Transport across the Blood-Brain Barrier of Pluronic Leptin

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ABSTRACT

Leptin is a peptide hormone produced primarily by adipose tissue that acts as a major regulator of food intake and energy homeostasis. Impaired transport of leptin across the blood-brain barrier (BBB) contributes to leptin resistance, which is a cause of obesity. Leptin as a candidate for the treatment of this obesity is limited because of the short half-life in circulation and the decreased BBB transport that arises in obesity. Chemical modification of polypeptides with amphiphilic poly(ethylene oxide)-poly(propylene oxide) block copolymers (Pluronic) is a promising technology to improve efficiency of delivery of polypeptides to the brain. In the present study, we determined the effects of Pluronic P85 (P85) with intermediate hydrophilic-lipophilic balance conjugated with leptin via a degradable SS bond [leptin(ss)-P85] on food intake, clearance, stability, and BBB uptake. The leptin(ss)-P85 exhibited biological activity

when injected intracerebroventricularly after overnight food deprivation and ^{125}I -leptin(ss)-P85 was stable in blood, with a half-time clearance of 32.3 min (versus 5.46 min for leptin). ^{125}I -Leptin(ss)-P85 crossed the BBB [blood-to-brain unidirectional influx rate (K_i) = $0.272 \pm 0.037 \mu\text{L/g} \cdot \text{min}$] by a nonsaturable mechanism unrelated to the leptin transporter. Capillary depletion showed that most of the ^{125}I -leptin(ss)-P85 taken up by the brain reached the brain parenchyma. Food intake was reduced when 3 mg of leptin(ss)-P85 was administered via tail vein in normal body weight mice [0–30 min, $p < 0.0005$; 0–2 h, $p < 0.001$]. These studies show that the structure based Pluronic modification of leptin increased metabolic stability, reduced food intake, and allowed BBB penetration by a mechanism-independent BBB leptin transporter.

Leptin, a product of the obese gene, is a 16-kDa protein that is synthesized in adipose tissue and secreted into the bloodstream but acts within the brain to regulate adiposity (Zhang et al., 1994). Circulating levels of leptin are directly correlated with the degree of adiposity in rodents and humans (Frederich et al., 1995), and administration of leptin to rodents, either centrally or peripherally, reduces food intake and increases energy expenditure (Ahima et al., 2000). Leptin is transported across the BBB by a saturable, unidirectional system located at the endothelium of the vasculature

and the epithelium of the choroid plexus to interact with its receptors to control feeding, thermogenesis, and other functions (Hileman et al., 2002; Banks, 2006). Leptin transport across the BBB has been shown to be impaired in obesity (Banks et al., 1999a, 2002; Kastin et al., 1999; Burguera et al., 2000). This impaired transport accounts in part for the leptin-resistant state found in obesity (Caro et al., 1996; Friedman and Halaas, 1998). This peripheral leptin resistance could be overcome were the BBB made permeable to leptin (Banks and Lebel, 2002). Leptin, as a therapeutic agent, has poor clinical efficacy because of its short circulating half-life, low potency, and poor solubility (Vilà et al., 1998).

To improve the therapeutic profile of peptides, protein conjugation with Pluronic block copolymers that consist of ethylene oxide and propylene oxide is a promising approach (Batrakova et al., 2005). Polymer conjugations can be ob-

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ABBREVIATIONS: BBB, blood-brain barrier; P85, Pluronic P85; leptin(ss)-P85, Pluronic leptin; HRP, horseradish peroxidase; MTr, 4'-methoxytrityl; TFA, trifluoroacetic acid; rleptin, recombinant murine leptin; MALDI-TOF, matrix-assisted laser desorption/ionization/time of flight; PAGE, polyacrylamide gel electrophoresis; LR-BSA, lactated Ringer's solution with 1% bovine serum albumin; Expt, exposure times; %Inj/ml, percentage of an injected dose in each milliliter of serum; %Inj/g, percentage of an intravenous dose taken up per gram of brain; TCA, trichloroacetic acid; ANOVA, analysis of variance.

tained by the covalent conjugation of peptides to polymers, and they are used to increase peptide stability, increase serum half-life, decrease elimination rate, and reduce immunogenicity (Reddy, 2000). In this study, leptin is conjugated with poly(ethylene oxide)-poly(propylene oxide) block copolymer, Pluronic P85 (P85), to attain the optimal modification via biodegradable disulfide links. P85 was shown previously to improve brain delivery of horseradish peroxidase (HRP). In particular, HRP modified with P85 enhances the transport of this protein across the BBB in vitro and in vivo compared with native HRP (Batrakova et al., 2005; Yi et al., 2008). In addition, P85 formulation has been shown to enhance the delivery of digoxin into the brain (Batrakova et al., 2001b) and to improve the analgesic profile of buprenorphine, [D-Pen², D-Pen⁵]-enkephalin, and morphine (Witt et al., 2002). In our previous work, we reported that block copolymers can induce ATP depletion in bovine brain microvessel endothelial cells in vitro (Batrakova et al., 2001a, 2003). Because ATP depletion induced by free Pluronics at the doses that are much higher than those used in these experiments in vivo, this is relevant here in this study. Therefore, in the present study, we describe for the first time the development of conjugation of leptin with Pluronic block copolymer P85 via biodegradable linker. The Pluronic leptin was shown to have markedly increased stability and diminished proteolytic degradation, a longer in vivo circulating half-life, an ability to cross the BBB of CD-1 mice, and biological activity after injection into the brain or blood.

Materials and Methods

Monoamine Pluronic P85. Monoamine P85 was prepared using a method described previously (Yi et al., 2008). In brief, 3 g of P85 (mol. wt., 4600) was incubated with 4'-methoxytrityl (MTr) chloride (200 mg, 1:1 M ratio) in 25 ml of anhydrous pyridine. Mono-MTr-P85 was eluted in a Silicagel column (3 × 20 cm) (Sigma-Aldrich, St. Louis, MO) using stepwise gradient elution of 200 ml of dichloromethane containing 2, 5, and 10% methanol. The collected mono-MTr-P85 was reacted with 5-fold molar excess of 1,1'-carbonyldiimidazole (530 mg) in 10 ml of anhydrous acetonitrile, followed by incubation with an excess of ethylenediamine in 20 ml of ethanol upon stirring. The mixture was concentrated in vacuo and redissolved in 50 ml of 2% trifluoroacetic acid (TFA) in dichloromethane. The resulting monoamine P85 was isolated by gel filtration on a Sephadex LH-20 column (2.5 × 30 cm) (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and analyzed by thin-layer chromatography in dichloromethane/methanol (9:1). The monoamine P85 develops blue color after spraying with 1% ninhydrin solution in ethanol (a test for an amino group). Quantification of the modification degree (number of amino groups per copolymer molecule) was conducted using ninhydrin assay as described previously (Yi et al., 2008).

