Original

Immunocytochemical Localization of Leptin Receptor in Rat Hypothalamus

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Abstract: The distribution and ultrastructural localization of leptin receptor in the rat hypothalamus was determined by immunocytochemistry. We used antiserum against a synthetic peptide that corresponds to the carboxy-terminal cytoplasmic domain to localize leptin receptor-like immunoreactivity in the rat hypothalamus. Strong leptin receptor immunoreactivity was detected in the arcuate, paraventricular and ventromedial nuclei of the hypothalamus, and lateral hypothalamic area. At the ultrastructural level, leptin receptor-like immunoreactivity appeared to be concentrated predominantly in neuronal perikarya and dendrites and strong immunostaining for the leptin receptor was found in plasma membrane, rough endoplasmic reticulum, Golgi apparatus, and cytoplasmic matrix. These results suggest possible functions for leptin in the rat hypothalamus.

Key words: obesity, leptin receptor, immunocytochemistry, hypothalamus, rat

Introduction

Leptin, the protein product of the recently cloned ob (obese) gene, is secreted by adipocytes and implicated in food intake and maintenance of energy balance. The leptin receptor, a single membrane-spanning receptor, was first demonstrated in the choroid plexus and hypothalamus. Since the first leptin receptor isoform was isolated, several splice variants have been found. The predominant "short" form is expressed in multiple tissues, including the kidney, adrenal gland and choroid plexus. An alternatively spliced isoform with a longer cytoplasmic domain, referred to as the "long" form, is most highly expressed in the hypothalamus. In situ hybridization in rat and mouse indicated strong leptin receptor gene expression in the arcuate, ventromedial, paraventricular and ventral premammillary nuclei of the hypothalamus, as well as in the choroid plexus and leptomeninges. In addition to the hypothalamus, leptin receptor mRNA is strongly expressed in the hippocampus, piriform cortex, and medial habenular nucleus of mouse and rat brain.

The distribution of leptin receptor has been recently examined in human and rat brains by immunohistochemistry and Western blotting. However the precise distribution and ultrastructural localization of leptin receptor remains unknown. We used antiserum against leptin receptor that recognizes the carboxy-terminal cytoplasmic domain found in

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Osamu MARUTA, et al

the “long” form of leptin receptor to localize leptin receptor-like immunoreactivity (LR-LI) in the rat hypothalamus by both light and electron microscopy.

Materials and Methods

Young adult male Sprague-Dawley rats (body weight 250 to 300 g) were housed in a temperature- and light-controlled room (lights on at 06:00 and off at 18:00) and supplied with standard laboratory chow and water ad libitum. All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of Showa University. The primary antiserum was affinity-purified goat polyclonal antibody raised against peptide corresponding to amino acids 877-894, mapping at the carboxy-terminus of the mouse leptin receptor (Santa-Cruz Biotechnology, Santa-Cruz, CA, USA). With this antiserum, immunoblot analysis yielded a strong single band at 120 kDa in all brain regions. In addition, when the antiserum used in the present study was tested using SKN-ML, HeLa, A431 and MCF-7 whole cell lysates, a strong band for leptin receptor was observed at 120 kDa (personal communication from Santa-Cruz Biotechnology, 1997). Thus, immunoblot analysis indicated that the antiserum reacted specifically only with leptin receptor.

The animals were anesthetized deeply with sodium pentobarbital (50 mg/kg, i.p.), and perfused transcardially with 50 ml of saline (37°C), followed by 250–300 ml of 2 % paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.2) for 20 min. The brains were removed immediately and post-fixed in 2 % paraformaldehyde in 0.1 M PB for 12 hours at 4°C. The brains were cut into coronal and sagittal sections 7–8 μm thick on a cryostat (MICROM HM 500; MICROM, Heidelberg, Germany). The sections were incubated with 2 % normal horse serum, and then placed in phosphate-buffered saline (PBS) containing 0.3% Triton X-100 for 30 min. The sections were rinsed with PBS, preincubated with 10% normal horse serum in PBS, and then incubated with the primary antiserum diluted at a dilution of 1 : 1000 for 48 hours at 4°C. After incubation, the sections were incubated with Cy3-conjugated donkey anti-goat antibody (1 / 200, Amersham International plc, Buckinghamshire, England) for 2 hours at 20°C. After rinsing in PBS, the sections were mounted in mounting medium (Perma Fluor Aqueous Mounting Medium, Immunon, Pittsburgh, PA, USA) and examined with an Olympus AX-70 fluorescence microscope (Olympus, Tokyo, Japan).

