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Upregulation of Astrocytic Leptin Receptor in Mice with Experimental Autoimmune Encephalomyelitis

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Abstract

The detrimental role of leptin in experimental autoimmune encephalomyelitis (EAE) is opposite to its neuroprotective role in other neuropathologies. We hypothesize that a shifted cellular distribution of leptin receptors underlies the differential effects of leptin. A robust increase of ObR immunoreactivity was seen along glial fibrillary acidic protein (GFAP)(+) intermediate filaments in reactive astrocytes in the hippocampus and hypothalamus of mice with EAE. Although astrocyte-specific GFAP mRNA and protein were both increased, ObRa mRNA was elevated only after resolution of EAE symptoms, and ObRb mRNA was even decreased at the peak time of symptoms of EAE. A cell type-specific action of leptin may underlie its differential effects.

Keywords

EAE; Reactive astrogliosis; Autoimmunity; Neuroinflammation; Leptin receptor

Introduction

Multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE), involve neuroinflammation, demyelination, and neurodegenerative changes in the brain and spinal cord. It is seldom remembered that glial fibrillary acidic protein (GFAP) was initially purified from plaques in the brain of MS patients in 1969, more than four

decades ago (Eng et al. 1971). GFAP is the principal 8–9-nm intermediate filament in mature astrocytes of the central nervous system (CNS). It modulates astrocyte motility and shape by providing structural stability to astrocytic processes. Reactive astrogliosis is seen after many different insults to the CNS, and it is characterized by rapid synthesis of GFAP (Eng et al. 2000). Increased GFAP and engorged astrocytic processes are seen in EAE within and around the inflammatory demyelinating lesions (Smith et al. 1983; Linington et al. 1984; Smith and Eng 1987). Higher levels of GFAP in cerebrospinal fluid (CSF) and serum of MS patients suggest increased astrocytic damage. Thus, GFAP can serve as a biomarker for disease progression (Malmstrom et al. 2003).

Leptin, a 16-kD protein mainly produced by adipocytes, shows immunomodulatory effects and is actively involved in MS. Leptin is transported across the blood–brain barrier (BBB) in normal mice (Banks et al. 2000; Kastin et al. 2001), a process which usually involves leptin receptors (LepR or LepR) (Kastin et al. 1999; Banks et al. 2002; Pan and Kastin 2007; Tu et al. 2007, 2010). As in patients with active MS, animals with EAE also show increased permeability of the BBB (Juhler et al. 1984) and region-specific changes of cerebral blood flow (Juhler and Paulson 1986). Patients with relapsing–remitting MS have higher serum concentrations of leptin (Frisullo et al. 2007) that precede the first episode of exacerbation, whereas successful resolution of symptoms is associated with a decrease of serum leptin (Batocchi et al. 2003; Angelucci et al. 2005). In EAE mice, a transient but distinct hyperleptinemia is seen before the onset of symptoms (Sanna et al. 2003). Exogenous leptin treatment worsens symptoms in female SJL/J mice and confers susceptibility to male mice that are usually resistant to EAE induction (Matarese et al. 2001). Although the involvement of CNS targets in the effects of leptin on EAE mice has not been previously tested, leptin concentrations are inversely associated with circulating CD4⁺CD25⁺ regulatory Treg (T) cells. The EAE-resistant ob/ob mice (no leptin production) and db/db mice (no functional LepRb receptor subtype) have high levels of Tregs, whereas patients with relapsing–remitting MS have low levels of Tregs during the leptin surge. Furthermore, leptin blockade with either anti-leptin antibodies or a soluble leptin receptor (LepR/Fc chimera) improves EAE, slows disease progression, reduces disease relapse, inhibits proteolipid protein fragment 139–151 (PLP_{139–151})-specific T cell proliferation, and switches cytokine secretion toward a Th2 and Treg profile (Matarese et al. 2005; De Rosa et al. 2006).