Synthesis of Leptin(ss)-P85. Monoamine P85 (9.3 mg) was activated by dithiobis(succinimidyl-propionate) (4.9 mg; 6-fold molar excess) in 0.5 ml of dimethylformamide and supplemented with 0.1 ml of sodium borate buffer (0.1 M; pH 8.0). After incubating for 20 min at 25°C, the reaction solution was eluted from Illustra NAP-25 columns (GE Healthcare) in 20% aqueous ethanol. The fraction containing activated polymer was immediately mixed with 2 mg of recombinant murine leptin (rleptin: R&D Systems, Minneapolis, MN) in 1.5 ml of 20% aqueous ethanol, and the pH of the reaction solution was kept at 7 to 8 by adding 0.2 ml of sodium borate (0.1 M; pH 8.0). The homogeneous reaction mixture was incubated overnight at 4°C and then purified by precipitating in acetone (25 ml) for 20 min at -80°C to remove excess of nonreacted copolymers. The white

precipitates containing leptin conjugates were collected at 3000 rpm for 10 min at 4°C and then redissolved in water followed by filtering using Amicon ultra-15 centrifuge filter (molecular mass cut-off, 10 kDa; Millipore, Billerica, MA) at 11,000 rpm at 4°C for 10 min. The final product was filtered-sterilized with a 0.2-μm filter and lyophilized for storage.

Leptin(ss)-P85 Characterization. Leptin(ss)-P85 was analyzed by matrix-assisted laser desorption/ionization/time of flight (MALDI-TOF) mass spectrometry in a 4800 MALDI analyzer (Applied Biosystems, Foster City, CA), with a laser power of 3000 V, in positive reflector mode. Samples were prepared by 1) plate coating with 0.5 μl of saturated sinapinic acid in 50% acetonitrile with 0.1% TFA, 2) depositing 0.5 μl of solution of salt-free leptin(ss)-P85 in water (10⁻⁴ M), and 3) then coating with 0.5 μl of saturated sinapinic acid in 50% acetonitrile with 0.1% TFA. The mass spectra were obtained in linear mode with the instrument calibrated externally using proteins, such as insulin (5729.61 Da) and albumin (66429.09 Da). In addition, a regular SDS-PAGE (15%) procedure was applied to determine the modification of leptin(ss)-P85. A 10-μg sample was prepared using denaturing loading buffer with or without reducing reagent and loaded to the well. After running, the gel was fixed in 50% methanol/10% acetic acid, stained in SYPRO Ruby solution (Bio-Rad Laboratories, Hercules, CA), and then scanned on a Typhoon gel scanner (GE Healthcare).

Mice. CD-1 male mice from an in house colony (VA-New Orleans), ~8 to 10 weeks of age, served as test subjects in experiments 1 to 3. This colony has been maintained as an outbred strain obtained from Charles River Laboratories, Inc. (Wilmington, MA). The mice are tested regularly to ensure that they are virus- and pathogen-free. All subjects were experimentally naive. Mice were on a 12:12-h light/dark cycle, with lights on at 6:00 AM. Water and food (PMI Nutrition LabDiet 5001; PMI Nutrition, St. Louis, MO) were available ad libitum. All experiments were conducted after institutional approval of the animal use subcommittee, which subscribes to the National Institutes of Health *Guide for Care and Use of Laboratory Animals*.

Intracerebroventricular Surgery and Drug Administration. Forty-eight hours before the induction of food, mice were anesthetized with tribromoethylene and placed in a stereotaxic instrument. The scalp was then deflected and a hole was drilled through the skull over the injection site. The injection coordinates for the intracerebroventricular injections were 0.5 mm posterior to the bregma and 1.0 mm to the right and left of the sagittal suture. The injection depth was 2.0 mm. Fifteen minutes before the induction of food, mice were lightly anesthetized with isoflurane, and a 0.5-ml solution of saline or drug [leptin(ss)-P85] was injected into each injection site over 60 s through a 30-gauge needle. After intracerebroventricular injection, the scalp was closed, and the mice were returned to their cages. For peripheral administration, 15 min before the induction of food, mice were given a 0.1-ml solution of drug or vehicle via tail vein (see experiment 3). Mice were then returned to their home cages.

Intracerebroventricular Experimental Design. In experiments 1 and 2, mice were food-deprived for 18 h before the start of the experiment. Mice were lightly anesthetized, and an injection of leptin(ss)-P85 (experiment 1) or rleptin (experiment 2; 0, 0.5, 2.0, or 4.0 μg) was given intracerebroventricularly. The mice were then returned to their home cages. Fifteen minutes after injection, a food pellet of known weight was placed on the bottom of the cage. Food was weighed at 2, 4, and 24 h after induction of food. At the 4-h mark, the pellet was not returned to the cage, and a small amount of food of a known weight was placed in the food hopper. Experiment 3 was conducted exactly like experiments 1 and 2 except that the mice were not anesthetized while given their tail-vein injection. All other parameters were the same.

Radioactive Labeling. Leptin(ss)-P85 conjugate was radioactively labeled with ¹²⁵I (PerkinElmer Life and Analytical Sciences, Boston, MA) by the chloramine-T method (i.e., excluding the sodium metabisulfite treatment; Banks et al., 1999b). In brief, 2 mCi of ¹²⁵I,

10 µg of chloramine-T, and 20 µg of protein were incubated together for 60 s. Radiolabeled material [^{125}I -leptin(ss)-P85] was purified by elution on a Sephadex G-10 column (Sigma-Aldrich).

