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Effects of chronic central leptin infusion on proopiomelanocortin and neurotensin gene expression in the rat hypothalamus

Abhiram Sahu

Department of Cell Biology and Physiology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA

Abstract

Leptin signaling in the hypothalamus is critical for normal food intake and body weight regulation. While hyperleptinemia in obese people suggests a state of leptin resistance, the mechanism is not clearly understood. In a rat model of central leptin infusion in which animals develop resistance to the satiety action of leptin, orexigenic peptide producing neuropeptide Y neurons in the hypothalamus develop leptin resistance. However, it is still unknown if increased hypothalamic leptin tone caused by central leptin infusion results in the development of leptin resistance in anorexigenic peptide producing proopiomelanocortin (POMC) and neurotensin (NT) neurons. To this end, male rats were infused chronically with leptin (160 ng/hr) or vehicle into the lateral cerebroventricle for 16 days. On day 4 of leptin infusion when food intake was decreased, POMC and NT mRNA levels, as determined by RNase protection assay, were significantly increased as compared to control. By contrast, on day 16 of leptin infusion, when food intake was mostly normalized, both POMC and NT mRNA levels remained unchanged compared with control. These findings suggest the development of leptin resistance in the POMC and NT neurons following chronic elevation of hypothalamic leptin tone, which may be involved in the development of resistance to the satiety action of leptin following central infusion of this peptide hormone.

Keywords

leptin; POMC; NT; hypothalamus; leptin resistance

Introduction

A large body of evidence suggests that leptin signals nutritional status to key regulatory centers in the hypothalamus and it has emerged as an important signal regulating energy homeostasis [11,35,40]. Leptin administration centrally or peripherally decreases food intake and body weight in a variety of animals [11]. The evidence that serum leptin levels are significantly higher in obese humans relative to non-obese humans [8,11], and that leptin administration shows very limited response in obese people [13], suggests that a state of leptin-resistance is present in obese individuals. However, the mechanisms behind the development of leptin resistance are not clearly understood. It has been known that human or rodents, made obese

Address for correspondence: Abhiram Sahu, Ph.D., Department of Cell Biology and Physiology, University of Pittsburgh School of Medicine, Magee-Womens Research Institute, 204 Craft Avenue; Room B303, Pittsburgh, PA 15213, Phone: 412-641-7669, Fax: 412-641-7676, Email: asahu@pitt.edu.

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by dietary manipulation, have elevated levels of circulating leptin but maintain a normal food intake [8,11,44]. Although a defective leptin transport is thought to be one of the many factors behind the development of leptin resistance [2,6,39], available data from diet-induced obese (DIO) rodents, which may represent the form of obesity seen in most humans, strongly suggests that central leptin resistance also contributes to the development of obesity [20,22,47]. Notably, rats fed with high-fat diet (HF) shows elevated leptin levels within 1 day of HF feeding [49]. Thus, it is possible that an extended period of exposure of the brain, especially the hypothalamus, to a high level of leptin may result in the development of central leptin resistance.

We have previously demonstrated that rats develop resistance to the satiety action of leptin following chronic central leptin infusion in association with the development of leptin resistance in neuropeptide Y (NPY) neurons within two weeks of leptin infusion [34]. However, the question still remains whether other leptin-sensitive neurons develop leptin resistance following chronic increase in hypothalamic leptin tone caused by central leptin infusion. In this regard, proopiomelanocortin (POMC) producing neurons are known to play a significant role in energy homeostasis and in transducing leptin action in the hypothalamus [7,9,31,35,40]. Furthermore, several reports have shown that the melanocortin system is itself intact during states of leptin resistance, with normal or enhanced responsiveness to melanotan II (MT-II) seen when this agonist is injected either peripherally [5] or centrally [38] in rodents. Recently, Enriori et al. [10] have shown that the MC4-R receptor is up-regulated in rats in the leptin-resistance states, perhaps explaining the enhanced responsiveness to experimentally administered agonists. However, the later group also showed a resistance to the effects of leptin on alpha-MSH secretion in DIO rats. Interestingly, there was no effect of leptin on POMC mRNA levels not only in DIO rats but also in control animals [10]. Several studies have reported neurotensin (NT) as an important centrally acting anorectic signal [21,23,41], which acts partially through histamine 1 receptor [29]. NT neurons are localized in the hypothalamus [17] and they are also the targets of leptin signaling [31]. NT antagonist or antibody reverses the anorectic effect of leptin [33]. These results suggest that NT may play a role in mediating leptin action in the hypothalamus. In the present study, we sought to examine whether POMC as well as NT neurons, like NPY, develop leptin resistance following chronic central leptin infusion, because if they do, then it would provide additional evidence in support of the development of leptin resistance in the hypothalamic neuropeptidergic circuitry, involved in regulation of food intake and bodyweight, following chronic central leptin infusion.