Although all the existing results seem to point to a detrimental role of leptin in EAE mice, the effects of leptin in the brain are cell-type specific. Endothelial leptin signaling has effects opposite to those of neuronal leptin signaling. This is illustrated in endothelial LepR knockout (KO) mice that show a better metabolic phenotype and partial resistance to diet-induced obesity (Pan et al. 2012), opposite to the known obesity and diabetes in neuronal-specific LepR KO mice (Chua Jr. et al. 1996; de Luca et al. 2005). Adult-onset obesity is associated with a reduction of neuronal LepR expression and an increase of astrocytic LepR in the hypothalamus (Pan et al. 2008a; Hsueh et al. 2009a). Astrocytic LepR also shows opposite effects from neuronal LepR. In mice receiving the metabolic inhibitor fluorocitrate by intracerebroventricular injection to decrease astrocytic activity, both the neuronal uptake of fluorescently conjugated leptin and signal transducer and activator of transcription (STAT3) signaling are enhanced (Pan et al. 2011b). Thus, a shift of cellular distribution of LepR from neurons to astrocytes might underlie the detrimental effect of leptin in EAE mice that is opposite to its usual neuroprotective role (Oomura et al. 2006; Farr et al. 2006). This is addressed by immunohistochemistry and confocal microscopic analysis and validated by Western blotting and qPCR measurement in this study.

Materials and Methods

EAE Induction

The animal protocol was approved by the Institutional Animal Care and Use Committee. SJL/J female mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed for 2 weeks before induction of monophasic EAE at age 6 weeks. Group design and group size are described in detail in the “Results” section. Each mouse received subcutaneous injection of 80 µg of PLP₁₃₉₋₁₅₁ (AnaSpec Inc, San Jose, CA) divided into three loci in the flank area under brief anesthesia by isoflurane inhalation. PLP₁₃₉₋₁₅₁ was emulsified in 210 µl of RIBI adjuvant (RIBI Immunochem Research Inc., Hamilton, MT). The time of induction is considered EAE day 0. The symptoms were monitored daily by use of a standard EAE score sheet (Pan et al. 1996; Wu et al. 2010b), with 0 being symptom free and 5 being the worst (moribund or dead).

Immunohistochemistry

The SJL/J mice were studied 16 days after PLP₁₃₉₋₁₅₁ immunization (EAE scores were 0.5–1), along with controls receiving RIBI adjuvant only ($n=3$ /group). Tissue preparation and immunohistochemistry (IHC) on 20-µm coronal sections were described previously (Pan et al. 2008a), except for variation of antibody concentration and incubation time. The primary antibodies were mouse monoclonal anti-GFAP (1:600, Sigma, St. Louis, MO) and rabbit polyclonal anti-LepR (1:100, M18 antibody, Santa Cruz Biotechnology, Santa Cruz, CA). The LepR antibody targets the membrane juxtapositional sequence of cytoplasmic LepR that is shared by LepRa, LepRb, LepRc, and LepRd. For comparison of cellular distribution, a rabbit polyclonal antibody against the β -subunit of the insulin receptor (InsR, 1:100, Santa Cruz Biotechnology) was also used. After overnight incubation at 4 °C and thorough washes, the floating sections were incubated with their respective secondary antibodies conjugated with Alexa-488 or Alexa-594 for 1 h at 23 °C. The specificity of immunostaining was verified by inclusion of several negative controls, including lack of signals in brain sections incubated with preadsorbed primary antibody and omission of primary antibody. Confocal images were captured on an Olympus FV1000 microscopic imaging station.

Western Blotting

Four groups of SJL/J mice were studied: naïve (no treatment or manipulation), treated with RIBI adjuvant by subcutaneous injection, and induced EAE with PLP₁₃₉₋₁₅₁ coinjected with RIBI ($n=2$ /group). The mice were sacrificed by decapitation after anesthesia. Bilateral hippocampi were pooled, and the hypothalamus was dissected for Western blotting, as described previously (Pan et al. 2008b; Wu et al. 2010a). The antibodies used for overnight incubation with the nitrocellulose membrane at 4 °C include goat polyclonal LepR antibody (1:500, M18, Santa Cruz Biotechnology), mouse monoclonal GFAP antibody (1:1,000, Sigma), rabbit monoclonal S100 β antibody (1:1,000, Abcam, Cambridge, MA), and the reference gene β -actin (1:20,000, Sigma). After thorough washes, the membrane was incubated with fluorescently conjugated secondary antibodies. Images were captured on an Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE). The relative integrated intensity of the signals was measured by use of the NIH ImageJ program, and the difference of target gene/ β -actin ratio among the groups was analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test.

