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Splenic Denervation Blocks Leptin-Induced Enhancement of Humoral Immunity in Siberian Hamsters *(Phodopus sungorus)*

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Photoperiod · Adrenal · Energy balance · Catecholamines · Spleen · Neuroimmune interactions · Hamster · Splenic denervation

Abstract
Nontropical rodents have evolved adaptations to maximize winter survival, including alterations in reproduction, energy balance and immunity. Short-day-housed Siberian hamsters display reductions in body fat and decreases in humoral immunity compared with long-day hamsters. The hormone leptin, secreted by adipose tissue, varies in response to changes in body fat and has been implicated in photoperiodic changes in immunity. In addition, the metabolic effects of this hormone appear to be mediated by the sympathetic nervous system (SNS). Very little is known, however, regarding the role of the SNS in regulating the effects of leptin on immunity. The goal of the present study was to examine the effects of splenic denervation on leptin-induced immune enhancement of short-day Siberian hamsters. Male hamsters were housed in long (LD 16:8) or short days (LD 8:16) for 10 weeks. Half of the animals in each photoperiod received surgical denervations of the spleen; the remaining animals received sham operations. In addition, animals in each group were implanted with osmotic minipumps containing either leptin or vehicle. Hamsters were then injected with keyhole limpet hemocyanin (KLH) and serum anti-KLH antibody production was assessed. Short-day hamsters displayed decreased humoral immunity in short versus long days; leptin attenuated the short-day decrease but did not enhance immunity of long-day hamsters. Furthermore, splenic denervation blocked the leptin-induced increase in immunity in short-day hamsters. Collectively, these data suggest that leptin plays an important role in regulating seasonal changes in humoral immunity of Siberian hamsters and the effects of leptin occur, at least in part, via changes in the SNS innervation of lymphoid tissue.

Introduction

Animals have evolved specific adaptations to maintain a balanced energy budget [1]. Maintaining a balanced energy budget, however, becomes challenging during winter when food supplies dwindle despite increasing thermoregulatory demands. Non-tropical animals have evolved specific adaptations to cope with winter energy shortages and these adaptations serve to partition energy

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to biological functions where it is needed most [1]. During winter most available energy is partitioned into thermoregulation and maintenance as opposed to growth, reproduction or other nonessential physiological functions. In addition to these well-known energetically demanding biological processes, the immune system also requires energy to maintain functioning. Cytokine activation elevates body temperatures and the energy requirements of inflammation and acute phase immune response likely increases metabolic rates in excess of 10% per 1°C of body temperature elevation [2]. The process of generating an antibody response to a non-inflammatory antigen also requires energy; house mice *(Mus musculus)* injected with keyhole limpet hemocyanin (KLH) display a ~40% increase in oxygen consumption and metabolic heat production [3]. Because total energy availability is finite, mounting an immune response requires using resources that could otherwise be allocated to other biological functions [4–6]. As a result, immune function, like other biological processes, should be 'optimized' so that individuals can tolerate small infections if the energetic costs of mounting an immune response outweigh the benefits. If prolonged energy shortages continue to deplete energy stores, then immune function can be suppressed to the point where survival may be compromised. Trade-offs among competing energetic demands exist, and strategies for allocation of energy to competing needs vary according to an individual's life history strategy, as well as other intrinsic and extrinsic factors [6].

The precise mechanisms by which energy availability is translated into a physiological signal indicating current energy balance are not fully understood. In the past few years alone, however, a variety of endocrine factors have been identified as potential candidates signaling energy availability (reviewed in [7]). One likely candidate is the peptide hormone leptin (Ob protein). Leptin is produced primarily by adipose tissue [8] and is released in direct proportion to body fat; thus, circulating leptin concentrations are a relatively accurate reflection of total body fat [7, 9]. In general, fasting reduces leptin concentrations, whereas exogenous leptin treatment reduces food intake [10]. Although some of the effects of leptin on body weight are due to the effects of this hormone on food intake, an increasing number of studies suggest that the actions of leptin on energy balance are due, at least in part, to activation of the sympathetic nervous system (SNS) and subsequent increases in metabolism [11–14]. For example, exogenous leptin treatment in rats increases gene expression of uncoupling protein 2 (UCP2) and UCP3, proteins expressed predominantly in adipose tissue that are involved in the regulation energy metabolism [14]. Surgical denervation of brown adipose tissue (which removes neural inputs to this tissue), however, blocks the effects of leptin on UCP2 and UCP3 gene expression [14]. Furthermore, stimulation of the leptin system increases sympathetic activity in a variety of peripheral tissues [13]. These results suggest an important role of the SNS in regulating the metabolic effects of leptin.

