The differences in feeding-inhibitory responses to peripheral and central leptin between non-lactating and lactating rats

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Abstract

This study was conducted to examine the contributions of central and peripheral leptin to hyperphagia in lactation. Lactating rats were mated at 7–8 weeks of age and housed singly with their litters. In experiment 1, food intakes were significantly ($P < 0.01$) greater (350% on average) in lactation than in non-lactation throughout a day. Cerebrospinal fluid (CSF) leptin levels remained constant despite plasma leptin levels being significantly ($P < 0.05$) greater in non-lactation than in lactation. In experiment 2, CSF leptin levels were not altered by i.v. injections of leptin (0.2 and 0.4 mg/kg body weight) despite that plasma leptin levels were dose dependently ($P < 0.01$) increased. Moreover, i.v. administration of leptin significantly ($P < 0.05$) decreased food intake in non-lactating rats but not in lactating rats. In experiment 3, nocturnal food intakes were temporarily ($P < 0.05$) reduced in

non-lactating and lactating rats. I.c.v. administration of a leptin antagonist (15 µg) blocked the reductions of food intakes. I.c.v. administration of leptin (10 µg) significantly ($P < 0.05$) decreased cumulative food intakes during 24 h in both the physiological states. In conclusion, this study has presented new evidence that the hyperphagia of lactating rats could be partly due to depressed sensitivity of neurons contacting blood leptin. In contrast, the responsiveness of leptin receptors contacting CSF leptin may not differ between non-lactating and lactating rats. Furthermore, the levels of CSF leptin remained constant independent of those of blood leptin. Therefore, the expression of hypothalamic leptin receptors contacting CSF could be involved in the difference in food intake between non-lactating and lactating rats.

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Introduction

Lactation prominently increases the nutrient requirements of mammals. The nutrient requirements of the lactating mammary gland exceed those of the whole body in non-lactating animals (Vernon & Flint 1984, Wade & Schneider 1992). Consequently, food consumption increases several fold during lactation. Compared to non-lactating rats, lactating rats display significantly lower serum concentrations of an anorexigenic peptide leptin (Vernon *et al.* 2002, Crowley *et al.* 2004).

Leptin, a hormone secreted from white adipocytes, has been implicated in the regulation of energy metabolism and energy expenditure (Cohen *et al.* 2002, Chilliard *et al.* 2005), food intake (Pelleymounter *et al.* 1995, Levin *et al.* 1996) and reproduction (Ahima *et al.* 1996). Leptin binds to the receptor Ob-Rb, preferentially expressed in the hypothalamus, suppressing food intake (Fei *et al.* 1997, Guan *et al.* 1997). Infact, i.c.v. leptin administration markedly reduces food consumption in lactating and non-lactating animals

(Schwartz *et al.* 1996, Mistry & Romsos 2002). A diurnal pattern of leptin levels in cerebrospinal fluid (CSF) reflects characteristics of feeding in lactating and non-lactating rats (Asakuma *et al.* 2004). Furthermore, lactating rats exhibit a decrease in leptin receptor mRNA expression in the hypothalamus (Brogan *et al.* 2000, Denis *et al.* 2003b).

On the other hand, leptin receptors and mRNAs encoding leptin receptor isoforms are detected in the rat nodose ganglion, which contains the cell bodies of the vagal afferent neurons (Buyse *et al.* 2001). A population of hypothalamic arcuate nucleus (ARC) neurons directly contacts the circulation and displays increased sensitivity to circulating leptin compared with neurons behind the blood–brain barrier (BBB) in the hypothalamus (Faouzi *et al.* 2007). Blood leptin levels in lactating rats were lower than those in non-lactating rats (Brogan *et al.* 1999, Denis *et al.* 2003a). Food-deprived lactating rats have lower circulating leptin levels than *ad libitum*-fed lactating rats (Woodside *et al.* 1998).

Both central and peripheral leptin are certainly involved in the regulation of food intake in non-lactation and lactation.

However, the contributions of central and peripheral leptin to the hyperphagia of lactation have not been fully elucidated. In this study, therefore, we examined the involvement of CSF and blood leptin in the regulation of food intake in non-lactating and lactating rats.

