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Differential Regulation of Leptin Transport by the Choroid Plexus and Blood-Brain Barrier and High Affinity Transport Systems for Entry into Hypothalamus and Across the Blood-Cerebrospinal Fluid Barrier*

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ABSTRACT

Leptin is a circulating hormone that controls food intake and energy homeostasis. Little is known about leptin entry into the central nervous system (CNS). The blood-cerebrospinal fluid (CSF) barrier at the choroid plexus and the blood-brain barrier (BBB) at the cerebral endothelium are two major controlling sites for entry of circulating proteins into the brain. In the present study, we characterized leptin transport across the blood-CSF barrier and the BBB by using a brain perfusion model in lean rats. Rapid, high-affinity transport systems mediated leptin uptake by the hypothalamus (K_M = 0.2 ng/ml) and across the blood-CSF barrier (K_M = 1.1 ng/ml). High affinity *in vivo* binding of leptin was also detected in the choroid plexus (K_D = 2.6 ng/ml). In contrast, low affinity carriers for leptin (K_M = 88 to 345 ng/ml) were found at the BBB in the CNS regions outside the hypothalamus (*e.g.* cerebral cortex, caudate nucleus, hippocampus). Our findings suggest a key role of high affinity leptin transporters in the hypothalamus and choroid plexus in regulating leptin entry into the CNS and CSF under physiological conditions. Low affinity transporters at the BBB outside the hypothalamus could potentially contribute to overall neuropharmacological effects of exogenous leptin. (*Endocrinology* **141:** 1434–1441, 2000)

LEPTIN, A PRODUCT of *ob* (obese) gene (1), is a circulating hormone that controls food intake and energy homeostasis (2). Peripheral or central administration of recombinant leptin is effective in reducing food intake, body weight and blood glucose in leptin deficient (*ob/ob*) mice (3, 4), in normal lean mice (5) and in obese mice (5, 6), but not in diabetic (*db/db*) mice with a mutation in the leptin receptor gene (7–10). Leptin homeostasis is disrupted in obesity (11), anorexia nervosa (12), Cushing's syndrome (13), and during aging (14). Obese humans show a decreased sensitivity to circulating leptin possibly due to defect in leptin transport into the brain (15).

Recent studies indicate that leptin may have widespread actions in the central nervous system (CNS) (16, 17). The primary site of its action is thought to involve hypothalamic nuclei associated with the control of feeding and energy expenditure (18, 19). The receptors for leptin were also found in thalamic and amygdaloid nuclei and cortical neurons (19, 20). Little is known about leptin entry into the CNS. The blood-cerebrospinal fluid (CSF) barrier at the choroid plexus and the blood-brain barrier (BBB) at the cerebral endothelium are two major controlling sites for the entry of proteins into the brain (21–24). Relative contributions of these two CNS transport pathways in maintaining leptin CNS homeostasis, and its access to the hypothalamic and/or other CNS targets are not known. Low uptake of leptin across the murine BBB (25) and restricted transport into the CSF in humans (26) have been reported.

It has been speculated that leptin receptors in the choroid plexus (27–30) and at the BBB (31–33) may serve to transport leptin in the CNS. Recent work in transfected cells, however, indicated that all known leptin receptor isoforms are linked to intracellular signaling, but none to the transcytosis across the blood-CSF barrier and/or across the BBB (34–36). Thus, the physiological significance of potential CNS/CSF transport pathways and the mechanisms involved in leptin transport at different CNS homeostatic sites remain unclear. The present study used an *in situ* brain perfusion model (21) to determine simultaneously the rates of leptin transport across the blood-CSF barrier, into the hypothalamus and at the BBB, and to define the kinetic properties of putative leptin transporters and/or receptors at the luminal side of the BBB and the basolateral side of the choroid plexus epithelium. This technique has been used extensively in CNS/CSF transport studies of several peptides and proteins (21, 22, 37).

Materials and Methods

Animals

Fifty-two adult male Wistar rats (250–300 g Charles River Laboratories, Inc., Ballardville, MA) were used for the experiments. The animals were housed in a vivarium on a 12-h dark, 12-h light cycle, at 21 ± 2 C and 70 ± 5% relative humidity. All experimental procedures were approved by the Institutional Animal Care and Use Committee for the University of Southern California, following the USDA Guidelines, PHS

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policy/NIH Guidelines, Animal Welfare Act and Guide for the Use and Care of Laboratory Animals.

In situ brain perfusion technique

An established *in situ* brain/choroid plexus perfusion technique was used for the measurement of leptin uptake in lean, normal rats (21). This method allows direct measurements of leptin CNS/CSF uptake under conditions where arterial inflow was controlled and the concentration of leptin in the perfusate clamped. Briefly, the rats were anesthetized (Nembutal, 50 mg/kg ip) and the right common carotid artery isolated and connected to an extracorporeal perfusion circuit. The perfusion medium consisted of 20% sheep red blood cells (oxygen carrier) suspended in mock plasma containing 48 g/liter dextran (FW 70 000). Radiolabeled leptin (^{125}I-leptin, Amersham Pharmacia Biotech, specific activity 87–275 cpm/pg; three different batches were used) was introduced in the perfusion medium at a constant tracer concentration *via* a slow-drive syringe at a rate of 0.2 ml/min. The rate of perfusion fluid was between 4 and 4.5 ml/min. The cerebrovascular tracer ^{99}Tc-Albumin (Medi-Physics, IL, Technetium Tc99m HSA Multidose Kit) was introduced simultaneously. Physiological parameters (*e.g.* perfusion arterial pressure, acid base status, cerebral perfusion flow etc.) were kept within the range found in the anesthetized rat.