In addition to its important role in energy balance, a link has been established recently between leptin and immune function. For example, mice that are deficient in the production of leptin (ob/ob mice) or lack functional leptin receptors (db/db mice) due to genetic defects display excessive obesity but impaired cell-mediated immunity [15]. Leptin appears to differentially regulate the proliferation of naive and memory T cells. Specifically, exogenous leptin added to T cell cultures enhances proliferation in response to allogenic stimulator cells in both naive and memory T cell types, but the effect of leptin is significantly more pronounced in naive compared with memory T cells [15]. These results are particularly intriguing given that these mice have an excess of energy stored as adipose tissue but abnormal leptin signaling pathways. Leptin also appears to regulate the actions of several cytokines that are involved in proinflammatory immune responses [16–19]. Furthermore, exogenous treatment with leptin counteracts the immunsuppressive effects of starvation [15]. Collectively, these results suggest that leptin is a likely candidate to mediate the interactions between energy balance and immune function.

Our laboratory has been investigating the role of energy balance in immune function of Siberian hamsters *(Phodopus sungorus)*. Siberian hamsters, like other non-tropical mammalian species, are seasonally breeding rodents that breed during the long days of summer but curtail reproduction during the short days of winter. In the laboratory, Siberian hamsters housed in short 'winter-like' photoperiods display significant reductions in body mass and inhibit reproductive functions compared with long-day-housed hamsters [20, 21]. Recently, it has been shown that short-day hamsters also display marked reductions in humoral immunity compared with long-day animals [22–24]. In addition, short-day reductions in humoral immunity appear to be due to decreased leptin concentrations; exogenous administration of long-day-like concentrations of leptin blocks short-day decreases in immune function and restores immunity to the level of long-day hamsters [9]. Exogenous leptin, in contrast, has no effect on immune function of long-day-housed hamsters. These results suggest that short-day suppression of hu-

moral immunity is due to reduced leptin concentrations [9]. Recently, it has been demonstrated that, similar to leptin's role in regulating energy balance, the effects of leptin on immune function may also involve the SNS [25]. In contrast to studies demonstrating leptin-induced enhancement of immunity, central leptin administration appears to *reduce* splenic cell-mediated immune function in mice [25]. Specifically, intracerebroventricular administration of leptin to mice reduces splenocyte proliferation in response to the T-cell mitogen concanavalin A (Con A). This effect, however, is abolished by surgical denervation of the spleen [25]. Despite these intriguing results, very little is known about the potential role of the SNS in mediating leptin-induced changes in immune function.

The goal of the present experiment was to test the hypothesis that the SNS is important for the enhancement of humoral immunity seen after exogenous leptin administration in short-day-housed Siberian hamsters. Specifically, if SNS innervation of the spleen is necessary for leptin-induced immune enhancement, then surgical denervation of the spleen should block the increase in humoral immunity seen in short-day-housed Siberian hamsters. If the biochemical actions of leptin on immune function are independent of the SNS, however, then exogenous leptin should increase humoral immunity in both splenic denervated and intact short-day hamsters.

Materials and Methods

Animals and Housing Conditions

Fifty-six adult (> 60 days of age) Siberian hamsters *(P. sungorus)* were obtained from the breeding colony maintained at Georgia State University and were housed individually in polypropylene cages (40 × 20 × 20 cm) in colony rooms with a 24 h LD 16:8 cycle (lights on 06.00 h EST). Temperature was kept constant at 20 ± 2°C and relative humidity was maintained at 50 ± 5%. Food (Purina Rat Chow) and tap water were available ad libitum throughout the experiment. All animals were treated in accordance with the Georgia State University Institutional Animal Care and Use Committee.

