

Acquired leptin resistance by high-fat feeding reduces inflammation from collagen antibody-induced arthritis in mice

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Abstract

Objectives

Rheumatoid arthritis (RA) patients with high body mass index (BMI) show lower mortality than thinner patients, indicating a paradoxical effect of body mass on mortality in RA. We considered that leptin might play some part in this mechanism. Leptin regulates not only body weight, but also inflammatory processes. Furthermore, hyperleptinemia decreases sensitivity to leptin (leptin resistance). This study examined whether high-fat diet-induced hyperleptinemic obese mice with acquired leptin resistance show reduced inflammation induced by collagen antibody-induced arthritis (CAIA).

Methods

Diet therapies were induced in mice by exposure to 50% fat to obesity for 6 weeks. We examined serum leptin concentrations and leptin responses after 6 weeks and induced CAIA. Leptin effects were examined by intraperitoneal (IP) or intracerebroventricular (ICV) leptin administration after CAIA. Hindpaw swelling was monitored daily. Histopathological features were also determined at sacrifice.

Results

Serum leptin concentrations were approximately 5-fold higher than in normal mice. IP leptin did not inhibit food intake, but ICV leptin did. Obese mice thus acquired peripheral leptin resistance. Arthritis was reduced approximately 30% compared with normal controls and was not exacerbated by IP leptin injection, but ICV leptin injection exacerbated arthritis to levels equal to normal controls. Histopathological assessment showed that cartilage damage was reduced by 76% compared to normal controls.

Conclusion

High-fat diet-induced obese mice acquired peripheral leptin resistance reducing the development of CAIA. Leptin sensitivity was associated with severity of arthritis. These results suggest that RA patients with high BMI who acquire leptin resistance may show reduced inflammation. However, the real function of leptin in the immune system remains partly unclear.

Key words

leptin resistance, collagen antibody-induced arthritis, rheumatoid arthritis, obesity

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Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease associated with increases in overall mortality as compared with people without RA (1). One of the most frequent causes of death in RA patients is cardiovascular disease (CVD) due to the development of premature, accelerated atherosclerosis (2, 3). As the risk of CVD is lower in RA patients treated with tumour necrosis factor blockers (4), inflammatory mechanisms may contribute to this increased cardiovascular risk. In the general population, total mortality and cardiovascular mortality reaches the highest rates among individuals with the highest body mass index (BMI) (5), but RA patients with high BMI show lower mortality than thinner RA patients (6). Body mass thus has a paradoxical effect on mortality in RA. The mechanisms protecting high BMI patients with RA from death are unclear, but adipokines might play a role in this phenomenon (7).

Leptin is a peptide hormone secreted by adipocytes (8, 9) and relayed to the central nervous system, where it acts to decrease appetite. Serum leptin concentrations are increased in obesity and circulating levels of leptin directly correlate with body fat mass. Although leptin-deficient ob/ob mice show severe obesity, the majority of obese humans do not have ob gene mutations. Most obese subjects have lost weight-reducing effects of leptin. The suggestion has thus been made that obese individuals acquire resistance to the anorectic effects of leptin (10, 11). Furthermore, several researchers have reported that consumption of a high-fat diet by obese rodents with hyperleptinemia resulted in the development of leptin resistance (12, 13).

Leptin has also been reported to promote T-helper (Th) 1 polarisation of cellular immune responses (14, 15) and can affect the proliferation and cytokine production by T-cells. Therefore, leptin appears to contribute to inflammation by regulating immune responses. The few studies have revealed some link between leptin concentrations and inflammation (7, 16, 17). Furthermore, Busso *et al.* demonstrated reduced se-

verity of antigen-induced arthritis in leptin-deficient ob/ob mice (18). However, whether any role played by leptin in leptin-resistant obesity is linked to changes in the immune system remains unclear. We have therefore adopted high-fat diet-induced obese mice that acquire leptin resistance as a model for examining the underlying causes of human obesity. We examined the effects of leptin resistance on collagen antibody-induced arthritis (CAIA) in these model mice.

