Peripheral but not central leptin prevents the immunosuppression associated with hypoleptinemia in rats

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Abstract

Leptin is a peripheral immunoenhancing reagent that directly activates splenic lymphocytes in mice. We found that a 48 h fast in rats resulted in a decrease in serum leptin that was accompanied by a lower delayed-type hypersensitivity (DTH) response. Peripheral leptin replacement completely restored this response in fasted animals. We employed a recombinant adeno-associated virus (rAAV) system to deliver leptin gene directly into rat brain to assess the effect of sustained long-term central expression of leptin on immune responses. The rAAV-leptin rats had elevated central leptin over the 60 day duration of the experiment, whereas body fat and circulating leptin fell to near zero levels. The DTH response was significantly reduced by 10–20% in rats receiving rAAV-leptin compared with the control rats, and the difference was

maintained for over 50 h. When the rats undergoing rAAV-leptin gene therapy were given either murine recombinant leptin or PBS s.c., rats receiving leptin had a 17% higher DTH response than rats receiving PBS. The isolated splenocytes from the former group also proliferated 34% more *in vitro* in response to the mitogen concanavalin A as compared with the latter group. These results suggest that peripheral leptin has a dominant role in maintaining T-cell-mediated immune responses in rats, and central leptin is unable to compensate for the immunosuppression associated with peripheral hypoleptinemia. Furthermore, preservation of normal cell-mediated immune responses does not require fat tissue as long as serum leptin levels are maintained.

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Introduction

Leptin, the *ob* gene product, is one indicator of the quantity of fat storage (Zhang *et al.* 1994, Ahima & Flier 2000). This adipocyte-derived hormone regulates whole body energy balance through its dual effects on food intake and energy expenditure (Friedman & Halaas 1998, Ahima & Flier 2000). More recently, leptin was shown to directly stimulate murine T-lymphocyte immune activity (Lord *et al.* 1998). Considering that leptin is structurally related to the long-chain helical cytokine family, and its receptors are one member of the hematopoietic cytokine receptor family (Fruhbeck *et al.* 2001), it is not surprising that leptin impacts T-cell functions. Diminished leptin levels associated with starvation or malnutrition result in immunosuppression including a reduced delayed-type hypersensitivity (DTH) response and lowered *in vitro* mitogenic proliferation in mice (Lord *et al.* 1998). Peripheral leptin replacement can effectively reverse these immunosuppressive

effects (Lord *et al.* 1998). Leptin administration also protects normal mice from starvation-induced lymphoid atrophy (Howard *et al.* 1999), and chronic leptin deficiency produces decreased T-helper 1 (Th1) cell responses and increased Th2 cell responses (O'Neill 2001). Leptin, therefore, provides one important link between nutritional state and T-cell functions.

The effects of leptin on food intake and energy expenditure are primarily mediated by the central nervous system (CNS) (Campfield *et al.* 1995, Stephens *et al.* 1995, Vaisse *et al.* 1996). Leptin enters specific regions in the brain, in particular the hypothalamus, interacts with its receptors, and subsequently activates specific neuroendocrine pathways (Campfield *et al.* 1995, Stephens *et al.* 1995, Vaisse *et al.* 1996). Evidence also points to direct actions of leptin on some peripheral tissues such as white adipose tissue, liver and pancreas (Shimabukuro *et al.* 1997, Siegrist-Kaiser *et al.* 1997, Ookooma *et al.* 1998). Some of these actions, however, may still require participation of the

CNS (Koyama *et al.* 1998). The direct stimulation of lymphocytes by leptin is presumably mediated by the long-form functional leptin receptor residing on the surface of T cells (Lord *et al.* 1998), but it is not clear whether central effects of leptin also influence T-cell immune responses. Although acute central leptin administration suppresses splenic lymphocyte functions in rodents (Okamoto *et al.* 2000), little is known thus far about how chronic central leptin therapy relates to peripheral immunity.

We previously reported that application of central leptin gene therapy through recombinant adeno-associated virus (rAAV) prevents growth related weight gain in young F344 × BN rats (Scarpace *et al.* 2002). This benign virus containing the rat leptin coding sequence is injected directly into the third ventricle, resulting in a near complete loss of fat mass by 40 days post injection. The disappearance of the fat mass is accompanied by an elevation in cerebral spinal fluid (CSF) leptin due to unabated central leptin transgene expression, whereas serum leptin is correspondingly reduced to below the detection limit because of near zero adiposity level. This unusual scenario, i.e. augmented CSF leptin vs undetectable circulating leptin, has permitted us to address whether peripheral or central leptin is more important in maintaining the T-cell-mediated immune responses.