Real-Time RT-PCR

Since the hippocampus of the SJL/J mice with monophasic EAE showed the most robust upregulation of LepR immunoreactivity, the hippocampi of these mice were used for

transcriptional analysis. At days 3, 7, 12, 16, and 24 after PLP immunization ($n=4$ /group), the EAE mice were sacrificed and the hippocampi were collected. A control group (3 days after adjuvant injection, $n=4$) was included in the study. Total RNA extraction and reverse transcription were performed as described previously (Wu et al. 2010b). qPCR used the SYBR master mix on an ABI 7900 HT system (Applied Biosystems, Carlsbad, CA). PCR primers were the same with those we have used previously, including those for GFAP (Wu et al. 2010a), the reference gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH), and LepR isoforms (Pan et al. 2008b; Hsueh et al. 2009a). The mRNA expression of each gene was quantified by the standard curve method with template plasmids containing target fragments. The results were normalized to that of GAPDH in the same sample.

Statistical Analysis

All data are presented as mean $\pm$ standard error of mean. In the study to determine the effects of EAE induction on EAE scores, repeated measures ANOVA was performed. Also, to determine the levels of protein or mRNA expression, one-way ANOVA was performed to compare differences among groups, followed by Tukey's post hoc test if a significant overall difference was detected.

Results

EAE Induces Reactive Astrogliosis and Increased LepR in Astrocytes: Morphological Evidence

To determine whether LepR levels are altered in activated astrocytes, we analyzed LepR immunoreactivity in GFAP-positive cells in the CNS of mice with EAE. SJL/J mice with monophasic EAE had the worst motor deficits around day 12. By day 16, mice were in the recovery stage with EAE scores of 0.5–1. Data from day 16 mice with an EAE score of 1 are shown below. At this time, GFAP immunoreactivity was increased in the hippocampus (Fig. 1), in several hypothalamic nuclei, including the dorsomedial hypothalamus (DMH), particularly in $\alpha 1$ tanyocytes outlining the third ventricle (Fig. 2), and the arcuate nucleus (Arc) of the hypothalamus (Fig. 3). Morphologically, the GFAP-positive processes showed mild engorgement. This is apparent in an enlarged image from the Arc (Fig. 3b) in which astrocytic LepR (double arrows) and neuronal LepR (single long arrow) are both present. The predominant astrocytic LepR immunoreactivity contrasts with the neuronal distribution of InsR β that persisted in the EAE mice (Fig. 4).

Patients with MS may show impairment of memory, mood, and other cognitive functions, suggesting involvement of the hippocampus. Therefore, we tested the prediction that EAE induces a shift of cellular distribution and expression of LepR in the hippocampus. In the hippocampus of normal mice, LepR immunoreactivity showed nearly complete colocalization with GFAP-positive cells, indicating that hippocampal astrocytes have substantially higher expression of LepR than neurons. In the hippocampus of EAE mice, not only GFAP but also LepR immunoreactivity had a striking increase in fluorescent intensity. Confocal microscopic analysis showed that the increased LepR and GFAP expressions were colocalized in the same cells (Fig. 1a). At higher magnification, the punctate LepR immunoreactivity contrasted with the continuous GFAP immunoreactivity, suggesting vesicular distribution of LepR in the GFAP(+) intermediate filaments within astrocytes (Fig. 1b).

Patients with MS also show alterations in feeding and thermoregulation as well as sickness behavior, suggesting hypothalamic involvement. Thus, we determined whether LepR expression in the hypothalamus had shifted cellular distribution and expression. In the DMH of normal mice, there were more LepR(+) cells than GFAP(+) cells. While all astrocytes

appeared to express LepR, there were more neurons with LepR immunoreactivity at a similar fluorescent intensity (short arrows, inset of upper middle panel in Fig. 2a). In the DMH of EAE mice, both GFAP and LepR were increased in intensity. The higher levels of LepR seemed to be present mainly in GFAP(+) cells, rather than neurons that show distinctive cell morphology and were verified by NeuN immunoreactivity in separate sections. This suggests selective upregulation of astrocytic LepR. The most apparent increase of both GFAP and LepR was seen in the adjacent $\alpha 1$ tanycytes (Fig. 2a). The GFAP(+) tanycyte processes that extend into brain parenchyma appear to be “hollow,” with GFAP immunoreactivity only present at the outer surface. LepR immunoreactivity, by contrast, filled the interior of the processes, creating the impression, illustrated in high magnification images, that GFAP encases LepR (Fig. 2b).