The rleptin was radioiodinated with ^{131}I by the lactoperoxidase method as described previously (Blasberg et al., 1983b). In brief, 10 µg of rleptin was mixed with 30 µl of 0.4 M sodium acetate, pH 5.6, 5 µl of lactoperoxidase (10 µg/ml), and 2 mCi of ^{131}I . The reaction was started by adding 0.02 ng of H_2O_2 in a volume of 2.5 µl. Ten minutes later, an additional 0.02 ng of H_2O_2 was added. At the end of this second 10-min period, the iodinated rleptin (^{131}I -rleptin) was separated from unincorporated iodine on a column of Sephadex G-10.

Multiple-Time Regression Analysis of Influx into the Brain. Multiple-time regression analysis was applied as detailed previously (Blasberg et al., 1983a; Patlak et al., 1983) to calculate the blood-to-brain unidirectional influx rate (K_i). Male CD-1 mice (8–10 weeks old) were anesthetized with an intraperitoneal injection of 0.2 ml of urethane (4.0 g/kg). The jugular vein and right carotid artery were exposed. Mice were given an injection into the jugular vein of 0.2 ml of lactated Ringer's solution with 1% BSA (LR-BSA) containing 300,000 cpm of ^{125}I -leptin(ss)-P85. Blood from the carotid artery was collected between 2 to 60 min after intravenous injection, and then the mice were immediately decapitated and the whole brain was removed and weighed. The arterial blood was centrifuged at 5400g for 10 min at 4°C, and the serum was collected. The levels of radioactivity in serum (50 µl) and brain samples were counted in a gamma counter for 3 min. The brain/serum ratios (microliters per gram) of ^{125}I -leptin(ss)-P85 in each gram of brain was calculated separately and were plotted against their respective exposure times (Expt). Exposure time was calculated from the following formula:

$$\text{Am/Cpt} = K_i \left[\int_0^t \text{Cp}(t) dt \right] / \text{Cp}(t) + V_i \quad (1)$$

where Am is cpm/g of brain, Cpt is cpm per microliter of arterial serum at time t , and Expt (minutes) is measured by the term

$$\left[\int_0^t \text{Cp}(t) dt \right] / \text{Cp}(t) \quad (2)$$

The linear portion of the relation between the brain/serum ratios versus Expt was used to calculate K_i (microliters per gram · minute) and initial volume of distribution for brain (V_i ; microliters per gram). The slope of the linearity measures K_i and is reported with its error term. The y -intercept of the linearity measures V_i , the initial volume of distribution in brain at $t = 0$.

To determine whether brain uptake of ^{125}I -leptin(ss)-P85 was saturable, either 1 µg/mouse nonradioactive Pluronic-modified, leptin(ss)-P85 (self-inhibition was tested) or nonradioactive recombinant leptin (1 µg/mouse) was included in the intravenous injection of some mice. Blood was collected from the right carotid artery, and the whole brain was removed, weighed at 10 min after intravenous injection, and processed. Results were expressed as brain/serum ratios.

Clearance of ^{125}I -Leptin(ss)-P85 from Serum. To determine the rate of clearance of ^{125}I -leptin(ss)-P85 from the serum, results are expressed as the percentage of the injected dose in each milliliter of serum (%Inj/ml), and these values are plotted against time (minutes). The %Inj/ml was determined by the equation

$$\% \text{Inj/ml} = 100(\text{CPM/ml serum}) / (\text{mean CPM/injection}) \quad (3)$$

The percentage of the injected dose entering each gram of brain tissue (%Inj/g) was calculated at each time from the equation

$$\% \text{Inj/g} = 100(\text{Am/Cpt} - V_i) \text{Cpt/Inj} \quad (4)$$

where Inj is the dose of ^{125}I -leptin(ss)-P85 injected intravenously. Subtracting V_i , here assumed to be 10 µl/g, from the brain/serum ratio corrects for ^{125}I -leptin(ss)-P85 in the vascular space of the brain. Thus, the quantities expressed more nearly represent only the ^{125}I -leptin(ss)-P85 that has entered the brain tissue. The values for %Inj/g were plotted against time (minutes).

Stability and Presence of Intact ^{125}I -Leptin(ss)-P85 in Blood and Brain. Mice were anesthetized with an intraperitoneal injection of 0.2 ml of urethane (40%), and the left jugular vein and right carotid artery were exposed. The mice were given an injection into the left jugular vein of 0.2 ml of LR-BSA containing 500,000 cpm of ^{125}I -leptin(ss)-P85. For acid precipitation, arterial blood and brain were collected at 10 min and 6, 18, or 24 h after intravenous injection. The whole blood was centrifuged at 5400g for 10 min at 4°C. The resulting serum (50 µl) was added to 500 µl of 1% bovine serum albumin in 0.25 M sodium phosphate buffer and then precipitated with 500 µl of 30% trichloroacetic acid (TCA). It was centrifuged at 5400g for 10 min at 4°C, and the resulting supernatant and pellet were counted in a gamma counter. The whole brain was homogenized in 3 ml of LR-BSA for 10 strokes, and 2 ml of brain homogenate was centrifuged at 5400g for 10 min at 4°C. Brain supernatant (1500 µl) was transferred to a new tube, and 1500 µl of 30% TCA was added. This solution was centrifuged at 5400g for 10 min, and the supernatant and the pellet were separated. The levels of radioactivity in acidified serum and brain supernatants and pellets were counted in a gamma counter for 3 min. To determine degradation of ^{125}I -leptin(ss)-P85 that occurred ex vivo, we performed processing controls. In brief, 100 µl of ^{125}I -leptin(ss)-P85 in LR-BSA solution was placed on the surface of a nonradioactive mouse brain or in a tube used to obtain carotid blood, and the samples were processed as described above. The percentage of radioactivity precipitated by acid in serum and brain was calculated by the following formula:

$$100[(\text{pellet cpm}) / (\text{pellet cpm} + \text{supernatant cpm})] \quad (5)$$

Correction for ex vivo degradation was made by dividing the values for the biological samples by the values for processing controls.

To further characterize the radioactivity in blood and brain, a male CD-1 mouse was given an injection into the jugular vein of 0.2 ml of 1% LR-BSA containing ^{125}I -leptin(ss)-P85. Blood from the carotid artery and brain were collected at 10 min after intravenous injection. The arterial blood was centrifuged at 5400g for 10 min at 4°C, and the serum was collected. The brain sample was processed with 0.25 M phosphate-buffered saline for homogenization on ice. The supernatant of the brain homogenate was obtained by centrifugation at 9000g for 15 min at 4°C. The supernatant was concentrated by a speed vacuum system. The supernatant and serum samples were heated at 60°C to denature proteins before 12% Bis-Tris NuPAGE electrophoresis (Invitrogen, Carlsbad, CA) was performed. The gel was dried and exposed to BioMax film (Eastman Kodak, Rochester, NY) overnight. The results were replicated three times.

