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Symposium on 'New perspectives on adipose tissue function'

The adipose organ: morphological perspectives of adipose tissues

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Anatomically, an organ is defined as a series of tissues which jointly perform one or more interconnected functions. The adipose organ qualifies for this definition as it is made up of two tissue types, the white and brown adipose tissues, which collaborate in partitioning the energy contained in lipids between thermogenesis and the other metabolic functions. In rats and mice the adipose organ consists of several subcutaneous and visceral depots. Some areas of these depots are brown and correspond to brown adipose tissue, while many are white and correspond to white adipose tissue. The number of brown adipocytes found in white areas varies with age, strain of animal and environmental conditions. Brown and white adipocyte precursors are morphologically dissimilar. Together with a rich vascular supply, brown areas receive abundant noradrenergic parenchymal innervation. The gross anatomy and histology of the organ vary considerably in different physiological (cold acclimation, warm acclimation, fasting) and pathological conditions such as obesity; many important genes, such as leptin and uncoupling protein-1, are also expressed very differently in the two cell types. These basic mechanisms should be taken into account when addressing the physiopathology of obesity and its treatment.

Adipose organ: Adipose tissues: Morphology: Adipocytes

The concept of the 'adipose organ' was introduced 60 years ago (Wells, 1940), but was only used as a new definition of white adipose tissue (WAT). The concept proposed here is totally new and starts from the minimum requirement for definition of an organ: the presence of two distinct tissues, WAT and brown adipose tissue (BAT). Another important aspect of this concept is that the organ is considered as a single structure with the unitary function of sharing energy contained in food (mainly lipids) between thermogenesis and other metabolic needs of the body. This unitary function implies the plasticity of the organ and, specifically, that interconversion of its two tissues is possible. Indeed, recent experimental evidence corroborates this hypothesis. Such interconversion can be induced also by drugs and genetic manipulations, and could serve as a means of treating obesity in the future.

Materials and methods

Animals

Dissections for the purpose of describing the gross anatomy of the organ were performed on C57BL/KS-db/+ mice aged 43 weeks. Mice and rats were housed in animal quarters and provided with food and water *ad libitum*. The animals were kept at 20°C; some animals were subsequently exposed (several hours) or acclimated (several days) at low temperature (4°C), while some animals were acclimated at warm temperature (34°C). Care of the animals was in accordance with institutional guidelines, and the experimental protocols were approved by the local Animal Care Ethical Committee.

The animals were anaesthetised with ketamine (Ketaset; Bristol-Myers, Syracuse, NY, USA; 100 mg/kg, intraperitoneally) in combination with xylazine (Rompum, Haver,

Abbreviations: BAT, brown adipose tissue; CGRP, calcitonin gene-related peptide; ir, immunoreactive; NPY, neuropeptide Y; SP, substance P; TH, tyrosine hydroxylase; UCP, uncoupling protein; WAT, white adipose tissue.

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Mobey Corp., Shawnee, KS, USA; 10 mg/kg intraperitoneally). They were perfused intra-aortically with a paraformaldehyde solution (40 ml/l 0·1 M-phosphate buffer, pH 7·4).

Techniques

For light microscopy, tissues were postfixed overnight in the same fixative by immersion, washed, dehydrated in ethanol and embedded in paraffin. Sections (3 µm thick) were stained with haematoxylin and eosin or processed for immunohistochemistry according to the avidin–biotin–peroxidase method (Hsu *et al*. 1981). The primary antibodies were as follows: polyclonal sheep anti-uncoupling protein (UCP)-1 (generously provided by Dr D Ricquier), and as no cross-reaction was observed in samples of liver, kidney, skeletal muscle and white adipose tissue, which are known to contain UCP2 and/or UCP3 (Boss *et al*. 1997; Fleury *et al*. 1997), our antibody can be considered specific for UCP; polyclonal rabbit anti-tyrosine hydroxylase (TH; Chemicon, Temecula, CA, USA); polyclonal rabbit anti-neuropeptide Y (NPY; Amersham International plc, Amersham, Bucks., UK); polyclonal rabbit anti-calcitonin gene-related peptide (CGRP; Amersham International plc); polyclonal rabbit anti-substance P (SP; Amersham International plc); polyclonal rabbit anti-NO synthase isoforms (Biomol, Plymouth, PA, USA); polyclonal rabbit anti-haem oxygenase (StressGen, Victoria, B.C., Canada); polyclonal rabbit anti-leptin, raised against the NH_2-terminal and the COOH-terminal peptides (made by Dr RC Frederich and purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Negative controls were obtained in each instance by omitting the primary antibody and using preimmune serum instead of the primary antiserum. When the antigen was available, adsorption tests were performed. To test the specificity of the antisera, immunohistochemical reactions were performed in parallel on tissues known to express those antigens.

