Localization of leptin receptor splice variants in mouse peripheral tissues by immunohistochemistry

R. De Matteis¹, K. Dashtipour¹, A. Ognibene² and S. Cinti*¹

¹Institute of Normal Human Morphology-Anatomy, University of Ancona, Ancona, Italy
²Laboratory of Cellular Biology and Electron Microscopy, IOR, Bologna, Italy

The recently discovered leptin hormone is produced by adipocytes and acts on the hypothalamus inducing satiety and an increase in energy expenditure (for review, see Friedman, 1997), as well as on a number of organs involved in reproduction, metabolism and glucose homeostasis (Ahima et al. 1996; Chehab et al. 1996; Levin et al. 1996). These findings suggest that leptin also acts directly on peripheral tissues. Even though several studies have investigated the localization of the various spliced isoforms of the leptin receptor (Lee et al. 1996; Wang et al. 1996) in peripheral tissues (Ghilardi et al. 1996; Fei et al. 1997; Hoggard et al. 1997), the techniques used in most of them do not allow the exploration of their precise cytological localization, which is a precondition to gaining a better understanding of the functional role of the receptor in these tissues.

The present immunohistochemical study was thus performed to obtain cytological details of the localization of the receptor splice variants in a number of peripheral tissues of lean and obese mice.

Materials and methods

Animals were twelve adult (10-14 weeks old) obese C57BL/6-ob(ob/ob) and C57BL/ks-db(db/db) mice and lean mice of the same strains, three in each group (Harlan Nossan, Correzzana-MI, Italy). They were caged under standardized laboratory conditions with tap water and regular chow available ad libitum in a 12 h–12 h light–dark cycle. Care and handling were in accordance with institution guidelines.

The mice were weighed and anaesthetized with ketamine (Ketavet; Farmaceutici Gellini, Aprilia-LT, Italy; 100 mg/kg, intraperitoneally) in combination with xylazine (Rompum; Bayer AG, Leverkusen, Germany; 19 mg/kg, intraperitoneally). Transcardiac perfusion was performed using paraformaldehyde in 0-1 m-phosphate buffer (PB; pH 7.4; 40 mg/l). After perfusion, the animals were dissected using paraformaldehyde in 0.1 M-phosphate buffer (PB; pH 7.4; 40 mg/l). After perfusion, the animals were dissected under a surgical microscope (Zeiss OPM 19; Carl Zeiss, Oberkochen, Germany) and the samples were fixed by immersion in the same fixative overnight at 4°C.

The tissues used for this investigation were: epididymal, interscapular brown adipose tissue, testis, ovary, pancreas, liver, kidney, adrenal gland, heart, lung, skeletal muscle, small intestine and lymph nodes.

The samples were washed rapidly with 0-1 m-PB at pH 7-4, dehydrated in ethanol and embedded in paraffin blocks. Sections (3μm thick) were used for light microscopy and immunohistochemistry.

Immunohistochemistry

Antibodies. Affinity-purified goat polyclonal antibodies directed to the peptides corresponding with amino acids 877-894 (M-18) and 32-51 (K-20) of leptin receptor OB-Ra of mouse origin (Santa Cruz Biotec, Santa Cruz, CA, USA) and an affinity-purified rabbit polyclonal antibody against an eighteen amino acid peptide near the C terminus of mouse leptin receptor OB-Rb (OB-Rb-13; Alpha Diagnostic International, San Antonio, TX, USA) were used (Fig. 1).

Procedure. Immunohistochemical demonstration of leptin receptors was performed with the avidin–biotin–peroxidase (EC 1.11.1.7; ABC) method. Dewaxed sections (3μm) were processed through the following incubation steps: (1) H2O2 (3 ml/l methanol) for 30 min to block endogenous peroxidase, (2) normal rabbit serum 1:75 (v/v; K-20 and M-18 protocol) and normal goat serum 1:75 (v/v; OBR-13 protocol) for 20 min to reduce non-specific background staining; (3) incubation with primary antibody against leptin receptor (K-20; M-18 and OBR-13) diluted 1:100 (v/v) to 1:300 (v/v) in PBS according to the type of tissue, overnight at 4°C; (4) biotinylated secondary antibody: rabbit anti-goat immunoglobulin G 1:200 (v/v; K-20 and M-18 protocol) and goat anti-rabbit immunoglobulin G 1:200 (v/v; OBR-13 protocol) for 30 min (Vector Labs, Burlingame, CA, USA); (5) ABC complex for 1 h (Vectastain ABC kit, Vector Labs); (6) histochemical visualization of peroxidase using 0-75 ml 3,3′-diaminobenzidine hydrochloride as chromogen (Sigma, St Louis, MO, USA) and 0-2 ml H2O2/l 0-05 m-Tris buffer, pH 7-6, for 5 min in a dark room. Sections were then rinsed in tap water, counterstained with haematoxylin, dehydrated and mounted in Entellan (Merck, Darmstadt, Germany).