Materials and methods

Animal and obesity induction

Seventy-two female C57BL/6J mice were obtained from SLC Laboratories (Shizuoka, Japan). Mice were housed 4–6 per cage in a temperature-controlled room with a 12-hour light/dark cycle. Mice were allowed *ad libitum* access to water and food. At 4 weeks old, to induce obesity, 44 mice were provided with a high-fat diet containing 10.8% protein, 50.3% fat, 38.9% carbohydrate and a total calorie content of 518 kcal/100 g (19) manufactured by Oriental Yeast (Tokyo, Japan). The remaining 28 mice were provided with a normal diet (Clea Rodent Diet CE-2; Clea Japan, Tokyo, Japan) containing 29.7% protein, 11.5 % fat, 58.8% carbohydrate and a total calorie content of 342 kcal/100 g as normal control. Both groups of mice were initially fed a standard diet (CE-2) before starting each diet therapy. All mice were maintained on the allotted diet for 6 weeks, and body weight was measured at the end of every week. Use of animals for all experiments was in accordance with the Guide for Animal Experimentation of Osaka City University.

Leptin reactivity after induction of obesity

After the 6-week feeding period, small blood samples were collected from the tail vein for measurement of serum leptin levels (mouse leptin ELISA; R&D Systems, Minneapolis, MN, USA). To evaluate the development of leptin resistance, body weight changes in mice were measured after intraperitoneal (IP) or intracerebroventricular (ICV) injection of leptin.

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The other co-authors have declared no competing interests.

Twenty-four mice in each diet group were divided into 4 subgroups ($n=6$ /subgroup). Two subgroups from each diet were administered either ICV injection or IP injection of leptin. The remaining two subgroups were administered phosphate-buffered saline (PBS) by each route as controls. The injection scheme for each group was as follow: ICV group, 5 μ g of leptin in 2 μ l of PBS or 2 μ l of PBS alone; and IP group, 10 μ g of leptin in 200 μ l of PBS or 200 μ l of PBS alone. The leptin used was recombinant murine leptin (R&D Systems). Mean body weight did not differ between diet subgroups before injection. Body weight was measured at 4, 8 and 24 h after leptin injection. To allow ICV injection, 24 h before injection mice were anaesthetised with diethylether and holes were bored into the right cranial bone at 1.0 mm lateral and 1.0 mm posterior from the bregma using a 0.5-mm steel drill. We then injected leptin or PBS with a 33-G internal cannula 2.5 mm in length attached to a micro-syringe (Hamilton, Reno, NV, USA) under sterile conditions.

Induction of CAIA and leptin administration

After the 6-week feeding period, 20 obese mice were then divided into 5 subgroups ($n=4$ /subgroup). Arthritis was induced in 4 subgroups, with 2 subgroups receiving IP injection of leptin or PBS and the other two subgroups receiving ICV injection of leptin or PBS. The remaining group was used for observational controls, so arthritis was not induced. As controls, arthritis was induced in normal-diet mice ($n=4$) without additional injection of leptin or PBS. All mice were assessed by daily monitoring of body weight and joint swelling. To induce arthritis, animals in the four obese subgroups and the one normal-diet subgroup received IP injection with 3 mg of a cocktail of four different type II collagen antibodies (CAIA system; Chondrex, Redmond, CA, USA) on days 0 and 1 and 50- μ g lipopolysaccharide stimulation on day 3. Obese mice were administered leptin or PBS by ICV injection (5 μ g of leptin per body in 2 μ l of PBS or 2 μ l of PBS alone) or by IP injection (1 μ g/g body

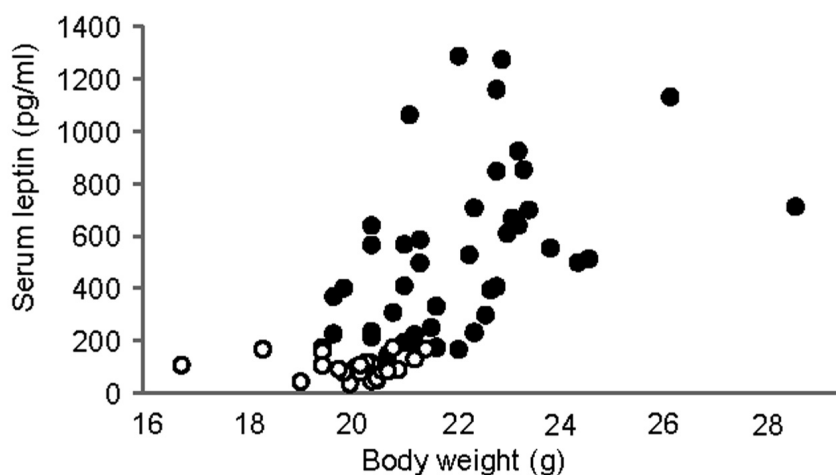


Fig. 1. Serum leptin concentration.

