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Leptin inhibits amyloid β-protein degradation through decrease of neprilysin expression in primary cultured astrocytes

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ABSTRACT

Pathogenesis of Alzheimer's disease (AD) is characterized by accumulation of extracellular deposits of amyloid β-protein (Aβ) in the brain. The steady state level of Aβ in the brain is determined by the balance between its production and removal; the latter occurring through egress across blood and CSF barriers as well as Aβ degradation. The major Aβ-degrading enzymes in the brain are neprilysin (NEP) and insulin-degrading enzyme (IDE), which may promote Aβ deposition in patients with sporadic late-onset AD. Epidemiological studies have suggested an inverse relationship between the adipocytokine leptin levels and the onset of AD. However, the mechanisms underlying the relationship remain uncertain. We investigated whether leptin is associated with Aβ degradation by inducing NEP and IDE expression within primary cultured astrocytes. Leptin significantly decreased the expression of NEP but not IDE in a concentration- and time-dependent manner through the activation of extracellular signal-regulated kinase (ERK) in cultured rat astrocytes. Furthermore, leptin inhibited the degradation of exogenous Aβ in primary cultured astrocytes. These results suggest that leptin suppresses Aβ degradation by NEP through activation of ERK.

1. Introduction

Alzheimer's disease (AD) is neuropathologically characterized by the accumulation of amyloid-β protein (Aβ) as extracellular plaques and the deposition of hyperphosphorylated tau in intracellular neurofibrillary tangles [1]. The life-long accumulation of Aβ in the brain is determined by the rate of Aβ generation versus clearance. Clearance can be accomplished via two major pathways: proteolytic degradation and receptor-mediated transport from the brain [2]. The two major endopeptidases associated with Aβ degradation in the brain are zinc metalloendopeptidases, referred to as neprilysin (NEP) and insulin degrading enzyme (IDE), and have been demonstrated in NEP- and IDE-knockout animals [3,4]. It was recently demonstrated that the oligomer form of Aβ is the main neurotoxin involved in early alterations occurring in an AD brain, and the levels of Aβ oligomers are well correlated with the extent of synaptic and neuronal loss as well as with severity of cognitive impairment [5]. NEP degrades not only the Aβ-monomeric form but also the Aβ-oligomeric form. Astrocytes express some of the Aβ-degrading proteases including NEP and IDE [6]. Moreover, we recently reported that the expression of NEP but not IDE was decreased by ketamine through a noncompetitive N-methyl-D-aspartate receptor in astrocytes [7].

Leptin is synthesized in the periphery and actively transported across the blood–brain barrier or blood–CSF barrier [8], and plays a major role in the regulation of appetite and body energy metabolism [9]. Leptin receptors are widely expressed in several extrahypothalamic brain regions including the cortex, hippocampus, brain stem, and cerebellum [10]. Recent studies indicate that leptin has a remarkable effect on hippocampal development and function, particularly learning and memory processes and leptin dysfunction has recently been linked to AD [11]. However, little is known about

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β-protein; CNS, central nervous system; IDE, insulin-degrading enzyme; NEP, neprilysin; NMDAR, N-methyl-D-aspartate receptor.

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the relationship of leptin with Aβ degradation occurring in the AD brain. Various studies have reported that leptin supplementation ameliorates Aβ and tau deposition and improves memory in AD mouse models [12,13]. Both *in vitro* and *in vivo*, leptin increases adult hippocampal neurogenesis [14] and reduces Aβ levels [15]. Moreover, Marwarha et al. reported that leptin augments the degradation of Aβ by increasing IDE expression through activation of the AKT pathway [16]. It was recently demonstrated that the leptin receptor is present on the astrocytes and plays a role in various responses, including neuroinflammation [17]. Astrocytes, which represent a major glial cell subpopulation in the brain, actively participate in various brain functions such as neuronal differentiation, protection, and migration.

We investigated whether leptin was involved in Aβ degradation of astrocytes through the induction of NEP and IDE expression. The present study suggests that leptin may be associated with the regulation of NEP but not IDE in astrocytes. In particular, leptin-treated astrocytes decreased the degradation of exogenously added soluble Aβ.