For transmission electron microscopy, fragments of about 1 mm^3 were immersed in glutaraldehyde and paraformaldehyde (20 ml/l 0·1 M-phosphate buffer, pH 7·4 in each case), at 4°C for 3 h. Specimens were then washed, postfixed in OsO_4 (10 g/l), dehydrated in ethanol and embedded in an Epon–Araldite mixture. Thin sections were obtained with a Reichert Ultracut E (Reichert, Vienna, Austria), stained with lead citrate and examined with a Philips CM10 Electron Microscope (Philips, Eindhoven, The Netherlands).

Results and discussion

Lean 20°C

The adipose organ consists of several depots, which in small mammals are mainly represented by the dermic, subcutaneous (upper and lower), mediastinic, mesenteric, perigonadal, perirenal and retroperitoneal depots (Girardier & Stock, 1983; Trayhurn & Nicholls, 1986; Cinti, 1999). In mice and rats maintained at 20°C most of these depots have a white-yellowish colour but, especially in young animals, some areas in the upper (interscapular, subscapular, axillary and cervical) and lower (inguinal) subcutaneous depots, and the perirenal and mediastinic depots are brown in colour (Fig. 1).

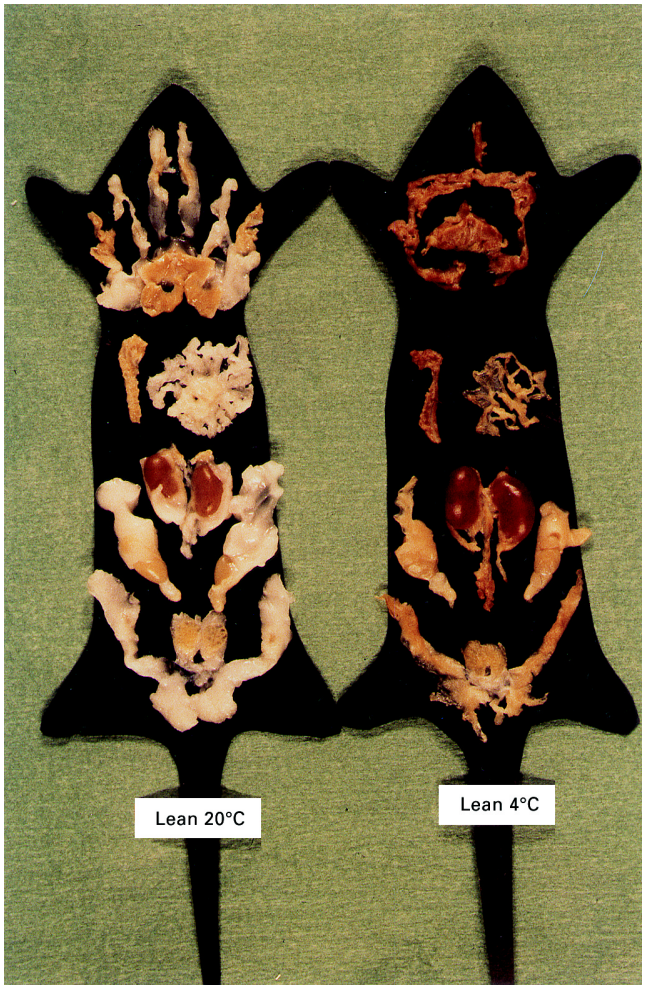

Fig. 1. Gross anatomy of the adipose organs of adult mice kept at 20°C and at 4°C. (Reproduced with permission from Cinti, 1999.)

Brown areas consist of BAT, with the typical multilocular adipocytes rich in characteristic mitochondria (large and with numerous cristae; Fig. 2) expressing the UCP1, which is responsible for the thermogenic activity of this tissue (Cannon *et al*. 1982; Cinti *et al*. 1989*b*; Klaus *et al*. 1991*b*).

The vascular and nerve supply is very rich, especially in the interscapular area (Nnodim & Lever, 1988), where large and mainly myelinated, and small and mainly amyelinated, nerves reach the tissue. The small nerves are intensely immunoreactive (ir) for TH, an enzyme marker of noradrenergic fibres, while the large nerves have many fibres ir for neuropeptides, such as the CGRP and SP (Norman *et al*. 1988), which are usually found in sensitive afferent fibres (Gibson *et al*. 1984; Skofitsch & Jacobowitz, 1985; Terenghi *et al*. 1985; Lee *et al*. 1986). Other NPY- and TH-ir nerve fibres reach the BAT via the peri-arterial plexus (Cannon *et al*. 1986). Brown adipocytes are electrically coupled by gap junctions (Schneider-Picard *et al*. 1980).

