Neuroprotective Effects of Leptin Against Ischemic Injury Induced by Oxygen-Glucose Deprivation and Transient Cerebral Ischemia

Feng Zhang, MD, PhD; Suping Wang, MD; Armando P. Signore, PhD; Jun Chen, MD

Background and Purpose—Leptin is the major adipose hormone that regulates body weight and energy expenditure by activating leptin receptors in the hypothalamus. Leptin receptors are also present in other cell types, and a potent antiapoptotic effect for leptin has recently been reported. We investigated whether leptin was neuroprotective against ischemic brain injury.

Methods—In vitro ischemic injury was induced in rat primary neuronal culture by oxygen-glucose deprivation for 90 minutes. In vivo ischemic brain injury was induced by middle cerebral artery occlusion in mice for 60 minutes.

Results—Leptin receptors were detected in cultured rat cortical neurons, as well as in the mouse cortex, striatum, and hippocampus. In vitro results showed that leptin, 50 to 100 μg/mL, protected primary cortical neurons against death induced by oxygen-glucose deprivation in a concentration-dependent manner. In vivo studies in the mouse brain demonstrated that the intraperitoneal administration of leptin, 2 to 8 mg/kg, dose-dependently reduced infarct volume induced by middle cerebral artery occlusion. Leptin was effective when injected 5 minutes before or 30 to 90 minutes after reperfusion, but not 2 hours after reperfusion. Leptin improved animal body weight recovery and behavioral parameters after cerebral ischemia. Leptin enhanced the phosphorylation of extracellular signal–related kinase 1/2. Both extracellular signal–related kinase 1/2 activation and neuroprotection were abolished by the administration of PD98059 in vitro and in vivo.

Conclusions—Leptin is neuroprotective against ischemic neuronal injury. Our findings suggest that leptin is a legitimate candidate for the treatment of ischemic stroke. (Stroke. 2007;38:2329-2336.)

Key Words: brain ischemia ■ extracellular signal–related kinase 1/2 ■ leptin ■ oxygen-glucose deprivation

Leptin is a 16-kDa adipose hormone of the secreted product of the ob gene1 that can cross the blood-brain barrier.2 Leptin is known to negatively regulate body weight and to facilitate energy expenditure through binding of leptin receptors in the hypothalamus.3 The leptin receptor is the product of the db gene.4 In addition to its abundant expression in the hypothalamus, the leptin receptor is also expressed in the forebrain.5 Secondary signaling pathways activated by leptin in neurons include the signal transducers and activators of transcription-3 (STAT3), extracellular signal–related kinase (ERK) 1/2, and Akt pathways,3,5,6 all of which have prosurvival roles in a variety of cell types.

A neurotrophic7 and an antiapoptotic role for leptin has recently been reported.5,6,8,9 The cell types in which the apoptosis-suppressing effect of leptin was characterized include human neuroblastoma cells,6 human osteoblasts,8 and T lymphocytes.9 Given that leptin receptors are also expressed in forebrain neurons, we hypothesized that exogenous administration of leptin could have a neuroprotective effect against ischemic neuronal injury. Our results indicate that leptin indeed protects against ischemic injury in both in vitro and in vivo models, and this effect of leptin is mediated mainly by activation of the ERK1/2 pathway.

Materials and Methods

Rat Primary Neuronal Culture and Oxygen-Glucose Deprivation

Primary cultures of rat cortical neurons were prepared from 17-day-old SD rat embryos as previously described with B27/Neurobasal.10 Experiments were conducted at 11 days in vitro, when cultures consisted primarily of neurons. To model ischemia-like conditions in vitro, primary cultures were exposed to oxygen-glucose deprivation (OGD).10 In brief, the culture medium was replaced 4 times with serum- and glucose-free medium. The glucose-deprived cultures were then placed in a Billups-Rothenberg modular incubator chamber, which was flushed for 5 minutes with 100% argon and then sealed. The chamber was placed in a water-jacketed incubator at 37°C for 90 minutes and then returned to 95% air, 5% CO₂, and glucose-containing medium for 24 hours. Control glucose-containing...
cultures were incubated for the same periods of time at 37°C in humidified 95% air and 5% CO₂.

