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Immunohistochemical Localization of Leptin and Uncoupling Protein in White and Brown Adipose Tissue*

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ABSTRACT

Leptin is synthesized exclusively by adipocytes and acts on the hypothalamus to regulate energy balance. Previous messenger RNA expression studies demonstrated that leptin is expressed in white adipocytes and also in brown adipose tissue, however expression in brown fat is markedly lower than in white fat. This suggests the possibility that leptin expression in brown adipose tissue is due to the presence of white adipocytes that reside within brown adipose tissue, and that brown adipocytes actually do not express leptin. To address this point, we performed immunohistochemistry on paraffin sections and studied leptin protein expression in different depots of white and brown fat of lean and obese (*db/db*) mice. To establish the cell type expressing leptin, we also assessed the size and organization of lipid droplets, the ultrastructural features of mitochondria, and the presence or absence of uncoupling protein, a brown fat-specific marker. In white adipose tissue of lean and obese (*db/db*) mice, leptin protein was expressed in adipocytes of various sizes (range examined: 19.67–200 μm), including adipocytes at the multilocular stage of differentiation. Leptin staining was more intense in some depots (retroperitoneal), and appeared to decrease with fasting. In brown adipose tissue of lean animals, multilocular uncoupling protein (UCP)-positive brown adipocytes had typical brown mitochondria and were leptin-negative, both in fed and fasted conditions. At the periphery of the interscapular brown adipose tissue depot, unilocular, UCP-negative adipocytes (mean diameter: 41.55 μm) with white-type mitochondria were observed, and these cells were leptin-positive. In obese (*db/db*) animals, brown fat was composed mainly of small unilocular, UCP-positive adipocytes (mean diameter: 40.08 μm), which were also leptin-positive. At the periphery of the organ, numerous large, unilocular, UCP-negative adipocytes (mean diameter: 73.65 μm) with white-like mitochondria were present. As expected, these cells were also leptin-positive. In summary, classical brown adipocytes differ from white adipocytes, not only by their morphology and UCP expression, but also by their apparent lack of detectable leptin expression. *db/db* brown adipocytes, however, were unilocular and leptin-positive. The molecular mechanisms mediating expression of leptin in white but not brown adipocytes of lean animals, and the significant expression of leptin in brown adipocytes of *db/db* mice will be the focus of future studies. (*Endocrinology* **138:** 797–804, 1997)

THE RECENT DISCOVERY of leptin and its receptor has opened important new avenues for obesity research (1–5). Leptin is synthesized by adipose tissue (1, 6, 7) and acts on the hypothalamus, causing decreased food intake and increased energy expenditure (8–10), and as such may be part of a feedback loop regulating body fat stores. Adipose tissue is composed of many cell types including endothelial cells, fibroblasts, vessel muscle cells, Schwann cells, preadipocytes, young adipocytes, and mature adipocytes containing variable amounts of lipid. Hitherto, published studies were unanimous in indicating that leptin messenger RNA (mRNA) in white fat is expressed exclusively by mature white adipocytes (6, 11–13). Leptin gene expression in brown fat, however, is somewhat controversial because some authors report minimal expression (7, 11, 14), whereas others report considerable levels of expression (15). Because brown adipose tissue is composed of numerous cell types, including varying numbers of white adipocytes (16–18), it is possible that leptin expression in this tissue is due to the presence of contaminating white adipocytes (as previously suggested by Trayhurn *et al.*, Ref. 7). This raises the possibility that brown adipocytes do not express leptin. This point merits further study because at present all known adipose-specific genes are expressed in both white and brown adipocytes (adipocyte lipid binding protein, adipsin, etc.) or in brown adipocytes only [uncoupling protein (UCP)], with none being expressed exclusively in white adipocytes (19–21). If studies ultimately reveal that leptin is expressed exclusively in white but not brown adipocytes, then further studies aimed at elucidating molecular mechanisms accounting for this unique pattern of expression should shed light on two important questions, namely the mechanism for regulation of leptin gene expression and the identity of factors controlling white *vs.* brown adipocyte differentiation.

In the present study, we used immunohistochemical techniques to localize leptin expression in white and brown adipose tissue of fed and fasted lean mice, as well as in genetically obese (*db/db*) mice. To characterize cell types expressing leptin, morphological features such as size of lipid droplets, organization of lipid droplets (multi- *vs.* unilocular), ultrastructural characterization of mitochondria (normal *vs.* densely packed cristae typical of brown adipocytes), and UCP immunolocalization were also assessed. Our

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results indicate that in white adipose tissue, leptin is present in adipocytes of all diameters of lean and obese animals, including adipocytes at a multilocular stage of differentiation, but not in any other cell types. Importantly, our data also indicate that in brown adipose tissue, leptin is not expressed at detectable levels in classical multilocular, UCP-positive brown adipocytes, but is expressed in unilocular, UCP-negative, white adipocytes that reside within brown adipose tissue. Of interest, brown adipose tissue of genetically obese (*db/db*) mice possessed numerous small, unilocular adipocytes bearing mitochondria ultrastructurally typical of brown adipocytes. These cells were absent from white or brown adipose tissue of normal mice. Remarkably, these cells were positive for both UCP and leptin, raising the possibility that these cells may represent an intermediate stage of development between classical brown and white adipocytes.

