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Hypothalamic phosphodiesterase-3B pathway mediates anorectic and body weight reducing effects of insulin in male mice

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Abstract

Background—Insulin action in the hypothalamus plays a critical role in the regulation of energy homeostasis, yet the intracellular signaling mechanisms mediating insulin action are incompletely understood. Although phosphodiesterase-3B (PDE3B) mediates insulin action in the adipose tissue and it is highly expressed in the hypothalamic areas implicated in energy homeostasis, its role, if any, in mediating insulin action in the hypothalamus is unknown. We tested the hypothesis that insulin action in the hypothalamus is mediated by PDE3B.

Methods—Using enzymatic assay, we examined the effects of peripheral or central administration of insulin on hypothalamic PDE3B activity in adult mice. Western blotting and immunohistochemistry also examined p-Akt and p-STAT3 levels in the hypothalamus. Effects of leptin on these parameters were also compared. We injected cilostamide, a PDE3 inhibitor, prior to central injection of insulin and examined 12–24 hr food intake and 24 hr body weight. Finally, we examined the effect of cilostamide on insulin-induced proopiomelanocortin (*Pomc*), neurotensin (*Nt*), neuropeptide Y (*Npy*) and agouti-related peptide (*Agrp*) gene expression in the hypothalamus by qPCR.

Results—peripheral or central injection of insulin significantly increased PDE3B activity in the hypothalamus in association with increased p-Akt levels but without any change in p-STAT3 levels. However, leptin–induced increase in PDE3B activity was associated with an increase in both p-Akt and p-STAT3 levels in the hypothalamus. Prior administration of cilostamide reversed the anorectic and body weight reducing effects as well as stimulatory effect of insulin on hypothalamic *Pomc* mRNA levels. Insulin did not alter *Nt, Npy* and *Agrp* mRNA levels.

Conclusion—Insulin induction of hypothalamic PDE3B activity, and the reversal of anorectic and body weight-reducing effects and stimulatory effect of insulin on hypothalamic *Pomc* gene expression by cilostamide suggest that activation of PDE3B is a novel mechanism of insulin signaling in the hypothalamus.

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Disclosure Statement

The authors have no conflicts of interest to disclose

Keywords

Insulin; PDE3B; p-Akt; POMC; hypothalamus; feeding

Introduction

Insulin signaling in the hypothalamus plays a significant role in regulation of food intake and energy homeostasis. The circulating level of insulin is positively correlated with body weight and adiposity [1–4] and relays information on peripheral energy stores to the central nervous system (CNS) by acting on diverse neuronal populations. Along this line, chronic or acute intracerebroventricular (icv) administration of insulin decreases food intake and body weight in baboons, rats and mice [5–7]. Intranasal application of insulin reduces food intake and body weight in men [8, 9]. Reduction of insulin activity in the brain, including the hypothalamus, results in hyperphagia and weight gain [10–13]. Central insulin action is also essential for peripheral fat and glucose metabolism [14–17]. Furthermore, obese rats [18], sheep [19] and humans [20] have elevated cerebrospinal fluid insulin concentration, and in rats, imposition of a high-fat diet induces within 72 hours, a central insulin resistance independent of adiposity [21]. Thus, improving our understanding of the mechanisms of insulin signaling in the brain, particularly in the hypothalamus, is a vital step in analyzing the molecular and neural basis of food intake and body weight regulation and, ultimately, for the development of novel therapeutic approaches for the treatment of obesity and related disorders.