2. Materials and methods

Cortical astrocyte cultures were isolated from Sprague–Dawley rats on embryonic day 20, as previously described [18]. In brief, trypsinized and dissociated cortical cells were cultured with Dulbecco's modified Eagle's medium (Wako) supplemented with 10% fetal bovine serum in 75-cm^2 culture flasks for a 7-day incubation. The cells were trypsinized and subcultured in 60- or 90-mm-diameter culture dishes. All cultures were maintained in 5% CO_2 and 95% air at 37 °C. The cell populations consisted of >95% astrocytes, which was determined by immunocytochemical examination with anti-glial fibrillary acidic protein (data not shown). The cells were pharmacologically treated with leptin (Wako), U0126 (Wako), LY294002 (Wako), or a vehicle (control).

The cells were lysed in a buffer containing 5 mM Tris–HCl (pH 7.4), 2 mM EDTA, 1% Triton X-100, and a protease inhibitor (Complete™ protease inhibitor cocktail, Roche Molecular Biochemicals, Penzberg, Germany). The protein concentration of each sample was determined using a BCA protein assay kit (Thermo Fisher Scientific). The samples were separated on a 10% polyacrylamide gel (Wako) and electrotransferred to polyvinylidene difluoride membranes (EMD Millipore). The transferred blots were incubated with 1:1000 dilutions of anti-NEP (Leica), anti-IDE (EMD Millipore), anti-phospho-ERK1/2 [Cell Signaling Technology (CST)], anti-phospho-ERK1/2 (CST), anti-ERK1/2 (CST), anti-phospho-AKT (CST), or anti-AKT (CST) antibodies. The bands were incubated with horseradish peroxidase-linked anti-mouse or anti-rabbit antibodies (CST) and were visualized using the SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific).

Synthetic wild-type Aβ 40 (Peptide Institute) was dissolved in a 0.02% ammonia solution at 500 μM. To obtain seed-free Aβ solutions, the prepared solutions were centrifuged at 540,000×*g* for 3 h to remove undissolved peptides that may act as preexisting seeds. The supernatant was collected and stored in aliquots at −80 °C until use.

Data are presented as the mean ± standard deviation of five independent experiments. Statistical analyses were performed using one-way factorial ANOVAs combined with Scheffe's test for all paired comparisons. *P* values <0.05 were considered statistically significant.

3. Results

To evaluate the effects of leptin on NEP and IDE expression, astrocytes were incubated with indicated concentrations of leptin for 48 h and each expression level was detected by Western blot analyses. Treatment of astrocytes with leptin significantly decreased the expression of NEP but not IDE in a concentration-dependent manner (Fig. 1A). A significant decrease in NEP expression levels was observed 24 h after leptin treatment. IDE levels were not affected by leptin treatment (Fig. 1B). β-actin (internal control) expression levels were similar in all samples (data not shown).

Leptin signaling induces the activation of ERK and PI3K [10]. To investigate whether treatment with leptin resulted in the same