The first study examined the time-dependent uptake of radio-iodinated leptin into different CNS regions using a single batch of leptin tracer for all experiments with a final concentration in the arterial inflow of 0.52 ± 0.09 ng/ml of ^{125}I-leptin, more than 97% intact by acid precipitation (TCA) and HPLC analysis. At predetermined times ranging from 1 to 20 min rats were killed by decapitation and the CNS tissues collected for radioactivity analysis in a Beckman Coulter, Inc. γ 4000 Counter. The following samples were prepared from the ipsilateral perfused hemisphere: choroid plexus and CSF from the lateral ventricle, hypothalamus, and different BBB regions (*e.g.* parietal cortex, caudate nucleus, hippocampus). The choroid plexuses were harvested from the lateral ventricles, and the wet weights of the choroid plexus samples were between 1.9 and 2.2 mg. The wet weight of the choroid plexus was determined immediately after the isolation using preweighted vials. The weights of the CSF samples from the lateral ventricles were between 2.1 and 2.5 mg. The CSF was sampled from the lateral ventricle by stereotactic puncture through a window in the skull using a 10 μl Hamilton syringe. The area dissected as hypothalamus was its ventromedial part with a standardized weight between 18 and 24 mg. This area consisted of nervous tissue, previously shown to be largely inside the BBB, but contained also the median eminence, a region that is not protected by the BBB (22). The weights of the parietal cortex, caudate nucleus and hippocampus samples varied between 20 and 38 mg.

All plasma and CNS tissue samples were TCA-precipitated (see below) and counted simultaneously. Each time point included three to six animals per point, and each experiment/per time point was performed on the same day.

In separate experiments, unlabeled leptin at different concentrations ranging from 0.6 to 300 ng/ml was introduced into the arterial inflow simultaneously with ^{125}I-leptin for either 1 min for measurements in the choroid plexus, CSF, and hypothalamus, or for 10 min for uptake studies at the BBB regions outside the hypothalamus. In these experiments concentration-dependent uptake of leptin was determined, and each concentration point included three to six animals per point. Leptin tracer was from two different batches of similar specific activity, and its final concentration in the arterial inflow was 0.15 ng/ml of ^{125}I-leptin, more than 97% intact as determined by TCA and HPLC analysis.

TCA precipitation assay

The recovery of intact ^{125}I-leptin radioactivity in the arterial inflow and CNS tissue and CSF samples was determined by TCA precipitation assay in all samples. Samples were mixed with 0.2 ml of 35% TCA, then centrifuged at 6,000 rpm at +4 C for 8–10 min, and the radioactivity in the precipitate, water fraction and chloroform fraction determined. The intactness of radio-iodinated leptin infused into the arterial inflow was > 97% by the HPLC analysis that was confirmed by the TCA assay. It has been also demonstrated that TCA assay provides reliable analysis of intact leptin in the CNS tissues as corroborated with the HPLC analysis (see below).

Binding to microvessels and transendothelial transport

To distinguish between leptin binding to brain microvascular endothelium *vs.* transport across the BBB, in some experiments brain tissue was subjected to capillary depletion step and microvessels separated from perfused brain, as we reported (21, 22). In these studies we used the ipsilateral forebrain tissue consisting of cortical tissue and subcortical regions (caudate, hippocampus), but did not contain rapid uptake regions such as the choroid plexus and hypothalamus that were discarded. Briefly, dextran (FW 70,000) was added to a final concentration of 13% to brain tissue homogenates in a physiological buffer (10 mM HEPES, 141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM $NaHPO_4$, and 10 mM D-glucose adjusted to pH 7.4). The aliquots of homogenates were centrifuged at 5,400 × *g* for 10 min at +4 C. The pellet containing brain microvessels and the supernatant containing capillary-depleted brain were carefully separated and the radioactivities of ^{125}I-leptin and ^{99m}Tc-albumin determined in these two brain fractions.

Calculations

Details of mathematical analysis were as we reported (21, 22, 37). Briefly, the uptake values for ^{125}I-leptin and ^{99}Tc-albumin by the choroid plexus, CSF and brain BBB regions are expressed as ratios of tracer concentrations in different tissues/fluids relative to the constant concentration of their respective isotopes in the plasma arterial inflow. The following equation was used to calculate the ratios: C_{IN}/C_{PL} = [cpm./g (tissue or CSF)]/[cpm/ml (plasma inflow)]. The uptake values for ^{125}I-leptin were based on TCA-precipitable radioactivity (intact leptin as confirmed by HPLC analysis) and were corrected for tracer distribution in the vascular brain space and choroid plexus vascular and extracellular space (*i.e.* the choroid plexus has leaky capillaries, but tight epithelium) by subtracting albumin values as: $C_{IN\ (LEPTIN)} - C_{IN\ (ALBUMIN)}$. Although, short infusion times of 1–3 min may preclude complete equilibration of ^{99m}Tc-albumin in the choroid plexus interstitial space, using different extracellular space markers (*e.g.* ^{14}C-inulin, FW 5,000 or ^{3}H-mannitol, FW 182) we obtained only slightly higher values than for albumin, and the respective uptake values at 1 min were 2.3%, 2.5% and 3.9% for albumin, inulin, and mannitol. As FW of leptin is 16,000, *i.e.* between albumin and inulin, it is likely that the amount of leptin tracer that remains in the interstitial space at 1 min is between 2.3% and 2.5%. Therefore, our applied correction with albumin would at most overestimate leptin epithelial uptake by less than 5%, which may not be significant given that the SD of leptin uptake rate by the choroid epithelium is close to 20% (Table 1).

