Positron Emission Tomography Shows that Intrathecal Leptin Reaches the Hypothalamus in Baboons


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ABSTRACT

Human obesity may be caused by a resistance to circulating leptin. Evidence from rodents and humans suggests that a major component of this resistance is an impairment in the ability of the blood-brain barrier (BBB) to transport leptin from the blood to the brain. One potential way to bypass the BBB is by administering leptin into the intrathecal (i.t.) space. To be effective, i.t. leptin would have to move caudally from the site of injection, enter the cranium, and reach the hypothalamic arcuate nucleus at the base of the pituitary fossa. However, many substances, especially small, lipid-soluble molecules, do not diffuse far from the site of i.t. injection but are resorbed back into blood. To determine whether i.t. leptin can move caudally, we injected leptin conjugated to diethylenetriaminepentaacetic acid (DTPA) and labeled with 68Ga (G-Ob) into the lumbar space of three baboons. We also studied unconjugated DTPA labeled with 68Ga, which did not move up the spinal cord but rapidly appeared in blood after i.t. injection. In contrast, G-Ob steadily moved toward the cranium and had reached the hypothalamus 91 and 139 min after i.t. injection in two baboons. We estimated the concentration of leptin in the hypothalamic region to be at least 8 ng/ml, which is about 40 times higher than cerebrospinal fluid levels in normal weight humans and about 4 times higher than the highest level ever recorded after the peripheral administration of leptin. In a third baboon, the leptin neither moved caudally nor appeared in the blood. We conclude that leptin administered i.t. can reach the hypothalamus in therapeutic concentrations, although there is considerable individual variation.

Leptin has emerged as a major regulator of adiposity. It is a 16-kDa protein secreted by fat cells (Zhang et al., 1994) and is transported into the central nervous system (CNS) by a saturable system (Banks et al., 1996) likely located at both the vascular blood-brain barrier and the choroid plexus (Devos et al., 1996; Golden et al., 1997; Zlokovic et al., 2000; Maresh et al., 2001; Thomas et al., 2001). Leptin acts at the arcuate nucleus and, probably at other sites (Funahashi et al., 1999; Schwartz et al., 2000) as well, to decrease feeding and increase thermogenesis, events that promote weight loss (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995). Leptin also has effects on reproduction, respiration, onset of puberty, bone density, brain development, and immune function (Ahima et al., 1996, 1997, 1999; Cheung et al., 1997; O’Donnell et al., 1999; Ducy et al., 2000) and reverses many of the starvation-induced changes in the endocrine system (Ahima et al., 1996; Lord et al., 1999). Many of these effects are also mediated through the CNS.

Obesity in humans and in many rodent models of obesity arises because of a resistance to leptin. Resistance could arise because of impaired transport of leptin across the BBB, impaired target organ sensitivity to leptin (e.g., receptor/post-receptor defects), or impaired response to the hormones that leptin affects (e.g., neuropeptide Y, cocaine- and amphetamine-regulated transcript, corticotrophin-releasing hormone). Evidence exists for impairment of each of these mechanisms, but the clearest evidence is for impaired transport at the BBB. Indirect evidence includes the demonstration that CSF/serum ratios for leptin progressively decrease as obesity and serum leptin levels increase (Caro et al., 1996; Schwartz et al., 1996) and the finding that obese animals that do not respond to leptin after peripheral administration can still respond to leptin given directly into the CNS (Halaas et al., 1997; van Heek et al., 1997). Transport of leptin across the BBB has been directly shown to be impaired in several types of brain pathology (Ahima et al., 1997; O’Donnell et al., 1999; van Heek et al., 1997).
of obese rodents (Banks et al., 1999; Kastin et al., 1999; Burguera et al., 2000; Nave et al., 2000).

The impaired transport of exogenous leptin in obese rodents is partly explained by the finding that the leptin transporter becomes saturated as serum leptin levels increase (Banks et al., 2000). Even at blood levels typically seen only in very thin individuals, the leptin transporter is partially saturated. This is consistent with the view that during most of evolution, leptin levels were much lower in active, nonstarving populations than they are today in domesticated animals and Western humans. Wild populations of baboons have serum leptin levels about one-third that of captive baboons (Banks et al., 2001). This has suggested that the ancestral role of leptin may have been to signal to the brain when fat reserves were adequate to support the initiation of puberty, reproduction, and other functions rather than to prevent obesity.

When the vascular level of leptin is raised in a normal weight mouse to that seen in an obese mouse (30 ng/ml), the transport rate decreases by about one-third. However, obese mice actually have a transport rate reduced twice as much, by about 70%, of that predicted by saturation alone (Banks et al., 1999). Brain perfusion studies can negate the differences in serum leptin levels by presenting to the BBB a common concentration of leptin. Brain perfusion studies show that even when vascular levels of leptin are the same, obese mice transport leptin at only 50% of the rate of thin mice (Banks et al., 1999). Therefore, two distinct mechanisms underlie the impaired transport of leptin by the BBB: partial saturation caused by the higher serum leptin levels seen in obesity and a decrease in transport capacity.