To address this question, we first examined the DTH response in F344 × BN rats that underwent a 48 h fast with or without peripheral leptin replacement. Secondly, we compared the DTH response of the rats receiving central rAAV-leptin gene therapy with the rats receiving only control vector. Thirdly, we investigated the effect of peripheral leptin supplementation on the DTH response and mitogenic activity of the splenic lymphocytes following central rAAV-leptin-induced hyperleptinorrachia. We found peripheral but not central leptin is essential for maintaining T-cell immune functions in rats.

Materials and Methods

Animals

Three-month-old male F344 × Brown Norway (F344 × BN) rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN, USA) under contract with the National Institute on Aging. Rats were housed individually under controlled temperature with a 12 h light:12 h darkness cycle (0700–1900 h) and allowed free access to a regular laboratory chow and water. The care and use of the animals were in accordance with the principles of the Guide to the Care and Use of Experimental Animals and approved by the local Institutional Animal Care Committee. The animals remained in quarantine for either 2 days before fasting or 1 week before administration of rAAV-leptin.

Preparation of rAAV vectors

The construction and packaging of rAAV vectors were described in detail elsewhere (Zolotukhin *et al.* 1999). Briefly, leptin gene is under the control of the chicken β -actin promoter that is connected to a CMV enhancer. A 'humanized' green fluorescent protein reporter gene is linked to leptin for coordinate expression and detection. The titer of rAAV-leptin vector used in this study was $1.38\text{--}8.28 \times 10^{13}$ physical particles/ml with a ratio of physical to infectious particles of 29.

Administration of rAAV-leptin vectors

Rats were given a single dose ($1.4\text{--}8.5 \times 10^9$ infectious particles in $3\text{--}5 \mu\text{l}$) of either control vector or rAAV-leptin by intracerebroventricular injection into the third cerebral ventricle (Scarpace *et al.* 2002). Rats were handled daily (starting one week before rAAV-leptin delivery and continuing from the time of delivery) for body weight (BW) and food intake measurement. The DTH response experiment was performed 60 days after rAAV-leptin administration.

Rodent fasting

Three groups of rats were included in this experiment and all were mock injected twice daily with PBS for 2 days before initiation of the experiment. Whereas rats in the control group had free access to food, the other two groups were fasted for 48 h during which period they received twice-daily i.p. injections of either PBS for the Fasted-PBS group or recombinant murine leptin ($1 \mu\text{g/g}$ BW) for the Fasted-leptin group. The first i.p. injection was given just prior to food withdrawal. The control rats also received PBS administration in the similar fashion. After 48 h, the fasted rats were allowed free access to food.

Measurement of the DTH response

The DTH response was assessed according to Dhabhar & McEwen (1999). Briefly, rats were sensitized on two consecutive days by applying $100 \mu\text{l}$ 1% (w/v) 2,4-dinitrofluorobenzene (DNFB) (Sigma) in acetone/olive oil (4:1 v/v) to the plucked dorsum ($3 \times 4 \text{ cm}$ area). Five days later, $50 \mu\text{l}$ 0.5% DNFB were applied to the dorsal surface of the right pinnae of all animals while $50 \mu\text{l}$ of vehicle were placed onto the left ear. Ear thickness of the same relative region was measured with a hand-held micrometer at specified times after rats were challenged. The sensitization was performed during the period of fasting in the starvation experiment.

Blood collection from tail vein

Conscious rats were placed in plastic cones. A $\sim 3 \text{ mm}$ piece was removed from the end of the tail with a razor

blade. The rat was held straight up and the tail was hanging naturally. The tail was massaged gently from the base all the way to the tip and the blood collected drop-wise in a 1.5 ml microcentrifuge tube until reaching ~500 µl in volume. A silver nitrate applicator (silver nitrate 75%, potassium nitrate 25%; local distributor) was then used to stop further bleeding from the tail.

Implantation of mini-pumps

Mini-osmotic pumps (Alzet Model 2001; DURECT Corp., Cupertino, CA, USA) were filled with either PBS or murine recombinant leptin in PBS (4 µg/µl) and primed in 0.9% NaCl at 37 °C overnight. The maximum pump volume was 200 µl and the solution was delivered at a rate of 1 µl/h over a 1 week period. Rats were anesthetized with ethrane and the pump was implanted s.c. on the dorsal side.