To calculate the rate of leptin entry (K_{IN}) into the CSF and different CNS regions, multiple-time uptake series were performed. The analysis of data were based on previously developed two-compartment mathematical model (21, 22, 37), using the equation: $d[C_{IN\ (LEPTIN)} - C_{IN\ (ALBUMIN)}]/dt = K_{IN} C_{PL} - K_{OUT} [C_{IN\ (LEPTIN)} - C_{IN\ (ALBUMIN)}]$ (Eq 1), where K_{OUT} is the exit transfer coefficient, and R is the steady-state or equilibrium ratio. Eq 1 is integrated to give $[C_{IN\ (LEPTIN)} - C_{IN\ (ALBUMIN)}]/C_{PL} = R(1\text{-}e^{-KOut\ T})$ (Eq 2). R is the steady state ratio, or the ratio

TABLE 1. The unidirectional rates, K_{IN}, of leptin entry across the blood-CSF barrier, hypothalamus and at different BBB regions

Region	K_{IN} (min^{-1})	Ratio
CSF	$0.105 \pm 0.040^{a,ns}$	
Choroid plexus	$0.079 \pm 0.003^{a,ns}$	1.30
Hypothalamus	$0.075 \pm 0.020^{a,ns}$	1.40
Hippocampus	0.002 ± 0.0001^{ns}	52.5
Cortex	0.006 ± 0.0001^{a}	17.5
Caudate nucleus	0.002 ± 0.0003	52.5

Values are mean ± SEM based on ^{125}I-TCA-precipitable radioactivity; n = 24–29. The K_{IN} values for choroid plexus, blood-CSF barrier and hypothalamus, and the BBB regions, were determined from Figure 1 by using Eq 4 and 5 and Eq 6, respectively.

[a] $P < 0.01$, CSF, choroid plexus and hypothalamus *vs.* hippocampus, cortex, and caudate nucleus; cortex *vs.* caudate nucleus and hippocampus.

[ns] Nonsignificant, CSF *vs.* choroid plexus *vs.* hypothalamus; caudate nucleus *vs.* hippocampus, by two-way post-ANOVA.

$[C_{IN\ (LEPTIN)} - C_{IN\ (ALBUMIN)}]/C_{PL}$ and the ratio K_{IN}/K_{OUT} at infinite time, and T is leptin infusion time. Numerical values for K_{OUT} may be obtained from the slope of the plot of ln $(R - [C_{IN\ (LEPTIN)} - C_{IN\ (ALBUMIN)}]/C_{PL})$ (Eq 3) against T, using the equation $K_{OUT} = -\ln(R - [C_{IN\ (LEPTIN)} - C_{IN\ (ALBUMIN)}]/C_{PL})/T$ (Eq 4). Finally, the value for K_{IN} is derived by substituting the number for K_{OUT} in: $K_{IN} = R\ K_{OUT}$ (Eq 5). In this study, the nonlinear regression analysis and Eq 4 and 5 were used to compute the K_{IN} values for leptin at the choroid plexus and at the blood-CSF barrier. In case when tracer uptake remains linear over a studied period of time, the exit constant K_{OUT} approaches zero and the rate of entry K_{IN} can be calculated from Eq 1 as: $K_{IN} = d\ [C_{IN\ (LEPTIN)} - C_{IN\ (ALBUMIN)}]/(dt\ C_{PL})$ (Eq 6). In the present study, the linear regression analysis and Eq 6 were used to compute the K_{IN} values at the BBB regions. The K_{IN} represents the fraction of circulating radioactive leptin that is taken up by a given CNS compartment in min^{-1}.

To determine concentration-dependent transport of leptin into the CSF and across the BBB, the uptake of ^{125}I-leptin was studied at different concentrations of unlabeled leptin. The unidirectional influx, J_{IN}, was calculated as $J_{IN} = K_{IN}C_{PL}$ (Eq 7), where K_{IN} is the entry constant of radio-labeled leptin in the presence of increasing concentrations of unlabeled leptin in the arterial inflow plasma, C_{PL}. (For details, see refs. 21, 22, 37.) Michaelis-Menten analysis was applied to compute the affinity constant (K_M) and maximal transport capacity (V_{MAX}) of putative leptin transporters. The possibilities for a single and/or multiple transport sites were considered using the following Eq 8, $J_{IN} = V_{MAX1}C_{PL}/(K_{M1} + C_{PL}) + V_{MAX2}C_{PL}/(K_{M2} + C_{PL}) + \ldots V_{MAXn}C_{PL}/(K_{Mn} + C_{PL})$. *In vivo* uptake of ^{125}I-leptin by the choroid plexus was also determined in the presence of different concentrations of unlabeled leptin and the J_{IN} values calculated using Eq 7. The J_{IN} values for leptin in the plexus reflect binding of leptin to the choroidal tissue (37), as opposed to transport across the blood-CSF barrier, the binding constant (K_D) and maximal binding capacity (B_{MAX}) were calculated. The possibility for a single or multiple binding sites was considered (Eq 9): $J_{IN} = B_{MAX1}C_{PL}/(K_{D1} + C_{PL}) + B_{MAX2}C_{PL}/(K_{D2} + C_{PL}) + \ldots B_{MAXn}C_{PL}/(K_{Dn} + C_{PL})$. Kinetic parameters were calculated with a nonlinear regression analysis using SAAMII program as reported (39). Advanced graphics software (SlideWrite Plus) was used to obtain graphic plots. Data are presented as mean values (± SEM) and compared by two-way post ANOVA.