Materials and Methods

Animals and tissues

Twelve NMRI (male and female) mice were obtained from Morini Laboratories (S.Polo d'Enza, RE, Italy); 6 female C57BLKsJ *db/db* and *db*/? mice were purchased from Jackson Laboratories (Bar Harbor, ME). All the animals were 13 weeks old and had access to standard lab chow and water *ad libitum* (fed condition), except 6 NMRI mice of both sexes that were deprived of food for 36 h before death (fasting condition). Mice were weighed and anesthetized with ether and chloral hydrate (Merck, Darmstad, Germany) (300 mg/kg, ip) (NMRI mice) or ketamine (Ketaset, Bristol-Myers, Syracuse, NY) (100 mg/kg, ip) in combination with xylazine (Rompum, Haver, Mobey Corp, Shawnee, KS) (10 mg/kg, ip) (C57BLKsJ mice). Care of the mice was in accordance with institutional guidelines.

Tissues used for our investigation were: interscapular brown adipose tissue and epididymal, parametrial, and retroperitoneal white adipose tissue. Liver, kidney, and uterus were used for control tissues. Transcardial perfusions were performed with paraformaldehyde 4% in PBS after washing in Ringer's solution. After perfusion, tissues were dissected under a surgical microscope (Zeiss OPM 19, Carl Zeiss, Oberkochen, Germany) and fixed by immersion in the same fixative overnight for light microscopy and immunohistochemistry, and in glutaraldehyde 2% and paraformaldehyde 2% in phosphate buffer 0.1 M, pH 7.4 for 4 h, for ultrastructural studies. Brown adipose tissue was carefully microdissected from the surrounding white adipose tissue, and this dissection required about 15 min for each organ. Tissues for light microscopy and immunohistochemistry were embedded in paraffin blocks. For ultrastructural studies small fragments were postfixed in osmium tetroxide 1%, dehydrated in ethanol, and embedded in an Epon-Araldite (Epon: Multilab Supplies, Fetcham, UK; Araldite: Fluka Chemie AG, Buchs, Switzerland) mixture. Semithin sections (2 μm) were stained with toluidine blue; thin sections were obtained with a Reichert Ultracut E (Reichert, Wien, Austria), stained with lead citrate, and examined with a transmission electron microscope, Philips CM10 (Philips, Eindhoven, The Netherlands).

Antibodies and immunohistochemistry

Polyclonal rabbit antibodies were raised against the NH2-terminal (QKVQDDTKTLIKTIVTRIND) and the COOH-terminal (RLQGSLQDILQQLDVSPEC) peptides of the predicted *Ob* gene product, termed leptin. Antibody against the COOH-terminal sequence was purified using the Sulfolink antibody purification (Pierce, Rockford, IL) (14). Polyclonal sheep antibodies were raised against purified UCP from rat brown adipose tissue (generously provided by Dr. D. Ricquier, Meudon, France). Immunohistochemical demonstration of leptin and UCP was performed with the avidin-biotin peroxidase (ABC) method. Dewaxed sections (3 μm) were processed through the following incubation steps: 1) hydrogen peroxide 0.3% in methanol for 30 min to block endogenous peroxidase; 2) glycine 0.02 M for 10 min, normal goat serum 1:75 (leptin schedule) and normal rabbit serum 1:75 (UCP schedule) for 20 min to reduce nonspecific background staining; 3) polyclonal rabbit antibodies against leptin diluted 1:3000 in PBS and sheep antirat UCP diluted 1:12000 in PBS, overnight at 4 C; 4) biotinylated secondary antibodies: goat antirabbit IgG 1:200 (leptin) and rabbit antisheep IgG 1:300 (UCP) for 30 min (Vector Labs., Burlingame CA); 5) ABC complex for 1 h (Vectastain ABC kit, Vector Labs); 6) histochemical visualization of peroxidase using 3′,3′diaminobenzidine hydrochloride cromogen (Sigma, St. Louis, MO). Methods specificity tests were performed as follows: omission of primary antibodies in the staining; use of preimmune serum instead of the first antiserum; incubation of sections with an antiserum saturated with the homologous antigen (5 μg/ml) for 48 h (adsorption test was performed only for leptin immunoreactivity).