Serum leptin concentration in normal mice ($n=28$ ○) and obese induced mice ($n=44$ ●) after the 6-week feeding period. Simple linear regression analyses of obese group indicated that serum leptin levels correlated positively with body weight ($r=0.48$, $p<0.01$). Normal mice showed no correlation between leptin levels and body weight ($r=0.006$, $p=0.975$). Similar results were obtained in independent three experiments.

weight of leptin in 200 μ l of PBS or 200 μ l of PBS alone). Leptin injections were started on day 2 and continued for 6 days. The animals were sacrificed on day 11 after inducing CAIA. Severity of arthritis was determined by examining each paw every day and assigning a score of 0 to 4 according to the Terato criteria (20), with some modifications: 0=no swelling; 1=redness and swelling of any digits or mild redness and swelling of ankle or wrist; 2=moderate to severe redness and swelling of the ankle and wrist; 3=redness and swelling of entire foot including the digits; 4=maximally inflamed limbs. Each limb was graded, and grades were totaled to yield the arthritis score for each animal (maximum possible score, 16 per animal).

Histopathological analysis

Mice were sacrificed by exsanguination under general anesthesia on day 11 after inducing CAIA. Knee joints were removed and then fixed in buffered formalin, decalcified, embedded in paraffin, sectioned, and stained with toluidine blue. Histological changes were scored based on cartilage proteoglycan depletion as determined using toluidine blue staining. Cartilage destruction was scored on a scale from 0 to 3, ranging from fully stained cartilage to destained cartilage or complete loss of articular cartilage (21). His-

topathological changes in knee joints were scored in the patella/femur region on 5 semi-serial sections of the joint. We have performed those experiments more than three times at least.

Statistical analysis

Statistical analyses were performed using StatView version 5.0 software (SAS Institute, Cary, NC, USA). Body weight differences between groups at each diet period (0–6 weeks) were analysed using the Mann-Whitney U-test, with values of $p<0.05$ considered significant. Linear regression analyses were performed to determine associations between leptin concentration and body weight. Chronological changes were analysed using analysis of variance (ANOVA) and post hoc pair-wise comparisons of mean differences using the Scheffé test, with values of $p<0.05$ again considered significant.

Results

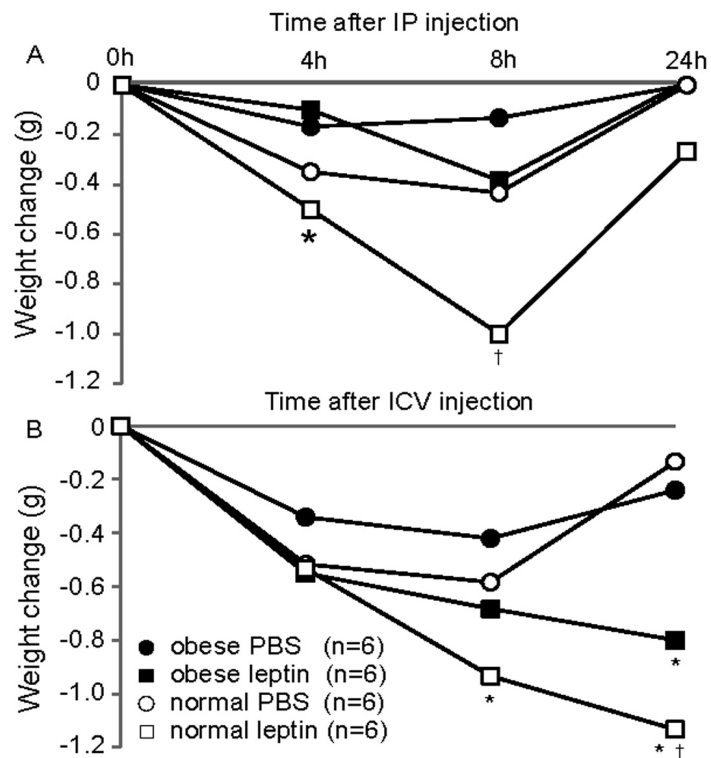
Body weight and leptin concentration

Obese mice showed a significantly higher body weight than normal mice from week 1 to week 6 ($p<0.05$). After the feeding period, mean body weight was 22.0 g in obese mice and 18.7 g in normal mice. Serum leptin concentrations were approximately 5-fold higher in obese mice (530 ng/ml) than in normal mice (107 ng/ml) and simple

Fig. 2. Acquisition of leptin resistance.