Although hypothalamic insulin action plays a critical role in the regulation of energy homeostasis, the intracellular signaling mechanisms mediating insulin action in the hypothalamus are incompletely understood. In this regard, (i) insulin is known to activate the phosphatidylinositol-3 kinase (PI3K)-Akt pathway in the hypothalamus [22], (ii) PI3K inhibitors reverse the anorectic and body weight reducing effects of insulin in the rat [22], and (iii) several signaling molecules downstream of Akt—including the mammalian target of rapamycin/S6 kinase [23, 24], and forkhead box O1 (FoxO1) [25]—have been implicated in insulin signaling in the hypothalamus and energy homeostasis [26–30]. In adipocytes, however, the anti-lipolytic effect of insulin [31], and insulin-induced glucose uptake and lipogenesis [32, 33] are mediated by activation of phosphodiesterase 3B (PDE3B) and regulation of the cAMP-signaling pathway. PDE3B, initially identified in adipose tissue and liver, was subsequently found in a variety of tissues including the hypothalamic arcuate nucleus (ARC), ventromedial nucleus (VMN), dorsomedial nucleus (DMN), lateral hypothalamic areas (LH), paraventricular nucleus (PVN), and perifornical hypothalamic areas that are implicated in food intake and body weight regulation [2, 34–40]. Furthermore, PDE3B is expressed in proopiomelanocortin- (*Pomc)* and neuropeptide Y (*Npy)*/agouti-related peptide *(Agrp)*-expressing neurons [34] that are the targets of both leptin and insulin signaling [1, 2, 40–43]. Thus, the question arises as to whether the PDE3B pathway mediates insulin signaling in the hypothalamus.

Whereas an appreciation of the role of the PDE3B pathway in mediating leptin signaling in the hypothalamus, particularly in the context of energy homeostasis, is gradually unfolding

[44, 45], the role of this pathway in mediating hypothalamic insulin action is completely unknown. Therefore, the aim of the present study was to elucidate whether PDE3B pathway mediates hypothalamic action of insulin particularly in food intake and body weight regulation. To this end, we first tested whether insulin stimulates PDE3B activity in the hypothalamus and then examined the effects of cilostamide, a specific PDE3 inhibitor [31, 46–48], on the anorectic and body weight-reducing effects of central insulin.

Materials and Methods

Male C57BL/6J mice were available in our breeding colony, which was established by mating male and female C57BL/6J mice obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). Mice were maintained in a light (lights on 0600 h to 1800 h) and temperature (22 °C)-controlled room with food (pelleted rodent chow) and water available *ad libitum*. Adult male mice of 12 to 16 weeks old were subjected to the following experiments, all of which were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Experiment 1: Effects of peripheral insulin on PDE3B activity, p-Akt, and p-STAT3 levels in the hypothalamus of male mice: a comparison with the effects of leptin

Because insulin is known to increase PDE3B activity in the liver and adipose tissues [31, 49], we first examined whether insulin also increases PDE3B activity in the medial basal hypothalamus (MBH) of mice. Mice were subjected to a prolonged fast (20 hours) and then injected intraperitoneally (ip) with insulin (15 IU/kg) [22] or saline, without glucose, and sacrificed at 15 or 30 min post-injection. Because central leptin injection increases hypothalamic PDE3B activity in rats [44], we also compared the effect of insulin with that of leptin on PDE3B activation in the mouse hypothalamus. Thus, simultaneously, another group of mice was injected ip with leptin (3.5 mg/kg; A. F. Parlow, National Hormone & Peptide Program, Torrance, CA) and sacrificed 30 min post-injection. Brains were removed immediately and the MBH were dissected out, frozen in liquid nitrogen and kept at −80 C until processed for protein extraction. The MBH tissue was bounded rostrally by the posterior border of the optic chiasma, laterally by the lateral sulcus, and caudally by the mammillary bodies and cut to a depth of approximately 1.5 mm, as described previously by us [50]. Because recent evidence from our laboratory showed that Akt might not be an upstream regulator of the PDE3B pathway of leptin signaling in the rat hypothalamus [51], we also examined Akt phosphorylation (p-Akt^{ser473}) and STAT3 phosphorylation (p-STAT3^{Tyr705}) in the MBH after insulin or leptin injection.