On brown adipose tissue, UCP and leptin tests were performed on adjacent serial sections to see the reaction results on the same cell or, at least, on the same cell type close to the one on the serial section.

Morphometric analysis and statistical analysis

One hundred random cell profiles from hematoxyline-eosin sections of each of the adipose tissues were drawn using projection light microscopy to estimate with test circles the area equivalent diameter (mean diameter) (22) of the unilocular adipocytes.

Morphometric data were subjected to statistical analysis (ANOVA test) for differences among the experimental groups.

Results

White adipose tissue (epididymal, retroperitoneal, and parametrial)

Lean animals: fed state. All mature adipocytes (sizes ranging from 19.67–141.3 μm) had a thin rim of cytoplasm that positively stained for leptin. Adipocytes from retroperitoneal depots appeared more intensely leptin-stained (Fig. 1a). Adipocytes at the multilocular stage of differentiation also appeared leptin-positive (Fig. 1, b and c). All other cell types were leptin-negative. No UCP-positive cells were found (data not shown).

Lean animals: 36 h fasting. After 36 h of fasting, striking differences in the morphology of the adipose tissues (mainly in the epididymal depot) were found (not shown). The main aspect was an increased variability of cell diameter and a decreased mean cellular diameter (50.43 μm *vs.* 81.04 μm, $P < 0.001$). All adipocytes were still leptin-positive, but the reaction was less intense (Fig. 1d). No UCP-positive cells were found.

Obese animals. Studies were restricted to obese animals (*db/db* mice) in the fed state. The mean adipocyte diameter was significantly higher than that of corresponding depots of lean animals (parametrial: 99 μm $\pm$ 2.2 *vs.* 67.33 μm $\pm$ 1.06, $P = 0.017$). In obese animals the range examined was: 45.9–200 μm. Although the cytoplasmic rim was very thin, it stained positive for leptin (Fig. 2, a and b). Adipocytes at the multilocular stage of differentiation were rare and leptin-positive (not shown). No UCP-positive cells were found.

Interscapular brown adipose tissue

Lean animals: fed state. Most of the interscapular brown adipose depot was composed of multilocular UCP-positive cells (Fig. 3a). Electron microscopy showed that the mitochondria of these cells possessed densely packed cristae, which are characteristic of brown adipocytes (Fig. 3c). These multilocular UCP-positive cells were leptin-negative (Fig. 3b). At the