Splenic Denervations

Prior to the start of the experiment, a subgroup of animals (n = 28) received surgical denervation of the spleen according to the method of Demas et al. [22] modified from Williams et al [26], whereas the remaining animals received sham denervations. Briefly, a small incision was made on the left dorsal surface of the animal. The underlying musculature was cut and the spleen was visualized and carefully inverted. The splenic nerve bundle innervating the spleen was identified under a dissecting microscope and carefully teased away from the nearby vasculature. Every effort was taken to leave the blood vessels intact. The nerve was cut in two locations with a pair of microscissors; the spleen was irrigated with sterile 0.9% saline, and returned to the body cavity. The muscle layer was then sutured, the skin was closed, and nitrofurozone antibacterial powder was applied to the incision. Animals were allowed to recover from surgery for 2 weeks and then transferred to their respective photoperiods. We have previously demonstrated that this surgical denervation technique consistently results in marked (~ 90%) reductions in splenic NE content, as determined by high pressure liquid chromatography [22].

Experimental Methods

After a 2-week recovery period, half of the surgically denervated hamsters (n = 14) and half of the sham-operated animals (n = 14) were selected randomly and transferred to a colony room with a short-day (LD10:14) photoperiod. The remaining animals (n = 28) were maintained in long days for the duration of the experiment. Animals were kept in their respective photoperiods for 10 weeks. After 10 weeks, half of the hamsters in each of the four experimental groups received surgically implanted osmotic minipumps (200 µl volume; 0.5 µl/h delivery rate; Alzet 2002, Alza Corp., Mountain View, Calif., USA) containing leptin, whereas the remaining animals received minipumps containing vehicle (0.5 *M* Tris buffer). Minipumps with leptin contained 2.6 µg/µl leptin (Pepro Tech, Inc., Rocky Hill, N.J., USA) dissolved in 0.5 *M* Tris buffer. We chose this concentration of leptin because it was demonstrated previously to result in a physiological, long-day level of serum leptin when administered to short-day Siberian hamsters [9]. All pumps were implanted s.c. in the intrascapular region of the animals.

Four days after implantation of minipumps, all hamsters received a single subcutaneous injection of 100 µg of the novel antigen keyhole limpet hemocyanin (KLH), suspended in 0.1 ml sterile saline (day 0) and were then returned to the colony room. KLH is an innocuous respiratory protein derived from the giant keyhole limpet *(Megathura crenulata)*. KLH was used because it generates a robust antigenic response in rodents, but does not make the animals ill (e.g. inflammation or fever) [27]. Blood was drawn from the retro-orbital sinus at two different sampling periods (days 5 and 10 postimmunization). These sampling periods were chosen in order to capture peak IgG production during the course of the immune response to KLH [3, 22]. On each sampling day, animals were brought into the surgery room individually, lightly anesthetized with methoxyflurane vapors (Metofane, Mundelein, Ill., USA), and blood samples (500 µl) were drawn from the retro-orbital sinus between 10.00 and 12.00 h EST. Samples were allowed to clot for 1 h, the clots were removed, and the samples centrifuged (at 4°C) for 30 min at 2,500 rpm. Serum aliquots were aspirated and stored in sealable polypropylene microcentrifuge tubes at –80°C until assayed for IgG.

On the last day of sampling (day 10) animals were killed by cervical dislocation. Paired testes and spleens were removed and cleaned of connective tissue at autopsy. All tissue was weighed to the nearest 0.001 g by laboratory assistants naive to the experimental hypotheses and treatment assignments.

Humoral Immunity

To assess humoral immunity, serum anti-KLH IgG concentrations were assayed using an enzyme-linked immunosorbant assay (ELISA) according to the method of Drazen et al. [23]. Microtiter plates were coated with antigen by incubating them overnight at 4°C with 0.5 mg/ml KLH in sodium bicarbonate buffer (pH = 9.6), washed with phosphate buffered saline (PBS; pH = 7.4) containing 0.05% Tween 20 (PBS-T; pH = 7.4), then blocked with 5% nonfat dry milk in PBS-T overnight at 4°C to reduce nonspecific binding, and washed again with PBS-T. Thawed serum samples were diluted 1:20