Results

In our initial study, ^{125}I-leptin was introduced into the cerebral arterial circulation at low physiological concentrations (*i.e.* 0.52 ± 0.09 ng/ml) in the absence of unlabeled plasma leptin. The uptake of intact ^{125}I-leptin by the choroid plexus (TCA-precipitable fraction) was rapid as well as transport of intact protein into the CSF (Fig. 1, A and B). The choroid epithelial uptake of leptin and its entry into the CSF exhibited significant departure from linearity after 1 min.

In the first minute, the uptake of ^{125}I-leptin by hypothalamus (Fig. 1C) was about 12.5–37.5-fold higher than in other BBB regions. Although the exact significance of the initial spike in the hypothalamus uptake curve is not clear (Fig. 1C),

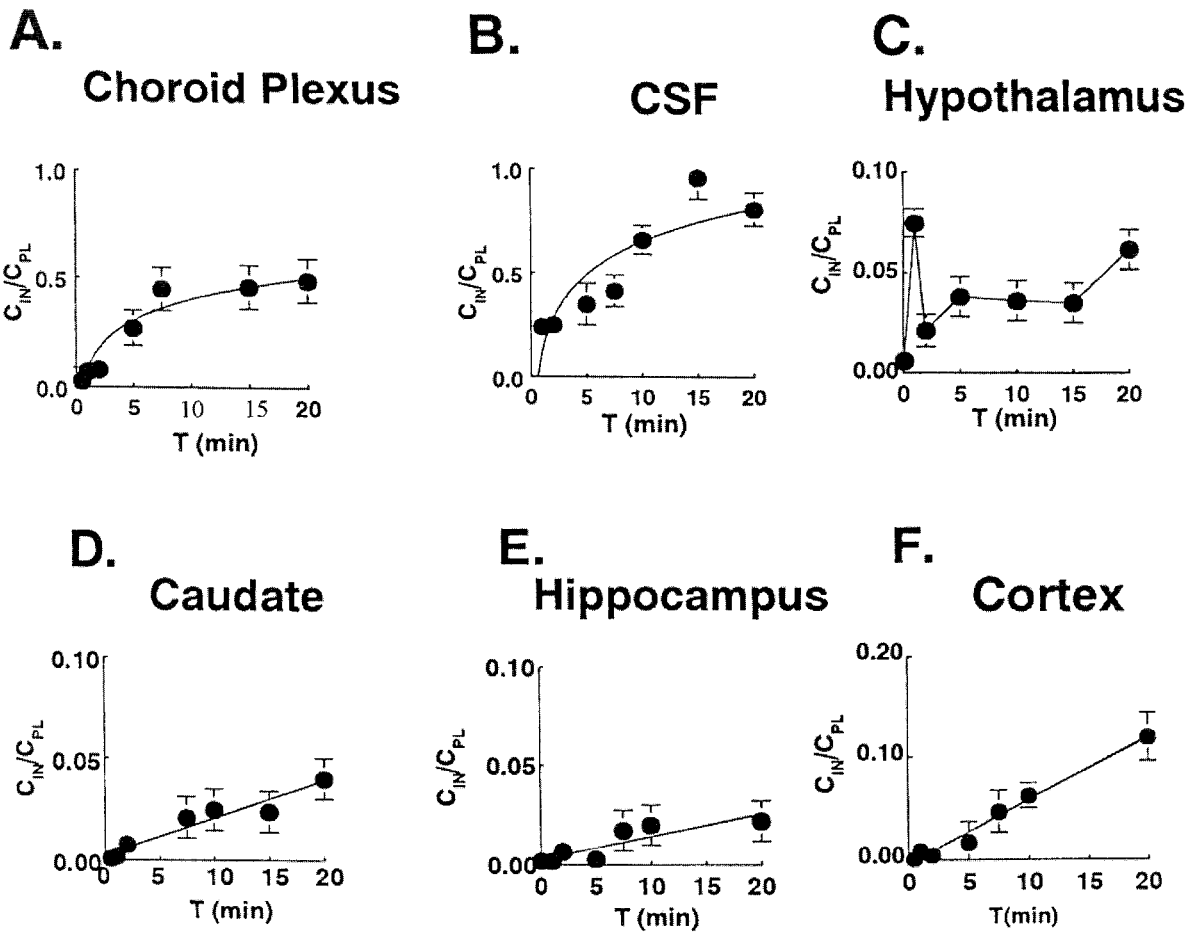

FIG. 1. Time-dependent uptake of circulating ^{125}I-leptin (0.52 ± 0.09 ng/ml) by the choroid plexus (A), across the blood-CSF barrier (B), hypothalamus (C), and at different BBB regions, *e.g.* caudate nucleus (D), hippocampus (E), and cortex (F); T is infusion time from 0.5 to 20 min. Each point represents the uptake of ^{125}I-TCA-precipitable radioactivity. Mean values ± SEM are from 3–6 rats; points without *bars* (SEM ≤ 5% of the mean). Each experiment with 3–6 rats/point was conducted on the same day.

