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A role of phosphodiesterase-3B pathway in mediating leptin action on proopiomelanocortin and neurotensin neurons in the hypothalamus

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Abstract

Leptin signaling in the hypothalamus is required for normal food intake and body weight homeostasis. Recent evidence suggests that besides the signal transducer and activator of transcription-3 (STAT3) pathway, several non-STAT3 pathways mediate leptin signaling in the hypothalamus. We have previously demonstrated that leptin stimulates phosphodiesterase-3B (PDE3B) activity in the hypothalamus, and PDE3 inhibitor cilostamide reverses anorectic and bodyweight reducing effects of leptin. To establish the physiological role of PDE3B signaling in the hypothalamus, we examined if leptin signaling through the PDE3B pathway is responsible for the activation of proopiomelanocortin (POMC) and neurotensin (NT) neurons, which are known to play a critical role in energy homeostasis. To this end, we assessed the effect of cilostamide on leptin-induced POMC and NT gene expression in the rat hypothalamus. Results showed that while central injection of leptin significantly increased both POMC and NT mRNA levels in the medial basal hypothalamus, cilostamide completely reversed this effect of leptin suggesting a PDE3B-activation dependent induction of POMC and NT gene expression by leptin. This result further suggests that the PDE3B pathway plays an important role in mediating leptin action in the hypothalamus.

Keywords

leptin; POMC; NT; hypothalamus; phosphodiesterase-3B

Introduction

Cumulative evidence suggests that leptin, a product of the obese gene (54), signals nutritional status to key regulatory centers in the hypothalamus and it has emerged as an important signal regulating energy homeostasis [16,18,19,47]. Leptin administration centrally or peripherally decreases food intake and body weight in a variety of animals [16,49]. The deletion of leptin receptor (LEPR) in neurons leads to an obese phenotype [9], and transgenic supplementation of the LEPR in neurons of $Lepr^{db/db}$ mice results in an amelioration of the obese phenotype [27]. The early recognition of the LEPR as a member of the class 1 super-family of cytokine

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receptors resulted in prompt identification of the Janus-kinase 2 (JAK2)-signal transducer and activator of transcription-3 (STAT3) pathway as a major leptin-signaling pathway in the hypothalamus [5,18,19,50,51]. However, we have demonstrated that, in addition to the STAT3 pathway, leptin action in the hypothalamus is also mediated by an insulin-like signaling pathway involving stimulation of phosphatidylinositol 3-kinase (PI3K) and phosphodiesterase 3B (PDE3B) activities and reduction in cAMP levels in the hypothalamus [55]. In addition, cilostamide, a selective PDE3 inhibitor, reverses the anorectic and body weight reducing effect of leptin. While these results suggest a potential role of the PDE3B pathway in mediating leptin action in the hypothalamus, the physiological role of this pathway of leptin signaling in energy homeostasis remains unknown.

To demonstrate physiological role of the PDE3B pathway, it is important to demonstrate if this pathway were involved in mediating action of hypothalamic leptin sensitive neurons that play critical role in energy homeostasis. 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 [2,10,14,33,37,38,47]. In addition, several studies have reported neurotensin (NT) as an important centrally acting anorectic signal [29,31,48], which acts partially through histamine 1 receptor [35]. NT neurons are localized in the hypothalamus [24] and they are the targets of leptin signaling [38]. NT antagonist or antibody reverses the anorectic effect of leptin [45]. These results suggest that NT may play a role in mediating leptin action in the hypothalamus. Thus, to begin to establish physiological role of the PDE3B pathway of leptin signaling we tested the hypothesis that leptin action on POMC and NT neurons is mediated by activation of PDE3B pathway in the hypothalamus. To this end, we examined the effect of cilostamide, a selective PDE3 inhibitor, on leptin-induced POMC and NT gene expression in the hypothalamus.

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 stereotaxically with 22-gauge stainless steel cannula into the third cerebroventricle under pentobarbital anesthesia [46]. After a recovery period of 14 days, rats were injected icv with cilostamide (10μg/1μl) in dimethyl sulfoxide (DMSO), or DMSO alone, and recombinant murine leptin (4 μg/2μl, Dr. A.F. Parlow, NHPP, Torrance, CA, USA) in artificial cerebrospinal fluid (aCSF, pH 7.4, Ref.20), or aCSF alone, at 1700–1800 hr, and food was withdrawn. One hour later, another injection of cilostamide or DMSO was given to the rats. After 24 hr, a similar injection protocol was used. Rats were killed by decapitation 15 hr after the last injection. Brains were removed immediately and the medial basal hypothalamus (MBH) were dissected out [36,40], frozen in liquid nitrogen, and kept at −80 C until processed for RNA extraction.

