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Leptin stimulates sympathetic axon outgrowth

Michael J. Pellegrino [a,*], Belinda H. McCully [b], Beth A. Habecker [a]

[a] Department of Physiology and Pharmacology, Oregon Health & Science University, Portland, OR 97239, USA
[b] Trauma Research Institute of Oregon, Division of Trauma, Critical Care & Acute Care Surgery, Department of Surgery, Oregon Health & Science University, Portland, OR 97239, USA

HIGHLIGHTS

- Leptin stimulates increased axon growth in sympathetic explants.
- STAT3 phosphorylation is required for leptin-stimulated axon outgrowth.
- This work links a high fat diet to increased risk of cardiac arrhythmias.

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ABSTRACT

The neurohormone leptin regulates energy homeostasis. Circulating levels of leptin secreted by adipose tissue act on hypothalamic neurons in the brain leading to decreased appetite and increased energy expenditure. Although leptin signaling in the central nervous system (CNS) is fundamental to its ability to regulate the body's metabolic balance, leptin also has a variety of effects in many peripheral tissues including the heart, the liver, and the sympathetic nervous system. Leptin stimulation of the hypothalamus can stimulate glucose uptake via the sympathetic nervous system in heart, muscle, and brown adipose tissue. Leptin receptors (Ob-Rb) are also expressed by peripheral sympathetic neurons, but their functional role is not clear. In this study, we found that leptin stimulates axonal growth of both adult and neonatal sympathetic neurons in vitro. Leptin stimulates acute activation of the transcription factor STAT3 via phosphorylation of tyrosine 705. STAT3 phosphorylation is required for leptin-stimulated sympathetic axon outgrowth. Thus, circulating levels of leptin may enhance sympathetic nerve innervation of peripheral tissues.

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1. Introduction

The neurohormone leptin controls energy homeostasis by relaying signals from adipose cells to the brain. Genetic studies have shown that removing leptin, or its receptor, from the central nervous system results in animals that are morbidly obese [1–3]. Not surprisingly, the majority of work in this field has been focused on understanding leptin's central effects [4–7]. However, leptin is secreted into the circulation and leptin receptors (Ob-Rb) are found in many peripheral targets including heart, liver, muscle, lungs, adrenals, lymph nodes, and the sympathetic nervous system [8–12]. It is well known that central neurons stimulated by leptin activate the sympathetic nervous system [13–18]. In addition, postganglionic sympathetic neurons express Ob-Rb and therefore can respond directly to changing leptin levels [19,20]. However, the effect of direct stimulation of sympathetic neurons by leptin has not been characterized.

Many studies show that obesity exacerbates cardiovascular disease states. Recently, we found that obese rats had increased axon growth and developed sympathetic hyperinnervation of the heart compared to rats fed normal chow [21]. A potential mediator could be leptin, which is elevated during the obese state. Interestingly, Ob-Rb is a member of the cytokine receptor class I super family and is most closely related to gp130 receptor subunit [22]. We recently reported that gp130 cytokines are required for maximal axon outgrowth in sympathetic neurons [23]. Due to leptin's closely related homology to gp130 cytokines, it is possible they share similar mechanisms as well. Leptin binding to Ob-Rb activates Jak2 – STAT3 and MAPK signaling pathways [24,25]. However, STAT3

Abbreviations: STAT3, signal transducer and activator of transcription 3; Ob-Rb, long form leptin receptor; MAPK, mitogen-activated protein kinase; SCG, superior cervical ganglion; JAK2, janus kinase-2; PI3K, phosphoinositide 3-kinase.

* Corresponding author at: Department of Physiology and Pharmacology, Mail Code: L334, 3181 S.W. Sam Jackson Park Road, Portland, OR 97239-3098, USA. Tel.: +1 503 494 0498; fax: +1 503 494 4352.

E-mail addresses: pellegri@ohsu.edu, mjp999@gmail.com (M.J. Pellegrino), houghtob@ohsu.edu (B.H. McCully), habecker@ohsu.edu (B.A. Habecker).

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activation is required for leptin's central effects regulating body weight and energy homeostasis [26,27,28]. Indeed, the chronic inflammatory state associated with obesity and cancer is characterized by increased circulating cytokines and activated STAT3 levels [29]. Phosphorylation of STAT3 is required for sympathetic axon outgrowth [23], but leptin activation of STAT3 has not been tested in this paradigm.

In the present study, we tested the hypothesis that leptin stimulation of sympathetic neurons regulates axon growth. We found that leptin increased sympathetic axon growth in adult and neonatal explants. This effect required phosphorylation of STAT3 on tyrosine 705. Thus, similar to related gp130 cytokines, leptin stimulated sympathetic axon outgrowth requires activation of STAT3. Leptin-stimulated increased sympathetic tone to peripheral tissue targets may help explain how circulating leptin levels contribute to obesity related diseases in humans.

2. Materials and methods

2.1. Materials

Matrigel™ was purchased from BD Biosciences (San Jose, CA). CNTF was from Preprotech (Rocky Hills, NJ). Recombinant mouse leptin was purchased from R&D Systems (Minneapolis, MN). Nerve growth factor (NGF) was purchase from Austral Biologicals (San Ramon, CA). Dispase was purchased from Boehringer Mannheim (Indianapolis, IN). Collagenase type II was purchased from Worthington Biochemicals (Freehold, NJ). Nitrocellulose membranes were from Schleicher & Schuell (Dassel, Germany). Protease inhibitor cocktail, phosphatase inhibitor cocktail (#2 & #3), and poly-L-lysine were purchased from Sigma–Aldrich (St. Louis, MO). Bovine Serum Albumin-Fraction V (BSA) was from Thermo Fischer Scientific (Waltham, MA). STATi/Stattic (STAT3 phosphorylation inhibitor) was from Calbiochem (Darmstadt, Germany).