**Neuronal Treatment With Leptin and PD98059**

Recombinant rat leptin was purchased from Sigma. The stock solution of leptin was prepared according to the manufacturer’s recommendation. For the studies of leptin-mediated neuroprotection, the cultures were pretreated with leptin for 0, 1, or 3 hours or overnight at concentrations of 10, 50, or 100 μg/mL according to experimental conditions. For the protective mechanism studies, the cultures were pretreated with leptin for 0, 1, or 3 hours at a concentration of 50 μg/mL. The ERK inhibition studies were performed in some experiments with the mitogen-activated protein kinase kinase-1 (MEK1) inhibitor PD98059 at a concentration of 10 μmol/L or U0126 at a concentration of 3 μmol/L. Neurons were treated with these inhibitors for 30 minutes, and leptin was then added to the medium to reach a final concentration of 50 μg/mL.

**Measurement of Cell Viability/Death**

The fluorescence of Alamar Blue, which changes from blue to red when reduced by cellular metabolic activity, and lactate dehydrogenase release were used. Results were averaged for 3 independent experiments, with each experiment containing 7 to 9 readings for each experimental condition. For propidium iodide (PI) uptake, cultures were incubated with PI solution at 5 μg/mL in phosphate-buffered saline (PBS) for 5 minutes. Cells were then photomicrographed by epifluorescence microscopy. The cultured neurons were then fixed with 4% paraformaldehyde, washed with PBS, and stained with Hoechst to count the total number of neurons. Cell death rate was calculated by dividing the number of PI-positive cells by the total numbers of cells.

**Mouse Model of Transient Focal Cerebral Ischemia**

Focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) in mice as described previously with slight modification. In brief, male 10- to 12-week-old C57BL/6j mice (Jackson Laboratory, Bar Harbor, Me) were anesthetized with 1.5% isoflurane in a 30% O₂-70% N₂O mixture through a face mask under spontaneous breathing. Rectal temperature was maintained at 37.0±0.5°C during and shortly after surgery with a temperature-regulated heating pad. Mean arterial blood pressure was monitored during MCAO through a tail cuff (ADInstruments). The animals underwent left MCAO for 60 minutes and then reperfusion for the indicated duration. Changes in regional cerebral blood flow (rCBF) before, during, and after MCAO were evaluated in mice by laser-Doppler flowmetry.

**Administration of Leptin and PD98059 in Mice**

Stock solutions of recombinant mouse leptin were prepared as described earlier. The amounts of leptin needed were then prepared by diluting the stock solution with PBS, bringing the total volume to 0.5 mL. Leptin solution was then injected intraperitoneally at the indicated time points. The vehicle-treated mice were injected intraperitoneally with 0.5 mL of saline. Stock solution of PD98059 in dimethyl sulfoxide was diluted with PBS to 0.2 mmol/L. With use of a stereotoxic device (Kopf), 2 μL of PD98059 solution or 2 μL of dimethyl sulfoxide/PBS was injected into the intracerebral ventricle of mice 20 minutes before MCAO.

**Measurement of Infarct Volume, Body Weight, and Neurologic Deficit**

At 48 hours after MCAO, brains were removed and sliced into 7 coronal sections 1 mm thick. Sections were immediately stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC). For detecting infarct areas 7 days after MCAO, brains were removed and freshly frozen in isopentane cooled with dry ice, and serial sections from anterior to posterior were selected for hematoxylin & eosin staining. Infarct size was measured with the MCID image analysis system (Imaging Research Inc). The body weight of each mouse for these purposes was measured before surgery, as well as on days 1, 3, and 7 after MCAO. Neurologic deficits were scored on a 0 to 4 scale.