POMC and NT mRNA levels were measured by ribonuclease protection assay (RPA) [36]. 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 [23] and NT [25] 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). Four μg of MBH RNA, ^{32}P-labeled POMC and NT (150,000 cpm) and β-actin (20,000 cpm) cRNA probes, and 16 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 vehicle control.

All values are expressed as means ± standard error (SE). Statistical significance of differences was analyzed by randomized one-way analysis of variance followed by Student-Newman-Keuls multiple range test. Comparisons with $p < 0.05$ were considered to be significant.

Results

The changes in POMC and NT mRNA levels in the MBH are presented in Fig 1. The bands representing stable hybrids for POMC, NT or β-actin mRNA levels in the MBH extract from one of the RNAse protection assays are presented in Figure 1A. It is evident that intensity of the bands for POMC and NT mRNA was increased in the leptin treated group as compared to all other groups, while β-actin mRNA remained unchanged among the groups. Quantitative analysis showed that intra-cerebroventricular injection of leptin significantly increased both POMC and NT mRNA levels in the MBH when compared with vehicle (aCSF +DMSO) control group ($p < 0.01$ for POMC, $p < 0.05$ for NT). Cilostamide completely reversed the stimulating effect of leptin on POMC and NT mRNA levels in the MBH. However, cilostamide alone had no effect on POMC and NT mRNA levels.

Discussion

The present study shows that POMC- and NT-producing neurons in the hypothalamus are activated by leptin, and PDE3 inhibition reverses this effect of leptin on POMC and NT gene expression. These results suggest that PDE3B activation plays a significant role in stimulation of POMC and NT neurons by leptin.

Leptin signaling in the hypothalamus is critical for normal energy homeostasis. The initial discovery of the leptin receptor as a member of the class 1 cytokine receptor super-family resulted in prompt identification of the JAK2-STAT3 pathway as a major pathway of leptin signaling in the hypothalamus [50,51]. Additionally, a defect in the STAT3 pathway has been identified in diet-induced obese (DIO) rats [28], and DIO mice [13] and brain-specific or leptin receptor- specific knockout of STAT3 causes obesity [4,17]. While the significance of the JAK2-STAT3 pathway is unequivocal, cumulative evidence strongly suggests that various non-STAT3 pathways including AMP-activated protein kinase (AMPK) [32], mammalian target of rapamycin (mTOR) [11], forkhead protein (FOXO1) [6,26], PI3K [34,55], and SHP2-GRB2-Ras-Raf-MAPK (mitogen-activated protein kinase) [3,7,8,53] pathways play significant role in transducing leptin action in the hypothalamus. We have demonstrated that leptin's action in the hypothalamus is also mediated through an insulin-like signaling pathway involving induction of PI3K and PDE3B activities and a reduction of cAMP levels [55]. Furthermore, we have also demonstrated that the PDE3B pathway interacting with the STAT3 pathway constitutes a critical component of leptin signaling in the hypothalamus, in that PDE3 inhibition by cilostamide reversed the leptin-induced STAT3 activation in the hypothalamus [55]. While these findings clearly suggest a potential role of the PDE3B pathway in transducing

leptin action in the hypothalamus, the physiological role of this pathway in energy homeostasis is still unknown.

The present study showed that leptin-induced stimulation of POMC and NT gene expression is dependent on PDE3B activation because cilostamide, a selective PDE3 inhibitor, completely reversed the stimulatory effect of leptin on these neurons. This is the first evidence suggesting a potential role of the PDE3B signaling pathway in mediating leptin action in these neurons. Because both POMC and NT neurons are implicated in energy homeostasis [9,26,28,41,43, 44], demonstration of a role of PDE3B in mediation leptin action on these neurons provides further support in favor of a physiological role of this pathway in leptin signaling. In addition, PDE3B is co-localized in POMC neurons [30], and indirect evidence such as all Ob-Rb (long form of the leptin receptor) expressing neurons coexpress PDE3B [A. Sahu, unpublished], and PDE3B is expressed in those hypothalamic areas where NT and Ob-Rb are expressed [15, 41], suggest that NT neurons may coexpress PDE3B. Thus, reversal of leptin-induced POMC and NT gene expression by cilostamide may suggest a direct role of PDE3B signaling in mediating leptin action in these neurons.