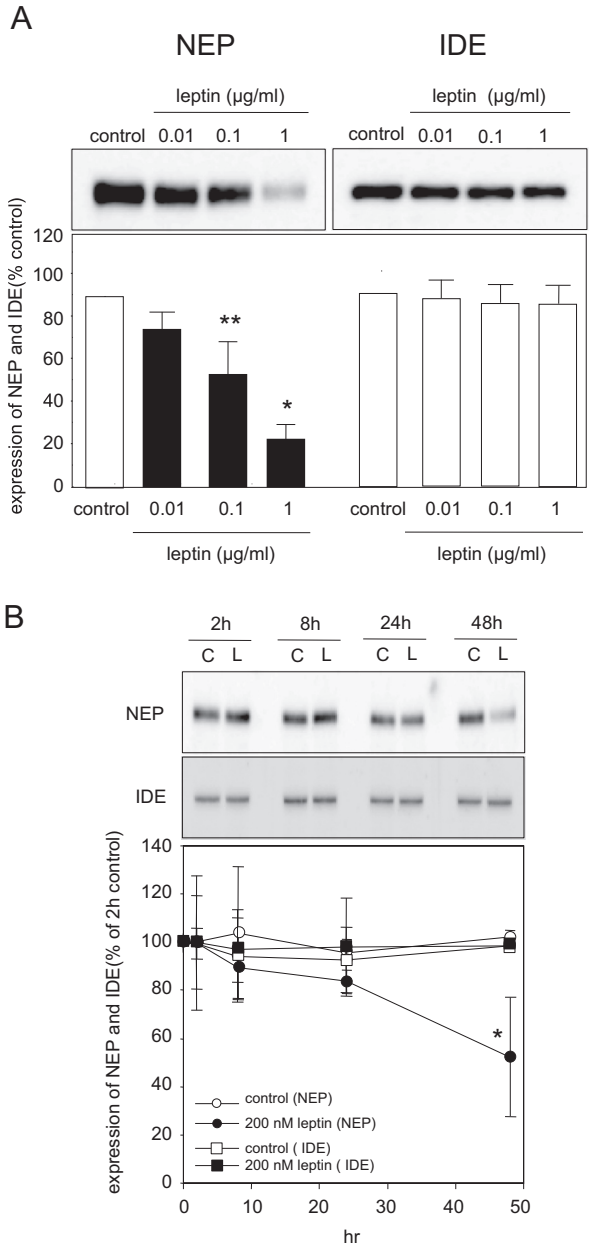

Fig. 1. Leptin decreases the expression of NEP in primary astrocytes. (A) Dose–response relationships of NEP and IDE expression in astrocytes treated for 48 h with the indicated leptin concentrations. (B) Time-course of NEP and IDE expression in astrocytes treated with 200 nM leptin. At the indicated times, the expression levels of NEP and IDE of cell lysates were subjected to Western blot analyses using anti-NEP or anti-IDE antibodies and were subsequently determined by densitometric scanning of the blots. The band densities are presented as the percentages of the control at each time. Each column indicates the mean of six values ± SD. *$P < 0.0001$ (one-way ANOVA combined with Scheffe's test, significant difference compared with control).

effects in astrocytes, we examined the phosphorylation of ERK1/2 and AKT in leptin-treated astrocytes. As expected, leptin markedly induced the phosphorylation of ERK1/2 and AKT for 4 h after treatment of leptin (Fig. 2A). To investigate the intracellular mechanism of leptin on NEP expression, neurons were incubated for 1 h with ERK (U0126) and PI3K (LY294002) inhibitors before the treatment of leptin. U0126 reduced the decrease on NEP expressions induced by leptin (Fig. 2B). However, the effect of leptin on NEP expression was not affected by pretreatment with LY294002 (Fig. 2B).

To investigate whether the leptin-induced decrease of NEP expression in astrocytes is on the membrane or within the extracellular space (medium), astrocytes were incubated in the presence or absence of leptin with or without pretreatment of U0126 and the membrane fraction and medium from cultured astrocytes were subsequently isolated. The isolation of the membrane fraction was confirmed by the membrane marker protein flotillin-1 (data not shown). NEP expression was localized in the membrane fraction and medium of astrocytes (Fig. 3). Furthermore, we compared the NEP levels in the membrane fraction or medium isolated from leptin-treated astrocytes. NEP levels in the membrane fractions and medium prepared from leptin-treated astrocytes significantly decreased compared with those from the untreated controls