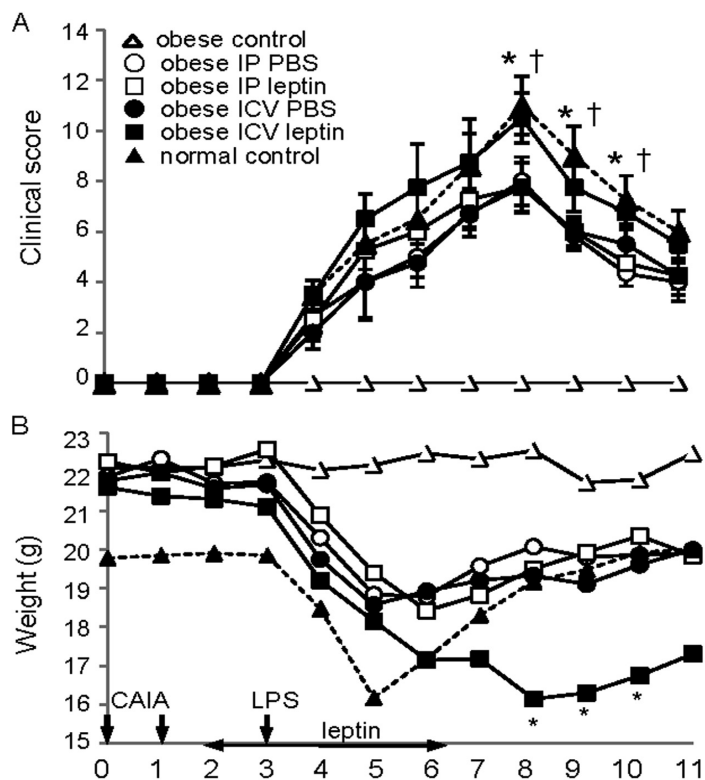
A) Effect of peripheral leptin administration on normal and high-fat diet mice. * $p < 0.05$, † $p < 0.01$ comparing other three groups.

B) Effect of central leptin administration on normal and high-fat diet mice. * $p < 0.05$ comparing obese and normal PBS subgroups; † $p < 0.05$ comparing obese leptin group. Data are presented as means. Obese PBS, mice fed high-fat diet followed by PBS injection; obese leptin, mice fed high-fat diet followed by leptin injection; normal PBS, mice fed normal-fat diet followed by PBS injection; normal leptin, mice fed normal-fat diet followed by leptin injection ($n = 6$ per subgroup). Experiments were done four times with similar results.

**Fig. 3.** Clinical score of arthritis and body weight changes after collagen antibody-induced arthritis.

A) Severity of clinical signs by Terato score. ICV leptin injections exacerbated arthritis to a level equal to normal controls. * $p < 0.05$ normal control vs. three subgroups (obese IP PBS, obese IP leptin, obese ICV PBS); † $p < 0.05$ obese ICV leptin vs. three subgroups (obese IP PBS, obese IP leptin, obese ICV PBS).

B) Changes in body weight after arthritis. The obese ICV leptin subgroup showed continued reductions in body weight during leptin injection and had significantly lower weight than other obese groups at days 8–10. * $p < 0.05$ obese ICV leptin vs. other groups except normal control. Obese IP leptin or PBS; obese mice with arthritis followed by IP leptin or PBS injection. Obese ICV leptin or PBS; obese mice with arthritis followed by ICV leptin or PBS injection. Normal control; mice fed a normal-fat diet with arthritis. Similar results were obtained in independent three experiments.



linear regression analyses of the obese group indicated that serum leptin levels correlated positively with body weight ($r = 0.48$, $p < 0.01$; Fig. 1). Normal mice group did not show that correlation between leptin levels and body weight

($r = 0.006$, $p = 0.975$) (Fig. 1). There were mice that showed similar body weights (in the range of 19–21 g) and different concentrations of leptin. It might be explained by the differences in the rate of fat tissue.