Whereas evidences such as leptin induces STAT3 in POMC neurons [22], 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 [4], gene expression of leptin-responsive POMC neurons in the hypothalamus requires STAT3 activation [33], selective deletion of STAT3 in POMC neurons causes mild obesity [52], and STAT3 plays a transcriptional role in the regulation of leptin-induced NT gene expression in N-39 neuronal cell line [12], suggest a role of STAT3 in mediating leptin action in these neurons, our study demonstrates that PDE3B pathway also mediates leptin's action on POMC as well as NT neurons. Notably, we have previously shown that the development of leptin resistance in POMC and NT neurons following chronic central leptin infusion was associated with a defective leptin signaling through the PDE3B pathway without compromising the STAT3 pathway [36,42,44]. These studies taken together further suggest that leptin signaling through the PDE3B pathway plays an important role in mediating leptin action in POMC and NT neurons. Recent studies have also implicated PI3K signaling in POMC neurons to play a significant role in mediating leptin-mediated suppression of food intake [1,21]. Additionally, PI3K is an upstream regulator of the PDE3B pathway of leptin signaling in the hypothalamus [43]. Thus, based on the available literature and current finding we hypothesize that both STAT3 and PDE3B pathways participate in transducing leptin action on POMC and NT neurons in the hypothalamus [Fig. 2].

In summary, we have demonstrated that PDE3 inhibition reverses leptin-induced stimulation of POMC and NT gene expression. This study along with recent evidence of co-localization of PDE3B in POMC and other Ob-Rb expressing neurons suggest that leptin's action on POMC and NT neurons is mediated, at least partly, through the activation of the PDE3B pathway.

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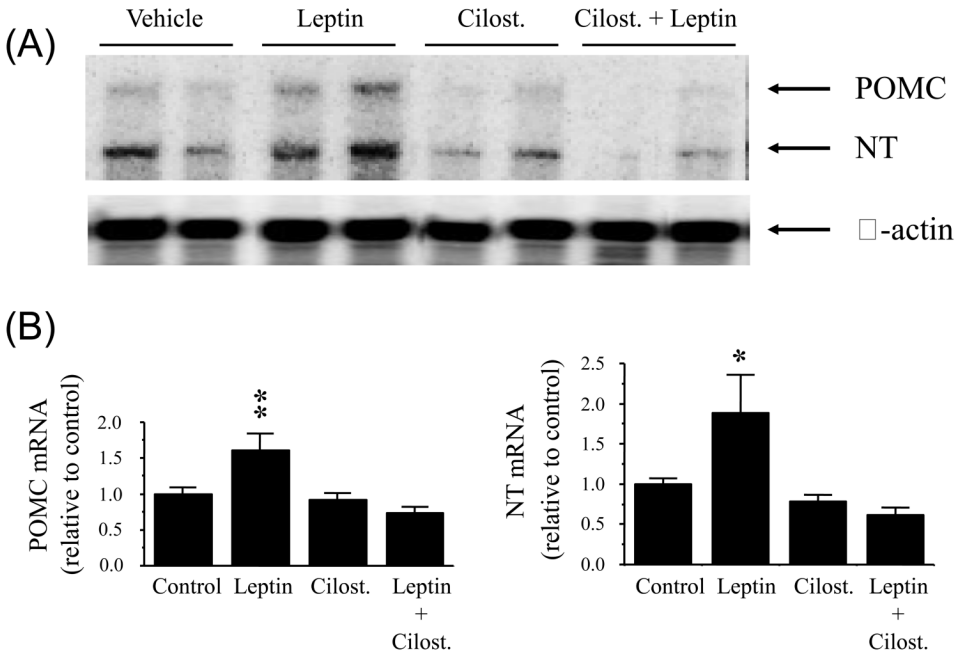

Fig. 1.

Proopiomelanocortin (POMC) and neurotensin (NT) gene expression as determined by ribonuclease protection assay in the hypothalamus following intra-cerebroventricular injection of leptin alone or in combination with cilostamide (Cilost.), a selective PDE3 inhibitor. (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 vehicle (artificial cerebrospinal fluid + dimethyl sulfoxide) control. Values represent the mean ± SEM. Control: $n = 4$, leptin: $n = 5$, cilost. : $n = 5$, and leptin + cilost. : $n = 7$. * $p < 0.05$ and ** $p < 0.01$ vs. all other groups.