with PBS-T, and 150 µl of each serum dilution was added in duplicate to the wells of the antigen-coated plates. Positive control samples (pooled sera from hamsters previously determined to have high levels of anti-KLH antibody, similarly diluted with PBS-T) and negative control samples (pooled sera from KLH-naive hamsters, similarly diluted with PBS-T) were also added in duplicate to each plate; plates were sealed, incubated at 37°C for 3 h, then washed with PBS-T. Secondary antibody (alkaline phosphatase-conjugated antimouse IgG diluted 1:2,000 with PBS-T; Cappel, Durham, N.C., USA) was added to the wells, and the plates were sealed and incubated for 1 h at 37°C. Plates were washed again with PBS-T and 150 µl of the enzyme substrate *p*-nitrophenyl phosphate (Sigma Chemical, St. Louis, Mo., USA: 1 mg/ml in diethanolamine substrate buffer) was added to each well. Plates were protected from light during the enzyme-substrate reaction, which was terminated after 20 min by adding 50 µl of 1.5 *M* NaOH to each well. The optical density (OD) of each well was determined using a plate reader (Bio-Rad: Benchmark; Richmond, Calif., USA) equipped with a 405 nm wavelength filter, and the mean OD for each set of duplicate wells was calculated. To minimize intra-assay variability, the mean OD for each sample was expressed as a percent of its plate positive control OD for statistical analyses.

Statistical Analyses

Hamsters that did not display the typical gonadal regression or changes in pelage (from summer gray to winter white) after 10 weeks in short days were considered to be photoperiodic nonresponders and were not included in subsequent statistical analyses. All experimental data was analyzed using a 2 (photoperiod) × 2 (surgery) × 2 (minipump) analysis of variance (ANOVA; Sigma SigmaStat, Jandel Scientific, San Rafael, Calif., USA). Post-hoc tests were performed on pair-wise means using Tukey HSD tests when the overall ANOVA was significant. Differences between group means were considered statistically significant if $p < 0.05$.

Results

Hamsters maintained in short days had significantly reduced paired testes and body masses compared with long-day animals ($p < 0.05$) (fig. 1). Neither leptin administration nor splenic denervation affected paired testes or body masses ($p > 0.5$) (fig. 1).

Serum anti-KLH antibody concentrations at day 10 were significantly higher in long- compared with short-day hamsters ($p < 0.05$) (fig. 2). Splenic masses were significantly smaller in long compared with short-day hamsters ($p < 0.05$); splenic mass was unaffected by either splenic denervation or leptin treatment ($p > 0.05$). Leptin treatment increased serum anti-KLH antibodies in short- but not long-day housed hamsters ($p < 0.05$). In addition, surgical denervation blocked the leptin-induced increase in anti-KLH antibodies in short days ($p < 0.05$); splenic denervation reduced immunity in long- but not short-day housed hamsters ($p < 0.05$) (fig. 2).