**Western Blot Analysis**

Western blotting was performed with standard methods as previously described. Cultured neurons or mouse cortical tissues from MCA regions were collected at the indicated time points after ischemia (n=4 per condition). The cultures were pretreated with leptin for 0 to 3 hours at a concentration of 50 μg/mL. In mice, 4 mg/kg IP leptin was injected 5 minutes before reperfusion. The proteins were extracted, and protein samples were subjected to Western blot analysis. Blots were probed with antibodies recognizing the leptin receptor (Santa Cruz Biotechnology); ERK 1/2, phospho (p)-ERK 1/2 at Thr202/Tyr204; Akt, p-Akt at Ser473; STAT3, p-STAT3 at Tyr705; cAMP-responsive element binding protein (CREB; Cell Signaling Technology, Danvers, Mass); and p-CREB at Ser133 (Upstate, Lake Placid, NY). Gel analysis was accomplished with the assistance of MCID.

**Immunofluorescence Staining**

Animals were injected with 4 mg/kg IP leptin 5 minutes before withdrawal of the suture. At the indicated time points after ischemia (n=3 per condition), brains were freshly frozen in cooled isopentane and then sectioned. The procedures for immunohistochemistry were the same as described previously. Rabbit polyclonal anti–p-ERK 1/2 (1:200, Cell Signaling Technology) or mouse monoclonal anti–NeuN antibody (1:500, Chemicon) was used as the primary antibody. For double-label immunofluorescence staining, sections were first incubated with the anti–p-ERK antibody at 4°C overnight, followed by incubation for 1 hour at room temperature with goat anti-rabbit Cy3 immunonjugate at 1:1000 dilution. Sections were then incubated for 1 hour with the mouse anti–NeuN antibody. This was followed by incubation with goat anti-rabbit Alexa Fluor 488 immunonjugate. Hoechst stain was also used for counterstaining.

**Data Analysis**

Data are presented as mean±SEM. Comparisons were made by ANOVA and post hoc Scheffe’s tests. A level of P<0.05 was considered statistically significant.

**Results**

The Leptin Receptor Is Expressed in Rat Cortical Neurons and in a Variety of Regions in the Mouse Brain

Previous studies have indicated that the intracellular effects of leptin are mediated by the leptin receptor. We first confirmed expression of the leptin receptor in rat cortical neurons and different regions of the mouse brain. As shown in Figure 1A, both the long and short isoforms of the leptin receptor were constitutively expressed in rat cortical neurons. Both isoforms were also readily detected in the hypothalamus, cortex, striatum, hippocampus, and cerebellum of the mouse brain, with the hypothalamus demonstrating the greatest levels and the cerebellum the least.

**Leptin Protects Primary Cortical Neurons From Cell Death Induced by OGD**

Neuronal cultures were incubated with leptin at 0, 10, 50, or 100 μg/mL for 3 hours, followed by 90 minutes of OGD. Alamar Blue reduction was performed 24 hours later to measure cell viability. As shown in Figure 1B, no obvious neuroprotection was observed when leptin was used at a concentration of 10 μg/mL; however, when the concentration
of leptin was increased to 50 or 100 μg/mL, significant neuroprotection was achieved. The ability of leptin to protect against OGD-induced neuronal death was confirmed by PI staining (Figures 1C and 1D) and lactate dehydrogenase release (Figure 2C). Taken together, these results demonstrate that leptin protected cultured rat cortical neurons from OGD-induced cell death in a concentration-dependent manner.

**The ERK 1/2 Signaling Pathway Plays a Critical Role in Leptin-Mediated Neuroprotection In Vitro**

To elucidate the neuroprotective mechanisms of leptin, neuronal cultures were incubated with leptin at the protective concentration of 50 μg/mL for 0, 1, or 3 hours, followed by protein extraction and Western blot analysis. ERK was phosphorylated at very low levels under control conditions. After leptin treatment for 3 hours, the level of p-ERK was increased by a factor of 12, as shown in Figure 2A. The levels of p-STAT3 were also increased significantly 3 hours after leptin treatment (Figure 2F). The levels of p-Akt, however, were not significantly increased (Figure 2E), indicating that ERK 1/2 and STAT3, rather than Akt, may play important roles in leptin-mediated neuroprotection against OGD-induced neuronal death.