Method-specificity tests were performed by omitting the primary antibodies in the staining and by incubation of...
sections with an antiserum saturated with the homologous antigen.

For the neutralization of the antibodies, they were incubated with a tenfold excess of homologous peptide (from 20 to 70 µg/ml, according to the antibody dilution) for 48 h (adsorption test).

Cell culture

Mouse C2C12 myoblasts from American Type Culture Collection (Rockville, MD, USA) were grown in Dulbecco’s modified Eagle’s medium supplemented with heat-inactivated fetal calf serum (100 ml/l) containing 100 U penicillin/ml and 0.1 mg streptomycin/ml.

Preparation of total cell homogenate

Subconfluent C2C12 cells were washed once in PBS and resuspended in lysis buffer (10 mM-Tris hydrochloride, pH 7.8, Nonidet P-40 (10 ml/l), 10 mM-mercaptoethanol, 1 mM-phenylmethylsulphonyl fluoride, 10 g leupeptin and aprotinin/ml, 10 g soyabean trypsin inhibitor/ml, 15 g calpain inhibitor I/ml and 7 g calpain inhibitor II/ml). After 10 min swelling on ice, cells were lysed by forty passages through a 25-gauge hypodermic needle. Protein content was determined using the Bio-Rad protein assay kit (Bio-Rad, Milano, Italy).

SDS-PAGE and Western blotting

Total cell homogenate (30 µg) in loading buffer was run on a 7.5% SDS-PAGE using a Bio-Rad Mini gel apparatus and transferred to a nitrocellulose membrane using a Bio-Rad wet blotting system overnight at 0.25 A (Towbin et al. 1979). The nitrocellulose was blocked in PBS containing (f) 1 ml Tween 20, 30 g bovine serum albumin and 50 g non-fat dried milk for 1 h at room temperature. The blot was incubated with primary antibody (1: 750, Ob-R (K-20); Santa Cruz Biotec) appropriately washed, incubated with secondary antibody conjugated to horseradish peroxidase (anti-goat 1:2000; Dako, Italy) and visualized using the ECL kit (Amersham, Milano, Italy).

Results

Results are summarized in Table 1.

In the adrenal gland (Fig. 2(a)), medullary cells were intensely positive only with the antibody recognizing the common part of the receptor (R). Zona fasciculata cells of the cortex stained for all three antibodies. In the pancreas, the exocrine portion was not stained while most of the cells in the Langerhans islets (Fig. 2(c and d)) were labelled, though more weakly for the long-splice variant (RL).

In the gonads, Leydig cells (Fig. 2(b)) in the testis and luteal and interstitial cells in the ovary (Fig. 3) were positive for all splice forms. Leydig cells were weakly positive for RL.

The heart (Fig. 4(d and e)) displayed diffuse positivity of myocardial fibres for all three antibodies which was more intense in atrial cells. Also, the striated cells of myocardial origin (Ludatscher, 1968; Hebel & Stromberg, 1986) in the wall of the main trunks of the pulmonary veins were intensely positive for the different splice forms of the receptor (Fig. 4(c)). The striated muscle cells of soleus (Fig. 5(c)) exhibited a focal staining pattern. The consistent positivity of different types of striated muscle cells prompted us to study by immunoblotting the expression of the receptor also in cultured cells. The present study (performed only with the antibody for R) revealed the presence of R in myoblasts of the C2C12 cell line (Fig. 6).
Fig. 2. (a) Adrenal gland of lean mouse showing intense labelling of zona fasciculata of cortical and medullary cells by the anti-R (intracellular part of leptin receptor common to all splice forms) antibody. Scale bar = 40 μm. (b) Testis of lean mouse showing Leydig cells labelled by the anti-R_S (the intracytoplasmic part of the leptin receptor common to all transmembrane forms) antibody. Scale bar = 20 μm. (c) Langerhans isle of the pancreas of lean mouse showing R_L-positive cells; negative exocrine pancreas is visible on the left of the figures. Scale bar = 10 μm. (d) Langerhans isle of the pancreas of obese db/db mouse negative for the anti-R_L antibody. Scale bar = 20 μm. For details of procedures, see pp.441–442. R_L, long form of the leptin receptor; R_S, the intracytoplasmic part of the leptin receptor common to all transmembrane forms.

