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In vivo but not *in vitro* leptin enhances lymphocyte proliferation in Siberian hamsters (*Phodopus sungorus*)

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ABSTRACT

Mounting an immune response requires a relatively substantial investment of energy and marked reductions in energy availability can suppress immune function and presumably increase disease susceptibility. We have previously demonstrated that a moderate reduction in energy stores by partial surgical lipectomy impairs humoral immunity of Siberian hamsters (*Phodopus sungorus*) and is mediated, in part, by changes in the adipose tissue hormone leptin. The goals of the present study were to assess the role of leptin in cell-mediated immunity and to determine if the potential effects of leptin on immunity are via the direct actions of this hormone on lymphocytes, or indirect, via the sympathetic nervous system (SNS). In Experiment 1, hamsters received osmotic minipumps containing either murine leptin (0.5 μl/h) or vehicle alone for 10 days and splenocyte proliferation in response to the T-cell mitogen Concanavalin A (Con A) was determined. In Experiment 2, Con A-induced splenocyte proliferation was tested in the presence or absence of leptin *in vitro*. In Experiment 3, exogenous leptin was administered to intact or sympathetically denervated hamsters. Hamsters treated with *in vivo* leptin displayed increased splenocyte proliferation compared with control hamsters receiving vehicle. In contrast, *in vitro* leptin had no effect on splenocyte proliferation. Sympathetic denervation attenuated, but did not block, leptin-induced increases in immunity. Taken together, these results are consistent with the idea that leptin can enhance cell-mediated immunity; the SNS appears to contribute, least in part, to leptin-induced increases in immunity. Importantly, these findings confirm previous studies that leptin serves as an important endocrine link between energy balance and immunity.

1. Introduction

A wide range of studies has established an important biological link between energy balance and immune function and thus, disease susceptibility (Chandra, 1996). Immunity, like all other physiological processes, requires adequate energy to sustain optimal functioning (reviewed in Lochmiller and Deerenberg, 2001; Demas, 2004). Despite this fact, the role of energy balance has only recently been considered in the context of immune function and disease (e.g., Chandra, 1996; Lochmiller and Deerenberg, 2001). Animals require a relatively steady supply of energy to sustain biological functions, including immunity. Energy, however, is not a limitless resource; finite energy reserves must serve all physiological processes and thus energy must be allocated to a wide variety of often competing physiological functions. Thus, during times of decreased energy availability, reductions in energy allocation to specific physiological functions, such as immune function, may result (Demas, 2004; Lochmiller and Deerenberg, 2001).

Although energy availability can affect immunity, relatively little is known regarding the physiological mechanisms by which energy regulates specific immune responses. A chronic positive imbalance between energy intake and expenditure leads to obesity and can impair immune function and increase disease susceptibility in both clinical populations and animal models of obesity (Marti et al., 2001). Alternatively, marked reductions in energy availability without concomitant reductions in energy output can also lead to substantial suppression of immunity (Chandra, 1996; Nova et al., 2002). For most mammalian species, and small rodents in particular, white adipose tissue (WAT) depots represent a substantial portion of the total energetic budget and thus WAT likely plays an important role in maintaining energetically expensive physiological processes, including immune function (Demas, 2004). Consistent with this idea, reductions in total body fat are correlated with impaired immunity in a wide range of species, including humans (Norgan, 1997; Klasing, 1998; Spurlock et al., 1997), and experimental reductions in body fat can impair antibody formation (Demas and Sakaria, 2005). Furthermore, immunological disorders (e.g., AIDS) trigger marked changes in whole-body lipid metabolism, suggesting an important role of adipose tissue in immunity (Pond, 1996).