To verify the role of ERK, we performed an ERK inhibition study with PD98059 and U0126. The neurons were first incubated with PD98059 at 10 μmol/L for 30 minutes; leptin was then added at a concentration of 50 μg/mL. The neurons were then incubated for another 3 hours. As shown in Figure 2A, administration of PD98059 blocked the increase in p-ERK levels in neurons stimulated by leptin. Treatment of neurons with PD98059 compromised the neuroprotective effect of leptin, as assessed by Alamar Blue reduction (Figure 2B), although PD98059 itself did not affect cell viability. The inhibiting effect of PD98059 on leptin-mediated neuroprotection was also confirmed by PI staining (Figures 1C and 1D) and by lactate dehydrogenase release (Figure 2C). Similar inhibition was observed when the cultures were pretreated with U0126 (data not shown).

One route by which ERK 1/2 can promote neuronal survival is to phosphorylate and activate the transcription factor CREB.14 We therefore measured the phosphorylation of CREB after leptin treatment. As shown in Figure 2D, the level of p-CREB was increased after 3 hours of leptin treatment, in parallel with the increase seen in p-ERK levels. Interestingly, the addition of PD98059 also blocked the increase in CREB phosphorylation, suggesting that
p-ERK was responsible for the phosphorylation of CREB after leptin treatment. Taken together, these findings suggest that the ERK 1/2 pathway plays a key role in leptin-mediated neuroprotection against OGD-induced neuronal death, whereas the STAT3 and CREB pathways may also be involved.

Leptin Decreases Infarct Volume Induced by Focal Cerebral Ischemia in Mice

We next determined whether leptin could be neuroprotective in a mouse model of MCAO. Vehicle or leptin (1 to 8 mg/kg IP) was injected 5 minutes before the onset of reperfusion. The infarct volume in the vehicle-treated control group was 32.4 ± 4.1 mm³ as measured with TTC staining (Figures 3A and 3C.) When leptin was injected at a dose of 1 mg/kg, no obvious reduction in infarct volume was observed; however, when the dose of leptin was increased to 2 mg/kg, the infarct volume was significantly smaller than in the vehicle-treated group. The maximal reduction in infarct volume was achieved when leptin was administered at a dose of 8 mg/kg, with an infarct volume of 15.8 ± 3.8 mm³, a 51% reduction compared with the vehicle-treated control group.

We then determined the protective time window of leptin. Leptin at a dose of 4 mg/kg IP was injected 30, 60, 90, or 120 minutes after the onset of reperfusion. As shown in Figure 3B, leptin protected the mouse brain from cerebral ischemia, even when leptin was administered 90 minutes after ischemia. However, when leptin was used 120 minutes after ischemia, the neuroprotection disappeared. These findings indicate that leptin is effective in reducing infarct...
volume in the mouse brain induced by MCAO in a dose- and time-dependent manner.

In another set of experiments, mice were allowed to survive for 7 days after MCAO to examine neurologic function, body weight changes, and the long-term neuroprotective effects of leptin. Figure 3D shows that leptin significantly reduced the neurologic deficit in mice 1, 3, and 7 days after ischemia. Leptin also improved the recovery of body weight at day 7 after MCAO (Figure 3E), although a single dose of leptin did not affect body weight in nonischemic mice (data not shown). After the final neurologic function tests and body weight measurements, the mice were humanely killed for hematoxylin & eosin staining and infarct volume measurements. Infarct volumes 7 days after ischemia were $32.9 \pm 4.4$ mm$^3$ in the vehicle-treated group and $20.6 \pm 3.7$ mm$^3$ in the leptin-treated group, a significant reduction (Figure 3F).