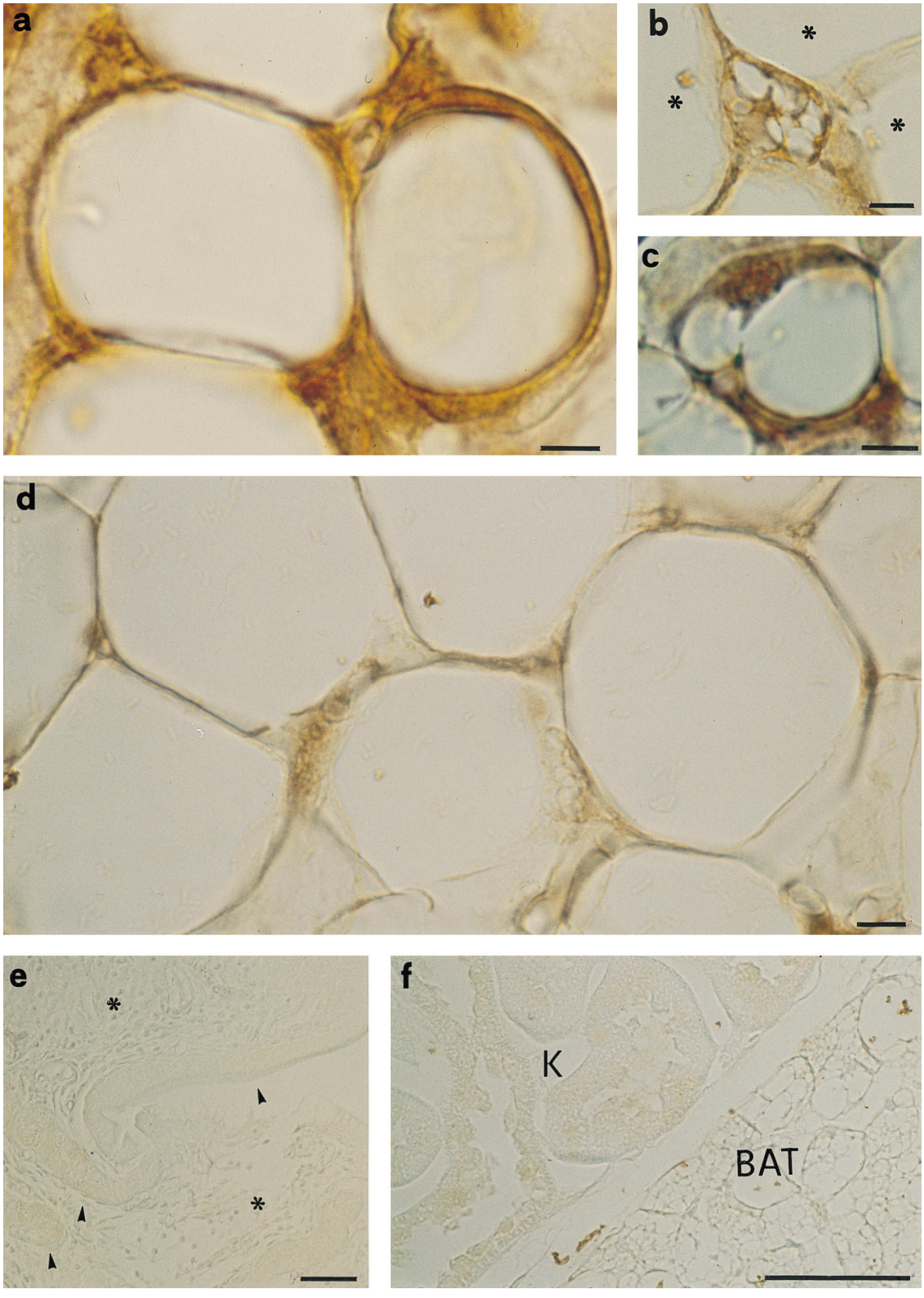

FIG. 1. Leptin immunolabeling of retroperitoneal white adipose tissue, lean-fed (a–c) and lean-36 h-fasted mice (d). a, Cytoplasmic rim of mature unilocular cells was intensely leptin-positive. b, Adipocyte at multilocular stage of differentiation among mature unilocular cells (*) was leptin-positive. c, Adipocyte at a later multilocular stage of differentiation showing intense leptin staining in cytoplasm. d, Adipocytes from fasted animals showing decreased leptin staining. e, Mouse uterus: epithelial (*arrows*) and stromal (*) tissues were leptin-negative. f, Mouse kidney (K) and perirenal brown adipose tissue (BAT). Both tissues were leptin-negative. *Bars*: a and c = 5 μm; b and d = 10 μm; e and f = 50 μm.