it is possible that it reflects high affinity initial binding of leptin to its hypothalamic receptors followed by ligand-induced rapid receptor down-regulation, as observed in cells transfected with leptin receptors (40). Leptin uptake at different BBB regions outside the hypothalamus was substantially lower and remained linear within the studied 20-min period (Fig. 1, D–F). Data given in Fig. 1 illustrate the uptake of intact leptin as determined by the TCA assay and confirmed by the HPLC analysis.

The HPLC analysis of radio-iodinated leptin used for internal carotid arterial infusions indicated that in all batches > 97% of ^{125}I-radioactivity was intact leptin (as supplied by manufacturer). This was confirmed by the TCA analysis of the infusion solution and the arterial plasma inflow, which indicated > 97% TCA-precipitable radioactivity. In the present model, there is no degradation of peptide and protein tracers in the arterial inflow as the radiolabeled material is directly infused into an enzyme free artificial plasma medium used for brain perfusions, and the tracer is completely separated from the systemic circulation and does not come into contact with the animal's own blood during passage through the brain circulation (21–24).

Previous studies specifically designed to determine leptin degradation in whole brain homogenates by the acid precipitation and the HPLC methods, and to validate the acid precipitation method by the HPLC, demonstrated an excellent correlation between the two methods if the acid precipitation was greater that 50% (25, 38). It has been reported based on incubation studies of the whole brain homogenates with radio-iodinated leptin that the relationship between the methods was: acid precipitation = 1.1 (HPLC) + 2.13 with an r = 0.983 (25). In the present study, we found with the TCA assay that the amount of intact leptin determined in studied CNS tissues was between 91% and 80%, for the shortest (1 min) and the longest (20 min) time point, respectively. Because the values by the TCA acid precipitation in our study were much higher than 50%, according to reported validation analysis of the acid precipitation by HPLC (25), the HPLC will detect similar amounts of intact leptin in the CNS tissues (25, 38).

The regional CNS uptake curves for ^{125}I-TCA precipitable leptin in the choroid plexus and CSF were analyzed by nonlinear regression analysis to compute the K_{IN} values (Table 1). The K_{IN} values at the BBB regions were calculated by linear regression analysis and are also shown in Table 1. The K_{IN} of 0.105 min^{-1} determined for intact leptin at the blood-CSF barrier, suggests transport/secretion that is 18- to 53-fold faster than simultaneously determined leptin uptake at the BBB where the regional K_{IN} values varied between 0.002 and 0.006 min^{-1}. The rate of leptin uptake by the hypothalamus as computed from the unidirectional linear phase was 0.076 min^{-1}, comparable with the rates observed in the choroid plexus and the CSF (Table 1).

In some experiments, a capillary-depletion step was performed to distinguish between binding of tracer to endothelial binding sites at the BBB *vs.* transport into brain parenchyma across the BBB. Our data indicate that, after 10 min, the majority of tracer is transported out of the vascular compartment across the BBB into brain parenchyma (Fig. 2). The value of leptin uptake into brain parenchyma was approximately 13-fold higher than sequestration by capillaries. Direct measurements of intact leptin tracer in the CSF confirmed leptin transport across the choroid plexus epithelium of the blood-CSF barrier into the CSF of lateral ventricles.

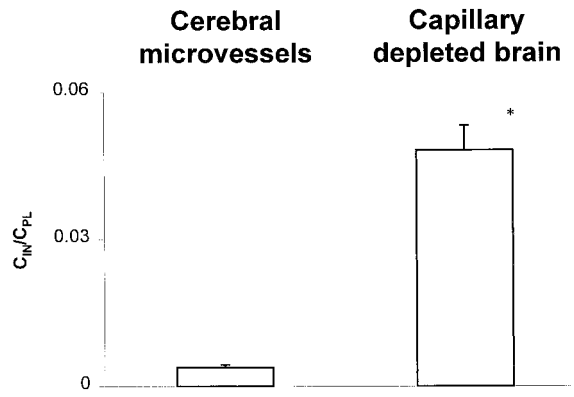

FIG. 2. The uptake of intact ^{125}I-leptin (0.50 ng/ml) by cerebral microvessels and capillary-depleted brain determined after 10 min of brain perfusion. Values are expressed as [cpm/g tissue]/[cpm/ml plasma] and corrected for distribution of vascular space tracer (albumin). Mean values ± SEM, are from four experiments conducted on the same day.

Introduction of gradually increasing concentrations of unlabeled leptin in the arterial inflow corresponding to lower and upper physiological concentrations of leptin in plasma, *i.e.* between 0.6 and 10 ng/ml, resulted in a saturable dose-dependent binding of ^{125} I-leptin (TCA-precipitable fraction) to the choroid plexus and concentration-dependent influx into the CSF and hypothalamus (Fig. 3, A–C). The measurements were performed within initial 1 min of the linear ^{125}I-leptin uptake to ensure inhibition of tracer during its unidirectional uptake and/or transport. In contrast, there was no detectable rapid (≤ 1 min) leptin influx at the BBB. However, with a longer infusion time of 10 min, a saturable leptin influx across the BBB could be demonstrated over a wide range of pharmacological concentrations, from 10 to 300 ng/ml (Fig. 3, D–F). It is noteworthy that lower physiological concentrations of unlabeled leptin failed to inhibit leptin tracer uptake at the BBB (not shown).