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Despite the established connection between body fat and immunity, much less is known regarding the endocrine mediators of this connection. One potential endocrine mediator of energetic trade-offs with immunity that has received recent experimental attention is leptin, a peptide hormone within the cytokine family that is secreted primarily by adipose tissue (Ahima and Flier, 2000). Circulating concentrations of leptin are directly proportional to adipose tissue mass such that high levels of the hormone indicate adequate energy stores, whereas relatively low leptin levels suggest an energy deficit (Woods and Seeley, 2000). Initial studies of leptin suggested that its primary function was that of a satiety factor, as treatment with mice with physiological levels of leptin triggered marked reductions in food intake and body fat (Zhang et al., 1994). More recent research has demonstrated an important pleiotropic role of leptin on diverse physiological functions, including the enhancement of a variety of immune parameters in rodents and humans (Lord et al., 1998; Faggioni et al., 2001). For example, changes in serum leptin concentrations are positively correlated with measures of immune function in rodents (Lord et al., 1998; Faggioni et al., 2001) and leptin deficiency can increase susceptibility to infections (Faggioni et al., 2001).

Recent data from our laboratory have provided initial support for the hypothesis that changes in immunity are mediated, at least in part, by changes in circulating leptin levels (Drazen et al., 2000). Specifically, Siberian hamsters housed in short "winter-like" days for 10 weeks display significant (~30%) reductions in body fat, as well as reduced antibody concentrations in response to specific antigenic challenge. Administration of exogenous leptin via osmotic minipump (to mimic long-day levels of the hormone), however, blocks short-day reductions in antibodies and restores humoral immunity to long-day levels (Demas, 2004). These results suggest that short day decreases in humoral immunity in Siberian hamsters are due, at least in part, to reductions in body fat and decreased leptin.

Despite these and other studies suggesting an important role for leptin in mediating immune responses, the precise physiological mechanisms by which leptin regulates immunity remain unknown. Leptin, like other hormones, may act directly on lymphocytes to affect immunity. Consistent with this idea, high-affinity leptin receptors have been identified on lymphoid tissues including monocytes, natural killer cells, and T lymphocytes (Martin-Romero et al., 2000; Siegmund et al., 2004). In addition, leptin can increase mitogen-stimulated T-cell proliferation *in vitro* (Martin-Romero et al., 2000), suggesting a direct effect of leptin on lymphocytes. Alternatively, the effects of leptin on immunity may be *indirect* (e.g., via the action of this hormone on the central nervous system). One known target for leptin is the sympathetic nervous system (SNS). Although many of the metabolic actions of leptin 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 SNS (Elmquist, 2001; Mizuno et al., 1998; Rayner, 2001; Scarpace et al., 2000). Furthermore, it is well established that the SNS can exert profound effects on a wide range of immune responses (reviewed in Madden, 2001; Sanders et al., 2001). Recent evidence suggests that the effects of leptin on immune function may also involve the SNS; sympathetic denervation of the spleen blocks leptin-induced changes in humoral and cell-mediated immunity in both house mice and Siberian hamsters (Okamoto et al., 2000; Demas, 2002).

The goals of the present study were (1) to test the hypothesis that leptin enhances cell-mediated immunity in Siberian hamsters, (2) to determine if the potential effects of leptin on immune function are direct or indirect, and (3) examine the contributions of the SNS to leptin-induced immunoenhancement. This species was chosen because individuals display robust and reliable seasonal fluctuations in both body fat and acquired immunity that are associated with changes in circulating leptin (Demas and Sakaria, 2005; Drazen et al., 2001).

2. Materials and methods

2.1. Animals and housing conditions

Adult (>60 days of age) male Siberian hamsters (*Phodopus sungorus*) were obtained from our breeding colony at Indiana University. Hamsters were weaned at 21 days of age and housed with same sex siblings. Two weeks prior to the initiation of the experiments, animals were housed individually in polypropylene cages (27.8 × 7.5 × 13.0 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 (PMI LabDiet 5012, Rat Diet, St. Louis, MO) and tap water were available *ad libitum* throughout the experiments. All animals were treated in accordance with the Bloomington Institutional Animal Care and Use Committee (BIACUC) and all experimental procedures meet NIH guidelines for the Care and Use of Experimental Animals.