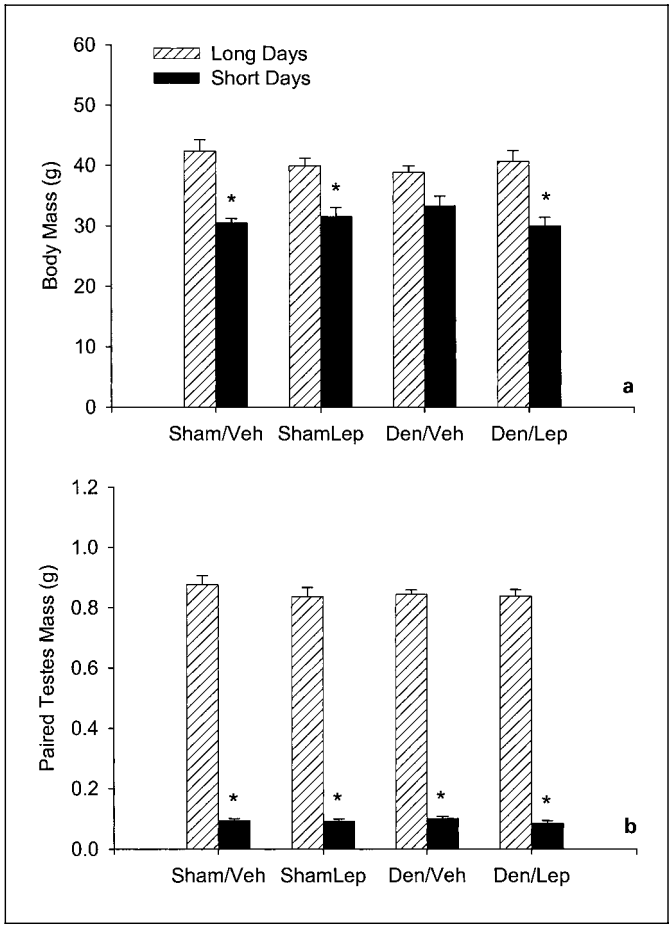

Fig. 1. Mean (± SEM) body masses (a) and paired testes masses (b) of splenic-denervated (Den) or sham-operated (Sham) hamsters housed in long or short days and treated with exogenous leptin (Lep) or vehicle control (Veh). Significant differences between pairwise means: * $p < 0.05$.

Discussion

The results of the present study confirm previous research [23] demonstrating that short days suppress humoral immunity and that administration of exogenous leptin can restore immune function to long-day levels. Specifically, maintenance in short days reduced anti-KLH IgG but administration of exogenous leptin to short-day animals restored immune function to long-day levels; leptin had no effect on long-day hamsters. In addition, we provide the first demonstration that the SNS is necessary for leptin-induced enhancement of humoral immunity of short-day hamsters. Surgical denervation of the spleen blocked the immunoenhancing effects of exogenous leptin

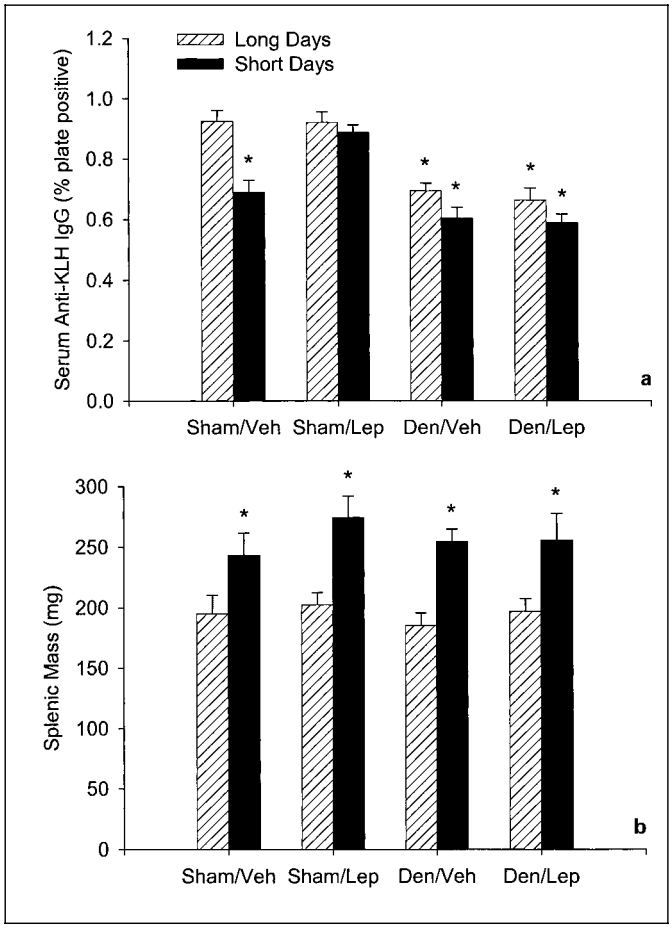

Fig. 2. Mean (± SEM) serum anti-KLH immunoglobulin G (IgG) at day 10 post-KLH injection (a) and splenic masses (b) of splenic-denervated (Den) or sham-operated (Sham) hamsters housed in long or short days and treated with exogenous leptin (Lep) or vehicle control (Veh). Significant differences between pairwise means: * $p < 0.05$.

in short-day hamsters. Splenic denervation also reduced humoral immunity of long-day-housed hamsters, confirming previous results [22]. Collectively, the results of the present study suggest that photoperiodic changes in humoral immunity in Siberian hamsters are due, at least in part, to changes in circulating leptin concentrations. In addition, the immunomodulating actions of leptin appear to act via changes in SNS activity.

