilkison WO, Willson TM and Kliewer SA, An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ). *J Biol Chem* **270:** 12953–12956, 1995.
32. Foellmi-Adams LA, Wyse BM, Herron D, Nedergaard J and Kletzien RF, Induction of uncoupling protein in brown

adipose tissue. Synergy between norepinephrine and pioglitazone, an insulin-sensitizing agent. *Biochem Pharmacol* **52:** 693–701, 1996.

33. Deng C, Paoloni-Giacobino A, Kuehne F, Boss O, Revelli J-P, Moinat M, Cawthorne MA, Muzzin P and Giacobino J-P, Respective degree of expression of β_1-, β_2- and β_3-adrenoceptors in human brown and white adipose tissues. *Br J Pharmacol* **118:** 929–934, 1996.
34. Champigny O and Ricquier D, 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* **37:** 1907–1914, 1996.