2.2. Experiment 1: Does in vivo leptin enhance splenocyte proliferation?

The goal of Experiment 1 was to determine the effects of *in vivo* leptin administration on lymphocyte proliferation in male Siberian hamsters. Hamsters ($n = 20$) were maintained in LD 16:8 and half of the hamsters were randomly selected and received surgically implanted osmotic minipumps (200 μl volume; 0.5 μl/h delivery rate; Alzet Model 2002, Alza Corp., Mountain View, CA) containing leptin. Leptin minipumps contained 2.6 μg/μl murine leptin (Pepro Tech, Inc., Rocky Hill, NJ) dissolved in 0.5 M Tris buffer. The remaining control animals received minipumps containing vehicle (0.5 M Tris buffer; Sigma Chemical, St. Louis, MO). We have previously demonstrated that this short-term leptin protocol results in significant changes in humoral immunity in Siberian hamsters (Demas and Sakaria, 2005). All animals were lightly anesthetized with anhydrous diethyl ether (Sigma Chemical, St. Louis, MO) and a small incision was made on the dorsal surface of the skin and pumps were implanted s.c. in the intrascapular region. The skin was closed with surgical staples and nitrofurazone antibacterial powder (W.A. Butler Co., Dublin, OH) was applied to the incision. Ten days later animals were brought one at a time into the surgery room, killed by cervical dislocation and inguinal white adipose tissue (IWAT), retroperitoneal WAT (RWAT), and epididymal WAT (EWAT) pads were removed, cleaned of connective tissue, and weighed at necropsy. Spleens were removed under aseptic conditions, weighed and immediately suspended in culture media (RPMI-1640/Hepes). All necropsies occurred between 10:00 and 12:00 h EST to control for potential circadian influences on lymphocyte proliferation. Although food intake was not assessed in the present study, previous research has demonstrated that Siberian hamsters, unlike laboratory rats and mice, do not experience decreased food intake following short-term treatment with exogenous leptin (Drazen et al., 2001; Rousseau et al., 2002).

2.3. Experiment 2: Does in vitro leptin enhance splenocyte proliferation?

The goal of Experiment 2 was to test whether the effects of exogenous leptin on splenocyte proliferation are due to the direct effects of this hormone on splenocytes. Hamsters ($n = 10$) were housed individually and maintained in long days (LD 16:8) for 2

weeks. Next, hamsters were killed by cervical dislocation and WAT pads and spleens were removed under aseptic conditions, cleaned of connective tissue, weighed as in Experiment 1. Additionally, spleens were immediately suspended in culture media (RPMI-1640/Hepes) as described in Experiment 1.

2.4. Experiment 3: Does chemical sympathectomy attenuate leptin-induced increase in proliferation?

The goal of Experiment 3 was to test the hypothesis that the effects of exogenous leptin on splenocyte proliferation are due to the indirect effects of this hormone on the SNS. Hamsters ($n = 32$) were housed individually and maintained in long days (LD 16:8) for 2 weeks. Half of the animals ($n = 16$) received global chemical denervations of the SNS via s.c. injections of 6-hydroxydopamine (6-OHDA). 6-OHDA (Sigma Chemical, St. Louis, MO) was dissolved in sterile 0.9% saline containing 0.001% of the antioxidant ascorbate and given at a dose of 250 mg/kg body weight, based on a previous protocol (Kruszewska et al., 1995). This experimental regimen has been shown previously to decrease splenic catecholamine concentrations, as assessed by high pressure liquid chromatography (HPLC), within 12 h and by at least 75% over at least 21 days post-injection (Kruszewska et al., 1995). The remaining control animals ($n = 16$) received injections of vehicle alone. Five days later, half of the animals in each group received minipumps filled with leptin, as described in Experiment 1, whereas the remaining hamsters received vehicle-filled pumps. Ten days later, animals were killed by cervical dislocation and spleens removed and processed as described in Experiment 1.