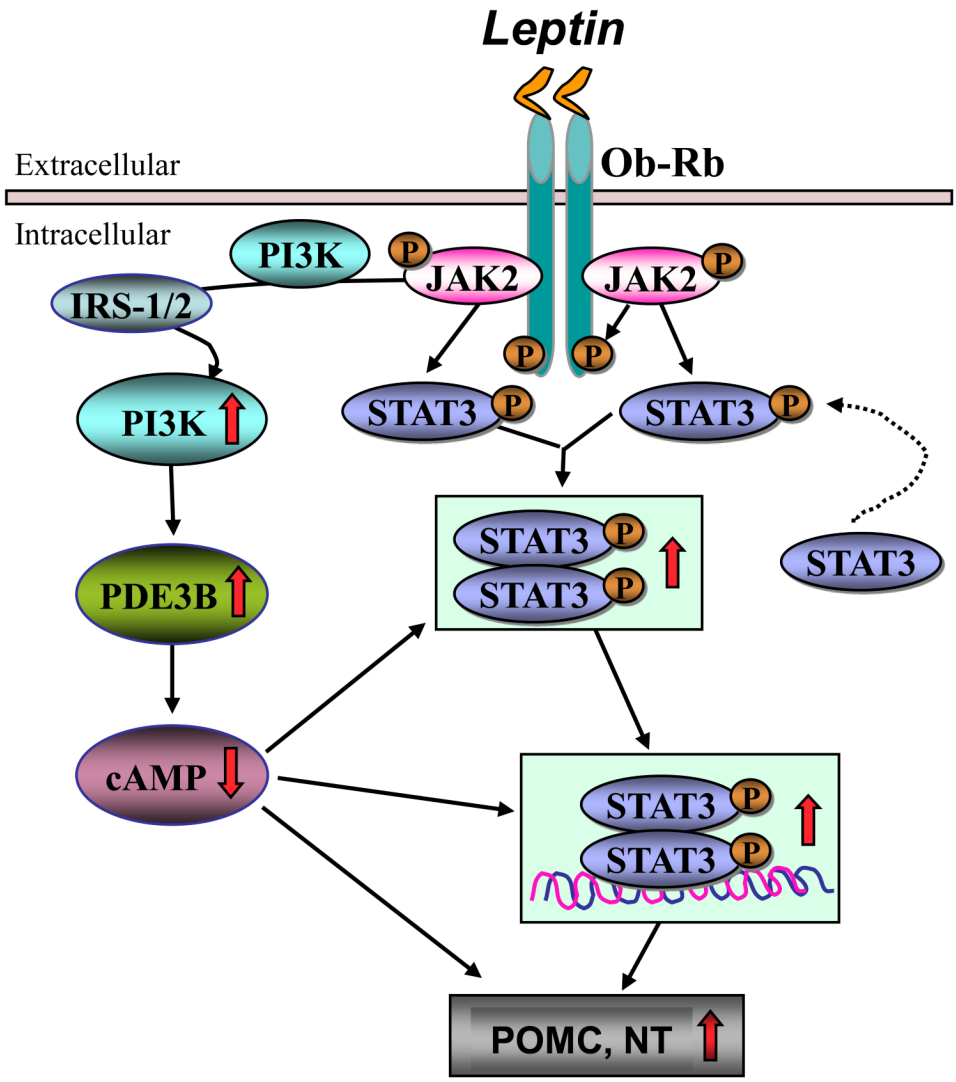

Fig. 2.

Schematic of leptin signaling through STAT3 and PDE3B pathways in POMC and NT neurons in the hypothalamus. Leptin binding to it's receptor (Ob-Rb) leads to activation of JAK2, receptor dimerization, JAK2-mediated Ob-Rb phosphorylation followed by phosphorylation and activation of STAT3. Activated STAT3 dimerizes, translocates to the nucleus, and trans-activate target genes including POMC and NT. Additionally, leptin activates PI3K and PDE3B, and decreases cAMP levels in the hypothalamus. Since PDE3 inhibition by cilostamide reverses the effect of leptin on STAT3 activation [Ref. 55] as well as leptin-induced POMC and NT gene expression (current study), it is possible that decrease in cAMP levels is necessary for STAT3 activation and subsequent stimulation of POMC and NT gene expression by leptin. Also, PDE3B-activation dependent decrease in cAMP levels may directly result in increased POMC and NT gene expression by leptin - a hypothesis need to be experimentally tested. IRS, insulin receptor substrate.