Exogenous leptin is effective in inducing weight loss in obese, leptin-expressing rodents and humans (Pellemounter et al., 1998; Heymsfield et al., 1999). However, resistance to leptin from impaired transport across the BBB raises the question of whether leptin will be effective in morbid obesity. One possible way of overcoming impaired transport would be to directly administer leptin into the CNS by, for example, the intrathecal route (i.t.). Administration of substances into the CSF of the spinal cord is often ineffective for the treatment of intracranial lesions because of rapid CSF-to-blood transfer (Bernards, 1999). Small, lipid-soluble molecules in particular are able to quickly cross the capillaries in the CNS-to-blood direction (McQuay et al., 1989; de Lange et al., 1993), just as they can rapidly cross in the blood-to-CNS direction. However, recent evidence has suggested that proteins administered i.t. may not be able to easily cross in the CNS-to-blood direction (LeBel, 1999), just as their movement is limited in the blood-to-CNS direction, and that leptin may be much more potent after i.t. than after peripheral administration (LeBel et al., 1999). Since the saturable transport system for leptin does not have an efflux component (Banks et al., 1996) and since very little CSF is reabsorbed into blood at the spinal cord (Davson and Segal, 1996), it may be that leptin would be retained in the CSF long enough to reach the brain. Therefore, we determined whether leptin directly injected into the CSF of the lumbar spinal cord was able to reach the brain.

**Materials and Methods**

**Conjugation of Leptin to DTPA.** DTPA-leptin was prepared using methods described previously for N-terminal-specific DTPA-protein conjugation (Ralph et al., 1995) with the following modifications. The conjugation was performed in 20 mM NaPO₄ buffer, pH 7.0. The reaction mixture was dialyzed against 20 mM Tris-HCl buffer, pH 7.2 (buffer A), and DTPA-leptin product was purified by anion-exchange chromatography using a high-performance Sepharose Q column (Amersham Biosciences, Piscataway, NJ) eluted with buffer A and a 0 to 0.5 M NaCl gradient. Isolated N-terminal-specific DTPA-conjugated leptin monomer product was confirmed by mass spectrometry, peptide mapping, and N-terminal sequencing attempts. The DTPA-leptin was shown to be biologically active and equipotent with native leptin in a weight loss bioassay. Final concentration of DTPA-leptin in buffer was 2.2 mg/ml.

**Preparation of Radiopharmaceuticals.** Carrier-free gallium-68 chloride (68GaCl₃) was eluted from a 100-mCi 68Ge/68Ga generator (SOURCE, DuPont Pharmaceuticals, Wilmington, DE) with 3 ml of 1 N hydrochloric acid. After evaporation to dryness under nitrogen with a 700°C drying gun, the residue was dissolved in 2 ml of 6 N HCl and extracted twice with diisopropyl ether (2 ml and 1 ml). The combined organic extracts were concentrated under nitrogen and used to label DTPA and DTPA-leptin.

To label DTPA, the 68Ga was dissolved in 1 ml of sodium acetate buffer (0.4 M, pH 4.55) and 700 μl of DTPA (3.93 mg of DTPA dissolved in 0.2 ml of 2 N NaOH and then diluted with distilled water to 1 ml). The resulting solution of 68Ga-DTPA (G-DTPA) was mixed and incubated at room temperature for 5 min and filtered into a sterile vial.

To label DTPA-leptin, the 68Ga was dissolved in 100 μl of HCl (0.5 N) and transferred to a plastic tube containing 110 μl of DTPA-leptin (2.2 mg/ml). The solution was incubated at room temperature for 10 min and then centrifuged at 5000g for 8 min with a 10,000 NanoSep 50- to 500-μl centrifuge concentrator (Fulfil Filtron Corp., Northborough, MA). Greater than 98% of the radioactivity was usually retained on the membrane. The 68Ga-DTPA-leptin (G-Ob) was removed from the NanoSep membrane by rinsing with phosphate-buffered saline (three washes of 100 μl each) and was sterilized by filtering through a 0.22-μm Millex-GV low protein binding Durapore sterile filter (Millipore Corp., Bedford, MA). A sample (20 μl) was removed for analysis by fast protein liquid chromatography (FPLC).

**FPLC Analysis.** FPLC was performed with a Pharmacia/LKB chromatograph with a Superose 12 HR 10/30 size-exclusion column. Material was eluted with 0.05 M NaOAc (pH 4.5) at a rate of 0.4 ml/min. The elution was monitored for UV absorption at 280 nm, and fractions of 0.4 ml were collected and measured for UV absorption.