2.5. Lymphocyte proliferation

In all experiments, lymphocyte proliferation in response to the T-cell mitogen, Concanavalin A (Con A), was determined using a colorimetric assay based on the tetrazolium salt 3-(4,5-demethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) (Cory et al., 1991). Splenocytes were separated from tissue by compressing the whole spleen between sterile frosted glass slides; separated cells were suspended in 4 ml of culture media (RPMI-1640/Hepes supplemented with 1% penicillin (5000 U/ml)/streptomycin (5000 μg/ml), 1% L-glutamine (2 mM/ml), 0.1% 2-mercaptoethanol (5×10^{-2} M/ml), and 10% heat-inactivated fetal bovine serum). Splenocyte counts and viability were determined with a hemacytometer and trypan blue exclusion. Viable cells (which exceeded 95%) were adjusted to 2×10^6 cells/ml by dilution with culture media. In Experiment 1, Con A (Sigma Chemical Co., St. Louis, MO) was diluted with culture media to concentrations of 0.6, 1.25, 2.5, 5, 10, 20, and 40 μg/ml based on a previous protocol (Demas et al., 1996); 50 μl of each mitogen concentration was added to the wells of the plate containing the spleen cell suspensions to yield a final volume of 150 μl/well (each in duplicate).

In Experiment 2, 50 μl aliquots of each cell suspension (i.e., ~100,000 cells) were added to each of three wells of sterile flat-bottom 96-well culture plates. One well then received 50 μl of 10 nM leptin (Pepro Tech), another well received 50 μl of 100 nM leptin dissolved in culture media, and the last well received 50 μl of culture media. Con A was diluted with culture medium to a concentration of 5 μg/ml; 50 μl of the diluted mitogen was added to the wells of the plate containing the spleen cell suspensions to yield a final volume of 150 μl/well (each in duplicate). This mitogen concentration was chosen because it resulted in optimal proliferation in Experiment 1. In Experiment 3, spleens were treated as described in Experiment 2, except that no leptin was added to the celled *in vitro*.

The plates from Experiments 1–3 were incubated at 37 °C with 5% CO_2 for 48 h prior to addition of 20 μl of MTS/PMS solution (Promega; 0.92 mg/ml of phenazine methosulfate [PMS] in sterile Dulbecco's phosphate-buffered saline) per well. Plates were then incubated at 37 °C with 5% CO_2 for an additional 4 h. The optical density (OD) of each well was determined with a microplate reader (Bio-Rad: Model #3550) equipped with a 490 nm wavelength filter. Mean OD values for each set of duplicates were used in subsequent statistical analyses. In Experiment 1, dose–response curves were constructed using group means of the mean OD values at each mitogen concentration and un-stimulated cultures. In Experiments 2 and 3, group means of the mean OD values in response to the single mitogen dose (5 μg/ml) are reported.

2.6. Statistical analyses

Differences in splenocyte proliferation in Experiment 1 were assessed via a two-way (hormone × Con A concentration) mixed model ANOVA. Differences in splenocyte proliferation in Experiment 2 were assessed by a one-way (hormone) between-groups ANOVA. Differences in Experiment 3 were determined by a two-way (denervation × hormone) between-groups ANOVA. Post hoc comparisons between pair-wise means were conducted using Tukey-HSD tests when the overall ANOVAs were significant. All statistical tests were conducted using SPSS statistical software (Chicago, IL). In all cases, differences between group means were considered statistically significant if $p < 0.05$.

3. Results

In Experiment 1, Siberian hamsters receiving exogenous leptin *in vivo* displayed significantly greater lymphocyte proliferation in response to the T-cell mitogen Con A compared with animals receiving vehicle ($F_{1,13} = 3.67$; $p < 0.05$). There was a significant does response effect of Con A, with "optimal" concentrations of Con A (i.e., 5 μg/ml) eliciting greater proliferation than either sub-optimal (i.e., 1.25 μg/ml) or supra-optimal (i.e., 40 μg/ml) concentrations ($F_{1,18} = 10.27$; $p < 0.05$). There were no significant differences in body, splenic or WAT pad masses between leptin- and vehicle-treated hamsters ($p > 0.05$ in all cases) (Table 1 and Fig. 1).