Two arteries reach the lateral ends of the interscapular BAT, and two veins drain the blood in the middle plane. The

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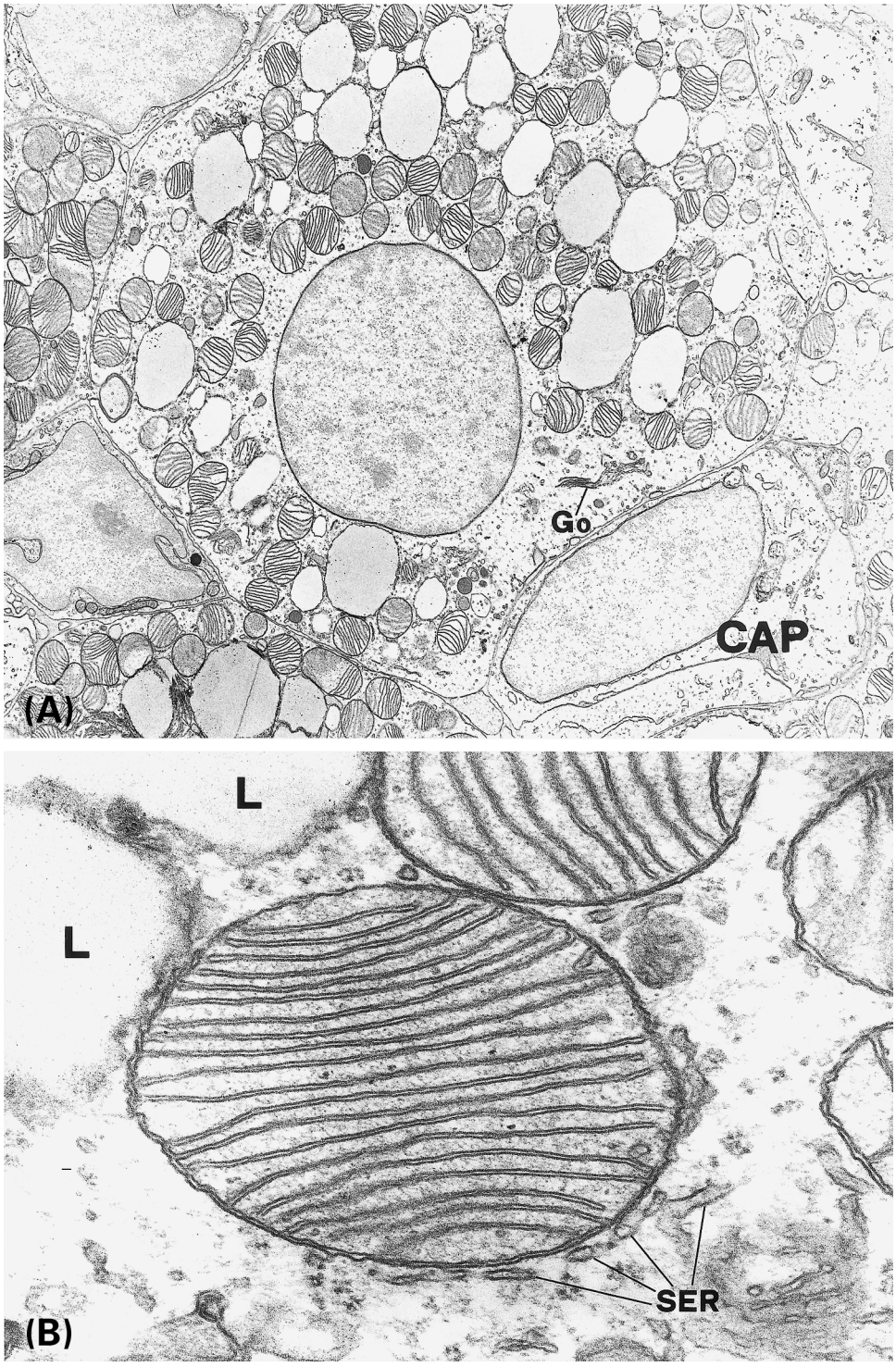