The Michaelis-Menten analysis indicated the presence of a single high affinity transport site for leptin at the blood-CSF barrier with the K_M of 1.1 ng/ml (Table 2). The K_M for leptin uptake and/or binding to hypothalamus was even lower (0.22 ng/ml). *In vivo* leptin binding to the choroid plexus exhibited K_D of 2.6 ng/ml. Michaelis-Menten analysis based on slow (≤ 10 min) leptin influx at the BBB regions, revealed a single leptin BBB transporter at the luminal side with a regional affinity varying from 88 to 345 ng/ml. Slow uptake of radio-leptin by the caudate nucleus may be affected by uptake across BBB capillaries as well as uptake from adjacent CSF, as shown previously for brain regions adjacent to the ventricles (40).

Discussion

In the present study, we characterized putative leptin transporters at the blood-CSF barrier, hypothalamus, and at the BBB by using a brain perfusion model in lean rats. We assumed that radiolabeled leptin behaves in front of the CNS

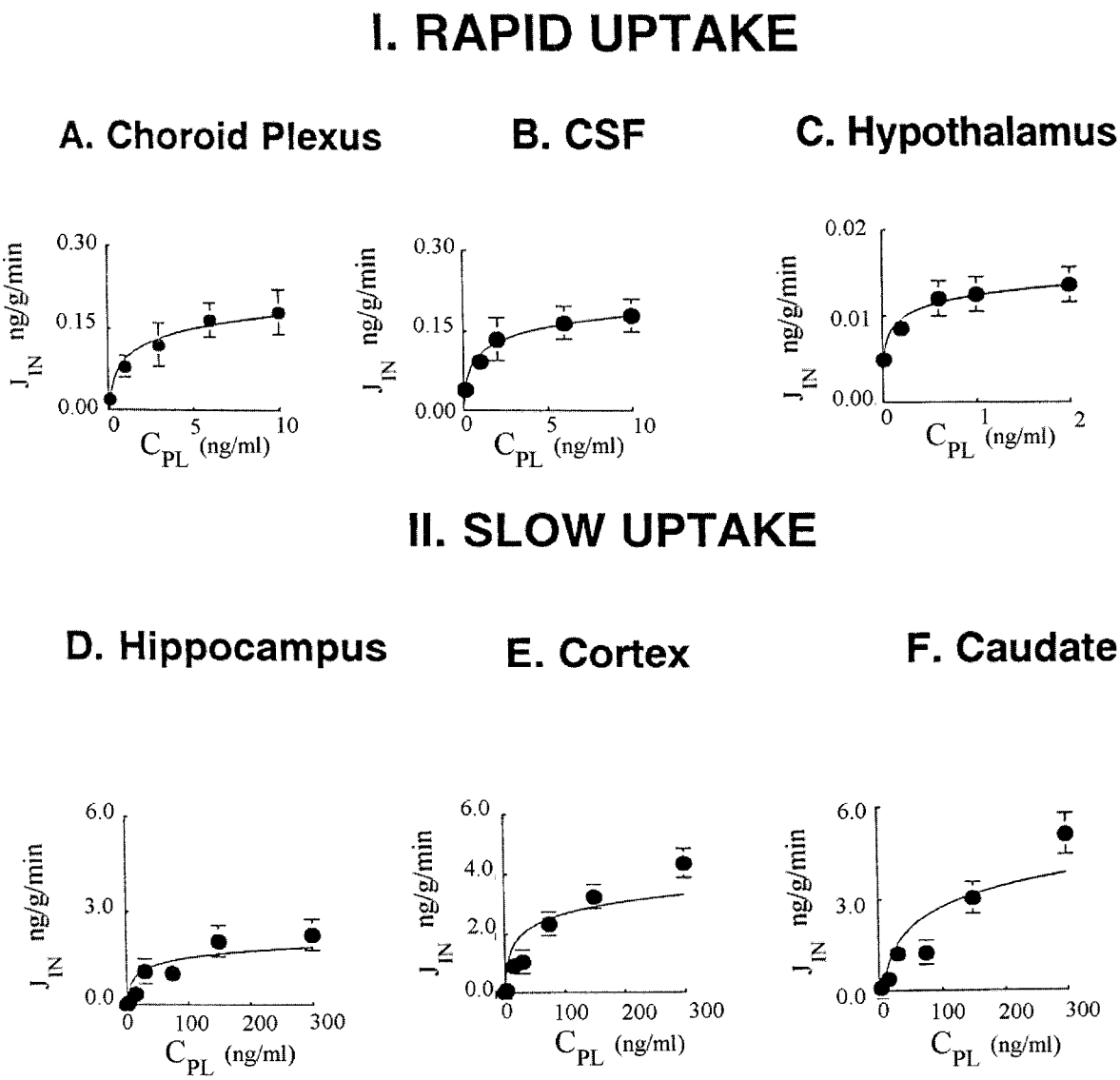

FIG. 3. I, Rapid uptake. Concentration-dependent uptake of ^{125}I-leptin by the choroid plexus (A), transport across the blood-CSF barrier (B) and uptake by the hypothalamus (C) in the presence of unlabeled leptin in the arterial inflow within a physiological 0.6 to 10 ng/ml range. ^{125}I-leptin (0.15 ng/ml) was infused simultaneously with varying concentrations of unlabeled leptin for 1 min. II. Slow uptake, Concentration-dependent uptake of ^{125}I-leptin (0.15 ng/ml) at the BBB regions: hippocampus (D), cortex (E), and caudate nucleus (F), in the presence of pharmacological concentrations of unlabeled leptin from 10 to 300 ng/ml. Because leptin tracer uptake was undetectable at the BBB regions within 1 min, in these studies ^{125}I-leptin (0.15 ng/ml) was infused simultaneously with varying concentrations of unlabeled leptin for 10 min. Each point represents the uptake of ^{125}I-TCA-precipitable radioactivity; mean ± SEM from 3 to 6 rats; points without bars (SEM ≤ 5% of the mean).

biological membranes in a manner comparable with unlabeled leptin, as reported in several recent *in vivo* and *in vitro* studies (25, 30, 36, 38, 41). Rapid, high affinity transport systems mediated leptin entry into the hypothalamus (K_M = 0.2 ng/ml), and across the blood-CSF barrier (K_M = 1.1 ng/ml) at rates 18- to 53-fold faster than across the BBB in other CNS regions. In contrast, low affinity carriers (K_M = 88 to 345 ng/ml) were found at the BBB outside the hypothalamus.