Materials and methods

Adult male Sprague-Dawley rats, weighing ~250 g, obtained from Taconic Farms (Germantown, NY) were housed individually in a light (lights on 0500 h to 1900 h) and temperature (22 °C)-controlled room with food (pelleted Purina rat chow) and water available *ad libitum*. After 7 days of acclimatization, rats were subjected to the following experiments according to an approved Institutional Animal Care and Use Committee protocol.

Rats were implanted stereotactically with 22-gauge osmotic pump connector cannulae (Plastic One, Roanoke, VA, USA) into the lateral cerebroventricle under pentobarbital anesthesia as described previously [34]. The lateral ventricle was then connected via Medical Vinyl tubing to an artificial cerebrospinal fluid (aCSF, pH 7.4, Ref.12)-filled Alzet osmotic pump (model#2002, DURECT Corp., Cupertino, CA) implanted sc in the back. Seven days later, aCSF pumps were replaced with new pumps to infuse with either recombinant murine leptin (Dr. A.F. Parlow, NHPP, Torrance, CA, USA) at a dose of 160ng/0.5µl/hr or aCSF vehicle for 16 days. A group of aCSF infused rats was fed the amount of food that was consumed on the day before by the leptin-infused group, and served as pair-fed (PF) group. Food intake and body weight were measured daily for 16 days. Rats were killed by decapitation between 0900

and 1200 h on day 4 or 16 of infusion. Brains were removed immediately and the medial basal hypothalamus (MBH) were dissected out [30,34], frozen in liquid nitrogen and kept at -80°C until processed for RNA extraction. Trunk blood was collected for glucose, insulin and leptin determination. Epididymal fat (WAT) was dissected out and weighed.

POMC and NT mRNA levels were measured by ribonuclease protection assay (RPA) [32]. Total RNA was isolated from MBH, using RNazol (RNA STAT 60) followed by precipitation with isopropanol and ethanol washes according to the manufacturer's instructions (TEL-TEST, Inc., Friendswood, TX). Rat POMC [16] and NT [18] cDNAs were kindly provided by Dr. J. L. Roberts (Mount Sinai School of Medicine, New York, NY) and P. R. Dobner (University of Massachusetts, Amherst, MA), respectively. A riboprobe generated from a plasmid containing a rat-specific β -actin cDNA fragment (Ambion Inc., Austin, TX) served as an internal control in all RPA. [α - ^{32}P]UTP-labeled antisense cRNA probes were synthesized using T7 RNA polymerases using a transcription kit (Ambion Inc., Austin, TX). Six μg of MBH RNA, ^{32}P -labeled POMC and NT (200,000 cpm) and β -actin (20,000 cpm) cRNA probes, and 12 μg yeast tRNA (Boehringer Mannheim, Indianapolis, IN, USA) were allowed to hybridize in solution at 45°C overnight, followed by combined RNase A and T1 digestion of non-hybridized probe at 32°C for 1 hour. Stable hybrids were extracted with phenol-chloroform followed by ethanol precipitation and then separated on 6% polyacrylamide-8M urea gels. The dried gels were exposed in a Bio-Rad Molecular Imaging Screen-K for 6 to 40 hours, and the image of each gel was acquired using a Molecular Imager FX (Bio-Rad Laboratories, Hercules, CA, USA). The volume analysis of each band was performed using Quantity One Software (Bio-Rad). POMC and NT mRNA values were first normalized with β -actin mRNA levels and then the values were expressed in relation to aCSF control.

Plasma leptin was determined with a rat leptin radioimmunoassay (RIA) kit (Linco Research, St. Charles, MO). Plasma insulin was measured with a rat insulin RIA kit (Linco). All samples were assayed in the same assay. Plasma glucose was determined by the Trinder method [43] using a kit (Sigma).

All values are expressed as means $\pm$ standard error (SE). Statistical significance of differences in food intake and body weight were analyzed using repeated measures one or two-way analysis of variance (ANOVA) with post-hoc testing using Student-Newman-Keuls multiple range test. All other data were analyzed by randomized one-way ANOVA followed by Student-Newman-Keuls multiple range test. Comparisons with $p < 0.05$ were considered to be significant.