Effect of leptin response on body weight

After the 6-week feeding period, obese mice showed no leptin effects on body weight following IP injection compared with PBS, but normal

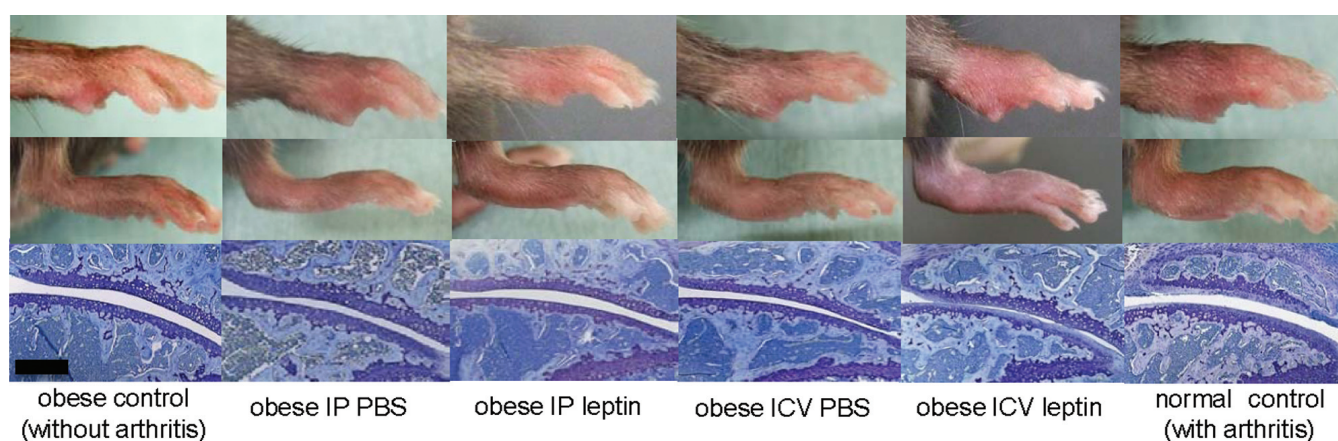


Fig. 4. Inflammation and cartilage destruction.

Representative photographs of the forepaws (top) and hindpaws (middle) on day 8 and histological examinations of knee joints harvested on day 11 (bottom). Normal control and obese intracerebroventricular (ICV) leptin groups show severe redness and swelling of the entire foot, including the digits. Obese IP PBS, leptin and obese ICV PBS subgroups show mild redness and swelling of paws. Knee joints of obese control mice: fully stained cartilage layers show no loss of proteoglycans. Knee joints of arthritic normal mice: note severe joint inflammation and complete loss of toluidine blue staining of the cartilage layers. Obese mice treated with ICV leptin: loss of matrix proteoglycan can still be seen. Knee joints of obese mice treated with intraperitoneal (IP) or ICV PBS: marked reductions in joint inflammation and matrix proteoglycan. Toluidine blue staining, bar represents 250 mm. Obese controls were high-fat diet obese mice group in which arthritis was not induced. Similar results were obtained in independent three series of studies.

mice showed a 2.7% reduction in body weight ($p < 0.01$) 8 h after injection of leptin (Fig. 2A). After ICV injection of leptin, obese mice showed a significant reduction (2.5%, $p < 0.05$) in body weight at 24 h (Fig. 2B), while normal mice showed 2.0% ($p < 0.05$) and 5.2% ($p < 0.001$) reductions at 8 and 24 h, respectively (Fig. 2B). IP injection of leptin showed significant effects on body weight reduction only in normal mice. Conversely, ICV injection of leptin was effective in producing body weight reductions for both normal and obese mice. These results indicate that obese mice on a high-fat diet for 6 weeks developed resistance to peripherally administered leptin, but remained responsive to leptin delivered directly to the central nerve system.