Similar to the DMH, the Arc also had more neurons than astrocytes showing LepR immunoreactivity. EAE induced an increase of fluorescent intensity of both GFAP and LepR. The upregulation of LepR was seen in many neurons and some astrocytes. Colocalization of LepR with GFAP-immunopositive cells was sparse. The $\alpha 2$ tanycytes also showed robust upregulation of GFAP and some increase of LepR, though less than that of $\alpha 1$ tanycytes adjacent to the DMH. By contrast, there was no increase of either GFAP or LepR in $\beta 1$ and $\beta 2$ tanycytes bordering the median eminence (Fig. 3a). A higher magnification image from the Arc of an EAE mouse shows a punctate distribution of LepR in many neurons and a few astrocytes in the same field (Fig. 3b). The pattern of mixed cellular distribution of LepR contrasts with the seemingly exclusive neuronal distribution of InsR β in neurons (Fig. 4).

EAE Induces Reactive Astrogliosis and Increased LepR: Semiquantitative Analysis by Western Blotting

To determine the relative increase of astrocytic LepR, we quantified fluorescent intensity of the immunostaining in representative sections by use of the thresholding method. The thresholded area for GFAP or LepR staining in the hippocampus, DMH, and Arc was analyzed by ImageJ. The data are presented as threshold area / total area of interest and showed higher levels in EAE mice for both GFAP and LepR (Fig. 5). More accurate quantification of the levels of protein expression was performed by Western blotting.

In comparison with the naïve control or mice treated with RIBI adjuvant only, hippocampal homogenates from the EAE mice showed a significant increase of GFAP protein expression. S100 β , another astrocytic protein, was increased both by adjuvant and EAE induction. The expression of LepR was also increased in mice receiving either adjuvant treatment or EAE induction (Fig. 6a, b).

In the hypothalamus, EAE induction also induced a significant increase of GFAP immunoreactivity. However, the increase of S100 β was seen only after adjuvant treatment. There was no significant difference of overall LepR expression in the hypothalamic homogenates that include neurons, glia, and microvessels (Fig. 7a, b).

Transcriptional Upregulation of LepR Lags Behind the Increase of LepR Immunoreactivity in the Hippocampus

To measure changes of LepR and GFAP mRNA, qPCR was performed. We chose the hippocampus as the region of interest to determine transcriptional upregulation because this region has uniform increases of both proteins by IHC as shown above. The mRNA for GFAP showed a significant increase not only on day 16 after EAE induction (time point chosen for protein expression studies above) but also between days 12 and 24 (Fig. 8a). By contrast, LepRa mRNA did not increase significantly until the mice had almost completely

recovered on day 24 after EAE induction (Fig. 8b). This contrasts with the earlier increase of LepR immunoreactivity on day 16 shown in Fig. 1. The mRNA for LepRb even showed a significant decrease on day 12, and the increase on day 24 (in comparison with day 12) was not significantly different from the control (Fig. 8c). The changes of LepRc and LepRe mRNA over the course of EAE were not statistically significant (data not shown). Thus, the regulatory changes of LepR in the EAE mice mainly occurred at the level of protein processing rather than transcriptional activation, and there were subtype differences.