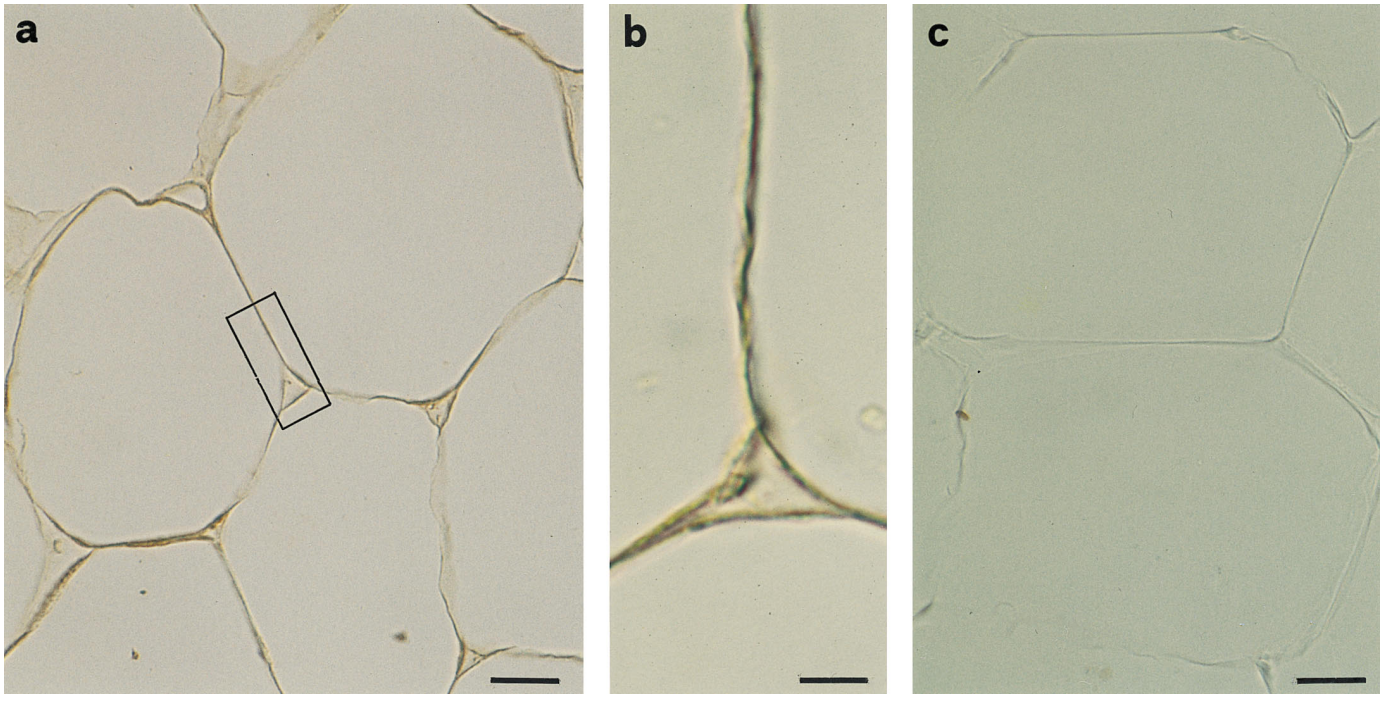

FIG. 2. Obese (*db/db*) retroperitoneal (a and b) and parametrial (c) white adipose tissues. a, Thin rim of cytoplasm was intensely leptin-positive. b, Enlargement of an area similar to *rectangle* in *a*. c, Absorption test: inhibition of specific staining was obtained by incubating rabbit antimouse leptin with homologous antigen (5 μg/ml). *Bars*: a and c = 20 μm; b = 5 μm.

periphery of the organ a few unilocular UCP-negative cells (mean diameter: 41.55 μm $\pm$ 9.02) were also found (Fig. 3d). These cells possessed mitochondria that were ultrastructurally typical of white adipocytes (Fig. 3g), and were often leptin-positive (Fig. 3, e–f).

Lean animals: 36 h fasting. The morphology of the organ changed strikingly, being composed predominantly of small, mainly unilocular cells (mean diameter: 17.99 μm $\pm$ 0.94). These cells were UCP-positive (Fig. 3h) and showed mitochondria with an ultrastructure different from that of fed animals but still consistent with brown mitochondria (Fig. 3l). These cells were leptin-negative (Fig. 3i). With fasting, the unilocular UCP-negative cells at the periphery of the organ were not found.

Obese animals. Only animals in the fed state were studied. The organ was composed of adipocytes showing three different morphologies: most of the organ was composed of unilocular, small cells (mean diameter: 40.08 μm $\pm$ 4.05); however, these cells were larger than the unilocular brown adipocytes found in fasted animals. Some multilocular cells were found in the central part of the organ and many large, unilocular cells were always found at the periphery (mean diameter: 73.65 μm $\pm$ 9.9). The multilocular and small unilocular adipocytes showed typical brown mitochondria (Fig. 4a) and were UCP-positive (Fig. 4c). The large unilocular adipocytes, in contrast, were UCP-negative (Fig. 4c) and showed white-like mitochondria (Fig. 4b). Multilocular adipocytes were leptin-negative (not shown), whereas small and large unilocular adipocytes were leptin-positive (Fig. 4d).

Controls. The specificity of the antibodies was confirmed by the absence of staining in adipose tissues under the following conditions: 1) omission of the primary antibody, 2) incubation with preimmune serum instead of the first antiserum, 3) absorption testing for leptin staining (Fig. 2c), and 4) incubation with the two antibodies on sections of tissues (kidney, liver, and uterus) not expressing the mRNAs for UCP (23, 24) and leptin (1, 6, 7) (Fig. 1, e–f) (liver not shown).

Discussion

Previous studies demonstrated that leptin is expressed exclusively in white and brown adipose tissue, with expression in white adipose tissue being restricted to mature adipocytes (6, 11–13). In the present study, we have used immunohistochemical techniques to assess leptin expression. These studies demonstrate that leptin is expressed by all adipocytes of white adipose tissue depots. Of note, this staining decreased with fasting in agreement with previous mRNA studies (7, 14, 25). With regard to genetically obese animals (*db/db* mice), the adipocytes observed in various white fat depots were large, possessing a large lipid droplet and, therefore, a very thin cytoplasmic rim. This thin cytoplasmic rim from obese adipocytes stained positive for leptin. As previously reported, all nonlipid-containing cells were leptin-negative (11, 26). Interestingly, adipocytes at a multilocular stage of differentiation located within white adipose tissue depots were also leptin-positive, demonstrating that leptin is expressed by all lipid-containing cells within white adipose tissue depots.