Fig. 2. Transmission electron microscopy. (A) Brown adipocyte of a neonatal rat filled with numerous small lipid droplets and typical mitochondria packed with cristae. Go, Golgi apparatus; CAP, capillary. Magnification ×8700. (B) High magnification of a typical brown adipocyte mitochondrion. L, lipid droplet; SER, smooth endoplasmic reticulum. Magnification ×80000.

capillaries have some peculiar characteristics, i.e. they can be completely surrounded by a single adipocyte, and a single endothelial cell can line the whole lumen of the capillary. Furthermore, electron microscopic evidence that endothelial cells are often hypertrophic and occlude the lumen suggests their involvement in the control of the blood flow. In this context, brown adipocytes contain NO synthase (Nisoli *et al*. 1997) and haem oxygenase (Giordano *et al*. 2000), the enzymes synthesising NO and CO respectively. Thus, they contribute to the control of the perfusion rate

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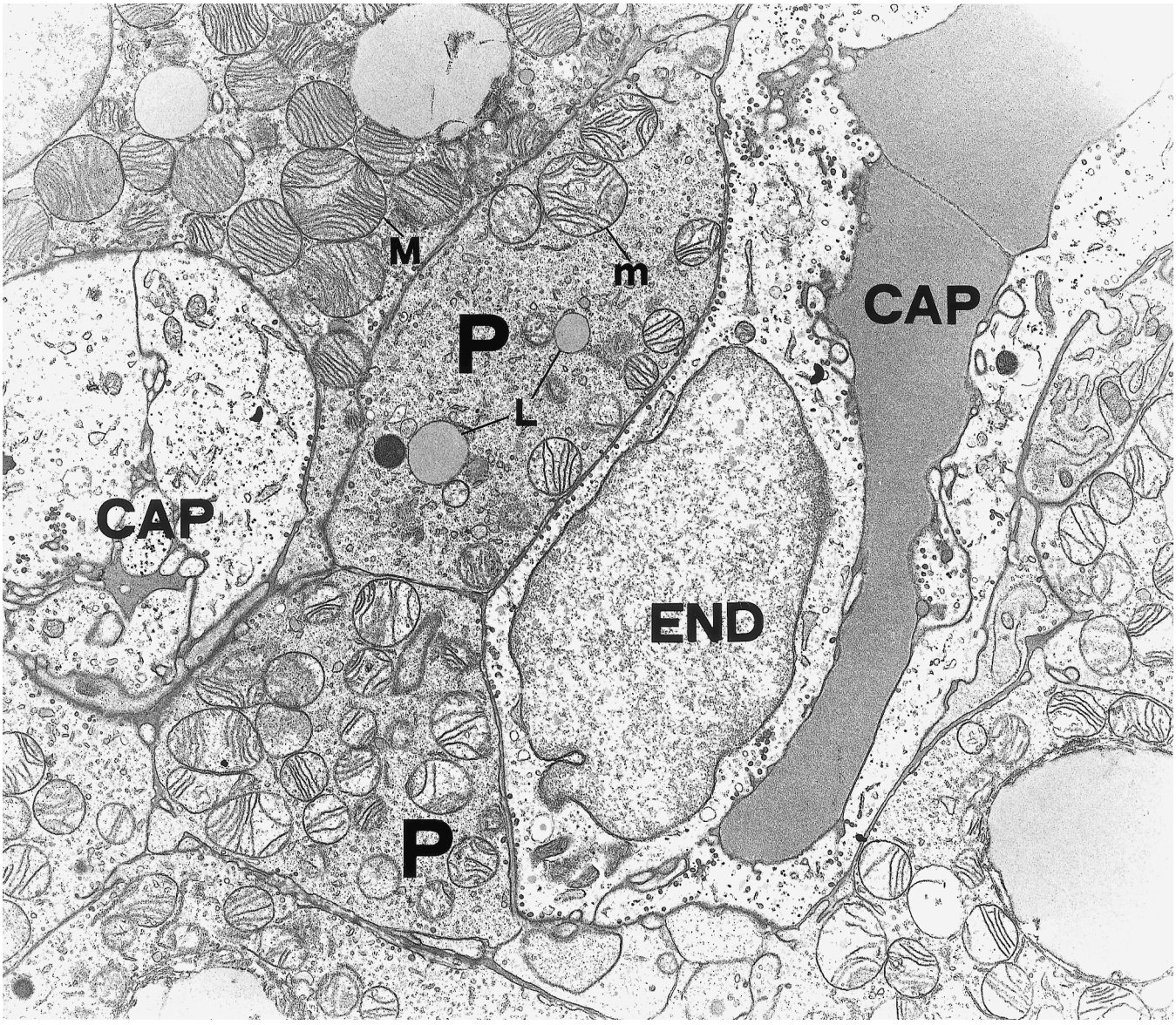