Discussion

Although reactive astrogliosis in the hippocampus and hypothalamus is expected for EAE mice, it has never been shown that LepR expression is increased or that the upregulation of LepR mainly occurs in reactive astrocytes in the hippocampus. Altered LepR distribution and expression suggests essential CNS effects of leptin in EAE. Leptin promotes neurogenesis (Lee and Piquette-Miller 2003), activates NMDA receptors in the hippocampus (Irving et al. 2006), and dose-dependently modulates long-term potentiation. As a result, it improves hippocampal-dependent memory not only in normal rats but also in SAMP8 mice with neurodegeneration resembling Alzheimer's disease (Oomura et al. 2006; Farr et al. 2006). Leptin-deficient ob/ob mice have increased seizure susceptibility, whereas leptin treatment inhibits epileptogenesis and is effective after intranasal delivery (Shanley et al. 2002; Xu et al. 2008; Erbayat-Altay et al. 2008). Another study, however, found that leptin dose-dependently potentiates penicillin-induced convulsions after intracerebroventricular injection. Lower doses of leptin (1 or 2 μ g) are most effective, seen 90 min after delivery to the rat, whereas a high dose (10 μ g) has no effect (Ayyildiz et al. 2006). A possible explanation would be cell type-specific activities of leptin. Higher astrocytic LepR suggests that leptin can induce greater astrocytic signaling. Overall, the effects of leptin on the hippocampus may be involved in the cognitive impairment and reduction of verbal and spatial learning observed in MS patients (Rao et al. 1991; DeLuca et al. 1994).

Signaling events induced by leptin in primary astrocytes include calcium influx (Hsuchou et al. 2009a), phosphorylation of mitogen-activated protein kinases, and activation of STAT3 (Pan et al. 2011a). Some of the effects may be autocrine, as both leptin and LepR can be produced by cultured C6 astrocytoma cells (Morash et al. 2000). Astrocytes play crucial roles in neuronal activities and regulation of cerebral blood flow and metabolism, actions summed as metabolic and gliovascular coupling (Tsacopoulos and Magistretti 1996; Nedergaard et al. 2003; Abbott et al. 2006; Iadecola and Nedergaard 2007). In vitro studies also suggest that the levels and subtypes of LepR in astrocytes affect leptin permeation across the BBB (Hsuchou et al. 2010). We found that EAE induces astrocytic LepR and facilitates LepR trafficking along GFAP(+) intermediate filaments within the cells. The presence of LepR along tanycyte processes suggests a potential role of the tanycytes in transporting leptin from CSF to the hypothalamus.

It is unusual that the protein regulation and accelerated LepR trafficking in astrocytes preceded transcriptional upregulation. We have shown that LepR mRNA is present in a small proportion of astrocytes as well as neurons in the normal rat hypothalamus (Hsuchou et al. 2009b). We also showed that the protein expression of astrocytic LepR is upregulated by the development of adult-onset obesity, as seen in both the agouti viable yellow mouse (Pan et al. 2008a), and diet-induced obesity (Hsuchou et al. 2009a). In EAE mice, we show here that astrocytic LepR expression is increased in the hippocampus as well as in the hypothalamus. This is the first time that extrahypothalamic astrocytic LepR regulation in the brain has been described.

Since leptin-deficient ob/ob mice and LepRb-defective db/db mice also show astrogliosis as well as defective neuronal functions (Ahima et al. 1999), we speculate that astrogliosis precedes or is at least independent of upregulated astrocytic LepR but that leptin signaling modulates the functions of these reactive astrocytes. We have shown that endothelial and neuronal leptin signaling has opposite effects on diet-induced obesity (Pan et al. 2012), whereas experiments involving treatment with fluorocitrate suggest that astrocytic and neuronal signaling antagonize each other in leptin distribution and pSTAT3 activation (Pan et al. 2011b). Leptin modulates the extent of astrogliosis after injury in an in vitro scratch model (Hsueh et al. 2012). The higher increase of astrocytic LepR may shift the signaling from neuronal to astrocytic. In EAE, astrocytic leptin signaling might also play a negative role. This may explain why leptin treatment worsens EAE symptoms, whereas ob/ob and db/db mice are resistant to EAE induction (Matarese et al. 2001; Sanna et al. 2003; Matarese et al. 2005; De Rosa et al. 2006).

In summary, we showed that EAE is associated with reactive astrogliosis in the hippocampus and hypothalamus. The reactive astrocytes show upregulation of LepR protein expression distributed along GFAP(+) processes, particularly in the hippocampus and around the third ventricle in the hypothalamus. In hippocampal homogenates, the regulation of LepR mRNA is subtype specific, with a late increase of LepRa that lags behind the increase of protein expression and a transient reduction of LepRb in the peak of the disease. This novel finding of astrocytic LepR changes might be pertinent for clinical intervention in patients with MS.