To examine whether p-Akt^{ser473} and p-STAT3^{Tyr705} were altered in specific regions of the hypothalamus, some mice from each group at 30 min post-injection were anesthetized with pentobarbital and perfused intracardially with 0.9% saline, followed by ice-cold 4% paraformaldehyde in 50 mM phosphate buffer. The brains were dissected out and post-fixed in the same fixative for overnight and then kept in 25% sucrose solution at 4°C until they sank. Thereafter, the brains were frozen on dry ice, and coronal 25-μm free-floating sections were cut in five series through the hypothalamus on a freezing microtome (Leica Sliding

Microtome; Leica Microsystems GmbH, Wetzlar, Germany), and stored in cryoprotectant at −20°C until use. One series was subjected to immunohistochemistry (IHC)

Measurement of PDE3B activity—PDE3B activity in the hypothalamus was measured according to a previously described protocol [52, 53], which has been used by us [44, 54]. The protocol used in the present study is essentially similar to that described recently by us [50]. MBH was homogenized in ice-cold extraction buffer (10 mM sodium phosphate, pH 7.2; 50 mM NaF, 150 mM NaCl; 3 mM benzamidine, 2 mM EDTA; 0.1% Triton X-100; 0.5% Igepal; containing cocktail of protease and phosphatase inhibitors from Roche Diagonostics, Indianapolis, IN, USA) and the homogenate was centrifuged at 38,000 g for 45 min at 4°C using an Optima TLX ultracentrifuge. The pellet was re-suspended in extraction buffer containing 1% NP-40 (Sigma Chemicals, St. Louis, MO, USA) and used for PDE3B assay, and supernatant was used to measure p-Akt and p-STAT3 levels by Western blotting. Protein was measured by Pierce BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA).

PDE3B assay was performed at 30°C in a total volume of 250 μl that included 12.5 μg of re-suspended MBH protein in a 50-μl sample volume and 200 μl reaction mixture [50 μl buffer A: 100 mM MOPS, pH 7.5, 4 mM EGTA, 1 mg/ml BSA; 50 μl buffer B: 100 mM MOPS, 75 mM Mg Acetate, ~100,000 cpm ^{3}H-cAMP (Perkin-Elmer, Boston, MA, USA); 1 μM cAMP; 100 μl H_2O] containing the specific PDE3 inhibitor cilostamide [31, 46–48] (100 mM) or no inhibitor. In this assay condition, cilostamide inhibited 30% of total PDE. PDE3B activity was calculated as pmol cAMP hydrolyzed per min/μg protein using an EXCEL Program (Microsoft Corp. Redmond, WA, USA). The values were then expressed in relation to the saline control.

Measurement of p-Akt and p-STAT3 levels in the hypothalamus by western blotting—Aliquots of supernatants from MBH protein extracts were resolved by SDS-PAGE (10% gel for p-Akt; 8% gel for p-STAT3) and transferred to polyscreen PVDF membranes. The membranes were blocked in 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween-20, and incubated overnight with primary antibody for phospho-Akt^{Ser473} (Cell Signaling Technology, Inc., Danvers, MA; 1:2,000; catalog# 4051), or phospho-STAT3^{Tyr705} (p-STAT3, Santa Cruz Biotechnology, CA; 1:900; catalog# Sc-8059) at 4 °C followed by incubation for 60 min with HRP-conjugated secondary antibodies at room temperature. Immunoreactive bands were visualized by Western Lightning chemoluminescence Reagent Plus-ECL as described by the manufacturer (Perkin Elmer). Immunoreactive bands were scanned and analyzed by NIH Image Software. The membranes were stripped and then blotted for total Akt (Cell Signaling Technology, 1:1200; catalog# 9272) or STAT3 (Santa Cruz Biotechnology, 1:1000; catalog# Sc-483) as appropriate to measure Akt and STAT3 levels. The levels of p-Akt and p-STAT3 levels were first calculated as ratio to total Akt and STAT3, respectively, and then expressed as relative to the control saline group.

Immunohistochemistry (IHC) for p-Akt^{ser473} and p-STAT3 localization in the hypothalamus—The IHC localization of p-Akt^{ser473} immunoreactive protein in the hypothalamus was done according to the method described previously by us [50, 51].