Materials and Methods

Animals

All experimental procedures involving animals were performed according to the guidelines on handling and care of animals by the committee for animal welfare of Kitasato University. Female Wistar rats of 11–12 weeks of age (CLEA Japan Inc., Tokyo, Japan) were used at the commencement of the experiments. The animals were housed under controlled lighting (12 h light:12 h darkness cycle, light was turned on at 0600 h) and temperature (22 °C) conditions with free access to food and water. Lactating rats were mated at 7–8 weeks of age and housed singly with their litters at 11–12 weeks of age. The litter size was adjusted to ten pups at birth. The control animals (non-lactating) were virgin, in the diestrous stage of the estrous cycle, and of the same age as the lactating rats. All animals were adapted to eat standard rat powder (CE-2, CLEA Japan Inc.) for more than 10 days.

Catheterization for i.v. injection and blood sampling

The lactating rats underwent surgery at 5 days *post partum*. The animals were anesthetized with i.p. injection of pentobarbital sodium (40 mg/kg body weight (BW)). The tip of a catheter (PE-50, inside diameter 0.58 mm and outside diameter 0.965 mm, Becton Dickinson, Franklin Lakes, NJ, USA) was inserted into the right jugular vein and preceded about 2.5 cm from the inserted point toward the heart.

Cannulation for i.c.v. injection

At the same time, a stainless steel guide cannula (24G) was inserted using the following coordinates from lambda: anterior, 6.5 mm; lateral, –1.3 mm. The guide cannula was held in place with stainless steel screws and dental cement. A stainless steel wire was inserted into the guide cannula to prevent blockage until injection. The rats were allowed to recover from surgery after 5 days. In the preliminary study, we confirmed that the cannulation had no effect on milk production by identifying daily gains of litters. When reagents were i.c.v. administered, the wire was removed from the guide cannula, and an injection cannula, a stainless steel tube (0.31 mm outer diameter) attached with an extension polyethylene tube (PE20), was inserted into the guide cannula aimed at the left lateral ventricle. For injections, the injection cannula was connected to a microsyringe.

Experimental procedures

Experiment 1: diurnal changes of plasma and CSF leptin concentrations in lactating and non-lactating rats

Twenty-four rats were used for each treatment (lactating and non-lactating condition). At each time point (0900, 1500, 2100, and 0300 h), the animals were anesthetized with i.p. injection of pentobarbital sodium (40 mg/kg BW). Blood (2 ml each animal) and CSF (15–20 µl each animal) samples were immediately obtained from the anesthetized animals ($n=6$ at each time) by puncturing the inferior vena and the cisterna magna respectively. Plasma and CSF samples were stored at –80 °C until analyzed.

Experiment 2: effects of i.v. administration of leptin on plasma and CSF leptin concentrations and food intake in lactating and non-lactating rats

Six rats were used for each group (lactating and non-lactating condition). Five days after the surgery, leptin (rat leptin, R&D Systems, Minneapolis, MN, USA) dissolved in saline at 0.06 and 0.12 mg/ml, was i.v. injected into the animals at 0.2 and 0.4 mg/kg BW respectively. Food was removed from cages immediately after the i.v. injections. Two hours later at 1800 h, light was turned off, and food was returned to the cages, and food intake was assessed 4 h into the dark phase (2200 h). Blood samples (2 ml each animal) were obtained from the jugular vein at 1800, 2000, and 2200 h. After the blood sampling at 2200 h, rats were immediately anesthetized, and CSF (15–20 µl) was obtained by puncturing the cisterna magna. The average BWs of lactating and non-lactating rats,

Figure 1 (A) CSF concentrations of leptin at every 6 h (0900, 1500, 2100, and 0300 h) in lactation and non-lactation. Values are the means and vertical lines represent the s.e.m. ($n=6$). (B) Plasma concentrations of leptin at every 6 h (0900, 1500, 2100, and 0300 h) in lactation and non-lactation. Values are the means and vertical lines represent the s.e.m. ($n=6$). Means with different superscript letters are significantly different ($^{a,b}P<0.05$). * $P<0.05$, ** $P<0.01$.

Figure 2 Food intakes in non-lactation and lactation at every 6 h (0300, 0900, 1500, and 2100 h). Values are the means and vertical lines represent the S.E.M. ($n=6$). Means with different superscript letters are significantly different ($^{A,B}P<0.01$, $^{A,C}P<0.01$, and $^{B,C}P<0.05$). $^{**}P<0.01$.

where no difference was observed among three doses of leptin, were 252.4 and 186.6 g respectively. Plasma and CSF samples were stored at -80°C until analyzed.