Lymphocyte proliferation assay (concanavalin A (Con A) response)

A splenocyte suspension was prepared according to Smialowicz (1995) with modifications. Basically, each spleen was placed in 10 ml cold RPMI-1640 medium (Gibco) supplemented with 5% heat-inactivated fetal bovine serum and 50 µg/ml gentamicin (complete medium), and then gently disrupted by pressing through a metal mesh using a flat-bottomed glass rod. Lymphocytes were collected by centrifugation after erythrocyte lysis and several washing cycles, and finally resuspended at 1×10^6 cells/ml in complete medium supplemented with 50 µM β-mercaptoethanol (reduced medium). Flat-bottomed microtiter 96-well plates were set up with either 100 µl/well reduced medium alone for background control, or 50 000 cells/100 µl per well with or without 8 µg/ml Con A mitogen (Sigma). The plates were kept at 37 °C for 72 h in a humidified incubator with 5% CO₂. The CellTiter 96 Aqueous One Solution Reagent (Promega) was then added at 20 µl/well, and the plates were further incubated for 4 h under the same conditions, and the absorbance at 490 nm was recorded using a 96-well plate reader.

Serum leptin analysis

Serum leptin levels were measured using a Linco Rat Leptin RIA kit (Linco Research, St Charles, MO, USA) following the procedures recommended by the manufacturer. The lower detection limit of the assay is 0.5 ng/ml of serum or plasma leptin.

Results

Peripheral leptin prevents fasting-induced immunosuppression

The effect of peripheral leptin replacement in response to fasting was determined in this experiment. By the end of a 48 h fast, the rats had lost ~7.4% of initial BW (Fig. 1).

Figure 1 The effect of a 48 h fast on BW of male F344 × BN rats. Values represent the mean of five or six rats per group ($n=6$, control and Fasted-leptin rats; $n=5$, Fasted-PBS rats). The 48 h fasting period from day 0 to day 2 is indicated. All three groups received i.p. injections of either PBS or leptin (1 µg/g BW) immediately before fasting, and twice daily during the course of fasting, as indicated by the arrows. All the animals were sensitized with DNFB on day 1 and day 2, and then were challenged with DNFB on day 7. (To ensure clarity of the figure, error bars are not included.)

Some experimental procedures, such as ethrane anesthesia and application of the skin irritant DNFB, caused a small degree of weight loss in control rats even though they were allowed to feed *ad libitum* (Fig. 1). Fasting significantly reduced serum leptin to one-quarter of that of the controls (Table 1), whereas twice-daily i.p. injections of recombinant murine leptin (1 µg/g BW) to fasted rats (Fasted-leptin rats) maintained serum leptin level above 10 ng/ml (Table 1). Upon completion of the 2-day fast, the fasted animals resumed free access to food, and the serum leptin levels in these rats correspondingly returned to the same levels as in control rats at the end of the DTH study (Table 1). Following the DNFB challenge on day 7, the control and Fasted-leptin rats showed a similar extent of pinna swelling at both 6 and 24 h post challenge. In contrast, the DTH response of the fasted rats administered PBS (Fasted-PBS rats) was only 10% of that of Fasted-leptin rats at 6 h, although at 24 h, pinna swelling increased to 84% (Fig. 2). If the values of the right pinna thickness of the fasted rats were considered individually, all six Fasted-leptin rats displayed right pinna swelling by 6 h, whereas three out of five Fasted-PBS rats experienced no pinna swelling during the same period, indicating that

Table 1 Peripheral leptin replacement prevents fasting-induced decrease in serum leptin. Data represent the means ± s.e. of six control, six Fasted-leptin and five Fasted-PBS rats. Leptin (1 µg/g BW) was provided twice a day only during the 2-day fasting period to the leptin treatment group

Treatment	Food consumption	Serum leptin (ng/ml)	
		End of the 48 h dieting period	Completion of the DTH study
Control	<i>Ad libitum</i>	2.40 ± 0.26	3.15 ± 0.63
PBS	Fasted	0.59 ± 0.20*	2.08 ± 0.50
Leptin	Fasted	12.27 ± 1.84*	1.44 ± 0.30

**P*=0.0002 for the difference between Fasted-PBS and control rats, *P*=0.0004 for the difference between Fasted-PBS and Fasted-leptin rats, and *P*=0.0001 for the difference with treatments by one-way ANOVA.

diminished serum leptin delayed the onset of the T-cell-mediated DTH response. Taken together, these data suggest that replenishment of circulating leptin during the period of fasting prevents fasting-induced immunosuppression in rats.