To verify the specificity of the staining, the sections were incubated with (1) non-immune serum instead of the primary antiserum, (2) the primary antisera without the second antiserum, or (3) the primary antisera that had been preabsorbed with the antigen (50 μg of peptide, Santa-Cruz Biotechnology in 1 ml of antiserum at the working dilution).

Vibratome (Oxford, Foster City, CA, USA) sections (40–50 μm) were incubated with primary antiserum overnight at 4°C and processed for peroxidase labeling by the avidin-biotin complex method (Vector, Burlingame, CA, USA), and were subsequently incubated with 3,3′-diaminobenzidine-4HCl kit (Vector) for 6–7 min. Blocks of tissue sections were postfixed in 1 % osmium tetroxide in 0.1 M phosphate buffer for 1 hour at 4°C, dehydrated in stepped ethanol solutions and flat embedded in an Epon-Araldite mixture. Ultrathin sections were cut on a SORVALL Porter-Blum ultra-microtome and examined with a JEOL JEM-1200 EX II electron microscope (Jeol, Tokyo, Japan) with or without counterstaining.
Fig. 1. Light micrographs showing distribution and localization of LR-LI in the hypothalamus (A). Immunoreactivity for leptin receptor is strong in the arcuate nucleus (B), ventromedial nucleus (C) and lateral hypothalamic area (D). The sections are immunostained with antiserum against the carboxy-terminus of the leptin receptor.
Scale bar (A)=200 μm, (B), (C), (D)=20 μm.

Results

The antiserum against leptin receptor clearly stained the rat hypothalamus with low background. Preadsorption of the antiserum with antigen completely abolished the immunostaining (data not shown). Immunoblot analysis of membranes from rat hypothalamus showed that the antiserum recognized a single polypeptide of 120 kDa$^{14}$; the
molecular mass of leptin receptor in the brain\textsuperscript{5).} Immunohistochemical staining with the antiserum yielded intense staining in the hypothalamus. Many neuronal cell bodies and dendritic processes showed LR-LI in many brain regions. The results showed extensive distribution of LR-LI in the hypothalamus with positively stained cells present in the arcuate nucleus, ventromedial nucleus, parvo-and magno-cellular parts of the paraventricular nucleus, supraoptic nucleus, periventricular nucleus and lateral hypothalamic area (Fig. 1).

Using an immunoperoxidase method, many neuronal cell bodies and dendritic processes stained positive in the arcuate nucleus (Fig. 2A, B). At the ultrastructural level, immunoreactive products representing LR-LI could be observed in the cytoplasm in the vicinity of rough endoplasmic reticulum (rER). As expected for an antibody directed against a cytoplasmic epitope, labeling was localized to the cytoplasmic face of the plasma membrane, rER cisternae, vicinity of the plasma membranes and in the cytoplasmic matrices (Fig. 3, 4). Interestingly, intense labeling of the Golgi apparatus was observed (Fig. 4). Immunoreaction products were not localized with either pre- or post-synaptic membranes at axo-somatic and axo-dendritic synapses.

**Discussion**

The distribution of LR-LI in the rat brain closely resembled that in human brain\textsuperscript{12} and expression of transcripts in rat and mouse brain\textsuperscript{3, 8, 10, 11}. In addition to the regions identified previously by in situ hybridization\textsuperscript{3, 8, 11}, strong LR-LI was demonstrated in many other nuclei throughout the brain\textsuperscript{13, 14}. A recent study indicated that the leptin receptor is approximately 97 kDa in the solubilized fraction of the human brain\textsuperscript{12} and 100 kDa and 179 kDa in Baf3 cells transfected with leptin receptor cDNA\textsuperscript{16}.
Fig. 3. Electron micrographs showing ultrastructural localization of LR-LI in the arcuate nucleus (A, B). LR-LI is visible at the plasma membranes (arrowheads). N, nucleus
Scale bars (A)=0.5 µm, (B)=0.1 µm.