**Animals.** Three baboons (Papio anubis) were studied. Baboon I was a young female weighing about 10 kg, baboon II was a young female weighing 12.5 kg, and baboon III was a 17-year-old male weighing 21.6 kg. The baboons were anesthetized with isoflurane and positioned on a bed in a Siemens ECAT HR + PET scanner for imaging after administration of the radiotracer as described below. This scanner is characterized by a 15.52-cm axial field of view divided into 63 transaxial slices (interslice distance of 2.46 mm), with slice 1 being the most rostral. Images were reconstructed with ordered subset expectation maximization (2 iterations, 16 subsets) with a zoom of 2. The image pixel size was 2.57 mm. The images were corrected for attenuation with a 10-min measured transmission acquisition. An [O¹⁵]H₂O scan was also performed in the PET camera for anatomical information. Computed tomography and MRI images were obtained as anatomical baselines in the bed positions that covered the spinal cord and brain.

A 22-gauge spinal needle was introduced at the level of L2 to L3, and 1 ml of CSF was allowed to drain off. At t = 0, a 0.2- to 0.7-ml of phosphate-buffered solution containing about 1.3 to 4.0 mCi of G-Ob (400–440 μg of Ob protein) was injected into the subarachnoid space. The dead space of the needle hub was cleared with a chase of 0.9% NaCl. Dynamic PET images were collected in frames of 5 min with field 1 extending from 2 cm below the site of injection (SOI) to 13.52 cm above it. Field 2 began at the caudal end of field 1, and subsequent fields were contiguous to the preceding field. The field positions for baboon I are shown in Fig. 1. Images were followed in real
time, and when a bed became filled with radioactivity, the position was advanced to the next field. Examples of these real-time images are shown for baboon I in Fig. 2. Baboons I and II required three field positions to cover the region from 2 cm rostral to the site of injection to the brain, and baboon III required four field positions.

The PET transmission image and the MRI were coregistered using six-parameter rigid body alignment and an objective function based on the method of Jesper and Andersson (1995). The PET transmission and emission data were assumed to be in register. The colored outlines were drawn on the PET data and superimposed on the coregistered MRI using analyze_avw (Biomedical Imaging Resource, Mayo Foundation, Rochester, MN). The time-activity data shown in Fig. 5 were measured in the region of interest immediately ventral to the hypothalamus. CSF sampled from the L2 to L3 region 2 h after i.t. injection for baboon II was submitted to analysis by FPLC. Two other baboons were injected with G-DTPA. These baboons received i.t. injections and were imaged as outlined above.

Results

For baboons I and III, radioactivity immediately filled field 1. Figure 3 shows the change over time of the amount of radioactivity in three slices of bed 2 expressed as microcuries and as percentage of the injected dose. A peak occurred in the most rostral slice at 30 to 35 min after injection and likely represents the concentration peak moving through the spine. Field 2 was completely filled by 75 to 80 min after i.t. injection for both baboons I and III.

The brain was contained in field 3 for baboon I and in field 4 for baboon III. Imaging of these fields began 91 min (baboon I) and 139 min after i.t. injection, and radioactivity was already present in these fields at those times. Figure 4 shows the transmission, water, and G-Ob images coregistered with the magnetic resonance for baboon III. These show that the radioactivity had reached the region of the pituitary fossa and hypothalamus. An almost identical image occurred for baboon I. Figure 5 shows the percentage of the injected dose...
and the micrograms of leptin calculated to be in the hypothalamic region during these imaging periods.

For baboon II, radioactivity moved little from the site of injection and never completely filled field 1. Radioactivity in field slice 60 (1.25 cm rostral to the SOI) changed little over time, did reach field slice 32 (5.63 cm caudal to SOI), but did not reach field slice 12 (10.5 cm caudal to SOI). Radioactivity had not reached field 2 when imaging ended 181 min after i.t. injection.

A sample of the radioactive material injected i.t. eluted as a single peak by FPLC in the position of G-Ob. The radioactivity in the CSF from baboon II also eluted as G-Ob with no degradation peaks seen.

G-DTPA advanced little from the site of injection. Intense radioactivity was noted in the aorta. Imaging of the head showed no radioactivity entering the brain, but did show intense uptake by nasal mucosa.

**Discussion**

These results showed that leptin injected i.t. into baboons can reach the hypothalamic region relatively rapidly and in amounts known to be biologically active. This was in marked contrast to DTPA, which had poor progression up the spinal cord but entered the circulation rapidly. The caudal movement of leptin after i.t. administration varied greatly among the three baboons studied, with no movement occurring in one baboon. Some of the factors causing this individual variation are discussed below, but other factors are likely to be identified. Overall, these results show that the i.t. route of administration can be a much more effective route for delivering biologically active proteins to the brain than it is for smaller molecules. The results also suggest that individual responses to those proteins may vary greatly, at least in the first few hours after administration.