Fig. 3. Transmission electron microscopy. Interscapular brown adipose tissue of a 10-d-old rat. Two brown adipocyte precursors (P) containing some lipid droplets (L) are located in close apposition to a capillary (CAP). Compare the typical mitochondria (M) of a mature brown adipocyte with pretypical mitochondria (m) of a brown precursor cell. END, endothelial cell. Magnification ×11200.

through the tissue by producing these two novel gaseous mediators (Moncada & Higgs, 1993), whose production increases after noradrenergic stimulation, probably in order to match thermogenesis in BAT with its perfusion.

The precursors of brown adipocytes already show specific morphological features in the early phases of development (Fig. 3; Cinti *et al*. 1984, 1986, 1987; Sbarbati *et al*. 1987). In primary cultures these cells acquire both the morphological (Cigolini *et al*. 1985, 1986) and the molecular (Rehnmark *et al*. 1989; Klaus *et al*. 1991*a*) characteristics of brown adipocytes *in vivo*.

The white areas of the organ consist of unilocular adipocytes, filled with mitochondria very different from those found in brown adipocytes (Fig. 4). These cells produce leptin (Zhang *et al*. 1994; Frederich *et al*. 1995), a hormone which acts both on peripheral targets (Cinti *et al*. 1997*b*; De Matteis *et al*. 1998*a*) and on hypothalamic and extrahypothalamic centres (De Matteis & Cinti, 1998). Its main function is to inform the brain of the individual's nutritional state, thus controlling energy intake and distribution to the other tissues in the intervals between meals (Flier, 1995), but it is also involved in fertility regulation and reproductive functions (Casanueva & Dieguez, 1999).

The vascular supply of WAT is extremely important on account of the functional relationships between endothelial cells and adipocytes and, according to several researchers, the origin of adipocytes, but capillary:adipocyte is lower than that in BAT tissues (Fawcett, 1952; Crandall *et al*. 1997).

White depots are reached by numerous nerves, but a varying amount of their collateral branches innervate these depots. Parenchymal fibres ir for TH (noradrenergic) and

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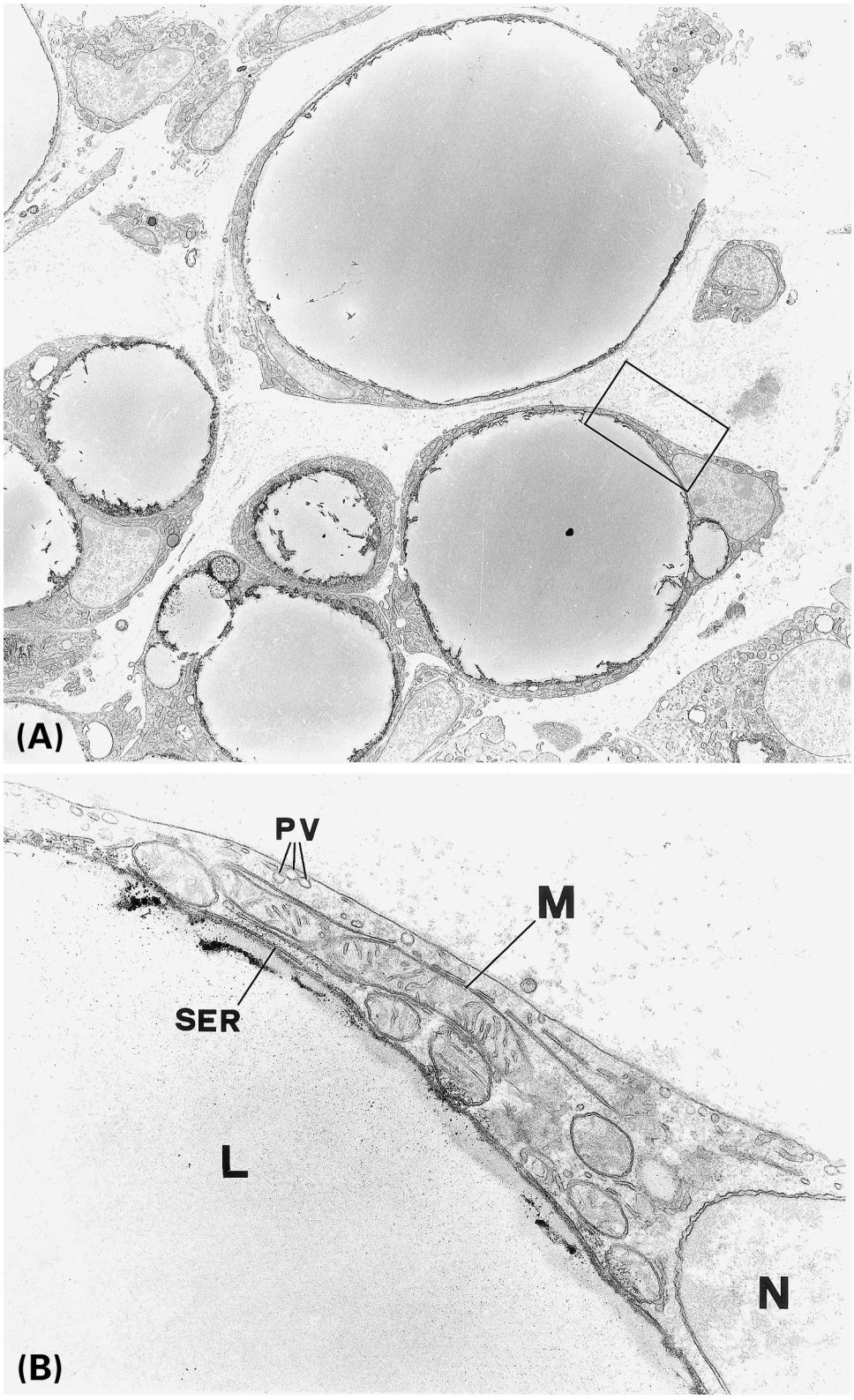