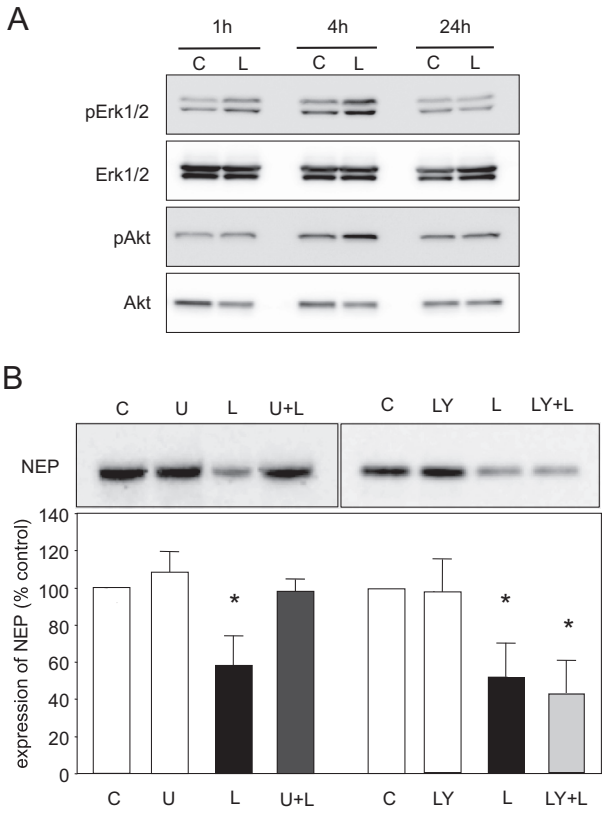

Fig. 2. Effects of leptin treatment on ERK and AKT signaling pathways involved in leptin-induced decrease of NEP expression levels. (A) Astrocytes were incubated with leptin for 1, 4, and 24 h. Leptin-treated cells were harvested and subject to Western blot analyses using antibodies for anti-phospho-ERK and anti-phospho-Akt antibodies. (B) Astrocytes were incubated for 48 h in the presence or absence of 300 nM leptin after pretreatment with or without pretreatment of U0126 (10 μM) or LY294002 (10 μM) for 1 h. The leptin-treated cell lysates were subjected to Western blot analyses using anti-NEP antibodies. NEP expression levels were determined by densitometric scanning of the blots and the band densities are presented as the percentages of the control at 48 h. Each column indicates the mean of six values ± SD. $^{*}P < 0.0001$ (one-way ANOVA combined with Scheffe's test, significant difference compared with control). C, L, U, and LY are the control, leptin, U0126, and LY294002, respectively.

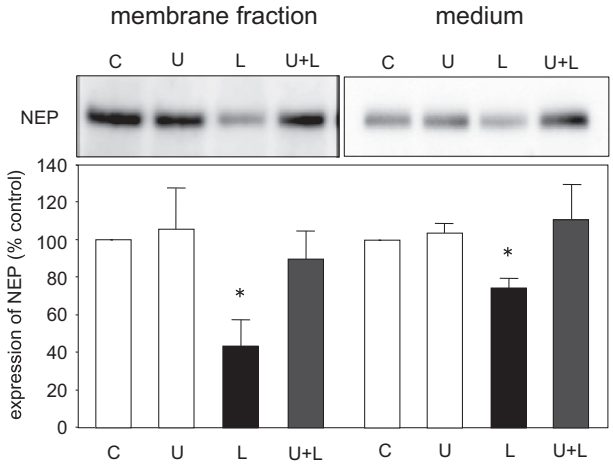

Fig. 3. Leptin decreases the expression of NEP in the cell membrane of astrocytes. Astrocytes were incubated for 48 h in the presence or absence of 300 nM leptin after pretreatment with or without pretreatment of U0126 (10 μM) for 1 h. Membrane fractions and mediums isolated from leptin-treated astrocytes were subjected to blot analysis using an anti-NEP antibody. NEP expression levels were determined by densitometry of the scanned blots following incubation with anti-NEP antibody and band densities are presented as percentages of the control (vehicle-treated astrocytes). Each column indicates the mean of four values ± SD. $^{*}P < 0.0001$ (one-way ANOVA combined with Scheffe's test, significant difference compared with control).

(Fig. 3). Moreover, U0126 reduced these leptin-induced decreases in NEP expression (Fig. 3).

We finally examined whether the leptin-induced decrease in NEP expression inhibited Aβ degradation of astrocytes. Exogenous Aβ was incubated for 12 h in astrocytes pretreated with leptin. Aβ levels in the incubated medium were significantly higher in the presence of leptin. The inhibition of Aβ degradation in the incubated medium was reduced by U0126 (Fig. 4). However, the induction of endogenous Aβ could not be detected in the medium of astrocytes incubated without Aβ in the presence of leptin or U0126 (data not shown).