To rule out the possibility that leptin might have altered rCBF and therefore infarct volumes, we also monitored rCBF in both vehicle- and leptin-treated animals. As shown in Figure 3G, no difference in rCBF changes was detected between the 2 groups before, during, or after MCAO. Taken together, these findings suggested that leptin improves neurologic
Figure 4. In mice, leptin decreased infarct volume induced by MCAO via the ERK 1/2 signaling pathway. A, Representative Western blots and semiquantitative analyses of p- and t-ERK 1/2 levels in MCA cortical tissues at serial time points. **P<0.01, *P<0.05 vs vehicle-treated groups at the sham time points; #P<0.05 vs sham group. B, Representative Western blots and semiquantitative analyses of p- and t-ERK levels show that PD98059 blocked the phosphorylation of ERK enhanced by leptin. The mice were treated with PD98059 by intracerebral ventricle infusion, followed by MCAO and leptin treatment. **P<0.01, *P<0.05 vs leptin-treated groups at the same time points. C, PD98059 inhibited the neuroprotective effects of leptin. The mice were treated with PD98059, followed by MCAO and leptin treatment. TTC staining was performed 48 hours later. n=9, *P<0.05 vs vehicle-treated group; #P<0.05 vs vehicle-treated group but P<0.05 vs leptin-treated group. D, Representative Western blots and semiquantitative analyses of p- and t-CREB levels in MCA cortical tissues at serial time points. *P<0.05 vs sham group. S indicates sham group; V, vehicle-treated group; L, leptin-treated group. E, Representative Western blots and semiquantitative analyses of p- and t-Akt levels in MCA cortical tissues at serial time points. *P<0.05 vs sham group. S indicates sham group; V, vehicle-treated group; L, leptin-treated group. F, Representative Western blots and semiquantitative analyses of p- and t-STAT3 levels in MCA cortical tissues at serial time points. **P<0.01, *P<0.05 vs sham group. S indicates sham group; V, vehicle-treated group; L, leptin-treated group.
function and body weight recovery after MCAO and provides long-term neuroprotection against cerebral ischemia.

The ERK 1/2 Signaling Pathway Plays a Critical Role in Leptin-Mediated Neuroprotection In Vivo

To investigate the possible mechanisms responsible for leptin neuroprotection in vivo, mouse cortical tissues from MCA regions were harvested and analyzed by Western blotting. A short-term increase in the phosphorylation of ERK 1/2 was detected as early as 30 minutes after MCAO and reperfusion in vehicle-treated animals, in agreement with previous reports. Noticeably, an 8-fold increase in the p-ERK level was detected in the leptin-treated group, as shown in Figure 4A. The increases in p-ERK levels in the leptin-treated groups lasted for 4 hours after MCAO, compared with 30 minutes in the vehicle-treated groups, albeit at relatively lower levels. In contrast, p-Akt (Figure 4E) and p-STAT3 (Figure 4F) were detected in the vehicle-treated groups, albeit at relatively lower levels. In contrast, p-Akt (Figure 4E) and p-STAT3 (Figure 4F) were not significantly altered. To detect the cellular distribution of p-ERK in the cortex, we performed immunofluorescence staining. As shown in Figure 5A, p-ERK demonstrated neuronal expression.

To confirm the neuroprotective role of leptin-induced p-ERK activity against cerebral ischemia, we performed an ERK inhibition study with PD98059. The intracerebral ventricles of the mice were infused with PD98059 20 minutes before MCAO. Shortly after reperfusion, brain tissues were removed and analyzed by Western blotting or immunohistochemistry. Direct infusion of PD98059 into the intracerebral ventricles largely blocked the phosphorylation of ERK enhanced by leptin (Figures 4B and 5B). Infusion of PD98059 also blocked the neuroprotective effects of leptin by diminishing the leptin-induced reduction in infarct volume (Figure 4C). Consequently, administration of leptin in vivo also resulted in enhanced phosphorylation of CREB (Figure 4D).