Results

The changes in food intake and body weight during the 16-day infusion period in aCSF, pair-fed and leptin-treated rats were essentially similar as described previously [34]. In control aCSF infused rats, body weight ($F_{14,240} = 221.70$; $p < 0.0001$) and food intake ($F_{14,240} = 3.30$; $p < 0.0001$) were progressively increased throughout the infusion period. In leptin infused rats, body weight gradually decreased to a nadir at day 10 and remained stabilized at lower level thereafter ($F_{14,240} = 25.08$; $p < 0.0001$). Food intake showed an initial dramatic decrease followed by a recovery by day 16, although it remained significantly ($p < 0.01$) lower than that of the aCSF control (Fig. 1). In pair-fed rats, body weight also changed significantly during the 16-day period ($F_{14,240} = 56.69$; $p < 0.0001$). Additionally, during the later period of infusion, the pair-fed rats showed significant increase in body weight as compared to that in leptin group.

As reported previously [34], plasma leptin levels were significantly increased (Day 4, aCSF: 3.01 ± 0.28 , pair-fed: 0.99 ± 0.10 , leptin: 18.29 ± 2.59 , $p < 0.0001$, $n = 12$ –14/group; Day 16, aCSF: 4.02 ± 0.25 , pair-fed: 3.58 ± 0.24 , leptin: 14.14 ± 2.21 , ng/ml, $p < 0.0001$, $n = 10$ –14/

group; mean $\pm$ SEM) and plasma insulin levels were significantly decreased in the leptin-infused rats (Day 4, aCSF: 2.58 ± 0.27 , pair-fed: 1.29 ± 0.23 , leptin: 0.71 ± 0.15 , $p < 0.0001$, $n = 12-14$ /group; Day 16, aCSF: 2.64 ± 0.23 , pair-fed: 2.15 ± 0.26 , leptin: 0.29 ± 0.04 ; ng/ml, $p < 0.0001$, $n = 10-15$ /group; mean $\pm$ SEM), compared with that of aCSF infused rats. Plasma glucose levels remained unchanged between the groups. On day 16 of infusion, epididymal fat weight was decreased in leptin treated group by 87% ($p < 0.0001$) and in pair-fed group by 24% ($p < 0.01$) as compared to that of aCSF control animals (aCSF: 5.51 ± 0.35 g; pair-fed: 4.41 ± 0.37 g; leptin: 0.79 ± 0.21 g, mean $\pm$ SEM, $p < 0.0001$); without any change on day 4 of infusion (aCSF: 4.43 ± 0.32 g; pair-fed: 3.97 ± 0.33 g; leptin: 3.36 ± 0.42 g, mean $\pm$ SEM).

On day 4 of leptin infusion, POMC and NT mRNA levels in the MBH were significantly increased when compared with aCSF control ($p < 0.01$ for POMC, $p < 0.05$ for NT) and pair-fed ($p < 0.01$ for POMC, $p < 0.05$ for NT) groups (Fig 2). In pair-fed rats, there was no change in either POMC or NT mRNA levels as compared to that of aCSF control. In contrast, on day 16 of infusion, both POMC and NT mRNA levels were comparable among aCSF, pair-fed and leptin groups (Fig 2).

Discussion

The present study shows that POMC- and NT-producing neurons in the hypothalamus become resistant to chronic leptin infusion in that on day 4 of leptin infusion POMC and NT mRNA levels were increased, but on day 16 of leptin infusion, when food intake was recovered, POMC and NT mRNA levels did not differ from that of aCSF control. Because α -melanocyte stimulating hormone, a product of POMC gene, and NT are known to inhibit food intake [7, 9,31,35,40], increased POMC and NT neuronal activity seen on day 4 of leptin infusion is most likely involved in decreased food intake during early period of leptin infusion. Similarly, leptin resistance in POMC and NT neurons may contribute in food intake recovery seen during later period of leptin infusion. Notably, although food intake in the leptin infused rats was mostly recovered by day 16 but it still remained significantly lower than control animals. Whereas the underlying mechanisms are currently unknown but if hyper-responsiveness of the melanocortin system as reported in case of DIO mice [10] did occur in our animals, then normal levels of POMC observed at this time could inhibit feeding. This requires further investigation. Nevertheless, these findings along with our previous demonstration of leptin resistance in the NPY neurons [34] suggest the development of leptin resistance in distinct orexigenic and anorectic peptide producing neurons of the hypothalamus, which may, at least in part, underlie the development of resistance to the satiety action of leptin following chronic leptin infusion.