Briefly, free-floating sections of fixed tissue were treated with 0.5% NaOH and 2% H_2O_2 in H_2O for 20 min at room temperature followed by 2 h with blocking solution [10% normal goat serum in potassium PBS (KPBS), 1% BSA, 0.4% Triton X-100] at room temperature. Sections were then incubated with p-Akt^{Ser473} antibody (Cell Signaling Technology, 1:2000; Catalog# 4060) in antibody dilution buffer (5% normal goat serum in KPBS, 0.5% BSA, 0.4% Triton X-100) for approximately 38 h at 4 °C followed by washing in KPBS and incubation with secondary antibody (goat anti-rabbit, 1:1200; Vector Laboratories, Burlingame, CA, USA) in antibody dilution buffer for 90 min at room temperature. Sections were washed and incubated in Vectastain Elite ABC reagent (Vector Laboratories) in KPBS containing 0.4% Triton X-100 for 90 min at room temperature. Finally, sections were washed and the signal was developed by incubating in 0.04% 3,3′-diaminobenzidine solution containing 0.01% nickel chloride, giving a brown precipitate. For IHC localization of p-STAT3^{Tyr705} immunoreactive protein in the hypothalamus, we essentially used the method described previously by us [51, 55]. Briefly, free floating tissue sections were pretreated with 1% NaOH and 1% H_2O_2 in H_2O for 20 min, 0.3% glycine for 10 min, and 0.03% sodium dodecyl sulfate for 10 min. Sections were then blocked for 1 h with blocking solution (5% normal goat serum in KPBS, 1% BSA, 0.4% Triton X-100), followed by incubation with p-STAT3^{Tyr705} antibody (Cell Signaling Technology, dilution 1:3000 in blocking solution; catalog# 9131) for overnight at 4°C. On the next day, the sections were washed, incubated with biotinylated secondary goat anti-rabbit antibody (Vector Laboratories, dilution1:4000 in blocking solution) for 90 min, and then treated with avidin-biotin-complex solution (Vector Laboratories) for 90 min. Finally, the signal was developed by diaminobenzidine solution containing 0.5% nickel sulfate, giving a dark-blue precipitate. The sections were then mounted on glass slide, dried, and processed through graded alcohol and clearing agent (Xylene), and then cover slipped with DPX.

For analysis of p-STAT3^{Tyr705}-positive cells, the sections were photographed under a bright field using a QImaging camera (MicroPublisher 5.0 RTV, Surrey, BC, Canada) attached to a Leica DMBRE microscope (Leica Microsystems) and the matched-sections from different groups were compared. For analysis of p-Akt^{ser473}-positive cells, the sections were photographed as described and all cells immunoreactive for p-Akt^{ser473} were counted in evenly-spaced, region matched sections (3 to 4) through the ARC of individual animals. Immunoreactive cells in the ARC, VMN and DMN areas were counted on both sides to determine the total number of p-Akt^{ser473}-positive cells per section.

Experiment 2: Effects of central insulin on PDE3B activity, p-Akt, and p-STAT3 levels in the hypothalamus of male mice: a comparison with the effects of leptin

Because in experiment 1 we observed peripheral insulin to increase hypothalamic PDE3B activity, which could be due to a direct or an indirect effect of insulin, we next examined whether central insulin administration increases PDE3B activity in the hypothalamus and also included a group receiving central leptin as a positive control [44]. For central injection, mice were anesthetized with pentobarbital (40 mg/kg) and then implanted stereotaxically with 28-gauge stainless steal cannula in the lateral cerebroventricle (LCV)(coordinates: AP: −0.5 mm, ML: 1 mm, DV: 2.5 mm) as described by Hill *et al.* [56]. After the recovery period of ten to 14 days, the icv location of the cannula was confirmed by drinking response after

angiotensin II injection [57]. Mice with indwelling lateral cerebroventricular (LCV) cannula were fasted 22–24 hours and injected icv with insulin (4.4 mU/2μl) [58,59], leptin (4 μg/2μl) or saline (2μl). After 30 minutes, the mice were sacrificed and the MBH were dissected out, frozen in liquid nitrogen and kept at −80°Cuntil processed for protein extraction to measure PDE3B activity and p-AKT and p-STAT3 levels as described above. Trunk blood was collected to measure circulating insulin levels.

To examine whether p-Akt^{ser473} and p-STAT3^{Tyr705} were altered in specific regions of the hypothalamus, some mice from each group were anesthetized with pentobarbital and perfused intracardially with 0.9% saline, followed by ice-cold 4% paraformaldehyde in 50 mM phosphate buffer. The brains were processed for IHC as described above.