Elevation of CSF leptin fails to maintain the T-cell-mediated DTH response

To evaluate whether high central leptin levels can maintain the normal DTH response in the presence of

peripheral hypoleptinemia, we compared the DTH response of the rats given central rAAV-leptin for 60 days with the rats given rAAV control vector. At day 60, in contrast to the plentiful amount of fat mass in all fat depots in the control rats, there was no visible body fat remaining in the rAAV-leptin rats. The serum leptin levels in these rats were less than 0.5 ng/ml (the detection limit of the RIA). In a separate but related study (Scarpace *et al.* 2002), leptin levels in CSF of both the control and rAAV-leptin rats that had undergone gene therapy for the same length were measured after killing. The rAAV-leptin rats had nearly a 2-fold higher CSF leptin (175 ± 25 pg/ml) compared with control rats (100 ± 8 pg/ml). The rAAV-leptin rats, therefore, have central hyperleptinorrachia but peripheral hypoleptinemia. Following a challenge with DNFB, the DTH response of the rAAV-leptin rats was lower throughout the entire 74 h time course post challenge, and the biggest difference amounted to a 10–20% decrease from control rats between 20 and 36 h (Fig. 3). Thus, central hyperleptinorrachia in rats with peripheral hypoleptinemia failed to maintain the T-cell-mediated DTH response.

The immunoenhancing effect of peripheral leptin is not obscured by central hyperleptinorrachia

To determine whether peripheral leptin replacement can enhance T-cell functions following induction of CSF leptin, two groups of rats both undergoing central rAAV-leptin treatment were given either s.c. infusion of PBS (PBS group) or recombinant murine leptin (leptin group). The DTH response and the splenic lymphocyte proliferative response to Con A were then compared between the groups. Again, 60 days of central rAAV-leptin treatment produced a near complete depletion of the body fat and a corresponding decrease in serum leptin to an undetected level (Table 2). However, the s.c. mini-osmotic pump delivery of recombinant leptin raised circulating leptin into the physiological range (Table 2). These data confirmed the effectiveness of the s.c. infusion of leptin at

Figure 2 Peripheral leptin replacement maintains normal DTH response in fasted rats. Data represent the means ± s.e. of five or six rats per group. *P*=0.007 (6 h) and *P*=0.009 (24 h) for difference with treatments by one-way ANOVA. ***P*=0.001 for difference between Fasted-PBS and Fasted-leptin rats at 6 h, and **P*=0.03 for difference between Fasted-PBS and Fasted-leptin rats at 24 h. The DTH response measured at both time points showed no statistical difference between the control and Fasted-leptin rats (*P*>0.05).

Figure 3 Central rAAV-leptin gene therapy results in an impaired DTH response. A 74 h time course of changes in the right pinna thickness of previously sensitized rats is presented. Data represent the means $\pm$ S.E. of four or five rats per group ($n=5$, control rats; $n=4$, rAAV-leptin rats). Rats with central rAAV-leptin treatment displayed a significant decrease in the DTH response compared with rats administered control vector. $P=0.0001$ for difference with rAAV-leptin treatment by repeated measures. $*P<0.05$ for difference between the rAAV-leptin and control rats by post-hoc analysis.

0.1 mg/day in restoring serum leptin levels. After receiving exogenous peripheral leptin replacement, the leptin rats displayed a 17% stronger DTH response at 24 h post challenge compared with the PBS rats (Fig. 4A). Although the unstimulated T lymphocytes from either the PBS or leptin-infused rAAV-leptin rats proliferated to a similar extent (data not shown), the stimulated T lymphocytes of the leptin-infused rats in response to Con A exhibited 34% higher mitogenic proliferation (Fig. 4B). These data demonstrate that peripheral leptin enhances T-cell immune activity in rats that are both fat-depleted and have central hyperleptinorrachia.

Discussion

Leptin is one modulator of immune functions in mice. Lord *et al.* (1998) demonstrated that starvation induces immunosuppression in mice and peripheral leptin replacement restores immune responses. Such studies have not

been reported for rats. Rats weigh substantially more than age-matched mice and lose much less weight for the same fasting period. For example, by the end of the 48 h fast in this study, the F344 $\times$ BN rats lost only $\sim 7.4\%$ of initial BW, whereas the mice used in Lord's study lost close to 30% of initial BW. These animals, therefore, may respond differently to nutritional deprivation and exogenous leptin administration. Nonetheless, our findings are consistent with published data in that fasting in rats induces immunosuppression and peripheral leptin replacement restores T-cell functions. During the period of the first 6 h after challenge in this study, only two out of five Fasted-PBS rats had evidence of pinna swelling, whereas swelling appeared in all six Fasted-leptin rats. By 24 h, however, pinna swelling in Fasted-PBS rats reached $\sim 84\%$ of that of Fasted-leptin rats. Thus, diminished serum leptin associated with fasting in our rat model appears to delay the onset of the DTH reaction rather than alter the magnitude of the response. It is not clear whether this would be the case in mice because only one measurement at 24 h post challenge was reported in the previous study with fasted mice (Lord *et al.* 1998).