Although the photoperiodic changes in anti-KLH IgG reported in the present study were modest (e.g. 10–15%), subtle changes in immunity can have important consequences for disease resistance. The precise correlation between changes in immune function and changes in disease susceptibility has not been fully elucidated, but recent evidence suggests that short-day decreases in immunity may reduce immune responsiveness to infection. For example, Siberian hamsters housed in short days display reduced immunity in response to treatment with lipopolysaccharide (LPS) [28]. LPS is found on the surface of most gram-negative bacteria and the presence of this carbohydrate 'alerts' the immune system to potential bacterial infection. Thus, the results discussed above suggest that short-day decreases in immunity may increase disease susceptibility to bacterial infection by reducing immune responsiveness to these pathogens. Although this hypothesis is intriguing, it remains to be tested empirically.

The important role of the SNS in regulating immune function has become increasingly well-established. For example, lymphocytes within the immune system are exposed to the catecholamines NE and EPI, the predominant post-ganglionic neurotransmitters of the SNS [29]. In addition, high-affinity α- and β-adrenergic receptors have been identified on lymphoid tissue, including the spleen and thymus [30]. Virtually all lymphoid cells express β-AR (primarily the β_2 subtype); the density of β-AR on B cells is greater than T cells. Destruction of sympathetic nerve fibers by treatment with the selective sympathetic neurotoxin 6-hydroxydopamine (6-OHDA) enhance antibody formation to a T-cell-dependent antigen [31] and infusions of catecholamines have been shown to inhibit IgM and IgG antibody formation in response to immunization with sheep red blood cells [32]. Chemical denervation of the spleen increased antibody titers of IgG and IgM in response to antigenic challenge with KLH, as well as KLH-induced interferon (IFN) production [33]. The effects of catecholamines on immune function, however, are complex [34]; humoral immune responses (e.g. antibody formation) can be *stimulated* by catecholamines. For example, 6-OHDA-induced denervation can reduce antibody responses to T-cell-dependent antigens in adult rats [35, 36].

It is important to note that the chemical denervation technique used in some of the cited studies (i.e. systemic injections of 6-OHDA) results in a global SNS denervation throughout the entire animal; subsequent changes in immunity may be the indirect result of disruptions in other physiological systems. Thus, precise interpretation of the results of these previous studies is limited. Surgical denervation of the spleen utilized in the present study, however, allows the *tissue-specific* effects of SNS innervation on immune function to be evaluated. Surgical denervation, in contrast, has the disadvantage of destroying not only SNS nerves, but also parasympathetic (PNS) and

sensory nerves because they are virtually indistinguishable from SNS nerves at the light microscopic level. Thus, although the results of the present study suggest a role of SNS nerves in the regulation of photoperiodic changes in immune function, future studies are needed to evaluate the relative contributions of SNS and sensory innervation. It is possible that some of the effects reported in the present study are due to changes in PNS activity, although this is unlikely. Unlike other lymphoid tissue, the majority (>98%) of the nerves innervating the spleen are sympathetic in origin; the spleen appears to receive very little innervation from the PNS [37]. It is possible, however, that some of the effects reported in the present study are due to sensory innervation of the spleen. The precise role of sensory afferent nerves in immune responses, however, has received much less experimental attention [37]. Future studies in which sensory-specific chemical denervation techniques (e.g., direct injection of the sensory neurotoxin capsaicin into the spleen) are required to tease apart the relative contributions of the SNS and sensory innervation of lymphoid tissue to the immunoenhancing effects of exogenous leptin.

Collectively, the results of the present study suggest that the immunoenhancing effects of leptin are mediated, at least in part, by SNS innervation of lymphoid tissue. In addition, these results support our previous findings [9] that photoperiodic changes in immune function are due to changes in circulating concentrations of leptin and suggest that the direct innervation of the spleen plays an important role in mediating this effect. Taken together, the results of the present study provide important and novel insights into the neural and endocrine mechanisms underlying environmentally influenced alterations in humoral immune function in Siberian hamsters specifically, and seasonally breeding rodents in general.

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