Fig. 4. Transmission electron microscopy. (A) Epididymal white adipose tissue of a 12-d-old rat. Magnification ×3300. (B) Enlargement of the framed area in A, showing the cytoplasm of a white adipocyte containing few and small mitochondria (M) with randomly-oriented cristae. PV, pinocytosis vesicles; SER, smooth endoplasmic reticulum; L, lipid droplet. N, nucleus. Magnification ×32000.

CGRP occur in all white depots, and in the periovarian depot of adult rats, specifically, the ir pattern of nerve fibres is very similar to that of the interscapular depot: TH, NPY, CGRP, SP (Giordano *et al*. 1996).

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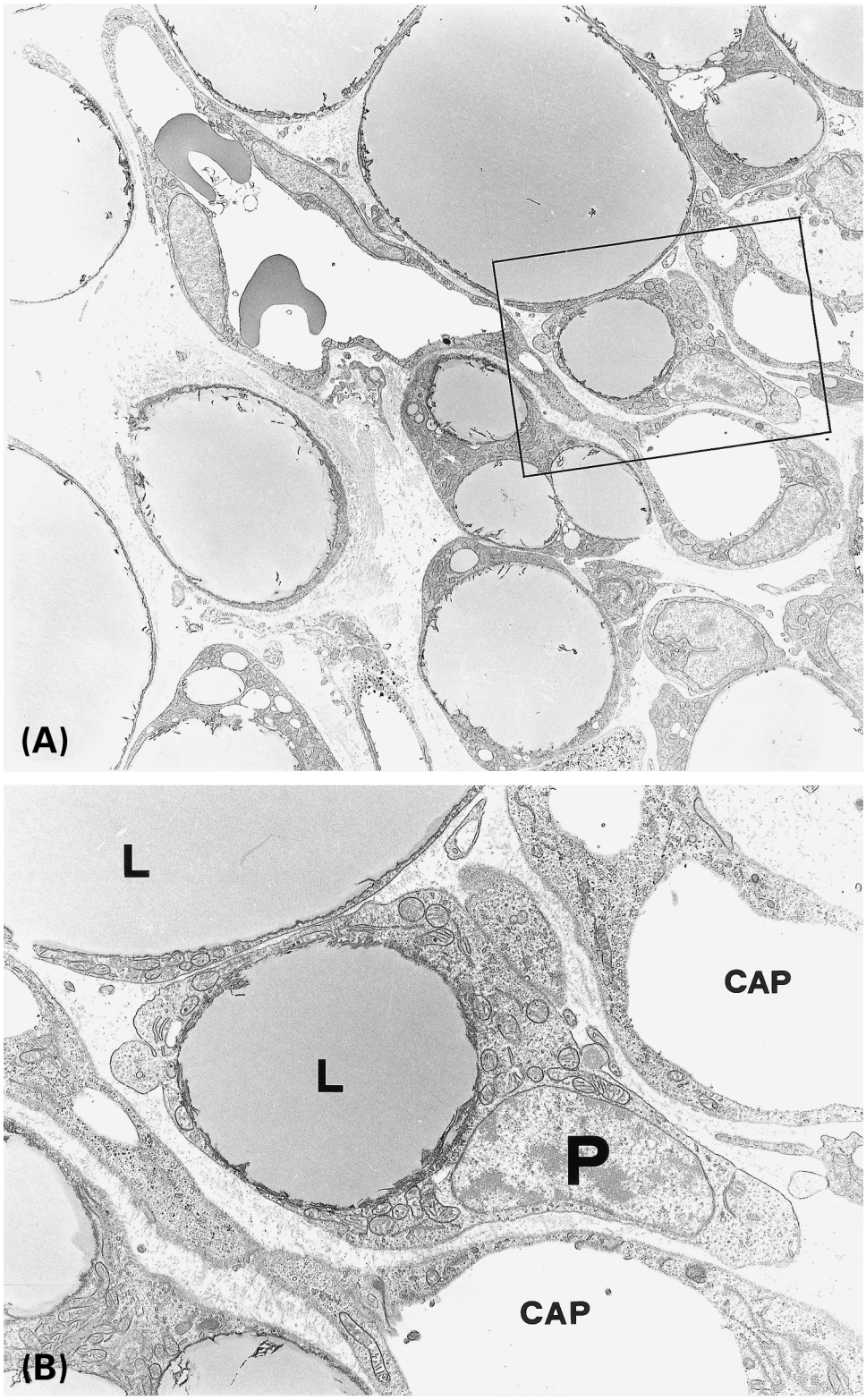