In the lung, in addition to the myocardial cells in the walls of the pulmonary veins, there were positive cells in the bronchiolar epithelium (Fig. 4(a and b)). These cells displayed a dome-like surface devoid of cilia and were identified as Clara cells.

In adipose tissues (Fig. 5(b)), the thin peripheral rim of white adipocytes stained for all three antibodies. In interscapular brown adipose tissue only the cells in the peripheral portion of the organ were positive (Fig. 5(a)). They differed from the majority of the cells of the organ in their few and large lipid droplets, sometimes gathered in a single vacuole mimicking the morphology of white adipocytes.

In the liver, the hepatocytes in the central part of the lobule were diffusely positive (Fig. 5(d)). Staining was more intense with the antibodies for R and the short splice variant (R_S). Small intestine and lymph nodes were negative.

In the tissues of ob/ob mice we obtained the same results as in lean mice, but Leydig cells and the endocrine cells of the pancreas stained intensely with all three antibodies.

With adsorption test (see pp. 441–442) the tissues were negative (not shown).
The same tissues, tested with R_L in db/db mice, were negative (Figs. 2–3), except the kidney, where there were some positive cells in the medullary elements of the nephron. Since this finding requires further investigation, kidney results were not included.

**Discussion**

The presence of leptin-receptor splice variants in peripheral tissues, described by several authors, suggests that leptin plays a direct role in peripheral organs (Tartaglia *et al.* 1995; Ghilardi *et al.* 1996; Fei *et al.* 1997; Hoggard *et al.* 1997; Lollmann *et al.* 1997). Thus, their cytological localization in these tissues must be known precisely if the role of the leptin receptor is to be fully understood. Since most published studies have been performed using techniques that do not achieve a precise cytological localization of the protein, the present work used immunohistochemistry to localize the splice variants in a number of peripheral tissues.

Our results indicate that most of the organs expressing Rs (adrenal gland, adipose tissues, heart, liver, lung, ovary, endocrine pancreas, skeletal muscle and testis) also express R_L in the same cell type, although in some of them (adrenal gland, liver, endocrine pancreas and testis) staining for R_L was weaker.

In the adrenal gland the medullary portion was intensely positive only with the antibodies for R, while the cortex (only the cells of zona fasciculata) was intensely positive also for Rs. Some authors (Cao *et al.* 1997) reported staining of the medulla and negative staining of the cortex by autoradiography and immunohistochemistry using an antibody recognizing both Rs and R_L in rats. Others (Hoggard *et al.* 1997), by in situ hybridization, showed a detectable signal in the medulla of mice that was stronger with the probe for Rs, and a weak signal also in the cortex.

In white and brown adipose tissue, our results confirm the recent data (Emilsson *et al.* 1997; Fruhbeck *et al.* 1997; Siegrist-Kaiser *et al.* 1997; Tanizawa *et al.* 1997; Kutoh *et al.* 1998), showing the presence of all isoforms, but cytological localization allows us to specify that only the unilocular and mainly unilocular cells at the periphery of interscapular brown adipose tissue were labelled. Interestingly, we obtained the same type of results for leptin expression in a previous study (Cinti *et al.* 1997). This suggests that only when brown adipocytes contain large lipid vacuoles, or are unilocular, do they express both leptin and its receptor.

Some of the cell types expressing the receptor isoforms were steroid-secreting cells: cells of zona fasciculata of the adrenal-gland cortex, luteal and interstitial (Blandau, 1977) cells of the ovary and Leydig cells of the testes. These results are consistent with the hypothesized interaction of leptin with the glucocorticoid system (Ahima *et al.* 1996; Campfield *et al.* 1997) and with its trophic role in the gonads of obese ob/ob mice (Mounzih *et al.* 1997; Zachow & Magoffin, 1997). On the other hand, they are only partially in line with those of other researchers, mainly because in our samples the receptor was not expressed in the spermatogenic epithelium of the testis (Hoggard *et al.* 1997) nor in the follicular cells of the ovary (Cioffi *et al.* 1997; Karlsson *et al.*
Fig. 4. (a) Pulmonary parenchyma and bronchiolar cells were completely negative in db/db mice with the anti-RL antibody. Scale bar = 20 μm. (b) Bronchiolar epithelial cells strongly labelled by the same antibody in lean mouse. Scale bar = 20 μm. (c) Pulmonary vein showing striated muscular fibres in the media Rs-positive (→). Scale bar = 20 μm. (d–e) Heart of lean mouse; atrial (A) and ventricular (Vt) fibres were RL-positive. Scale bar = 40 μm. For details of procedures, see pp. 441–442. RL, long form of the leptin receptor; Rs, the intracytoplasmic part of the leptin receptor common to all transmembrane forms.