Experiment 3: effects of i.c.v. administrations of leptin and the leptin antagonist on food intake in lactating and non-lactating rats Six to seven rats were used for each treatment in non-lactating and lactating conditions. On the experimental day, food and water were freely available until 1600 h. In total, 10 μg of leptin (rat leptin, R&D Systems) or 15 μg of leptin triple antagonist (Acris Antibodies, Herford, Germany), dissolved in 10 μl of saline, were i.c.v. injected into the animals at 1600 h. Control rats received saline alone. Food intakes during the nighttime were measured every 4 h at 2200, 0200, and 0600 h, and those during the daytime (0600–1800 h) were also measured.

Analysis of plasma and CSF leptin concentration

Leptin concentrations in plasma and CSF were determined using rat leptin ELISA kit (YK051, Yanaihara Institute Inc., Shizuoka, Japan). The assay sensitivity was 39 pg/ml. The inter- and intra-assay coefficients of variation were 6.2–9.5% and 3.9–4.5% respectively.

Statistical analysis

All data were expressed as a mean with S.E.M. In experiment 1, changes of CSF and plasma leptin levels among different time points within non-lactation and lactation were evaluated using Tukey's test. Statistical significance of difference between non-lactation and lactation at each time point was analyzed by Student's t -test. Changes of food intakes among different time periods within non-lactation and lactation were evaluated using Tukey's test. Statistical significance of difference between non-lactation and lactation during each time period was analyzed by Student's t -test. In experiment 2, changes of CSF and plasma leptin levels and of food intake among different doses within non-lactating and lactating group were evaluated using Tukey's test. Statistical significance of difference between non-lactation and lactation within the same dose group was analyzed by Student's t -test. Changes of plasma leptin concentrations in saline treatment were evaluated among different time periods using Tukey's test. In experiment 3, changes of food intake among different time periods within the same treatment were evaluated using Tukey's test. Statistical significance of difference among treatments in the same time period was analyzed by Tukey's test.

Results

Experiment 1

Figure 1A shows CSF leptin levels in non-lactation and lactation. CSF leptin levels remained constant throughout the daytime (0900 and 1500 h) and nighttime (2100 and 0300 h) in both physiological states. There was no significant

Table 1 Changes in plasma concentrations of leptin (at 1800, 2000, and 2200 h) after i.v. administrations of leptin (0, 0.2, and 0.4 mg/kg body weight (BW) at 1600 h) in non-lactation and in lactation. Values are the means $\pm$ S.E.M. ($n=6$)

Treatment		Saline	Leptin (0.2 mg/kg BW)	Leptin (0.4 mg/kg BW)
Time (h)				
Non-lactation (ng/ml)	1800	0.084 ± 0.004^A	21.912 ± 3.843^B	40.787 ± 5.569^C
	2000	0.105 ± 0.010^D	$1.466 \pm 0.287^{D,E}$	3.528 ± 0.918^F
	2200	$0.113 \pm 0.013^{G,*}$	0.706 ± 0.117^H	1.097 ± 0.090^I
Lactation (ng/ml)	1800	0.084 ± 0.008^a	20.785 ± 4.375^b	36.725 ± 7.330^c
	2000	$0.108 \pm 0.008^{d,*}$	$2.862 \pm 0.931^{d,e}$	3.981 ± 1.462^e
	2200	0.101 ± 0.015^f	0.682 ± 0.045^g	1.103 ± 0.121^h

Means with different superscript within the same row are significantly different in non-lactation ($^{A,B,C}P<0.01$, $^{D,E}P<0.01$, $^{F,G,H,I}P<0.05$, and $^{G,H,I}P<0.01$) and in lactation ($^{a,b,c}P<0.01$, $^{d,e}P<0.05$, $^{f,g,h}P<0.05$, and $^{i,g,h}P<0.01$). Means with asterisk in saline treatment are significantly ($P<0.05$) different from those at 1800 h within non-lactation and lactation.

difference in CSF leptin levels between non-lactation and lactation at each time point. Figure 1B shows plasma leptin levels in non-lactation and lactation. Plasma leptin levels were significantly ($P<0.05$ and $P<0.01$) greater in non-lactation than in lactation at 0900, 2100, and 0300 h. Plasma leptin levels were significantly ($P<0.05$) increased at 2100 h in non-lactation but not in lactation. Food intakes were significantly ($P<0.01$) lower in non-lactation than in lactation during each time period (Fig. 2). Food intakes were significantly ($P<0.01$) elevated during 1500–2100 h and 2100–0300 h in non-lactation but not in lactation.