Our findings indicate that choroid plexus plays a key role in regulating leptin entry into the CSF under physiological conditions. Plasma levels of leptin in normal lean rats vary between 0.6 to 10 ng/ml (42), and the K_M value of 1.1 ng/ml determined for leptin transport across the choroid epithelium of the blood-CSF barrier was close to the lower end of this range. Because leptin transport across the blood-CSF barrier is fully saturated at higher leptin physiological plasma concentrations, it is possible that the choroid epithelium acts as a rate-limiting step to prevent increases in CSF leptin concentrations. This epithelial barrier could also be responsible for "leptin resistance" reported in obese individuals where the increases in plasma leptin were not followed by parallel increases in the CSF leptin levels (11, 15). In the present study the C_{IN}/C_{PL} ratio for leptin in the CSF approaches 1 at the steady state, whereas in normal humans (11, 15) and rats (42) leptin levels are considerably higher in

TABLE 2. Kinetic parameters, K_M and V_{MAX}, of putative leptin transporters at the blood-CSF barrier (*i.e.* at the choroid epithelium), hypothalamus and different BBB regions, and *in vivo* binding constants, K_D and B_{MAX}, for leptin in the choroid plexus

Region	Rapid uptake (≤1 min)		Slow uptake (≤10 min)	
	K_M (ng/ml)	V_{MAX} (ng/g/min)	K_M (ng/ml)	V_{MAX} (ng/g/min)
CSF	1.10 ± 0.07[a]	0.20 ± 0.007[a]	None	
Hypothalamus	0.23 ± 0.04	0.014 ± 0.003	None	
Hippocampus	None		88 ± 10[a]	2.97 ± 0.56[a]
Cortex	None		130 ± 27[a]	6.20 ± 0.50[ns]
Caudate nucleus	None		345 ± 29	10.80 ± 2.60
	K_D (ng/ml)	B_{MAX} (ng/g/min)		
Choroid plexus	2.57 ± 0.48	0.23 ± 0.014	None	

Values are mean ± SEM; $n = 22–26$; The K_M and V_{MAX} values for rapid (*i.e.* the blood-CSF barrier, hypothalamus) and slow (other BBB regions) transport of leptin, and the K_D and V_{MAX} values for leptin binding to the choroid plexus were determined from Fig. 3 by using eq 8.

[a] $P < 0.01$, CSF *vs.* hypothalamus, hippocampus *vs.* cortex, hippocampus *vs.* caudate nucleus, and cortex *vs.* caudate nucleus.

[ns] Nonsignificant, cortex *vs.* caudate nucleus, by two-way post-ANOVA.

plasma than in CSF. This discrepancy could be possibly attributed to the differences in the CSF sampling sites. In the present study, a small volume of the CSF was obtained from the lateral ventricle (< 5% of the total CSF volume), whereas in previous studies the CSF was sampled either from the spinal subarachnoid space in humans (11, 15) or from the cisterna magna in rats (42). In the spinal and cisternal CSF samples (11, 15, 42), leptin secreted by the choroid plexuses would be considerably diluted into a larger CSF volume that may account for reported differences. A descending CSF gradient of radiolabeled leptin along the axis lateral ventricle-cisterna magna was also observed in the present study (data not shown). In contrast to choroid plexus and CSF, the steady-state values for leptin could not be determined for other brain regions because the uptake was linear over studied period of time.