Effects of leptin resistance on arthritis severity

Injection of the CAIA cocktail on days 0 and 1 and LPS on day 3 resulted in disease onset on day 4 with marked swelling or redness of the limb joints in normal control mice. Arthritis peaked on day 8 (Fig. 3A). In the C57BL/6 strain, as low responders to CAIA, a 6-mg dose of cocktail was able to maximally induce moderate arthritis. Arthritis in normal control mice progressed on day 8, with a mean (\pm standard deviation) score of 11.0 ± 1.2

(Fig. 3A). In contrast, arthritis score decreased in obese mice following IP injection of PBS, with a mean score on day 8 of 8.0 ± 1.0 ($p < 0.05$ vs. normal control; Fig. 3A). Obese mice with IP injection of leptin showed no exacerbation of arthritis score on day 8 compared to obese mice with IP injection of PBS (7.8 ± 1.0 vs. 8.0 ± 1.0 ; $p = 0.99$; Fig. 3A). Obese mice that received ICV injection of leptin showed a 26% exacerbation of peak score compared with obese mice treated with ICV injection of PBS (10.5 ± 1.0 ; $p < 0.05$; Fig. 3A). Peak score in obese mice receiving ICV leptin reached a level similar to that of normal controls (Fig. 3A). Actual photographs of paws in each group on day 8 are presented in Figure 4.

The effect of leptin was also assessed by daily monitoring of body weight in all groups. At the initiation of arthritis, no significant differences in body weights were seen for the 5 obese subgroups. No significant differences in change of body weight were seen between IP injection of leptin or PBS during arthritis (Fig. 3B). However, mice with ICV injection of leptin continued to show reductions in body weight during leptin injection and displayed significantly lower weight than mice in other obese groups on days 8–10 ($p < 0.05$; Fig. 3B).

We then examined the histological fea-

tures of arthritic knee joints on day 11. Radiographs of knee joints in all mice showed no joint space narrowing, bone erosion, or severe cartilage destruction (data not shown). Mild joint inflammation and complete loss of toluidine blue staining of the cartilage layers was seen in normal control mice (Fig. 4). Loss of matrix proteoglycan could also still be seen in obese mice treated with ICV leptin, while marked reductions in swelling and redness and in matrix proteoglycan were identified in obese mice treated with IP or ICV PBS. The damage score for cartilage after arthritis was significantly higher in normal mice (1.58 ± 0.13) than in obese mice for both IP injection groups (PBS, 0.37 ± 0.11 ; leptin, 0.34 ± 0.14) and the ICV injection of PBS group (0.32 ± 0.23 ; $p < 0.01$). Scores for mice with ICV injection of leptin were equal to those for normal mice (Fig. 5).

Discussion

Leptin is one of the adipocytokines, produced mainly by adipocytes and controlling body weight through a hypothalamic relay activating the sympathetic nervous system (8, 22, 23). Leptin can also modulate the T-cell immune response and increase inflammation, as a result of enhanced production of pro-inflammatory cytokines (Tumour necrosis factor-alpha

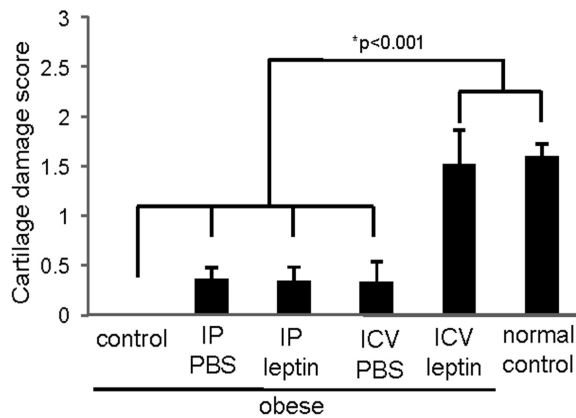


Fig. 5. Histopathological score of arthritis after collagen antibody-induced arthritis.

Histopathological score for arthritis. Histopathological scores for arthritic knee joints were determined from mice sacrificed on day 11. * $p < 0.001$ normal control and obese ICV leptin vs. other groups. Obese IP leptin or PBS; obese mice with arthritis followed by IP leptin or PBS injection. Obese ICV leptin or PBS; obese mice with arthritis followed by ICV leptin or PBS injection. Normal control; mice fed a normal-fat diet with arthritis.