Octanol/Buffer Partition Coefficient. Lipid solubility of leptin(ss)-P85 was measured by adding 1×10^5 cpm of ^{125}I -leptin(ss)-P85 to duplicate tubes each containing 0.5 ml of 0.25 M chloride-free sodium phosphate buffer, pH 7.5, and 0.5 ml of octanol. This solution was vigorously mixed for 1 min and then centrifuged at 5400g for 10 min to separate the two phases. Aliquots of 100 µl were taken in triplicate from each phase, and total radioactivity was counted in a gamma counter. The mean partition coefficient was expressed as the ratio of cpm in the octanol phase to the cpm in the buffer phase as a measurement of lipophilicity.

Capillary Depletion with Vascular Washout. The capillary depletion method (Triguero et al., 1990) as modified for mice (Gutiérrez et al., 1993) was performed to determine whether the ^{125}I -leptin(ss)-P85 completely crossed the capillary wall of the BBB to enter the brain parenchyma. Anesthetized mice received an intravenous injection of 0.8 ml of LR-BSA and 1×10^6 cpm of ^{125}I -leptin(ss)-P85. To remove ^{125}I -leptin(ss)-P85 in the vascular space or loosely adhering to the luminal side of capillaries of the brain microvasculature, the vascular space was washed out as follows. At 10 min, the abdomen was opened, and arterial blood was collected from the abdominal aorta. The thorax was then opened to expose the heart. The descending aorta was clamped, both jugular veins were severed, and 20 ml of LR-BSA was perfused over 1 min into the left ventricle

of the heart. Then, the mouse was decapitated, and the whole brain was removed, weighed, and placed in an ice-cold glass homogenizer. The brain was homogenized (10 strokes) in 0.8 ml of physiological buffer (10 mM HEPES, 141 mM NaCl, 4 mM KCl, 2.8 mM CaCl₂, 1 mM MgSO₄, 1 mM NaH₂PO₄, and 10 mM D-glucose, pH 7.4). Dextran solution (1.6 ml of a 26% solution) was added to the homogenate, mixed, and homogenized a second time (three strokes). The homogenate was centrifuged at 5400g for 15 min at 4°C. The resulting supernatant (brain parenchymal fraction) and pellet (capillary fraction) were separated, and the level of radioactivity for each fraction was determined using a gamma counter.

The parenchyma/serum and capillary/serum ratios (microliters per gram) were calculated by the equation

$$\text{Ratio} = (\text{cpm/g of tissue})/(\text{cpm}/\mu\text{l of serum}) \quad (6)$$

Brain Perfusion in a Blood-Free Buffer. Brain uptake of ¹²⁵I-leptin(ss)-P85 was determined by the brain perfusion method of Banks et al. (1999a). Mice were anesthetized with an intraperitoneal injection of 0.2 ml of urethane (40%). ¹²⁵I-Leptin(ss)-P85 was diluted in Zlokovic's buffer at pH 7.4 (7.19 g/l NaCl, 0.3 g/l KCl, 0.28 g/l CaCl₂, 2.1 g/l NaHCO₃, 0.16 g/l KH₂PO₄, 0.17 g/l anhydrous MgCl₂, 0.99 g/l D-glucose, and 10 g/l bovine serum albumin added on the day of perfusion). The thorax was opened, the heart was exposed, and the descending thoracic aorta was clamped. Both jugular veins were severed. A 26-gauge butterfly needle was inserted into the left ventricle of the heart, and the buffer containing ¹²⁵I-leptin(ss)-P85 (100,000 cpm/ml) was infused at a rate of 2 ml/min. Perfusion was stopped after 1 to 10 min for each mouse, followed by decapitation ($n = 3/\text{time point}$). The brain was collected, weighed, and counted in a gamma counter for 3 min. The brain/perfusion ratio (microliters per gram) was calculated by the following formula:

$$\text{Brain/perfusion ratio} = (\text{cpm/g of brain})/(\text{cpm}/\mu\text{l of perfusion}) \quad (7)$$

After the brain/perfusion ratio of ¹²⁵I-leptin(ss)-P85 was plotted against time (minutes), the slope of the regression line represented the influx rate (K_i).

To test inhibition, three groups of mice were studied by brain perfusion: 1) mice perfused with only ¹²⁵I-leptin(ss)-P85, 2) mice perfused with ¹²⁵I-leptin(ss)-P85 + nonradioactive rleptin at 100 ng/ml, and 3) mice perfused with ¹²⁵I-leptin(ss)-P85 + nonradioac-

tive leptin(ss)-P85 at 100 ng/ml ($n = 5$ mice/group). The brain/perfusion ratio (microliters per gram) of radioactivity was calculated.

Tissue Distribution of ¹²⁵I-Leptin(ss)-P85. Mice were anesthetized with an intraperitoneal injection of 0.2 ml of urethane (4.0 g/kg). The jugular vein and right carotid artery were exposed. In total, 0.2 ml of LR-BSA containing 300,000 cpm of ¹²⁵I-leptin(ss)-P85 was injected into the left jugular vein. Blood from the carotid artery was collected at 5, 10, 15, 30, 45, and 60 min after intravenous injection; the mice were immediately decapitated; and liver, kidney, lung, heart, stomach, and thigh muscle were removed and weighed. The whole blood was centrifuged at 5400g for 10 min at 4°C. The level of radioactivity was measured in serum (50 μ l) and each tissue sample by counting in a gamma counter. The tissue/serum ratios (microliters per gram) for all samples were calculated.

Statistics. Statistical analysis was performed with the use of Prism 5.0 (GraphPad Software Inc., San Diego, CA). Regression lines were calculated by the least-squares method and are reported with their correlation coefficient, r , n , and p values. Regression lines were compared statistically with Prism 5.0. One-way analyses of variance (ANOVA) with Newman-Keuls multiple comparison test and Dunnett's multiple comparison test were calculated by using Prism 5.0. Means are reported with their standard error terms and n values. Two means were compared by t test analysis.