With regard to brown adipose tissue, published studies differ regarding the relative expression of leptin in white *vs.* brown adipose tissue, with some studies reporting 20- to 50-fold lower levels in brown adipose tissue (7, 11, 14), or as much as 40% of white adipose tissue expression being found

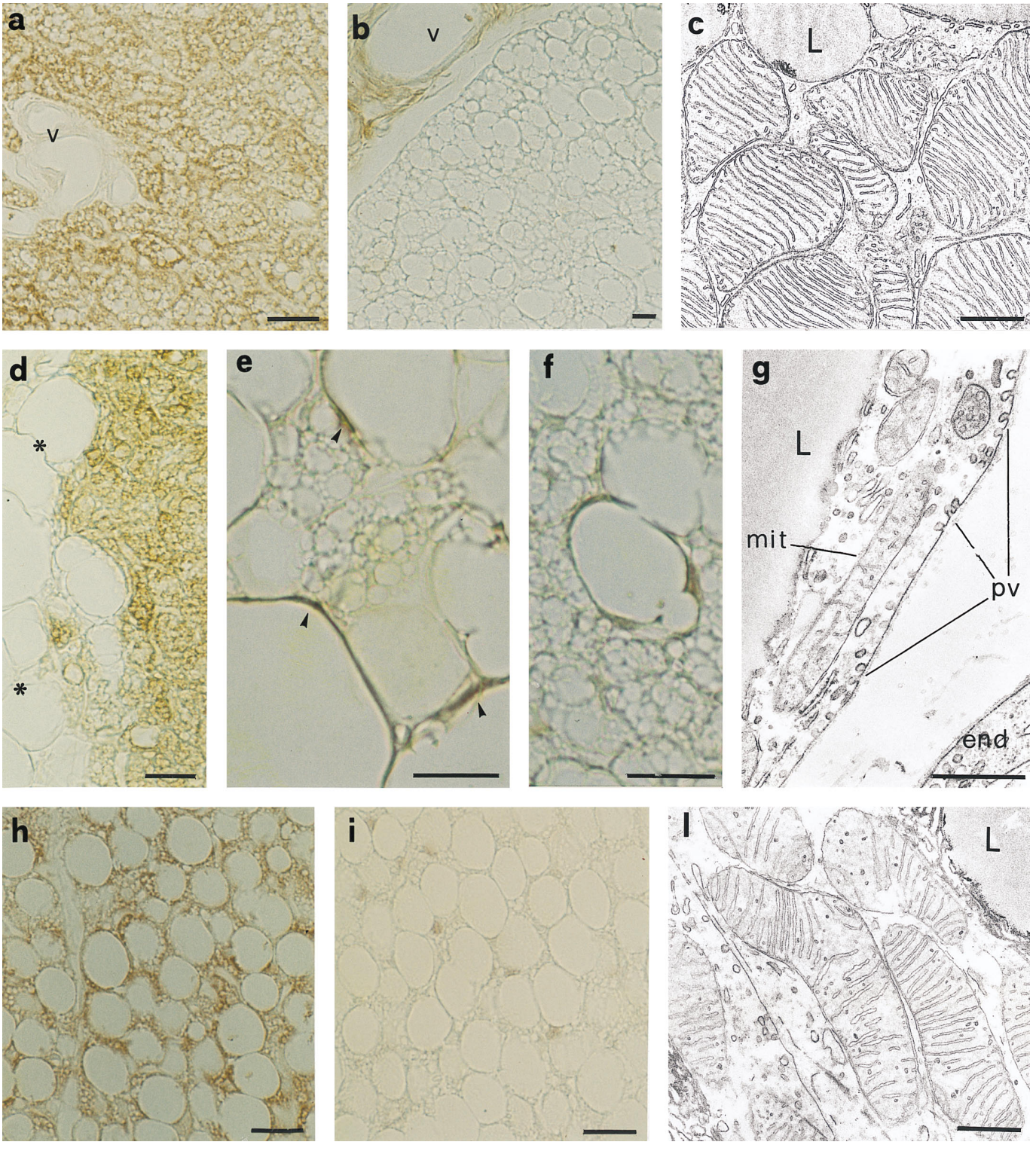

FIG. 3. Interscapular brown adipose tissue (BAT) in lean-fed (a-g) and lean-fasted (h-l) mice. a, d, and h, Stained for UCP; b, e, f, and i, stained for leptin. Multilocular adipocytes of fed animals were UCP-positive (a), showed typical brown mitochondria (c), and were leptin-negative (b). Unilocular cells (*) at periphery of BAT (d) showed white-like mitochondria (mit) (g) and were often leptin-positive (*arrows*) (e and f). BAT of 36 h-fasted mice was composed mainly of small unilocular cells that were UCP-positive (h) and leptin-negative (i). The ultrastructure of their mitochondria was changed but was still consistent with that of brown mitochondria (l). V, Vessels were not stained (a, b) but adventitia showed background staining when leptin antibodies were used (b); L, Lipid droplets; pv, pinocytotic vesicles; end, capillary endothelium. *Bars*: a, b, d, e, f, h, and i = 20 μm; c, g, and l = 0.5 μm.