The molecular nature of putative leptin transporters at the choroid epithelium is not known. Specific leptin receptors and the short splice variant of the receptor have been identified in the choroid plexus (27–29, 32). It has been speculated that short isoform may serve a transport function moving leptin from the peripheral circulation into the CSF and CNS (27). However, more recent work in transfected cells failed to confirm the transcytotic function of the short form of leptin receptor (36). It has been suggested that all leptin receptor isoforms are linked to cellular signaling (34, 35), leptin internalization, and degradation by a lysosomal pathway (36, 41). The K_D of 2.6 ng/ml determined for *in vivo* leptin binding to the choroid plexus was comparable with K_D of 3.3 ng/ml typically determined for leptin binding to cells transfected with the short form of receptor (36, 41). Thus, it is possible that binding of leptin to its short receptor isoform in the choroid plexus and transport across the blood-CSF barrier that exhibits somewhat higher affinity ($K_M = 1.1$ ng/ml) are two distinct processes. Binding to the short receptor isoform may lead to activation of afferent neural inputs to the network that regulates feeding behavior and energy balance as previously suggested (27, 43). On the other hand, a new class of molecularly yet nonidentified transcytotic leptin receptors could be involved in transport across the choroid epithelium supplying the CSF with leptin. The rate of leptin transport across the blood-CSF barrier was somewhat faster that previously reported rates of receptor-mediated transport of vasopressin, transthyretin, ceruloplasmin, and growth factors (21, 37, 44), as well as some water-soluble vitamins (45). The K_{IN} value for CSF uptake of leptin could be compared with K_{IN} values for Na and Cl, suggesting that uptake of leptin is very rapid, similar to that of Na and Cl in the brisk CSF secretory process (46).

Our results indicate that transport of leptin through the BBB has different properties than across the blood-CSF barrier. The rates of leptin regional BBB transport were similar to previously determined BBB permeability of whole brain to circulating leptin in mice (25) and were within a range of slow to moderate transport determined for several peptides and proteins in rodents including insulin, transferrin, insulin-like growth factors, vasopressin, enkephalins, endorphins, and MSH (21–24). Although there was no rapid uptake of leptin at the BBB (≤ 1 min), with longer infusion times of 10 min, slow but saturable leptin influx across the BBB was demonstrated over a wide range of higher pharmacological concentrations, from 10 to 300 ng/ml. In contrast, physiological concentrations of leptin failed to inhibit ^{125}I-leptin BBB transport, confirming an absence of sensitive transport mechanism for leptin at the BBB under physiological conditions. However, the importance of the BBB in transporting leptin into the CNS at higher pharmacological concentrations and at a slow rate should not be underestimated.

Present findings at the BBB *in vivo* revealed a single low affinity transporter with a regional K_M of 88 to 345 ng/ml. An earlier report on human brain capillary plasma membranes suggested two binding sites for leptin with K_D of 90 and 16,000 ng/ml (31). Leptin binding to isolated cerebral capillaries demonstrated in a previous study was highly suggestive of the abluminal localization of leptin binding sites at the BBB (31). In contrast, the present study described transport of leptin at the luminal side of the BBB and across the BBB into brain parenchyma. Although short and long isoforms of leptin receptors were shown at the BBB (32, 33), it is unlikely that previous (31) and/or the present study were able to confirm that either of these receptors is transcytotic. On the contrary, the binding kinetic properties of long and short isoforms of leptin receptors determined in transfected cells, *i.e.* K_D from 3.3 to 10 ng/ml (35, 36), were far below the K_M for leptin BBB transport determined in this study and/or binding to BBB plasma membranes (31). It is possible that a

novel class of leptin transporters and/or receptors at the BBB may regulate leptin supply to brain cells by analogy to transport mechanisms described for other peptides and proteins (21–24, 44).

The uptake of leptin by hypothalamus that predominantly expresses the long receptor isoform (19, 47) seems to be a special case. Leptin uptake by the hypothalamus was 12.5- to 37.5-fold faster than in other BBB regions and appeared to be mediated by high affinity transporters with K_M of 0.2 ng/ml that was significantly lower than the K_D determined for leptin binding to a long receptor isoform in cell transfection studies (35, 36, 41). The exact mechanisms remain unclear. Previous study in isolated brain slices also suggested extremely sensitive binding of leptin to hypothalamic nuclei (K_D approximately < 1 ng/ml), but the exact binding constants were difficult to determine due to too low maximal binding capacity (48). Many neurons in the hypothalamic nuclei are typically isolated from the circulation by the BBB and express high number of leptin receptors (18, 19). An enhanced autoradiography signal detected in the region of the arcuate nucleus following systemic injection of radiolabeled leptin was interpreted as direct leptin transport from blood to the hypothalamus (25). Alternative possible route is that blood-borne leptin is transported across the blood-CSF barrier and than carried *via* CSF bulk flow to its CNS targets similar to several major regulatory hormones and micronutrients (21, 43, 45). A third possibility is that leptin may enter the hypothalamus at the median eminence, which lacks the BBB (22) and then reaches its neuronal targets in hypothalamic nuclei by diffusion. Perhaps a combination of diffusion at the median eminence, transport across the BBB as well as transport from CSF across the ependyma mediate hypothalamic leptin uptake.

In conclusion, our study points to differential regulation of leptin CNS transport by the choroid plexus and the BBB. Specific high affinity transport systems for leptin in hypothalamus and across the choroid epithelium of the blood-CSF barrier play a key role in regulating leptin entry into the CNS and CSF under physiological conditions. On the other hand, at higher pharmacological concentrations of leptin and over longer periods of time, transport across the BBB takes over, which in turn could be important for neuropharmacological effects of exogenous leptin.

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