Results

Preparation of Leptin(ss)-P85. P85 has a triblock structure, poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide), with average molecular weight of 4600 (Fig. 1A). We selected this molecule for modification of leptin based on the previous work suggesting that such modification of HRP results in increased delivery of this polypeptide to the brain (Batrakova et al., 2005). At the first stage, P85 was converted to a monoamine derivative using the chemistry described under *Materials and Methods*, with approximately 70 to 80% yield by weight. The resulting reagent contained 0.74 primary amino groups per copolymer molecule as determined by ninhydrin assay. At the second stage, monoamine P85 was linked to leptin through a disulfide bond, which is stable in extracellular environments but degradable in reducing environments, such as intracellular glutathione (Colcher et al.,

Fig. 1. A, structure of Pluronic P85. B, conjugation of monoamine Pluronic P85 to leptin through a biodegradable linker.

Fig. 2. Mass spectra of leptin and leptin(ss)-P85 conjugates obtained by MALDI-TOF. Leptin or leptin(ss)-P85 was prepared in water or treated with 25 mM dithiothreitol (DTT) for 10 min at 100°C. The sample was then deposited on the plate using sinapinic acid matrix as described under *Materials and Methods*. The monomer form of leptin has a molecular mass of 16 kDa, whereas leptin(ss)-P85 conjugates include nonmodified leptin and leptin that was attached with 1 up to 3 P85 chains (peak corresponding to 21, 26, and 31 kDa). Only leptin was detected for leptin(ss)-P85 after incubating with DTT.

1998; Fig. 1B). The conjugation reaction was conducted in borate buffer, pH 8.0. The excess of free P85 was removed by precipitating the protein in acetone at -80°C . The resulting modified protein sample was further analyzed by MALDI-TOF mass spectrometry, which suggested that it contained a mixture of nonmodified leptin and leptin(ss)-P85 modified with from 1 to 5 P85 chains through a degradable disulfide linker (Fig. 2). The modified leptin (Fig. 3, lane B) showed a smeared band in SDS-PAGE, whereas the unmodified leptin (Fig. 3, lane A) or its nonconjugated mixture with P85 (Fig. 3, lane C) did not. In addition, only leptin was detected (Fig. 3, lane E) after incubating leptin(ss)-P85 with DTT (25 mM), which further indicated that the attached P85 chain in the conjugate was cleavable upon reducing environment (Fig. 3). Altogether, the MALDI-TOF mass spectrometry and SDS-PAGE suggest protein modification with P85 via cleavable bond, although mixture of unmodified leptin and leptin(ss)-P85 with various degrees of modification was obtained.

Effects of Rleptin and Leptin(ss)-P85 Intracerebroventricular on Food Intake in CD-1 Male Mice. The one-way ANOVA for food intake during hours 2 to 4 after induction of food was significant for leptin(ss)-P85 [$F(3,24) =$

4.754; $p < 0.01$] but not for rleptin (Fig. 4, A and B). Dunnett's multiple comparison test indicated that the mice that received 4.0 μg of leptin(ss)-P85 ate significantly less than the mice that received vehicle. The ANOVA for food intake during hours 4 to 24 after induction of food was significant for leptin(ss)-P85 [$F(3,24) = 5.469$, $P < 0.005$] and rleptin [$F(2,23) = 4.197$, $P < 0.05$]. Dunnett's multiple comparison test indicated that the mice that receive 2.0 and 4.0 μg of leptin(ss)-P85 or 0.5 μg of rleptin ate significantly less than the mice that received vehicle (Fig. 4, C and D). The ANOVA for cumulative food intake for the 24-h period was statistically significant for leptin(ss)-P85 [$F(3,24) = 6.169$; $p < 0.005$] and for rleptin [$F(3,23) = 3.707$; $p < 0.05$]. Dunnett's multiple comparison test indicated that the mice that received 4.0 μg of leptin(ss)-P85 or 0.5 μg of rleptin ate significantly less than the mice that received vehicle (Fig. 4, E and F).

Brain Uptake (Influx) of ^{125}I -Leptin(ss)-P85 after Intravenous Injection. The blood-to-brain unidirectional influx rate of ^{125}I -leptin(ss)-P85 was linear during the study period (2–60 min after intravenous injection in mice). As shown Fig. 5, the influx rate of ^{125}I -leptin(ss)-P85 into brain was $0.272 \pm 0.037 \mu\text{L/g} \cdot \text{min}$ ($r = 0.92$, $p < 0.0001$; $n = 2$ mice/time point). The initial volume of distribution in brain was $19.21 \pm 1.69 \mu\text{L/g}$.

To determine the mechanism of brain uptake, we injected ^{125}I -leptin(ss)-P85 with or without nonradioactive leptin(ss)-P85 at 1 $\mu\text{g}/\text{mouse}$. Inclusion of nonradioactive leptin(ss)-P85 at 1 $\mu\text{g}/\text{mouse}$ 10 min after intravenous injection did not significantly reduce the brain/serum ratio of ^{125}I -leptin(ss)-P85 (radioactive only; Fig. 5, inset). Recombinant leptin, 1 $\mu\text{g}/\text{mouse}$, also did not affect the brain/serum ratio of ^{125}I -leptin(ss)-P85, which was $21.34 \pm 0.82 \mu\text{L/g}$ without excess leptin and $19.26 \pm 0.64 \mu\text{L/g}$ in the presence of excess leptin 10 min after intravenous injections (Fig. 6A). However, ^{131}I -rleptin that was coinjected with ^{125}I -leptin(ss)-P85 was inhibited by the unlabeled rleptin. In particular, for ^{131}I -rleptin with nonradioactive rleptin, the brain/serum ratio was $14.27 \pm 0.5 \mu\text{L/g}$; and for ^{131}I -rleptin and without nonradio-

Fig. 3. SDS-PAGE electrophoresis of leptin(ss)-P85 conjugates. Lane A, leptin alone; lane B, P85(ss)-leptin; lane C, mixture of leptin and Pluronic P85 (4:1 by weight); lane D, leptin + dithiothreitol (DTT); and lane E, leptin(ss)-P85 + DTT. Ten micrograms of each sample was loaded to the well. The samples in lanes A, B, and C were prepared using sample loading buffer without reducing reagent, whereas samples in lanes D and E were incubated with DTT (25 mM) and heated for 10 min. After running, the gel was fixed in 50% MeOH/10% acetic acid for 15 min, stained overnight with SYPRO Ruby protein gel stain, and imaged on a Typhoon gel scanner.