Interleukin-1beta, interleukin-6) (24, 25). Although effects of leptin on the immune system use both central and peripheral pathways, central nervous system is inherent to integrate information from throughout the organism and mainly control immune function (26, 27). In our pilot study, severity of CAIA in leptin-deficient ob/ob mice was significantly reduced compared with wild-type mice and it did not contradict Busso's report (18). Furthermore, peripheral administration of leptin to ob/ob mice being induced CAIA exacerbated arthritis and central administration of leptin also exacerbated arthritis (unpublished data). We confirm that leptin may regulate some part of the immune system by central pathways using hypothalamic relays in antigen-induced arthritis models.

We demonstrated that obese mice with hyperleptinemia after high-fat feeding showed reductions in the inflammation induced by CAIA. These results are paradoxical given the efficacy of leptin on immune system functions described in previous reports (18). Most obese individuals are not leptin-deficient, but actually show increased serum leptin concentrations (28). Large amounts of leptin are thus not signaling sufficiently to maintain a healthy weight. Leptin-deficient ob/ob mice exhibit the immunological changes, *i.e.* thymic atrophy and significantly decreased maturation of T- and B-cells (29-31). Previous studies (32-34) had reported that human obesity and diet-induced obese mice induced atrophy of lymphoid tissues such as thymus and spleen and decreased the number

of circulating lymphocytes despite of elevations in serum leptin. Therefore, impaired leptin sensitivity in obesity is related to the change of sensitivity of immune system. Leptin hyporeactivity may have several possible underlying mechanisms. The first part of the feedback loop to fail in the so-called leptin resistance may be due to defective transport of serum leptin across the blood-brain barrier (BBB) and an inability of leptin to reach target sites in the hypothalamic arcuate nucleus (11). Peripheral leptin resistance is defined as a case of a mouse that does not respond by peripherally administered leptin but preserves central leptin sensitivity. Central leptin resistance is defined as a case of a mouse that does not respond by central administered leptin. Indeed, IP leptin administration in obese mice results in no change to body weight, whereas ICV injection results in weight loss (Fig. 2A, B) (10). The BBB appears to be a site for leptin regulation and resistance. C57BL/6J mice are obesity-prone strains by high-fat feeding. Furthermore, Prpic *et al.* demonstrated the model mice with peripheral leptin resistance that C57BL/6J mice fed for 4 or 8 weeks became unresponsive to peripheral leptin administration but responded to central leptin at these time points (35). The present study also found that arthritis in hyperleptinemic obese mice on a high-fat diet was only affected by ICV leptin administration. In obese mice, leptin does not seem to reach target sites within the brain to allow sufficient function. Peripheral leptin resistance in obese mice might

thus reduce the inflammation induced by CAIA compared with that in normal mice and systemic administration of leptin did not increase arthritis severity. However, central administration of leptin exacerbated arthritis and cartilage damage. Leptin sensitivity might thus be related to the progression of arthritis in the same manner as effects on food appetite. Leptin may regulate the immune system by hypothalamic relay similar to body weight control (36).

A limitation of the study was that the arthritis score and histologic damage score for all animals were not very high even in the control group. C57BL/6J mice are obesity-prone strains by high-fat feeding, but this strain is resistant to CIA by an ordinary immunisation schedule, and low responder to CAIA. It is imperative to use mice between 7-8 weeks (not more than 12 weeks) of age for CAIA model. We used 10-11-week-old mice in this study, because mice needed feeding periods for 6 weeks. Thereby, all mice were not able to induce high arthritis and persist of the response; the damage score for all mice are also not so high.

High-fat diet-induced obese mice showed decreased sensitivity or resistance to leptin and inhibited progression of inflammation. Leptin sensitivity was associated with severity of arthritis. These results suggest that RA patients with high BMI who acquire leptin resistance may show reduced inflammation. However, the real function of leptin in the immune system remains partly unclear.

A few studies have revealed some link between leptin concentrations and inflammatory markers (7, 37), and thus have not considered sensitivity to leptin. However, the notion of leptin resistance gives rise to different interpretations, and the complex nature of any potential effects would result in several definitions in human models. Mechanisms of leptin signalling beyond the brain remain unclear. Further studies are required to clarify the contribution of the sympathetic pathway or reveal other unknown mechanisms. Longitudinal studies are needed to clarify the potential influence of leptin on disease outcomes.

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