Experiment 2

In both lactating and non-lactating rats, plasma leptin levels were significantly ($P<0.01$) increased by i.v. infusion of 0.2 mg/kg BW of leptin at 1800 and 2200 h (Table 1). Plasma leptin levels were significantly ($P<0.05$ and $P<0.01$) increased by i.v. infusion of 0.4 mg/kg BW of leptin at all time points (Table 1). There was no significant difference in plasma leptin concentration between lactating and non-lactating rats at the same time point (Table 1). Furthermore, plasma leptin concentrations in saline treatment were significantly ($P<0.05$) greater at 2200 and 2000 h than those at 1800 h in non-lactation and lactation respectively (Table 1). In non-lactation and lactation, plasma leptin levels were dose dependently ($P<0.01$) increased by i.v. injections of leptin (0, 0.2, and 0.4 mg/kg BW) at 2200 h (Fig. 3B).

Figure 3 (A) Effects of i.v. administrations of leptin (0, 0.2, and 0.4 mg/kg BW at 1600 h) on CSF concentrations of leptin 6 h after the administration (at 2200 h) in non-lactation (open bars) and lactation (solid bars). Values are the means and vertical lines represent the S.E.M. ($n=6$). (B) Effects of i.v. administrations of leptin (0, 0.2, and 0.4 mg/kg BW at 1600 h) on plasma concentrations of leptin 6 h after the administrations (at 2200 h) in non-lactation (open bars) and lactation (solid bars). Values are the means and vertical lines represent the S.E.M. ($n=6$). Means with different superscript letters are significantly different in non-lactation ($^{A,B,C}P<0.01$) and in lactation ($^{D,E,F}P<0.01$).

Figure 4 Effects of i.v. administrations of leptin (0, 0.2, and 0.4 mg/kg BW at 1600 h) on 4 h food intakes (at 2200 h) in non-lactation (open bars) and lactation (solid bars). Values are the means and vertical lines represent the S.E.M. ($n=6$). Means with different superscript letters are significantly different in non-lactation ($^{A,B}P<0.05$, $^{A,C}P<0.01$). $^{**}P<0.01$.

However, there was no difference in CSF leptin levels among doses of leptin (Fig. 3A). Moreover, there was no difference in CSF and plasma leptin levels between non-lactation and lactation within the same dose (Fig. 3A and B). Figure 4 shows 4 h food intakes after i.v. injections of 0.2 and 0.4 mg/kg BW of leptin in non-lactation and lactation. In non-lactation, food intakes were significantly ($P<0.05$ and $P<0.01$) decreased by 0.2 and 0.4 mg/kg BW of leptin. In lactation, however, there was no difference in food intakes among three doses of leptin. Food intakes were significantly ($P<0.01$) greater in lactation than in non-lactation within each dose.

Experiment 3

Figure 5 shows the effects of i.c.v. injections of leptin and a leptin antagonist on food intakes in the nighttime (1800–0600 h) and daytime (0600–1800 h) in non-lactation and lactation. In both the physiological states, leptin did not affect food intakes significantly in every time period. In non-lactation, food intake was significantly decreased during 2200 to 0200 h in saline- and leptin-injected animals (Fig. 5A). A leptin antagonist significantly ($P<0.05$) increased food intake during 2200–0200 h and 0600–1800 h. In lactation, food intake was significantly decreased during 0200–0600 h in saline (Fig. 5B). A leptin antagonist significantly ($P<0.05$) increased food intake during 0200–0600 h. Figure 6 shows the effects of i.c.v. injections of leptin and a leptin antagonist on cumulative food intakes after the commencement of feeding in non-lactation and lactation. In non-lactation,

Figure 5 (A) Effects of i.c.v. injections (at 1600 h) of leptin (10 µg) and a leptin antagonist (15 µg) on food intakes in the nighttime (1800–0600 h) and daytime (0600–1800 h) in non-lactation. Values are the means and vertical lines represent the S.E.M. ($n=7$). Means with different superscript letters are significantly different ($^{a,b}P<0.05$, $^{c,d}P<0.05$). * $P<0.05$ versus saline. (B) Effects of i.c.v. injections (at 1600 h) of leptin (10 µg) and a leptin antagonist (15 µg) on food intakes in the nighttime (1800–0600 h) and daytime (0600–1800 h) in lactation. Values are the means and vertical lines represent the S.E.M. ($n=6$). Means with different superscript letters are significantly different ($^{a,b}P<0.05$). * $P<0.05$ versus saline.

cumulative food intakes were significantly ($P<0.05$ and $P<0.01$) decreased by leptin after 8, 12, and 24 h (Fig. 6A). In lactation, cumulative food intakes were significantly ($P<0.05$) decreased by leptin after 24 h (Fig. 6B). Cumulative food intakes were significantly ($P<0.05$) increased by a leptin antagonist after 24 h in non-lactation and lactation (Fig. 6A and B).