Fig. 4. Comparison of rleptin and leptin(ss)-P85 in reduction of food intake after their intracerebroventricular injections. A, for the first 4 h after injection, there were no differences among the groups that received rleptin. B, mice that received leptin(ss)-P85 4.0 µg i.c.v. ate significantly less during the time period of 2 to 4 h after induction of food. C and D, mice that received rleptin at 0.5 µg i.c.v. ($p < 0.01$; C) and leptin(ss)-P85 2.0 at 2.0 and 4.0 µg i.c.v. ($p < 0.05$ and $p < 0.01$, respectively; D) ate significantly less than the vehicle controls during hours 4 to 24 after induction of food. E and F, mice that received rleptin at 0.5 µg i.c.v. ($p < 0.05$; E) or leptin(ss)-P85 at 4.0 µg i.c.v. ($p < 0.01$; F) ate significantly less than the mice that received vehicle during the 24 h after induction of food ($n = 7$).

active rleptin, the brain/serum ratio was 22.25 ± 1.82 µl/g 10 min after intravenous injections ($p < 0.0005$; $n = 10$ mice/group; Fig. 6B).

Clearance from Serum. Figure 7A shows the clearance of 125 I-leptin(ss)-P85 from the serum for the first 60 min after injection into the jugular vein. The early phase of clearance from the serum for 125 I-leptin(ss)-P85 followed first-order kinetics. The inset shows the relation between the log of levels of radioactivity in arterial serum expressed as the %Inj/ml versus time after intravenous injection. Linear regression analysis showed a statistically significant relation between log (%Inj/ml) and time t ($r = 0.88$, $p < 0.0001$; $n = 2$ mice/time point; Fig. 7A, inset). The half-time disappearance rate from serum was calculated from the inverse of the slope of this relation to be 32.3 min.

The percentage of the intravenous injected dose of 125 I-leptin(ss)-P85 taken up per gram of brain is shown in Fig. 7B. The results were fitted to a one-site binding (hyperbola) model and indicate that the maximal value for %Inj/g approached 0.263 (Fig. 7B).

Stability of 125 I-Leptin(ss)-P85. To determine the in vivo stability of 125 I-leptin(ss)-P85 in serum and brain, the method of acid precipitation was used. Acid precipitation of radioactivity recovered from serum averaged 101% intact at 10 min, 92% intact at 6 h, 92% intact at 18 h, and 87% intact at 24 h. In brain, 98% was intact at 10 min, 67% at 6 h, 75% at 18 h, and 69% at 24 h (Table 1).

The radioactivity of the starting sample and samples extracted from brain and serum 10 min after intravenous injection of 125 I-leptin(ss)-P85 was shown in the NuPAGE

Fig. 5. Multiple-time regression analysis of ^{125}I -leptin(ss)-P85 transport across the BBB. The unidirectional influx rate, K_i (slope), was $0.272 \pm 0.037 \mu\text{l/g} \cdot \text{min}$. The V_i (intercept) was $19.21 \pm 1.69 \mu\text{l/g}$ ($r = 0.92$, $p < 0.0001$; $n = 2$ mice/time point). The brain/serum ratio of ^{125}I -leptin(ss)-P85 and ^{125}I -leptin-P85 + nonradioactive leptin(ss)-P85 10 min after intravenous injection is shown in the inset (means $\pm$ S.E.).

Fig. 6. A, nonradioactive rleptin ($1 \mu\text{g}/\text{mouse}$) did not significantly inhibit the brain/serum ratio of ^{125}I -leptin(ss)-P85 at 10 min after intravenous injections. B, self-inhibition of ^{131}I -rleptin by $1 \mu\text{g}/\text{mouse}$ nonradioactive rleptin at 10 min after intravenous injections (*, $p < 0.0005$; $n = 10$ mice/group).

gel (Fig. 8). The sample from serum mainly contained leptin monomer (band corresponding to 15 kDa). A light band at 30 and 60 kDa was also observed in the film exposed for a longer time (data not shown). Notably, sample from brain showed a smear band (as arrow indicated) that was thought to be leptin(ss)-P85 and leptin monomer band at 15 kDa. Similar gel profile was observed when the starting sample was eluted (Fig. 8B). No degradation products of ^{125}I -leptin(ss)-P85 were seen on the SDS-PAGE gel, demonstrating that ^{125}I -leptin(ss)-P85 was intact in brain and blood.

Fig. 7. A, clearance of ^{125}I -leptin(ss)-P85 from blood after intravenous injection. The inset shows the initial distribution phase was linear, and the half-time disappearance from blood was 32.35 min. B, percentage of intravenously injected dose of ^{125}I -leptin(ss)-P85 taken up by each gram of brain tissue from 0 to 60 min after injection. The maximal value was estimated by a one-site binding model to approach 0.263% Inj/g.

TABLE 1

Acid precipitation of ^{125}I -leptin(ss)-P85 by brain and serum

Time	Brain	Serum
	%	
10 min	$98.27 \pm 4.49^*$	$101.61 \pm 0.34^*$
360 min	67.66 ± 5.84	92.43 ± 3.03
1080 min	75.62 ± 1.77	92.78 ± 0.94
1440 min	69.21 ± 7.86	87.36 ± 4.09

* $p < 0.05$ compared with 6-, 18-, and 24-h groups ($n = 3$ mice/group).

Octanol/Buffer Partition Coefficient. The mean octanol/buffer partition coefficient calculated to be 0.0120 ± 0.00059 ($n = 6$). This gave a log value of -1.92 , showing that ^{125}I -leptin(ss)-P85 is very hydrophilic.

Capillary Depletion Study with Vascular Washout. To determine whether ^{125}I -leptin(ss)-P85 was sequestered by brain endothelial cells or entered into the brain parenchyma space, we performed capillary depletion with washout of the vascular space 10 min after intravenous injection. The brain parenchyma/serum ratio was $3.78 \pm 0.26 \mu\text{l/g}$ ($n = 8$ mice) and was significantly higher than the capillary/serum ratio of $3.04 \pm 0.18 \mu\text{l/g}$ ($p < 0.05$; $n = 8$ mice; Fig. 9). These results show that ^{125}I -leptin(ss)-P85 crossed the brain endothelial barrier of the cerebral cortex to enter the parenchymal space of the brain.

