Original Article

MOLECULAR METABOLISM

Peripheral cannabinoid-1 receptor blockade restores hypothalamic leptin signaling

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ABSTRACT

Objective: In visceral obesity, an overactive endocannabinoid/CB_1 receptor (CB_1R) system promotes increased caloric intake and decreases energy expenditure, which are mitigated by global or peripheral CB_1R blockade. In mice with diet-induced obesity (DIO), inhibition of food intake by the peripherally restricted CB_1R antagonist JD5037 could be attributed to endogenous leptin due to the rapid reversal of hyperleptinemia that maintains leptin resistance, but the signaling pathway engaged by leptin has remained to be determined.
Methods: We analyzed the hypothalamic circuitry targeted by leptin following chronic treatment of DIO mice with JD5037.
Results: Leptin treatment or an increase in endogenous leptin following fasting/refeeding induced STAT3 phosphorylation in neurons in the arcuate nucleus (ARC) in lean and JD5037-treated DIO mice, but not in vehicle-treated DIO animals. Co-localization of pSTAT3 in leptin-treated mice was significantly less common with NPY^{+} than with $POMC^{+}$ ARC neurons. The hypophagic effect of JD5037 was absent in melanocortin-4 receptor (MC4R) deficient obese mice or DIO mice treated with a MC4R antagonist, but was maintained in $NPY^{-/-}$ mice kept on a high-fat diet.
Conclusions: Peripheral CB_1R blockade in DIO restores sensitivity to endogenous leptin, which elicits hypophagia via the re-activation of melanocortin signaling in the ARC.

Keywords Peripheral CB1 blockade; Leptin resistance; POMC; NPY; Diet-induced obesity

1. INTRODUCTION

Visceral obesity and its metabolic complications, commonly called the metabolic syndrome, represent a growing public health concern worldwide [1]. Accumulating evidence supports the pathogenic role of an overactive endocannabinoid/CB_1 receptor (CB_1R) system in obesity/metabolic syndrome [2–4]. Indeed, the CB_1R antagonist/inverse agonist rimonabant was effective in reducing body weight in obese/overweight people and also improved the associated insulin resistance, fatty liver, and dyslipidemia [5], but the therapeutic development of this class of compounds was halted due to neuropsychiatric side effects mediated by blockade of CB_1R in the central nervous system [6]. More recent evidence indicates that activation of CB_1R in peripheral tissues, including adipose tissue [7], liver [8], skeletal muscle [9], the endocrine pancreas [10], and proinflammatory macrophages [11], contributes to visceral adiposity and its metabolic complications, and its selective blockade by CB_1R antagonists/inverse agonists with limited brain penetrance can improve the obese phenotype in animal models of diet-induced metabolic syndrome without eliciting behaviors attributable to blockade of CB_1R in the CNS [11–13].

In a previous study, we reported that the peripherally restricted CB_1R inverse agonist JD5037 was as effective as its brain penetrant parent compound SLV319 (ibipinabant) in normalizing all of the metabolic consequences of a high-fat diet (HFD), including the normalization of body weight as well as causing transient but pronounced hypophagia [13]. The latter effect was surprising in view of the dominant role of central neural circuits in the control of food intake. To resolve this paradox, we posited that the hypophagic effect of chronic blockade of peripheral CB_1R is mediated by endogenous leptin, as a result of the rapid reversal of the hyperleptinemia and associated leptin resistance of diet-induced obese (DIO) mice [13]. Reversal of the hyperleptinemia, in turn, could be attributed to reduced leptin production due to direct inhibition of CB_1R in adipocytes and sympathetic nerve endings in adipose tissue, as well as increased leptin clearance in the kidney, due to inhibition of CB_1R in renal proximal tubular cells [13].

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The ability of peripherally generated leptin to reach its hypothalamic receptors reconciles the paradox of modulating a centrally regulated function using a drug restricted to the periphery. The question remains: which neural circuits are activated by endogenous leptin, once the leptin resistance in DIO mice is reversed by treatment with a peripherally restricted CB_1R inverse agonist. Generally, leptin coordinates the activity of the appetitive neural circuitry primarily via promoting the activity of pro-opiomelanocortin (POMC)/cocaine- and amphetamine-regulated transcript (CART) neurons [14–16] and subsequent anorexigenic signaling by α-melanocyte-stimulating hormone (α-MSH) via the melanocortin 4 receptor (MC4R) [17] and simultaneously inhibiting the activity of orexigenic neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons in the arcuate nucleus (ARC) [18,19]. Therefore, we examined the relative role of these two pathways in the anorexigenic effect of endogenous leptin in DIO mice chronically treated with the peripheral CB_1R antagonist JD5037. The results indicate that resensitization of DIO mice to leptin is reflected in increased leptin-induce phosphorylation of STAT3 in both POMC and NPY neurons, with the former playing a key role in the anorexigenic and weight reducing actions of endogenous leptin, as reflected in the absence of these effects in MC4R knockout mice or DIO mice treated with an MC4R antagonist.