Discussion

Our results demonstrate that leptin receptors are constitutively expressed in cultured rat cortical neurons and in several regions of the mouse brain, including the cortex and striatum. The administration of exogenous leptin attenuated OGD-induced neuronal death in vitro and decreased infarct volume induced by MCAO in mice. The ERK 1/2 signaling pathway plays a critical role in leptin-mediated neuroprotection, whereas the CREB and STAT3 signaling pathways may also be involved.

There is evidence that leptin is involved in opposing cell death mechanisms. The protective mechanisms of leptin may include STAT3, Akt, and ERK 1/2 signaling pathways, as the Janus kinase-2 inhibitor AG490, the phosphatidylinositol 3-kinase inhibitor LY294002, and the MEK inhibitors U0126 and PD98059 have all been demonstrated to abolish the antiapoptotic effects of leptin. Leptin can upregulate the expression of Bcl-2 and Bcl-xL and to delay the cleavage of caspases. Our findings support an important role for the ERK 1/2 pathway in leptin-mediated neuroprotection against ischemic neuronal injury.

The phosphorylation of ERK was increased quickly and shortly after ischemia and reperfusion. The short-term phosphorylation of ERK is also an important and essential component in inducing neuroprotection by other well-characterized mechanisms, as p-ERK1/2 levels are enhanced after a variety of protective agents, including brain-derived neurotrophic factor, erythropoietin, and ischemic tolerance. ERK was necessary for the neuroprotective effects of the aforementioned agents, because blocking the ERK signaling pathway via MEK1 inhibition abolished their neuroprotective effects. The neuroprotective mechanisms of ERK 1/2 may involve inhibition of cell death machinery as well as transcriptional regulation. ERK 1/2 phosphorylates a series of proapoptotic molecules, including Bcl-EL at Ser69, facilitating its degradation; and caspase-9 at Thr125, blocking its cleavage and activation. In addition, ERK 1/2 phosphorylates and activates a set of transcription factors, including CREB, which plays a prosurvival role. In contrast, the molecular mechanisms of ERK’s proapoptotic effects are still controversial.

In support of previous reports, p-STAT3 was barely detectable in the cortex of sham-operated mice but was significantly increased after MCAO. We detected slight enhancement of p-STAT3 4 hours after MCAO in the leptin-treated group compared with the vehicle-treated group; however, the dif-
ference was not statistically significant. This finding cannot rule out the possibility that STAT3 may contribute to leptin-mediated neuroprotection in vivo, as this could be caused by the predominant glial distribution of p-STAT3 in the mouse brain after MCAO.\textsuperscript{20,21} The high level of p-STAT3 in glia may mask the leptin-mediated increase of p-STAT3 in neurons. Further investigation is required to clarify the role of STAT3 in leptin-mediated neuroprotection against ischemic brain injury.

Leptin itself is an endogenous natural protein and may thus be safe for the treatment of human diseases. In fact, leptin has been successfully used to treat patients who were morbidly obese due to a homozygous leptin deficiency.\textsuperscript{22} In our study, leptin was effective in reducing experimental ischemic injury induced by MCAO in mice, even after a significant delay of 90 minutes, and this suggests that leptin may be useful in treating ischemic incidents in humans.

Acknowledgments

We thank Carol Culver for editorial assistance and Pat Strickler for secretarial support.

Sources of Funding

This project was supported by National Institutes of Health/National Institute of Neurological Disorders and Stroke grants NS43802, NS45048, and NS36736 (Dr Chen). Dr Chen was also supported in part by the Geriatric Research, VA Pittsburgh Health Care System, Pittsburgh, Pa.

Disclosures

None.

References

Neuroprotective Effects of Leptin Against Ischemic Injury Induced by Oxygen-Glucose Deprivation and Transient Cerebral Ischemia
Feng Zhang, Suping Wang, Armando P. Signore and Jun Chen

Stroke. 2007;38:2329-2336; originally published online June 28, 2007;
doi: 10.1161/STROKEAHA.107.482786
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/38/8/2329

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/