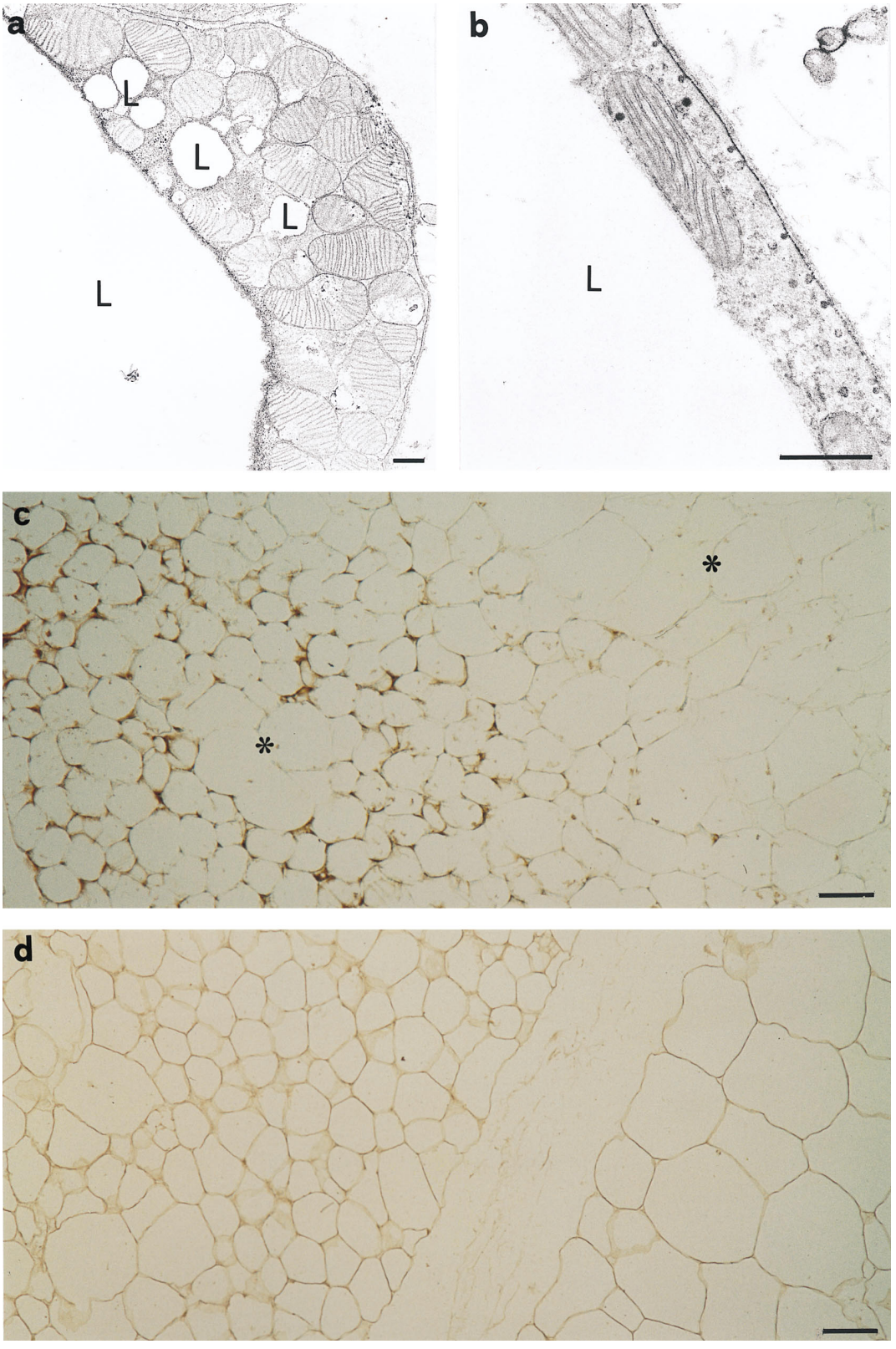

FIG. 4. Interscapular brown adipose tissue of obese (*db/db*) mice (a–d). organ was composed mainly of small unilocular cells with brown mitochondria (a). These cells were UCP-positive (c). Many large unilocular, UCP-negative (c) adipocytes (*) with white-like mitochondria (b) were also found. Both small and large unilocular adipocytes were leptin-positive (d). L, Lipid droplets. *Bars*: a, b = 0.5 μm; c and d = 50 μm.