Fig. 5. Transmission electron microscopy. (A) Epididymal white adipose tissue of a 12-d-old rat. Magnification ×3300. (B) Enlargement of the framed area in A, showing a white adipocyte precursor (P). L, lipid droplet; CAP, capillary. Magnification ×8700.

The precursors of white adipocytes have a characteristic electron microscopic morphology distinct from that of brown adipocyte precursors (Fig. 5; Cinti *et al*. 1989*a*; Barbatelli *et al*. 1993). In primary cultures these precursors

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develop into cells with the morphological and molecular features of the unilocular cells of WAT (Cinti *et al*. 1985; Ailhaud *et al*. 1992; Maffei *et al*. 1995).

Most white depots in young (15- to 30-d-old) rats and some white depots (parametrial, periovarian and retroperitoneal) in adult rats show areas made up of multilocular adipocytes ir for UCP1 (Young *et al*. 1984; Loncar, 1991; Cousin *et al*. 1992). In these areas (mainly in young animals) adipocyte precursors can be found. In their less-differentiated stages these cells are similar to brown adipocyte precursors, but in the later stages of differentiation they show an ultrastructure intermediate between that of white and brown adipocytes.

Cold-acclimated animals

In cold-acclimated (4°C) animals the brown areas increase in number (Fig. 1) mainly due to the development and proliferation of brown adipocyte precursors (Geloen *et al*. 1992), via the noradrenergic pathway (Lafontan & Berlan, 1993), that also influence the functional activation of the brown fat cells (Arch & Kaumann, 1993; Giacobino, 1995).

In the interscapular area of old (2 years) rats, the number of brown adipocytes increases threefold in 4 weeks (Sbarbati *et al*. 1991; Morroni *et al*. 1995). Also the morphology of mature adipocytes appears modified, mostly because mitochondria increase in number, size, cristae density and UCP1 content. Noradrenergic parenchymal fibres become denser (De Matteis *et al*. 1998*b*) and gap junctions become larger (Barbatelli *et al*. 1994).

Among the WAT depots, the periovarian adipose tissue has a high preadipocyte content and is particularly reactive to cold (Cousin *et al*. 1993); when rats are kept in thermoneutral conditions it contains rare brown adipocytes scattered into lobules of white fat, but in cold-acclimated animals their number increases, and brown fat areas appear in the periovarian fat lobules (Cousin *et al*. 1992). The periovarian brown adipocyte increase following cold acclimation is matched by a major increase in noradrenergic and peptidergic nerves (Giordano *et al*. 1996). To ascertain whether periovarian fat is provided with sensory nerves, and whether any relationship exists between such nerves (in particular, the CGRP-containing fibres found in close association with brown adipocytes) and brown fat recruitment, we have studied the effect of capsaicin desensitization on neuropeptide-containing nerves and brown adipocyte density in the periovarian adipose tissue of rats kept at 20°C and on a group acclimated to 4°C for 14 d. In both groups capsaicin, a neurotoxin acting specifically on a subpopulation of primary afferent nerves (Caterina *et al*. 1997), considerably reduced the expression of SP and CGRP in vascular-nerve bundles and parenchyma, thus confirming that these nerves are sensory peptidergic nerves (Holzer, 1988). In addition, in cold-acclimated rats the increase in brown adipocyte density was significantly ($P<0{\cdot}01$) checked by capsaicin pretreatment (i.e. sensory denervation). Finally, ultrastructural investigation showed the occurrence of brown adipocyte precursors filled with aggregates of glycogen and poorly-differentiated multilocular adipocytes (with large lipid droplets, abundant aggregates of glycogen and smaller often elongated mitochondria with few cristae) in capsaicin-treated cold-acclimated rats. On the whole, these data suggest that sensory nerves and/or neuropeptides play a role in the recruitment and differentiation of visceral brown adipocytes (Giordano *et al*. 1998).