1997), although the latter localization has been found in human specimens. Our findings confirm the presence of Rs and RL in Langerhans islets, in agreement with the results obtained with different techniques by other authors (Kieffer et al. 1996; Leclercq-Meyer et al. 1996; Emilsson et al. 1997; Fei et al. 1997; Tanizawa et al. 1997).

The present work is the first to show the presence of Rs and RL in the epithelial cells of the bronchioles and in striated muscle cells (Ludatscher, 1968; Hebel & Stromberg, 1986) of the pulmonary veins. The positive cells in the bronchioles had a dome-like surface and were devoid of cilia, which suggested their identification as Clara cells. Interestingly, the striated muscle cells of the wall of the pulmonary veins are of cardiac origin (Ludatscher, 1968; Hebel & Stromberg, 1986) and the striated cardiac cells of the heart were positive, more intensely in atrial cells. Previous studies have shown the presence of only Rs in lung and heart (Tartaglia et al. 1995; Fei et al. 1997; Hoggard et al. 1997) but, in agreement with
our results, Lollmann et al. (1997) reported high levels of R_L in the lung. The presence of the receptor in cultured C2C12 myoblasts suggests their precocious appearance during development and confirms the presence of R_S in this cell line, providing evidence that this form could mediate the functional activities observed in these cells after leptin administration (Berti et al. 1997; Kellerer et al. 1997; Muoio et al. 1997). In the soleus muscle, the receptor expression was in agreement with the studies showing important effects of glucose intake and glycogen synthesis in this muscle after leptin administration (Liu et al. 1997; Muoio et al. 1997).

In the liver, a major concentration of the receptor (mainly R_S) was observed near the centrolobular vein. Other authors (Hoggard et al. 1997) described the presence of R_S adjacent to the larger hepatic blood vessels, although some authors also found R_L (Emilsson et al. 1997).

The absence of immunoreactivity in the exocrine pancreas is in agreement with the results of other authors (Emilsson et al. 1997; Fei et al. 1997), but our negative results in small intestine and lymph nodes contrast with some previous reports (Cioffi et al. 1996; Ghilardi et al. 1996; Lollmann et al. 1997).
Table 1. Immunohistochemical localization of leptin receptor OB-R splice variants*

<table>
<thead>
<tr>
<th>Organ</th>
<th>R</th>
<th>R_S</th>
<th>R_L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal gland</td>
<td>BAT: Medulla and zona fasciculata of cortex</td>
<td>Zona fasciculata of cortex and a few medullary cells (weak)</td>
<td>Zona fasciculata of cortex and a few medullary cells (weak)</td>
</tr>
<tr>
<td>Adipose tissues: BAT</td>
<td>Only unilocular cells at the cortex</td>
<td>Only unilocular cells at the periphery</td>
<td>Only unilocular cells at the periphery</td>
</tr>
<tr>
<td>Adipose tissues: WAT</td>
<td>Unilocular cells</td>
<td>Unilocular cells</td>
<td>Unilocular cells</td>
</tr>
<tr>
<td>Heart</td>
<td>Atrial and ventricular (weak)</td>
<td>Atrial and ventricular (weak)</td>
<td>Atrial and ventricular (weak)</td>
</tr>
<tr>
<td>Liver</td>
<td>Bronchiar Clara cells and striated cells</td>
<td>Bronchiar Clara cells and striated cells</td>
<td>Bronchiar Clara cells and striated cells</td>
</tr>
<tr>
<td>Lung</td>
<td>Bronchiar Clara cells and striated cells</td>
<td>Bronchiar Clara cells and striated cells</td>
<td>Bronchiar Clara cells and striated cells</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Ovary</td>
<td>Insular cells</td>
<td>Luteal and interstitial cells</td>
<td>Luteal and interstitial cells</td>
</tr>
<tr>
<td>Pancreas: Endocrine (soleus)</td>
<td>Negative</td>
<td>Most fibres</td>
<td>Most fibres</td>
</tr>
<tr>
<td>Pancreas: Exocrine (soleus)</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Skeletal muscle (soleus)</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Small intestine</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Testis</td>
<td>Leydig cells</td>
<td>Leydig cells</td>
<td>Leydig cells</td>
</tr>
</tbody>
</table>

R, the extracellular part of the receptor common to all known splice variants; R_S, intracytoplasmic part common to all short transmembrane forms, short-splice variants; R_L, long form of the receptor, i.e. long-splice variant; BAT, brown adipose tissue; WAT, white adipose tissue.

*For details of procedures, see pp. 441–442.

Further work is in progress in our laboratory to study other organs and provide further details on the cellular localization of the leptin-receptor splice variants.

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References


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