G-Ob reached the brain in two of three baboons. Radioactivity moved rapidly through field 2, which consisted mostly of the thoracic spine in both baboons. Radioactivity reached the brain of both baboons, but took longer and was at a lower concentration in baboon III than in baboon I. This is not surprising, as baboon III was bigger, with a spine about 10 cm longer than that of baboon I. In contrast, the concentration of radioactivity was higher in field 2 for baboon III. This suggests that concentration may not only be affected by time after injection and distance from site of injection, but might also be influenced by total spinal cord length. The main driving force in spine-to-brain movement of a protein injected i.t. is likely to be the rate of CSF reabsorption at the arachnoid villi. Since the rate of CSF reabsorption is a function of body size, a correlation between spinal cord length and concentration could arise.

Reasons for the lack of movement in baboon II are unclear. An injection into the subdural, rather than the subarachnoid, space is a distinct possibility, despite good return of CSF after placement of the needle. The needle has a beveled edge and, consequently, it is possible to get CSF from the subarachnoid space and to have injected fluid dissect between the arachnoid and the dura. Sex is unlikely to be an explanation as both baboon I and II were female, and baboon II had a weight, age, and spinal cord length that was between those of baboon I and III. It is unclear whether slow distribution as observed here would result in a decreased clinical response or whether distribution and a therapeutic effect would eventually occur. If slower distribution resulted in slower clearance, a chronic therapeutic effect might even be
enhanced. Either way, these results suggest that therapeutic responses could vary significantly among subjects.

The fate of G-Ob differed from that of G-DTPA: 1) G-DTPA did not move up the spinal cord, but 2) did rapidly enter the blood stream. Even though G-Ob did not move up the spinal cord in baboon II, it did not enter the blood. It is well known that small molecules injected i.t. can rapidly cross the vascular endothelium to enter the blood. The results here clearly show that proteins act very differently from small molecules after i.t. injection.

Both in vivo evidence and FPLC show that the radioactivity reaching the hypothalamus represented G-Ob. In vivo, the distinct patterns of behavior of G-DTPA and G-Ob are radically different, with the former not ascending the spinal cord but rapidly entering the blood. Therefore, material degradation to free label did not occur. Results with FPLC confirm this conclusion, with 100% of the radioactivity in the lumbar CSF still eluting as G-Ob 2 h after injection.

These studies were limited by the half-life of our isotope, which was about 69 min. The results presented here mostly reflect the movement of the wave front. The peak concentration moves much slower, as can be seen in Fig. 2 and is quantified in Fig. 3. In baboon I, for example, Fig. 3, upper panel, shows that 25 min after injection, the wave front has traveled over 28 cm from the site of injection. In comparison, the peak concentration has only traveled 13.5 cm after 35 min. These results, therefore, do not indicate the ultimate concentrations that would have been reached in the hypothalamus or how long those concentrations would have lasted. It is obvious from the results that it would take hours to clear leptin from the CSF and probably that the peak concentration passing through the hypothalamus would be much higher than those directly observed. Even the observed levels, however, had reached therapeutic concentrations.

The most conservative estimates of the concentration in the hypothalamic area would be made with baboon III, who had the lower concentration of leptin in the hypothalamus (about 6.8 ng in the hypothalamic slice) and the largest volume of CSF (being the larger baboon). The diameter of the radioactive column in this slice was about 2.1 cm, and with a slice thickness of 2.46 mm, the volume of the cylinder was 0.85 ml. This gives a concentration for the hypothalamic slice of 6.8 ng/0.85 ml, or about 8 ng/ml. This compares to a level of leptin in the CSF of about 0.2 to 0.26 ng/ml in normal weight subjects with the highest reported level being 0.6 ng/ml (Caro et al., 1996; Schwartz et al., 1996). Infusions of leptin lasting 1 week, which raised serum leptin levels to 40 times greater than baseline to an average of about 470 ng/ml, increased CSF levels about 5-fold to an average of 1.26 ng/ml, with the highest level in the CSF being 2.15 ng/ml (Fujioka et al., 1999). This is likely near the maximal achievable level in CSF when leptin is administered peripherally, because the BBB transporter is nearly saturated at blood levels of about 100 ng/ml (Banks et al., 2000). The infusion study administered 1 mg/kg every 24 h, whereas the i.t. study reported here injected 400 to 440 µg per baboon, a dose 20 to 50 times less on a per kilogram basis. Therefore, the i.t. route can rapidly achieve therapeutic and probably long-lasting levels of leptin in the region of the hypothalamus. These levels are higher than the maximum levels likely achievable in CSF with peripheral administration of leptin.

References