Fig. 4. Electron micrographs showing ultrastructural localization of LR-LI in the arcuate nucleus. LR-LI is visible at the rough endoplasmic reticulum and cytoplasmic matrix. Intense LR-LI is visible in the Golgi apparatus (A,B) (arrowheads). ER, rough endoplasmic reticulum; G, Golgi apparatus Scale bars (A)=0.2 µm, (B)=0.5 µm.
The hypothalamic arcuate nucleus is the main target of circulating leptin. When $^{125}$I-leptin has been injected intravenously, leptin is transported intact from blood to brain by a saturable system. Autoradiographic data have shown that radiolabeled leptin is specifically taken-up at the choroid plexus, arcuate nucleus of the hypothalamus, and median eminence after intravenous infusion. The arcuate nucleus is a primary site of the satiety effect of leptin. There is evidence that the ventral part of the arcuate nucleus lacks a blood-brain barrier (BBB). These studies suggest that leptin secreted in adipocytes is transported to the arcuate nucleus via the general circulation and acts on leptin receptors in the arcuate nucleus which lacks the BBB. Immunohistochemical studies have shown that the arcuate nucleus contains numerous Neuropeptide Y (NPY)-containing neurons and fibers. NPY is a peptide involved in stimulation of food intake and energy balance. Leptin is shown to inhibit NPY synthesis and release and leptin inhibits NPY gene expression through a specific action in the arcuate nucleus. Leptin receptor- and NPY-like immunoreactivity are expressed in the same cells in the arcuate nucleus. Our data and other studies strongly suggest that leptin receptor is expressed in NPY neurons, and that leptin inhibits food intake by inhibiting NPY neurons through its specific receptors. LR-LI is present in NPY-, adrenocorticotropic hormone-, proopiomelanocortin-, galanin- and growth hormone-releasing hormone-containing neurons in the arcuate nucleus. We have shown co-localization of leptin receptor- and tyrosine-hydroxylase-like immunoreactivity in the same arcuate neurons. LR-LI is also demonstrated in vasopressin- and oxytocin-containing neurons in the supraoptic and paraventricular nuclei. The wide distribution of LR-LI in many different neurons suggests that leptin may participate in the control of other unidentified neuroendocrine systems in addition to food intake and thermoregulation.

We have observed LR-LI in the neuronal rER cisternae and over the Golgi apparatus in neurons in the arcuate nucleus by electron microscope. These observations suggested that leptin receptor accumulates in the Golgi apparatus via rER during the course of biosynthesis. Among members of the ligand-gated ion channel superfamily, $\alpha_4$ and $\beta_2$ subunits of nicotinic acetylcholine receptors accumulated in the somata in the cerebral cortex and were associated with rER, although a few were found in the Golgi apparatus. Together, these observations support the idea that the rate-limiting steps of leptin receptor biosynthesis occur in the rER and comprise amino acid polymerization, conformational maturation, and possibly macromolecular assembly. Based on the present observations and other studies, it is possible that leptin receptor is produced on the rER and transported via Golgi apparatus to the cytoplasm and is active in the plasma membrane. Further studies of post-embedding immunostaining using low-temperature resins or cryoultramicrotomy are underway to study the biosynthesis and transport of leptin receptor.

Leptin was recently shown to activate an ATP-sensitive potassium channel in neurons of the arcuate and ventromedial nuclei. Furthermore, administration of leptin induced neuronal activities in the hypothalamus as well as in the brainstem of rat and mouse. We have also found LR-LI in the hippocampus and neocortex. Leptin may play a very important role in the regulation of food intake, appetite, endocrine and autonomic function in rats as well as in humans, but it may also play some important role in learning and memory.
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