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Fig. 1. IHC in hippocampus. **a** In the hippocampus of a control mouse, LepR immunoreactivity (*red*) is almost exclusively seen in GFAP(+) astrocytes (*green*), as shown in the insets (*arrows*). In an SJL/J mouse 16 days after induction of monophasic EAE, there was a diffuse increase of immunoreactivity of both GFAP and LepR. Scale bar 100 μ m. **b** Higher magnification confocal image shows that LepR had a vesicular expression and was unevenly distributed along the GFAP(+) processes in SJL/J mouse 16 days after induction of EAE. $n=3$ /group. Scale bar 10 μ m

Fig. 2.

IHC in DMH of the hypothalamus. **a** In a control mouse, there were more cells immunopositive for LepR (*arrow heads*) than for GFAP (*arrows*), indicating that astrocytes were not the only type of cells expressing LepR. At 16 days after EAE induction, both GFAP and LepR immunofluorescent intensity was increased in the DMH. The most prominent increase of GFAP occurred in tanycytes (*diamond-headed arrows*) along the third ventricle. The increase of LepR was seen in both astrocytes (*arrows*) and other types of cells (*arrow heads*). *3 V* third ventricle. Scale bar 100 μm. **b** Higher magnification confocal image from the mouse 16 days after EAE induction showed that the GFAP(+) tanycyte processes appeared to be hollow and encased LepR. *n*=3/group. Scale bar 10 μm

Fig. 3. IHC in Arc of the hypothalamus. **a** In a control mouse, there were abundant LepR-immunopositive cells, and only a few were astrocytes with colocalization of GFAP (*arrow*). In the EAE mouse, there was an increase of both GFAP and LepR immunofluorescent intensity. Most LepR(+) cells were not astrocytes (examples indicated by *arrow heads*). Scale bar 100 μm . **b** Higher magnification image from the mouse 16 days after EAE induction showed that while the GFAP(+) astrocytes expressed LepR, there were more neurons showing cytoplasmic expression of LepR. $n=3/\text{group}$. Scale bar 10 μm

Fig. 4.
The immunofluorescence of InsR β differs from that of LepR and appears to show exclusive neuronal staining, distributed at cell surface and filament processes

Fig. 5. Quantification of fluorescent intensity of GFAP and LepR immunoreactivity was performed on brain sections shown in Figs. 1-3a. In all three regions (hippocampus, DMH, and Arc), EAE mice showed higher fluorescent intensity of both proteins than control mice

Fig. 6.

Western blotting of hippocampal tissue showed that the expression of astroglial markers GFAP, S100β, and LepR was increased 16 days after EAE induction. Mice treated with only RIBI adjuvant also showed an increase of S100β and LepR. The reference gene β-actin served as a loading control and was unchanged by treatment. The gel images (**a**) was quantified by densitometric analysis and the target gene/β-actin ratio presented. $n=2/\text{group}$. * $p<0.05$; ** $p<0.01$

Fig. 7.

Western blotting shows that EAE mice had increased GFAP expression in hypothalamic homogenate, whereas adjuvant treatment increased S100β 16 days after treatment. The expression of LepR (summation of glial, neuronal, and vascular components) was unchanged. $n=2/\text{group}$. * $p<0.05$

Fig. 8.

Transcriptional regulation in the hippocampus of the EAE mice was determined by qPCR at 0, 3, 7, 12, 16, and 24 days after EAE induction ($n=4$ /group). **a** A significant increase of GFAP mRNA in comparison with the adjuvant-injected control was seen at 12, 16, and 24 days. **b** LepRa mRNA did not show a significant increase until day 24, after nearly complete resolution of EAE symptoms. The EAE scores are shown by the *line graph* with the *right y-axis*. **c** LepRb mRNA was not increased at any time point but instead showed a reduction on day 12 that coincides with a high EAE score. * $p<0.05$; ** $p<0.01$; *** $p<0.005$ in comparison with the control