In Experiment 2, Con A significantly increased splenocyte proliferation ($F_{3,\ 36} = 29.74$; $p < 0.05$). *In vitro* treatment of splenocytes with low and high concentrations of leptin, however, did not significantly affect either basal or Con A-induced splenocyte proliferation ($p > 0.05$) (Fig. 2).

In Experiment 3, splenocyte proliferation in response to Con A was significantly increased in animals that received exogenous leptin ($F_{1,32} = 9.77$; $p < 0.05$); however, sympathetic denervation with 6-OHDA attenuated the leptin-induced increase in proliferation, with 6-OHDA-denervated hamsters treated with leptin displaying intermediate proliferation values (i.e., these values were not significantly different from either non-denervated, leptin-treated or vehicle-treated hamsters). There were no significant differences in body, splenic or WAT pad masses between leptin-

Table 1
Mean (±SEM) initial and terminal body masses, inguinal (IWAT), epididymal (EWAT), retroperitoneal (RWAT) white adipose tissues and spleen masses (g) in control and leptin-treated hamsters in Experiment 1.

	Control	Leptin
Initial body mass	41.09 ± 1.03	41.42 ± 1.00
Final body mass	41.61 ± 1.03	41.72 ± 1.09
IWAT	0.673 ± 0.039	0.613 ± 0.025
EWAT	1.112 ± 0.060	1.072 ± 0.086
RWAT	0.127 ± 0.017	0.171 ± 0.025
Spleen	0.064 ± 0.053	0.069 ± 0.044

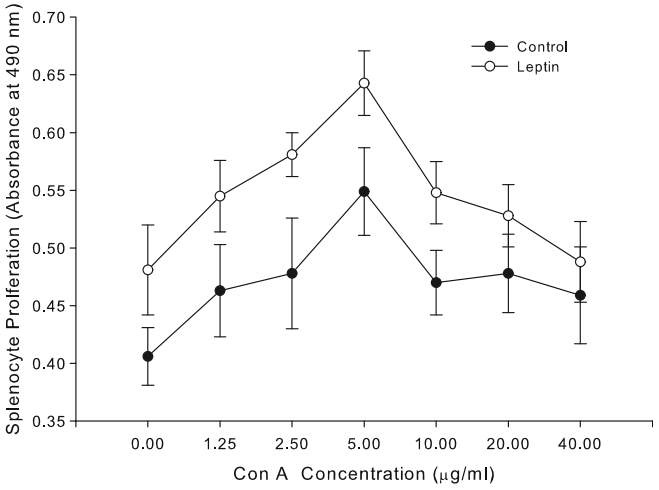

Fig. 1. Mean (±SEM) splenocyte proliferation in response to 0, 1.25, 2.5, 5, 10, 20, and 40 μg/ml of the T-cell mitogen Concanavalin A (Con A) of hamsters treated with leptin or vehicle alone (control) *in vivo*.