The mechanism(s) behind the development of leptin resistance in the hypothalamic neurons, particularly in the POMC and NT neurons, following chronic increase in hypothalamic leptin tone is not clearly understood. In this regard, JAK2-STAT3 pathway is established to be the major pathway of leptin signaling in the hypothalamus [11,15,42]. Bates et al [3] have shown that disruption of long-form of leptin receptor (Ob-Rb)-STAT3 signaling by mutation of Tyr1138 in Ob-Rb results in reduction of POMC gene expression. Munzberg et al. [25] have suggested that gene expression of leptin-responsive POMC neurons in the hypothalamus requires STAT3 activation. However, the possibility of an impaired STAT3 signaling as a cause of leptin resistance, at least, in the POMC neurons is not supported by our previous demonstration of intact STAT3 activation in the hypothalamus during 16 days of central leptin infusion [30]. Amongst other signaling pathways, leptin signaling through the phosphatidylinositol-3 kinase (PI3K)-phosphodiesterase 3B (PDE3B)-cAMP pathway has gained significant importance [27,35,48]. We have recently demonstrated that this pathway is impaired following chronic leptin infusion [36] in association with an increased expression of suppressor of cytokine signaling 3 (SOCS3) [30], a negative regulator of cytokine signaling including that of leptin [4,19]. Notably, SOCS3 has been implicated in the development of

leptin resistance in the hypothalamus [26], and brain specific SOCS3 deficiency [24] or haploinsufficiency in SOCS3 [14] resists the development of leptin resistance in DIO. Also, SOCS3 overexpression in hypothalamic neuronal cell line expressing POMC reverses the stimulating effect of leptin on PI3K activity [37]. It remains to be seen if impaired PI3K-PDE3B-cAMP pathway and increased SOCS3 contribute to the development of leptin resistance in the POMC and NT neurons (present study) and NPY neurons [34]. However, activation of JAK2-STAT3 pathway [30] may be responsible for regulation of energy expenditure and body weight, since the body weight remained decreased despite a recovery in food intake.

In addition to changes in the hypothalamus, the animals showed hyperleptinemia, hypoinsulinemia and reduced WAT weight, and maintained reduced body weight following chronic leptin infusion as described previously [34]. Whereas the reason behind the maintenance of reduced body weight despite normalization in food intake is not known, the direct inhibitory effect of leptin in lipogenesis and stimulation of lipolysis and fatty acid oxidation in adipocytes [1,45,46] may contribute to the reduced body weight following chronic leptin infusion. Previous studies have shown that chronic leptin administration (or overexpression) leads to a selective loss of adipose tissue while lean body mass is preserved [12,28]. Thus, decrease in WAT weight suggests that this type of response may be occurring in our rat model of chronic central leptin infusion, and which may explain the development of “leptin resistance” to the satiety action. Alternatively, melanocortin/SNS-mediated leptin-induced lipolysis could occur and remains to be examined. Notably, during the later period of leptin infusion, pair-fed animals gained weight as compared to leptin group, which may be due to decreased energy expenditure in pair-fed animals as opposed to increased energy expenditure in leptin group.

In summary, we have demonstrated that POMC and NT neurons develop leptin resistance in association with the development of resistance to the satiety action of leptin following chronic central leptin infusion. This study along with previous evidence of leptin resistance in NPY neurons suggest the development of leptin resistance, at least in some distinct neuronal system, in the hypothalamus in this rat model of chronic leptin infusion.

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Fig. 1.

Changes in food intake and body weight during central recombinant murine leptin (160 ng/0.5 μ l/hr) infusion for 16 days. Rats were infused into the lateral cerebroventricle with artificial cerebrospinal fluid (aCSF) via Alzet osmotic minipump (0.5 μ l/hr) for 7 days before infusion with leptin or aCSF. One group of aCSF infused rats was paired-fed to that of leptin-infused group. Values represent the mean $\pm$ SEM for $n = 13$, 11 and 15 in aCSF, pair-fed and leptin groups, respectively.

Fig. 2.

Proopiomelanocortin (POMC) and neurotensin (NT) gene expression as determined by ribonuclease protection assay in the hypothalamus after 4 or 16 days of leptin infusion. (A): representative phosphorimages showing the level of POMC mRNA, NT mRNA and β -actin mRNA in the hypothalamus. (B): results obtained by phosphor imaging showing the changes in POMC and NT mRNA levels. The values were first normalized to β -actin mRNA levels and then expressed as relative to aCSF control. Values represent the mean $\pm$ SEM. Day 4: $n = 7, 8$ and 7 in aCSF, pair-fed and leptin groups, respectively. Day 16: $n = 9, 7$ and 10 in aCSF, pair-fed and leptin groups, respectively. * $p < 0.05$ and ** $p < 0.01$ vs all other groups on day 4.