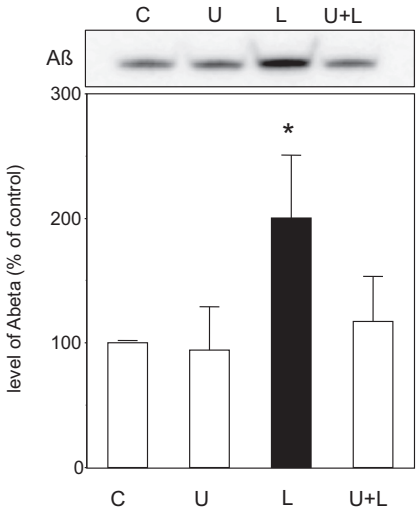

Fig. 4. Effects of leptin on the exogenous Aβ degradation induced by astrocytes. Leptin-treated astrocytes were incubated with 5 μM of soluble Aβ40 in cultured media for 12 h. The media of leptin-treated astrocytes and Aβ40 were subjected to Western blot analyses with anti-Aβ antibody (6E10). Aβ levels were determined by densitometric scanning of the blots and the band densities are presented as percentages of the control. Each column indicates the mean of four values ± SD. $^{*}P < 0.0001$ (one-way ANOVA combined with Scheffe's test, significant difference compared with control).

4. Discussion

Our findings indicate that leptin decreased Aβ degradation in astrocytes. This decrease of Aβ degradation may have been due to the suppression of NEP through activation of the ERK signaling pathway but not the PI3K/AKT signaling pathway; however, leptin did not induce the expression of IDE.

NEP, a zinc metalloendopeptidase, has been identified as a critical Aβ degrading enzyme in the brain that could play an important role in developing therapies for AD [19]. Pathological downregulation of NEP, IDE, or other Aβ-degrading enzymes in humans could predispose the individual to accumulation of Aβ and the development of AD [20]. Although NEP can degrade both the monomeric and oligomeric forms of Aβ40 and Aβ42, IDE is only able to degrade the monomeric form of these Aβs [21]. However, molecular mechanisms of the Aβ degradation and the regulation of NEP and IDE expression in astrocytes remain unclear. We recently reported that ketamine, which is a general anesthetic, inhibits Aβ degradation through the decrease of NEP expression in astrocytes [7]. Our present study observed that leptin downregulated NEP expression in astrocytes and inhibited the degradation of exogenous Aβ.

Leptin, the product of the obese gene, is implicated in obesity, food intake, glucose homeostasis, energy expenditure, and neuroendocrine responses [9]. The serum levels of leptin are correlated with the development of AD, suggesting a potential pathogenic role of leptin [10]. However, the relationship of leptin and the Aβ cascade and degradation pathways in the AD brain are unknown. Leptin decreases Aβ levels by targeting all facets of Aβ metabolism such as production, clearance, and degradation. Leptin activates four major signal transduction pathways, including the ERK, PI3K/AKT/mTOR, JAK/STAT, and AMPK pathways [10]. Leptin increases the expression levels of IDE in rabbit organotypic slices by activating the PI3K/Akt pathway [14]. We recently suggested that ketamine decreased the expression of NEP but not IDE through the reduction of p38/mitogen-activated protein kinase in astrocytes [7]. This study indicates that in astrocytes, leptin downregulated the expression of NEP but not IDE through activation of the ERK pathway. IDE in primary cultured astrocytes was not involved in the activation of the PI3K/AKT pathway by leptin. Taken together with our results, leptin may be associated with Aβ accumulation and fibrillogenesis in the brain through its ability to reduce Aβ degradation by decreasing NEP expression.

In conclusion, our present findings indicate that leptin reduces extracellular Aβ degradation in primary cultured astrocytes. It may be possible that leptin facilitates the formation of the Aβ fibril and oligomer in the brain and has adverse effects on patients at a high risk of developing AD. Thus, the present study may help to create a novel mechanism to treat AD.

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