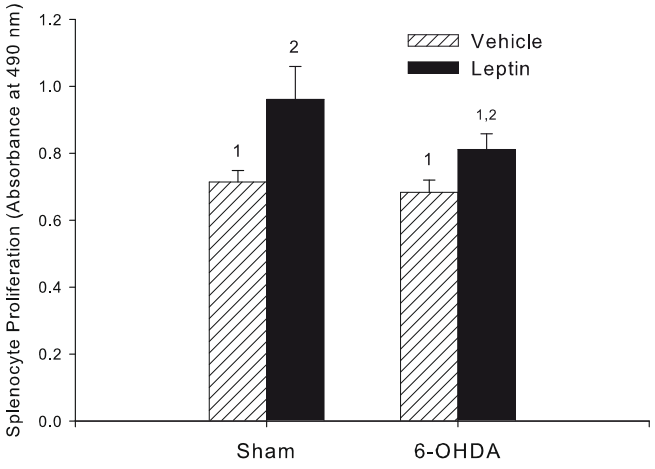

Fig. 3. Mean (±SEM) Con A-stimulated splenocyte proliferation of intact or chemically denervated (6-OHDA) hamsters treated with *in vivo* leptin or vehicle alone. Columns sharing at least one same number are statistically equivalent. Columns with different numbers are statistically different ($p < 0.05$).

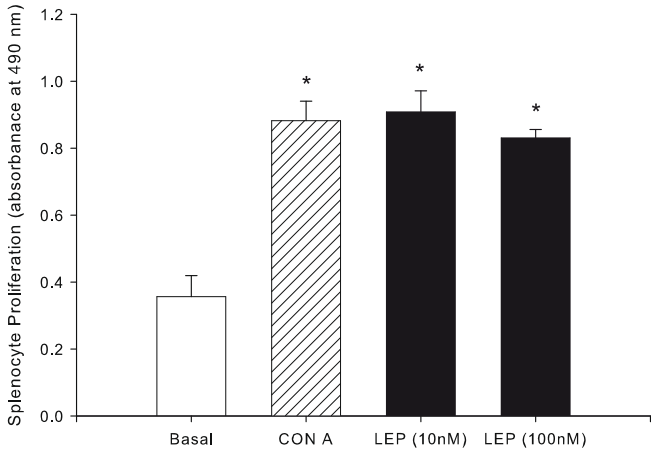

Fig. 2. Mean (±SEM) basal (no Con A) and Concanavalin A-stimulated splenocyte proliferation of long-day-housed hamsters receiving no leptin (Con A), 10 nM or 100 nM leptin added *in vitro*. Statistically significant differences among pair-wise means are indicated by an asterisk (*) when $p < 0.05$.

treated and control hamsters ($p > 0.05$ in all cases) (data not shown) (Fig. 3).

4. Discussion

The present findings demonstrate that exogenous leptin significantly altered splenocyte proliferation in Siberian hamsters, consistent with previous reports in other species (Martin-Romero et al., 2000; Okamoto et al., 2000). In contrast, *in vitro* leptin had no effect on splenocyte proliferation. Global sympathetic denervation by means of treatment with 6-OHDA attenuated, but did not block, the leptin-induced increase in splenocyte proliferation. Collectively, these results support previous findings that leptin enhances cell-mediated immunity (Lord et al., 1998; Lohmus et al., 2004); these results are consistent with both direct and indirect actions of leptin on lymphocyte proliferation. Consistent with an indirect effect, neither low nor high concentrations of *in vitro* leptin affected lymphocyte proliferation. Furthermore, SNS denervation attenuated the effects of *in vivo* leptin on proliferation. It remains possible, however, that leptin does act directly to increase splenocyte proliferation in Siberian hamsters as has been reported for other species (Lohmus et al., 2004), but that the concentrations used in the Experiment 2 were too low to elicit increased proliferation. Although this possibility cannot be ruled out, exogenous leptin as low as 20 μM has been shown to affect neural firing *in vitro* in rats (Inyushkin et al., 2009). Whether these findings generalize to peripheral structures, including lymphoid tissues, remains to be tested. Lymphocytes were exposed to leptin for only 2 days in Experiment 2 compared with 10 days in Experiments 1 and 3; the lack of a significant effect of *in vitro* leptin in Experiment 2 may be due to the short exposure time to the hormone. In fact, the failure of chemical sympathectomy to completely block leptin-induced increases in proliferation in Experiment 3 provides potential support for a SNS-independent effect of leptin on cell-mediated immunity. It is possible that the small effect of leptin on splenocyte proliferation in sympathetically denervated animals is due a *direct* effect of this hormone on cell-mediated immunity. Alternatively, it may represent an indirect effect regulated by physiological systems *other* than the SNS (e.g., cytokines). The present study, however, cannot resolve this issue; future studies will be needed to test these and other possibilities experimentally. Taken together, the results of these studies suggest that leptin mediates cell-mediated immunity, possibly through both direct actions of the hormone on splenocytes, as well as indirect actions via the SNS.