Discussion

This study has presented new evidence that the blood–CSF barrier function homeostatically controls CSF leptin levels. Rapid, high-affinity transport system of the choroid plexus mediates leptin uptake across the blood–CSF barrier (Zlokovic *et al.* 2000). In this study, however, the changes in plasma leptin levels were not reflected in CSF leptin levels. In experiment 1, there was no difference in CSF leptin levels between lactating and non-lactating rats, despite that plasma leptin levels were lower in lactating rats than in non-lactating rats at every time point. In experiment 2, CSF leptin levels were not altered, even though plasma leptin levels had been dose dependently elevated by i.v. administrations of leptin. There was no difference in plasma leptin concentrations between lactating and non-lactating rats at the same time point. Short-term fasting decreases circulating levels of leptin in rats (Kmiec *et al.* 2005, Palou *et al.* 2009). Therefore, fasting just before blood sampling may lead to the low leptin levels in

non-lactation. Although the possibility that blood sampling itself affects leptin transport across the BBB and CSF leptin remains, these results suggest that levels of CSF leptin cannot explain the difference in food intake between non-lactation and lactation. On the other hand, lactating rats exhibit a decrease in leptin receptor mRNA expression in the hypothalamus (Brogan *et al.* 2000, Denis *et al.* 2003b). Therefore, the expression of hypothalamic leptin receptors contacting CSF could be involved in the difference in food intake between non-lactating and lactating rats.

Food intakes were temporarily decreased in the nighttime in both non-lactating and lactating states. The temporal decreases in food intake were blocked by i.c.v. administration of a leptin antagonist in both states, suggesting that CSF leptin induced the temporal decrease in food intake in the nighttime. However, the time periods with the temporal decrease in food intake were different between both states. As demonstrated in experiment 1, CSF leptin levels remained constant throughout the day. Therefore, the changes in nocturnal food intake could depend on the expressions of hypothalamic leptin receptors contacting CSF. On the other hand, the i.c.v. administration of leptin reduced 24 h food intake in both lactating and non-lactating rats as has been reported (Schwartz *et al.* 1996, Mistry & Romsos 2002). Therefore, the responsiveness of leptin receptors to CSF leptin may not differ between non-lactating and lactating rats.

We confirmed that plasma leptin levels were consistently lower in lactating rats than in non-lactating rats as has been reported (Denis *et al.* 2003a, Asakuma *et al.* 2004,

Figure 6 (A) Cumulative food intakes from 1800 h after i.c.v. administrations (at 1600 h) of leptin (10 µg) and a leptin antagonist (15 µg) in non-lactation. Values are the means and vertical lines represent the S.E.M. ($n=7$). * $P<0.05$ versus saline. ** $P<0.01$ versus saline. (B) Cumulative food intakes from 1800 h after i.c.v. administrations (at 1600 h) of leptin (10 µg) and a leptin antagonist (15 µg) in lactation. Values are the means and vertical lines represent the S.E.M. ($n=6$). * $P<0.05$ versus saline.

Crowley *et al.* 2004). Low circulating leptin levels would contribute to the hyperphagia of lactating rats. On the other hand, we observed that i.v. leptin administration suppressed food intake in non-lactating rats but not in lactating rats, suggesting that responsiveness to circulating leptin is reduced in lactating rats. A population of hypothalamic ARC neurons directly contacts the circulation and displays increased sensitivity to circulating leptin compared with neurons behind the BBB in the hypothalamus (Faouzi *et al.* 2007). Therefore, depressed sensitivity of these neurons contacting circulating leptin could contribute to the hyperphagia of lactating rats. Chronic prolactin treatment induces central leptin resistance in pseudopregnant rats (Augustine & Grattan 2008), suggesting circulating prolactin may cause the depressed sensitivity of these neurons in lactating rats.

In conclusion, this study has presented new evidence that the hyperphagia of lactating rats (in mid-lactation) could be partly due to depressed sensitivity of neurons contacting blood leptin. In contrast, the responsiveness of leptin receptors contacting CSF leptin may not differ between non-lactating and lactating rats. Furthermore, the levels of CSF leptin remained constant independent of those of blood leptin. Therefore, the expression of hypothalamic leptin receptors contacting CSF could be involved in the difference in food intake between non-lactating and lactating rats. Further research will be required to investigate the expressions and responsiveness of leptin receptors directly contacting blood leptin.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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