Multilocular mitochondria-rich adipocytes appear in the WAT of rats treated with the β_3-adrenoceptor agonist, CL 316,243. The use of bromodeoxyuridine to identify cells which have undergone mitosis during this treatment has shown that most multilocular cells are derived from pre-existing cells. Electron microscopy has confirmed that at least a subpopulation of unilocular adipocytes underwent conversion to multilocular mitochondria-rich adipocytes (Himms-Hagen *et al*. 2000).

Not all brown adipocytes in the interscapular area and in the brown areas of white depots stain for UCP1. This phenomenon, which we have called Harlequin's secret, is particularly evident in older animals and in conditions of prolonged cold stimulation, and is not observed in warm-acclimated animals (Cinti *et al*. 1997*a*).

Warm-acclimated animals

In warm-acclimated (34°C) animals the brown areas are less coloured and their histology is profoundly modified. Brown adipocytes are mainly unilocular, even though they are still ir for UCP1. The mitochondria are smaller with less-developed cristae. These cells express both leptin (which is not expressed in multilocular brown adipocytes of cold-acclimated animals) and its mRNA. These data suggest that UCP1 and leptin genes are reciprocally regulated by temperature (Cancello *et al*. 1998).

Obese animals

In obese animals the organ increases in volume due to white adipocyte hyperplasia and hypertrophy (Himms-Hagen, 1979; Rothwell & Stock, 1979; Lowell *et al*. 1993). These phenomena are differently regulated in the various depots, and the subcutaneous deposits are those that undergo the more conspicuous enlargement.

The brown areas appear less coloured and, as in warm-acclimated animals, their histology evolves into unilocular cells still ir for UCP1. These brown adipocytes are also ir for leptin (Cinti *et al*. 1997*c*).

Fasted animals

In fasting conditions the volume of the adipose organ diminishes as a function of duration of fasting and environmental temperature. After fasting for 48 h at 19°C volume diminishes to about one-third of that of animals maintained in the same fasting conditions at 28°C (R de Matteis and S Cinti, unpublished results), suggesting that a greater amount of fuel (lipids) has been mobilised than that required to maintain the body temperature. Accordingly, the typical morphological aspect of brown adipocytes in the interscapular area is clearly suppressed (unilocularity with small mitochondria containing few cristae) in warm-acclimated fasting animals. These brown adipocytes are UCP1-positive, but leptin-negative (Cinti *et al*. 1997*c*).

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The 'slimmed' white adipocytes are elongated with numerous irregular cytoplasmic projections and the cell surface is particularly rich in pinocytotic vesicles. The tannic acid technique (Blanchette-Mackie & Scow, 1981, 1983) allows visualisation of many myelinic structures which have been interpreted as fatty acids leaving the cell. These myelinic structures can be seen either in association with the internal membranes of adipocytes (endoplasmic reticulum, mitochondria, pinocytotic vesicles) or apparently free in the cytosol and in interstitial spaces. Capillaries appear in greater number, in agreement with the increased blood flow observed in this condition (Crandall *et al*. 1997).

Conclusion

WAT and BAT are organised into a real organ, with a complex diffuse multi-depot organisation. Each depot has its own discrete vascular and nerve supply. The characteristics of the organ can be adapted to the functional requirements, mainly in relation to the energy balance of the whole organism.

The two tissues seem to derive from precursors with different morphological and functional characteristics that are defined early, but with clear possibilities of reciprocal conversion, with an important role played by the nervous system. Both cytotypes produce a series of molecular factors that can influence the tissue pattern, adapting it to the functional needs.

These basic concepts of functional anatomy seem necessary for a better understanding of the pathophysiology of obesity.

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