The kinetics of the DTH response was apparently different depending upon whether this immune reaction was associated with transient peripheral leptin deficiency or chronic leptin deficiency. In the case of transient leptin deficiency, pinna thickness of Fasted-leptin rats was already substantially greater than that of Fasted-PBS rats 6 h after challenge (Fig. 2). In the case of chronic leptin deficiency (rAAV-leptin rats), however, it was not until 20 h after challenge that there was a significant difference in pinna swelling between the rAAV-leptin rats and controls (Fig. 3). Examination of the physiology of these experimental rats may provide a possible explanation for such a difference in the DTH reaction. The rAAV-leptin rats have stable but near zero serum leptin levels associated with the depletion of body fat, whereas Fasted-PBS rats have only transiently reduced serum leptin levels, and the body fat in these rats is still plentiful compared with the rAAV-leptin rats. Considering these physiological distinctions, it is not hard to imagine that these animals may display DTH responses with different kinetics and magnitude.

Fasting-induced changes in T-cell responses are presumably due to changes in serum leptin. However, when serum leptin levels fluctuate in response to an ever-changing nutritional status of an animal, CSF leptin levels alter accordingly. Thus, it is unclear whether peripheral leptin, central leptin, or both together affect T-cell functions. Furthermore, if both peripheral and central leptin can impact T-lymphocyte functions, are the effects additive, synergistic or opposing, and does one form of leptin, central or peripheral, have a predominant effect over the other? Although this study did not address all these questions, our long-term central rAAV-leptin-treated rats represent an ideal model to address some. In

Table 2 Mini-pump delivery of leptin restores physiological level of circulating leptin. Data represent the means ± s.e. of three PBS and three leptin rats administered rAAV-leptin for 60 days

	Serum leptin (ng/ml)		
	Before rAAV-leptin treatment	rAAV-leptin (60 days)	End of the 7-day leptin or PBS infusion
rAAV-leptin; PBS	2.61 ± 0.95	<0.5*	<0.5*
rAAV-leptin; leptin	1.93 ± 0.68	<0.5*	9.27 ± 2.51

*The 60-day central rAAV-leptin therapy induced a depletion of body fat in both the PBS and leptin rats; as a consequence, the serum leptin levels in these rats were below the detection limit.

Figure 4 Peripheral leptin enhances cell-mediated immune responses in rats with central rAAV-leptin therapy. Data represent the means ± s.e. of three rats per group. Both the PBS and leptin groups had received central rAAV-leptin treatment for 60 days, and were then given either PBS or murine recombinant leptin (0.1 mg/day) by s.c. mini-pump delivery 2 h before the initiation of the immune function study and for the remaining 8 days over the course of the study. The leptin-treated rats showed significant increases in both the DTH response at 24 h post challenge (A) and the degree of lymphocyte proliferation in response to mitogen Con A stimulation (B). **P* = 0.04 (A) and *P* = 0.01 (B) for difference between the leptin and PBS rats by Student's *t*-test.

these rats, CSF leptin is increased by nearly 2-fold over the control animals, whereas serum leptin remains undetected over the duration of the experiment. Such a model allows us to separate, at least partially, the contribution of central and peripheral leptin to the regulation of T-lymphocyte functions.

There are three salient findings from this study. First, T-cell functions correlate with peripheral leptin levels. When serum leptin is reduced to below the detection limit, the DTH response correspondingly diminishes. When serum leptin is replenished to physiological levels by leptin replacement, both the DTH response and mitogenic activity of splenic lymphocytes are enhanced. Secondly, elevation in CSF leptin fails to maintain T-cell

immune responses. The near 2-fold higher CSF leptin is unable to compensate for the immunosuppression associated with fat depletion and peripheral hypoleptinemia. This observation suggests that either central leptin has little effect on lymphocyte functions, or its effect, whether positive or negative, is trivial compared with the predominant immunoenhancing effect of peripheral leptin. Thirdly, the maintenance of normal cell-mediated immune responses does not require the presence of fat tissues. T lymphocytes are stimulated with peripheral leptin replacement in the rAAV-leptin-treated rats that have basically no body fat. This last finding is rather unexpected. It points to the potential clinical application of using leptin supplementation to preserve a normal immune system in human subjects who experience a substantial loss of body fat resulting in diminished serum leptin. In summary, our data indicate that peripheral rather than central leptin is necessary to maintain T-cell-mediated immune responses.

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