It is important to note that, although exogenous leptin increased splenocyte proliferation, consistent with previous findings, the functional consequences of increased immunity on disease resistance were not evaluated in the present study. In other words, an increase in the number of T cells in response to leptin does not necessarily indicate that these cells are any more capable of attacking and destroying a potential pathogen. Thus, it is premature to conclude from these results whether or not leptin has any functional consequences on actual disease resistance. Future studies in which the effects of leptin on pathogen-specific immune responses are necessary to address this issue.

The present findings support an indirect effect of leptin on cell-mediated immunity. The precise mechanisms by which leptin mediates immune function, however, are not known. One likely candidate, supported by the results of the present study, is via the actions of this hormone on the SNS. Although many of the effects of leptin on the regulation total body fat 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 SNS and subsequent

increases in metabolism (Elmquist, 2001; Mizuno et al., 1998; Rayner, 2001; Scarpace et al., 2000). 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 (Okamoto et al., 2000). Specifically, intracerebroventricular administration of leptin to house mice reduces splenocyte proliferation in response to the T-cell mitogen Con A. This effect, however, is abolished by surgical denervation of the spleen (Okamoto et al., 2000).

More recently we evaluated the role of sympathetic denervation of lymphoid tissue in exogenous leptin-induced increases in immunity in Siberian hamsters (Demas, 2002). Specifically, hamsters were housed in long or short days and half of the animals in each photoperiod were administered exogenous leptin via osmotic minipumps. Half of the animals in each hormonal condition received surgical sympathetic denervation of the spleen (Demas, 2002) whereas the remaining animals were left intact. As previously reported (Drazen et al., 2000) anti-KLH antibodies were reduced in short days and the short-day reduction in humoral was reversed by exogenous leptin administration. Surgical denervation of the spleen, however, attenuated the immune-enhancing properties of exogenous leptin and short-day denervated hamsters receiving leptin displayed suppressed immunity comparable with short-day control animals not receiving the hormone (Demas, 2002). Because >95% of the efferent innervation of the spleen is sympathetic in origin (Elenkov et al., 2000) the results of these studies are consistent with the idea that leptin acts via the SNS to mediate immune responses.

The finding that chemical sympathectomy in the present study only partially blocked leptin-induced increases in cell-mediated immunity in the present study suggests that leptin can also affect immune response by SNS-independent mechanisms, possibly via the direct actions of this hormone on lymphoid tissues. As mentioned above, leptin receptors have been identified on circulating lymphocytes (Martin-Romero et al., 2000) and exogenous leptin treatment can affect *in vitro* immune responses (Lord et al., 1998; Martin-Romero et al., 2000). The present findings provide some support for these previous findings suggesting a direct effect of leptin on lymphocytes. Future studies, however, are needed to determine to what extent leptin acts directly on lymphoid tissues to mediate immune responses.

Collectively, the results of the present study suggest that the leptin plays an important role the regulation of cell-mediated immunity. Additionally, these results provide further support for the idea that leptin acts as an endocrine signal between body fat and immune function. Specifically, the ability of exogenous leptin to enhance mitogen-induced proliferation, coupled with the finding that chemical sympathectomy attenuated, but did not block, leptin-induced increases in immunity, suggest that the effects of leptin on immunity may be both direct and indirect (via the SNS), although the precise mechanisms remain to be elucidated. Regardless of the precise physiological mechanisms, these data provide novel and important insights into the role of the adipose tissue hormone leptin in mediating cell-mediated immunity in seasonally breeding rodents, and likely other species.

Acknowledgments

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