in brown adipose tissue (15). Because brown adipose tissue is known to possess some white adipocytes, it is possible that the signal detected in brown adipose tissue is from these contaminating white adipocytes, and that brown adipocytes do not express leptin. Using an immunohistochemical approach, the present study indicates that UCP-positive, multilocular cells with typical brown mitochondria do not express leptin. Of course, the possibility of very low expression below the level of immunohistochemical detection cannot be excluded. Of interest, unilocular, UCP-negative cells located at the periphery of interscapular brown fat depots possessed mitochondria typical of white adipocytes and, as might be expected, were markedly leptin-positive. The presence of these cells could explain the small amount of leptin mRNA detected by some authors in brown fat (7, 11, 14). However, it may be difficult to account, on this basis, for the significant amount of leptin mRNA detected in brown fat in another study (15). One possible explanation is that in the present study brown fat was isolated from the surrounding white adipose tissue with the aid of a dissecting microscope, with an accurate microdissection requiring at least 15 min per each animal, whereas the samples prepared for mRNA studies were most likely performed quickly to obtain intact RNA. Also, as the study of Moinat *et al.* (15) involved rats, and the present study as well as those of Trayhurn *et al.* (7), Maffei *et al.* (11), and Frederich *et al.* (14) involved mice, it is formally possible that rat brown adipocytes express leptin, whereas mouse brown adipocytes do not. It is our opinion that this is unlikely, however, because we find that rat brown fat, like mouse brown fat, expresses leptin mRNA at very low levels (S. Cinti and R. Sarzani, unpublished data).

Previous biochemical analyses demonstrated that fasting induces atrophy of brown adipose tissue (27–30). The effects of acute fasting on brown fat morphology, however, have not been previously reported. In the present study, 36 h of fasting produced significant changes, with a transition from typical multilocular cells to mainly unilocular cells, which remained UCP-positive. Ultrastructural analyses demonstrated that cristae within mitochondria became less densely packed with fasting but were still distinguishable from the loosely packed cristae typical of white adipocyte mitochondria. Despite the observed fasting-induced transition in brown adipocyte morphology, all cells remained leptin-negative. The fasting-induced changes in brown adipocyte morphology are similar to those seen following sympathetic denervation of brown adipose tissue (31), and are probably mediated by fasting-induced decreases in sympathetic nerve activity (32). Recently it has been shown that leptin positively regulates sympathetic stimulation of brown fat (33). Therefore, it can be postulated that the fasting-induced unilocular appearance of brown fat is due to low sympathetic activity resulting from fasting-induced reductions in circulating leptin (14, 25, 34).

Genetically obese (*db/db*) mice are also characterized by low sympathetic stimulation of brown adipose tissue (35), presumably mediated by defective leptin receptor signaling within the hypothalamus (3–5). Prior histological assessment of brown fat in obese animals has been primarily restricted to mitochondrial morphology, whereas brown adipocyte mitochondria in obese animals have been found to have less densely packed cristae (36, 37). In the present study, the histology of brown fat of obese animals was strikingly different from that of lean fed animals, but was similar to that of lean fasted animals because it was composed mainly of small unilocular, UCP-positive brown adipocytes. Their size was small compared with white adipocytes but more than twice the diameter of fasted unilocular brown adipocytes. Of note, unlike observations in fasted animals, these cells were leptin-positive. The morphological changes in brown fat morphology with obesity are probably due, at least in part, to the lack of leptin-mediated sympathetic stimulation (33). The leptin positivity of UCP-positive unilocular cells in brown fat of obese animals most likely accounts for the marked increase in leptin mRNA expression observed in brown fat of *ob/ob* obese mice (14). At present, it is not known whether leptin expression by unilocular, UCP-positive adipocytes in obese animals represents a partial interconversion between brown and white adipocytes, or whether brown adipocytes possess a latent capacity for leptin expression that is brought out by increased fat storage within brown adipocytes.

In conclusion, the present study indicates that classical brown adipocytes, demonstrating all the morphological and immunohistochemical characteristics of a thermogenically functioning brown adipocyte, *i.e.* numerous small lipid droplets, typical brown mitochondrial morphology, and UCP expression, differ from white adipocytes, not only in these characteristics, but also by their lack of detectable leptin expression. Thus, leptin appears to be the only known fat-specific gene that is expressed in white but not in classical brown adipocytes. Understanding the molecular mechanisms accounting for this may provide insight into the pathways controlling white *vs.* brown adipocyte differentiation, as well as factors regulating leptin gene expression. For example, at present the CCAAT/enhancer-binding protein, C/EBPα, is the only known positive regulator of leptin gene expression (38–41). However, this transcription factor cannot by itself account for the white fat specificity of leptin expression because C/EBPα is abundant in both white and brown adipocytes (42–44). Other regulatory factors must also exist, and these factors may play critical roles in determining other important characteristics of white *vs.* brown adipocytes. Thus, understanding the molecular mechanisms that account for tissue specificity of leptin gene expression should provide important insights into control of leptin gene expression and regulation of white *vs.* brown adipocyte differentiation.

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