2. MATERIALS AND METHODS

2.1. Animals and experimental protocol

The experimental protocol was approved by the Institutional Animal Care and Use Committees of the NIAAA and Hebrew University of Jerusalem. Male 6-week old $NPY^{-/-}$ mice (*129S-Npy^{tm1Rpa}/J*) and their littermate controls were obtained from the Jackson Laboratory. Adult, male, genetically obese $MC4R^{-/-}$ mice (*MC4R^{tm1Lowl}/J*) were generated by heterozygote mating using wild-type littermates as controls. Mice were maintained under a 12 h light/dark cycle and fed *ad libitum*. To generate DIO, C57Bl6/J and $NPY^{-/-}$ mice were fed a HFD (Research Diet, D12492; 60% calories from fat, 20% from protein, and 20% from carbohydrates), with age-matched lean controls receiving a standard laboratory diet (STD, NIH-31 rodent diet) for 12–14 weeks. To achieve normoleptinemia in DIO mice, we adapted a protocol described by Knight et al. [20]. Briefly, leptin-deficient *ob*/*ob* mice were implanted with an osmotic minipump (model 2001D, Alzet Osmotic Pumps; Durect, Cupertino, CA) delivering leptin dissolved in phosphate-buffered saline (PBS) at a rate of 150 ng/h for 12 weeks, during which time they were fed a HFD to induce DIO. Control groups of wild-type mice on HFD and *ob*/*ob* mice on STD were also implanted with minipumps delivering PBS at the same rate. Pumps were replaced every 28 days. HFD-induced obese C57Bl/6J, $NPY^{-/-}$ and normoleptinemic *ob*/*ob* mice, and genetically obese $MC4R^{-/-}$ mice on STD were treated daily with JD5037 (3 mg/kg/day, po) or vehicle (Veh; 4% DMSO + 1% Tween80 in normal saline) for 7 days. Body weight and food intake were monitored daily. Mice were euthanized by cervical dislocation under anesthesia, and their brains and trunk blood were collected for further analyses.

2.2. Leptin sensitivity

Leptin sensitivity was assessed in lean and DIO mice and DIO mice treated daily with JD5037 (3 mg/kg po.) for 7 days followed by twice daily treatment with leptin (3 mg/kg, ip) or vehicle for an additional 4 days. One hour after the last dose of leptin or vehicle, mice were anesthetized, perfused via the left ventricle with 5 mL of 0.9% saline for 1 min followed by 60–80 mL of cold 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde for 15 min at room temperature. Then, the perfused brains were removed, post-fixed in the same fixative for 6 h at 4 °C and further processed for immunohistochemical analyses.

2.3. Immunohistochemistry

After fixation, the brains were cryoprotected with 0.1 M phosphate buffer (pH 7.4) containing 20% sucrose for 72 h and then rapidly frozen in isopentane pre-cooled to −70 °C with dry ice. Serial coronal sections (30 μm) were cut using a cryostat through the brain region containing the ARC. After inactivating the endogenous peroxidase activity with 0.6% hydrogen peroxidase (Sigma–Aldrich, St. Louis, MO), sections were incubated separately with avidin and biotin solutions (Vector Lab, Burlingame, CA) for blocking nonspecific binding of endogenous biotin, biotin-binding protein, and lectins. Then, the sections were incubated free-floating in 0.01 M PBS (pH 7.4) containing 2% normal donkey serum (Jackson ImmunoResearch Labs, West Grove, PA), 0.3% Triton X-100 (Sigma–Aldrich, St. Louis, MO) and rabbit anti-pSTAT3 antibody (1:1,500; Cell Signaling, Beverly, MA) or rabbit anti-c-Fos antibody (1:10,000; Santa Cruz Biotechnology, CA) for 43 h at 4 °C. The immunoreaction product was visualized using the Vectastain elite ABC kit (Vector Lab., Burlingame, CA) and 3′,3′-diaminobenzidine (Sigma–Aldrich, St. Louis, MO) as a chromogen. After thorough washes, sections were mounted on gelatin-coated slides. Following dehydration in ethanol, sections were cleared in xylene and coverslipped in Permount® (Fisher Scientific, Fair Lawn, NJ). Staining for pSTAT3 and c-Fos were visualized using a bright field light source and captured with a digital camera mounted on an Olympus BX41 microscope.

Sections earmarked for double labeling with pSTAT3+POMC or pSTAT3+NPY were processed according to the indirect immunofluorescence method of Coons [21]. Briefly, following washes in PBS, the sections were incubated free-floating in PBS containing Triton X-100, blocking serums and two primary antibodies: rabbit anti-pSTAT3 (1:500; Cell Signaling, Beverly, CA) and chicken anti-POMC (1:1,000; Abcam, Cambridge, MA) or rabbit anti-pSTAT3 (1:500; Cell Signaling) and chicken anti-NPY (1:2,000; Novus Biologicals) for 43 h at 4 °C. Then, the sections were incubated at room temperature in PBS containing Triton X-100, blocking serum, and donkey anti-Rabbit Alexa Fluor 594 (1:250; Invitrogen) for 1 h, and then Triton X-100, blocking serum, and goat anti-chicken Alexa Fluor 488 (1:250; Invitrogen) for another hour. After thorough washes in PBS, all sections were mounted on gelatin-coated microscope slides, coverslipped with Vectashield (Vector Lab.), and analyzed using a Zeiss LSM700 confocal microscope. The localization of the immunoreactive signals was identified using Hof's mouse brain atlas. Six corresponding sections in the ARC, organized in a consecutive rostral to caudal sequence from −0.94 mm to −2.92 mm relative to the bregma, were counted for pSTAT3 and c-Fos positive cells and analyzed for co-localization of pSTAST3 with POMC or NPY positive cells. Cell counts from 4 mice per group were obtained from both sides of the brain in each section. pSTAT3-, c-Fos-, POMC-, and NPY-positive cells as well as double labeled cells were scored in each ARC section from vehicle and leptin-treated animals. The percentage of double-positive $pSTAT3^+$ POMC or $pSTAT3^+$ NPY cells was also determined by cell counting.

2.4. Blood biochemistry

Serum leptin levels were measured by an ELISA kit (Millipore, Billerica, MA, and R&D Systems, Minneapolis, MN).

2.5. Chronic infusion of SHU-9119

To assess whether hypothalamic MC4R mediates the response to peripheral CB_1R antagonism in DIO mice, vehicle (saline) or the MC4R