2.2. Animals

Pregnant adult Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). Animals were anesthetized using isoflurane and decapitated prior to harvest of ganglia. All procedures were approved by the OHSU Institutional Animal Care and Use Committee and comply with the Guide for the Care and Use of Laboratory Animals published by the National Academies Press (8th edition).

2.3. Western blotting and antibodies

Total STAT3 (#9132) and phospho-STAT3 (Tyr705) (#9131) antibodies were from Cell Signaling Technology (Danvers, MA). Species-specific secondary antibodies conjugated to horseradish peroxidase were from Pierce (Rockford, IL). Neonatal sympathetic neurons (postnatal day 1–4) were dissociated and cultured in serum free medium as described previously [23]. Cells were treated with 200 ng/ml leptin for the times specified, and lysates subjected to western blotting as described previously [23]. Bands were captured using the ChemiDoc™ XRS+ system and image lab software (Bio-Rad Laboratories, Hercules, CA). Immunoreactive bands were quantified using Image Lab Software 4.0.1 from Bio-Rad Laboratories.

2.4. Sympathetic axon outgrowth from SCG explants

Both SCGs were removed from neonatal (postnatal days 1–4) or adult rats, halved, and explanted into a 12-well tissue culture plate and covered with 35 μL Matrigel (BD Biosciences, San Jose, CA). Serum-free DMEM/F12 media (Life Technologies, Grand Island, NY) + 1% penicillin/streptomycin was layered over the solidified Matrigel, and wells were treated with 2 ng/ml NGF. Culture plates were placed in a humidified incubator of 95%O_2/5%CO_2 at 37 °C. Axon outgrowth was visualized with phase contrast microscopy, and axon length was measured in the images using Nikon Elements AR 3.0 software (Melville, NY). Initial images for "time-zero" were taken 24-h after plating. At time-zero, explants were either treated with vehicle or leptin (Sigma, 100 ng/ml), and additional images were taken 24-h later (time 24-h). For the Stattic experiments neonatal ganglia were used and Stattic (20 μm) was added to all explants for 6 h after the 24 h pictures were taken. The rate of axon outgrowth (μm/h) was calculated from the difference in axon growth length before and after treatment, and then dividing this value over the duration of the treatment (24 h for leptin or 6 h for Stattic). Multiple sites of growth (3–6) from were taken for each explant and averaged. Each condition had 4 replicates and these experiments were performed 4 times. For adult explants, SCGs were bisected and axon growth was measured in two wells for each animal ($n = 3$). The values from each well were averaged to determine the mean rate of axon outgrowth for each animal.

2.5. Statistics

Student's t-test was used for a comparison between two groups. Statistics were carried out using Prism 5.0 (GraphPad Software, La Jolla, CA).

3. Results

3.1. Leptin enhances sympathetic outgrowth

To test the hypothesis that leptin stimulates sympathetic axon outgrowth, we measured axon outgrowth from explanted SCGs from adult rats ($n = 3$) treated with either vehicle or leptin. At time-zero, axon length was similar between SCGs treated with vehicle (205 ± 30 μm) or leptin (253 ± 12 μm). After the 24-h treatment period, axon length was longer in leptin (701 ± 90 μm) compared to vehicle (484 ± 30 μm), and the rate of axon outgrowth was 39% higher in SCG treated with leptin compared to vehicle-treated SCG (Fig. 1A–C, $p < 0.05$).

3.1.1. Leptin stimulates phosphorylation of STAT3 in sympathetic neurons

To investigate the signaling pathway activated by leptin in sympathetic neurons, we examined levels of STAT3 phosphorylation using SDS-PAGE. We treated neonatal sympathetic neurons with leptin (200 ng/ml) over a 30 min time course using ciliary neurotrophic factor (CNTF) as a positive control (Fig. 2A). Leptin stimulated phosphorylation of STAT3 on Y705 at all time points tested. Leptin stimulated a maximum 163.72% increase in the ratio of phospho-STAT3 to total STAT3 at 30 min and we used this time point to test the STAT3 inhibitor Stattic's ability to block leptin-stimulated STAT3 phosphorylation. We pretreated sympathetic neurons with Stattic and then stimulated the neurons with leptin for 30 min. Stattic pretreatment blocks basal and leptin-stimulated STAT3 phosphorylation (Fig. 2B).

3.1.2. STAT3 phosphorylation is required for sympathetic axon outgrowth

To test the role of phosphorylated STAT3 in sympathetic axon outgrowth, we measured axon outgrowth of neonatal SCG explants. We compared untreated ($n = 4$, 37.48 ± 2.30 μm) to leptin treated ($n = 4$, 48.13 ± 1.42 μm) explants. After the 24-h treatment period, leptin stimulation increased growth rates 28.41% compared to untreated explants (Fig. 3A, $p < 0.01$). After 24 h of leptin treatment, Stattic was added to all explants for 6 h to test the requirement of

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