Brain Perfusion of ^{125}I -Leptin(ss)-P85. The unidirectional influx rate of ^{125}I -leptin(ss)-P85 measured during brain perfusion was found to cross the BBB, with a K_i value

Fig. 8. A, SDS-PAGE gel electrophoresis of ^{125}I -leptin(ss)-P85 extracted from blood and brain 10 min after intravenous bolus injection. Arrow indicates where ^{125}I -leptin(ss)-P85 standard in buffer appeared. B, molecular mass markers (in kilodaltons) are shown on the left. S, serum; B, brain. We do not observe exactly the same gel profile of leptin conjugates in Figs. 3 and 8, possibly due to different sample preparation. In Fig. 3, lane E, the conjugates were first incubated with DTT and heated for 10 min and then loaded onto the gel with loading buffer without reducing reagent; in Fig. 8, each sample was prepared using loading buffer containing reducing reagent.

Fig. 9. Capillary depletion. Results are reported for experiments that used vascular washout 10 min after intravenous injection. The brain parenchyma/serum ratio (microliters per gram) was $3.78 \pm 0.26 \mu\text{l/g}$ ($n = 8$ mice) and was significantly higher than the capillary/serum ratio of $3.04 \pm 0.18 \mu\text{l/g}$ ($p < 0.05$; $n = 8$ mice).

of $0.892 \pm 0.236 \mu\text{l/g}$ and V_i value of $15.68 \pm 1.202 \mu\text{l/g}$ ($p = 0.019$; $n = 3/\text{time point}$; Fig. 10A).

The brain/perfusion ratio of ^{125}I -leptin(ss)-P85 was measured with or without the addition of 100 ng/ml nonradioactive rleptin or leptin(ss)-P85 in the perfusion buffer at 5 min. As shown in Fig. 10B, there was no significant difference between the three groups.

Tissue Distribution of ^{125}I -Leptin(ss)-P85. The tissue/serum ratios of ^{125}I -leptin(ss)-P85 accumulation in each organ (liver, kidney, lung, heart, stomach, and thigh muscle) at various times was calculated, and the results are shown in Fig. 11. Correlations were determined between tissue/serum ratios and time ($n = 3/\text{time point}$). There were statistically significant correlations between radiolabeled Pluronic leptin accumulation and time in heart ($r^2 = 0.4013$, $p = 0.0048$), stomach ($r^2 = 0.6020$, $p = 0.0002$), and thigh muscle ($r^2 = 0.8313$; $p < 0.0001$). However, no significant correlations were observed in liver, kidney, and lung. The distribution of ^{125}I -leptin(ss)-P85 was the highest in the highly vascularized organs such as liver, lung, and kidney, whereas it was lower in the heart and stomach and lowest in thigh muscle and brain.

Effect of Leptin(ss)-P85 on Food Intake after Venous Administration. The t test for food intake 0 to 30 min after the induction of food intake was significant ($t = 4.70$, $p < 0.0005$), indicating that the mice that received leptin(ss)-P85 (0.91 ± 0.05) ate significantly less than the mice that received saline (1.40 ± 0.10 ; Fig. 12A). The t test on food intake 0 to 2 h after induction of food was significant ($t = 4.346$, $p <$

Fig. 10. A, time courses of the brain/perfusate ratio of ^{125}I -leptin(ss)-P85. The K_i value for ^{125}I -leptin(ss)-P85 was $0.892 \pm 0.236 \mu\text{l/g} \cdot \text{min}$ ($r = 0.88$, $p < 0.05$; $n = 3/\text{time point}$). B, inclusion of nonradioactive leptin(ss)-P85 or rleptin (100 ng/ml) in perfusion buffer did not alter the brain/perfusion ratio of ^{125}I -leptin(ss)-P85 at 5 min after intravenous injection. The brain/perfusion ratio as measured by brain perfusion was $21.82 \pm 4.04 \mu\text{l/g}$ with ^{125}I -leptin(ss)-P85 only, $19.32 \pm 3.34 \mu\text{l/g}$ with + nonradioactive rleptin, and $21.88 \pm 5.35 \mu\text{l/g}$ with + nonradioactive leptin(ss)-P85 ($n = 5/\text{group}$).

Fig. 11. Uptake of ^{125}I -leptin(ss)-P85 and relation between time and the log of the tissue/serum ratios in various tissues, including stomach, kidney, and liver (A) and lung, thigh muscle, and heart (B) after intravenous injection ($n = 3$ mice/time point).

Fig. 12. Effect of leptin(ss)-P85 administered via tail vein on food intake at 0 to 30 min (A) and 2 h (B) after induction of food that occurred 15 min after tail-vein injection. *, $p < 0.05$ and **, $p < 0.01$ ($n = 7$).

0.001), indicating that the mice that received leptin(ss)-P85 (1.13 ± 0.05) ate significantly less than the mice that received saline (1.71 ± 0.14 ; Fig. 12B). There was a rebound effect after 4 h, and the t test for food intake during hours 4 to 24 was significant ($t = 2.704$, $p < 0.02$), indicating that the mice that received leptin(ss)-P85 (7.09 ± 0.18) ate significantly more than the mice that received saline (6.53 ± 0.18). The t test for total food intake for the 24-h period was not significant ($t = 0.191$).

Discussion

Conjugation of Pluronic block copolymers to peptides and proteins has become a popular approach to improve their therapeutic potential in diagnostics and treatment of diseases of the central nervous system. Enhanced delivery of chemically modified proteins across the BBB is a successful technology to use them as a drug in therapeutic purposes (Banks and Lebel, 2002). These polymers are used to protect peptide drugs from enzymatic degradation, prolong exposure of drugs to tissues, and enhance the transport of drugs into cells as biologically stable components (Kabanov and Gendelman, 2007). One such polymer formulation is the Pluronic copolymer P85, and this technology was applied to recombinant leptin to produce conjugated Pluronic leptin via biodegradable linker, which was used in this study.

We first determined whether leptin(ss)-P85 retained bio-

logical activity by injecting it intracerebroventricularly and measuring effects on feeding. We found that, in comparison with the parent compound, its onset of action was sooner, detectable within the first 4 h after administration. Leptin only worked at the 0.5- μ g dose intracerebroventricularly and not at higher or lower doses. This inverted U-shaped curve is typical of leptin and many other feeding hormones. In comparison, leptin(ss)-P85 was active at higher doses but produced a more reliable dose-response curve. We estimate that the lowest effective dose of leptin(ss)-P85 was four to eight times greater than that for leptin.

Then we determined whether the circulating pharmacokinetic parameters of radiolabeled leptin(ss)-P85 (conjugated Pluronic leptin) were improved and whether it could enter into the brain. Multiple time-regression analysis (Banks and Kastin, 1993b) showed that radiolabeled leptin(ss)-P85 entered the brain from the blood, with an influx rate of $K_i = 0.272 \pm 0.037$ μ l/g $\cdot$ min. This entry rate did not differ significantly from that of recombinant leptin ($K_i = 0.587 \pm 0.52$ μ l/g $\cdot$ min) measured previously (Banks et al., 1996). The influx rate also was consistent with relatively low lipophilicity for leptin(ss)-P85 as measured by the octanol/buffer partition coefficient. Administration of excess unlabeled leptin(ss)-P85 or unmodified rleptin had no effect on the entry of radiolabeled leptin(ss)-P85 into brain after intravenous injection. However, excess unlabeled rleptin that was coinjected with leptin(ss)-P85 did inhibit the influx of radiolabeled rleptin. These results suggest that transport of conjugated Pluronic leptin was by a nonsaturated manner. More importantly, leptin(ss)-P85 seems to enter the brain through a route other than leptin transporter at the BBB. This is a critical finding because if leptin(ss)-P85 can cross the BBB by a mechanism independent of the leptin transporter, then it would not be subject to the resistance at the level of the BBB that arises in obesity. To investigate this further, we used a brain perfusion method with a blood-free perfusion buffer to eliminate the influence of certain factors on the transport of conjugated Pluronic leptin across the BBB. These factors include the binding the circulating proteins, degradation in blood, and sequestration by peripheral tissues (Shimizu et al., 2007). Brain perfusion of leptin(ss)-P85 in the buffer also did not show saturation. The rate of influx of perfused leptin(ss)-P85 ($K_i = 0.892 \pm 0.236$ μ l/g $\cdot$ min) was faster than when Pluronic leptin was injected intravenously, suggesting that there may be intravascular factors that alter leptin(ss)-P85 penetration of the BBB.

Leptin has a short half-life due to its predisposition for enzymatic degradation in blood in animals and humans (Cumin et al., 1996; Lau et al., 1996), and it has been reported that recombinant leptin does not circulate more than a few minutes in blood (Banks et al., 1996; Banks et al., 2002). We previously measured the half-life of radioactively labeled leptin to be 5.46 min, whereas here we found the half-time disappearance for radioactively leptin(ss)-P85 to be 32.3 min.

We then determined the amount of the intravenously injected dose that entered the brain. The percentage of the intravenously injected dose of Pluronic leptin entering a gram of brain was 0.27%/g, which was greater than what was found previously for native leptin (0.17%/g) (Banks et al., 1996). This value for leptin(ss)-P85 is approximately 2, 3, 10, and 100 times higher than the values for acetaminophen,

interleukin-1 α , morphine, and domoic acid, respectively (Banks et al., 1991; Preston and Hynie, 1991; Banks and Kastin, 1994; Courad et al., 2001). All are known to exert therapeutic effects on the brain after peripheral administration. It is more important to note that the uptake by brain of leptin(ss)-P85 was much more sustained than of leptin, which was due in large part to improved pharmacokinetics. Therefore, the amount of leptin(ss)-P85 transported across the BBB is well within the therapeutic range of compounds known to exert effects on the brain. This is consistent with results for HRP conjugated with Pluronic P85, indicating that the %Inj/g was almost twice as great for HRP-P85 as for unmodified HRP (Banks et al., 2004; Batrakova et al., 2005).

Capillary depletion studies with vascular washout confirmed that, by 10 min, most of the leptin(ss)-P85 did not remain bound to the endothelial cells, comprising the BBB but reached the brain parenchyma. Capillary depletion showed that 56% leptin-P85 reached brain parenchyma.

Electrophoresis for larger polypeptides is the usual technique for determination of the intact nature of the injected material. As shown by SDS-PAGE, the radioactivity reaching the brain from the blood represented intact leptin(ss)-P85 with no degradation products. Previous work has shown that TCA acid precipitation accurately determines the amount of radioactivity that represents intact leptin (Banks et al., 2002). We found that 69% leptin(ss)-P85 recovered from the brain and 87% from blood after a 24-h injection was precipitated by acid, indicating that its proteolytic degradation is diminished. For comparison, the recovery was found to be 36 and 53% of native leptin from brain and serum at 60 min after intravenous injection, respectively (Banks et al., 1996). Pluronic modification of leptin extended the enzymatic stability in both brain and blood after intravenous injection and extended the circulating half-life in the blood. Tissue distribution studies also showed that Pluronic modification significantly increased the distribution of leptin in the heart, stomach, and thigh muscle. It was reported earlier that Pluronic were excreted primarily through the kidneys (Batrakova et al., 2004). Although Pluronic modification of leptin did not increase leptin permeability across the BBB compared with native leptin, it was transported by a mechanism independent of the leptin transporter (Banks and Kastin, 1993a; Batrakova et al., 2005).

Thus, leptin(ss)-P85 was found to retain its biological activity when directly injected into the brain and penetrate the BBB. We, therefore, determined whether leptin(ss)-P85 could affect food intake after its peripheral administration. We found that leptin(ss)-P85 at 3 mg/mouse given subcutaneously had an immediate, statistically significant effect on feeding.

In the previous work, we reported that selected block copolymers can induce ATP depletion in bovine brain microvessel endothelial cells (Batrakova et al., 2001a, 2003). We do not believe that the issues of ATP depletion in the BBB are relevant here, because ATP depletion is induced by free Pluronic at the doses that are much higher than those used in these experiments. Furthermore, the block copolymer would have to be liberated from the conjugates to a free form to deplete ATP.

In summary, these results show that Pluronic block copolymer modification of leptin results in a compound that retains biological activity, has improved peripheral pharma-

cokinetics, penetrates the BBB at a rate similar to native leptin but not dependent on the leptin transporter, and can decrease food intake when administered by peripheral injection. Optimization of this strategy should result in a leptin analog effective in the treatment of obesity.

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