Insulin determination—Plasma insulin levels were determined using Mercodia Mouse Insulin ELISA kit (Mercodia AB, Uppsala, Sweden). It is to be noted that this particular insulin ELISA kit detects both mouse and human insulin.

Experiment 3: Effects of cilostamide, a specific PDE3 inhibitor, on the anorectic and body weight-reducing effects of insulin

To learn whether the insulin-induced increase in hypothalamic PDE3B activity has any physiological significance, we investigated whether prior injection with cilostamide reversed the effects of central insulin on food intake and body weight in mice with indwelling LCV cannula. We used cilostamide because it is one of the well-established PDE3 inhibitors that are being used for PDE3 inhibition in many studies including ours (31, 44, 46–48, 60–62). After 10 to 14 days of recovery from the surgery and the confirmation of the icv location of the cannula by drinking response after angiotensin II injection [57], mice were injected icv with cilostamide (10 μg/μl) or DMSO (vehicle) five hours (i.e. at 13:00h) before the lights were turned off and food was withdrawn. Forty minutes later, the mice were injected icv with insulin (4.4 mU/2μl) or saline (2μl). Food (rodent chow) was given at 6:00 PM and food intake was measured at 12 and 24 hours; body weight was measured at 24 hours after the injection.

Experiment 4: Examine whether cilostamide reverses hypothalamic Pomc, Npy, Agrp and neurotensin (Nt) gene expression response to central insulin

Adult male mice with indwelling lateral cerebroventricular cannula, fasted for 24 hours, were injected icv with cilostamide (10 μg/μl) or DMSO, followed 40 minutes later with icv insulin (4.4 mU/2μl) or saline (2μl) injection. Two hours later, mice were sacrificed and the MBH were processed for *Pomc*, *Nt*, *Npy* and *Agrp* gene expression by qPCR, using a SYBR green master mix (Invitrogen) and run on the Applied Biosystems Prism 7900HT real-time PCR machine. *Pomc*, *Nt*, *Npy* and *Agrp* mRNA levels were expressed as relative to a house keeping gene, *cyclophilin,* in each sample.

RNA isolation and RT-qPCR—RNA isolation and RT-qPCR were done as described previously [63]. Briefly, total RNA from MBH was isolated by Trizol reagent according to manufacturer's protocol (Life Technologies) and subjected to DNase treatment using RNase-Free DNase (Promega, Madison, WI) according to the manufacturer's protocol and re-

extracted with Trizol. cDNA was synthesized from 2 μg RNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). The RT reaction consisted of 10 min incubation at 25 °C, 120 min incubation at 37 °C, followed by a 5 min 85 °C termination step, and the resulting cDNA was stored at −20 °C. qPCR reactions were performed in duplicate with 100 ng of cDNA using 2X Power SYBR Green PCR master mix (Life Technologies) containing 500 nm each primer and run on the Applied Biosystems Prism 7900HT real-time PCR machine at 95 °C for 10 min followed by 40 cycles of 95 °C (15 s) and 60 °C (60 s). The primer sequences are as follows: *Pomc*: sense-5′-atgccgagattctgctacagtcg-3′; anti-sense-5′-ttcatctccgttgcc-aggaaacac-3′; *Nt*: sense-5′-aatgtttgcagcctcataaataac-3′; anti-sense-5′-tgccaacaaggtcgtcatc-3′; *Npy*: sense-5′-cagaaaacgcccccagaa-3′; anti-sense-5′-aaaagtcgggagaacaagtttcatt-3′; *Agrp*: sense-5′-cggaggtgctagatccacaga-3′; anti-sense-5′-aggactcgtgcagccttacac-3′; *cyclophilin*: sense-5′-aaggtgaaagaaggcatgaac-3′; anti-sense-5′-agctgtccacagtcggaaatg-3′. The relative quantification of mRNA levels (fold change) normalized to cyclophilin was calculated using the ΔΔCT method from the Ct (threshold cycle) values obtained from ABI SDS software package.

Statistical analysis

All values are expressed as means ± standard error (SE). Statistical significance was determined using randomized one-way ANOVA with Fisher's least significant difference (LSD) (protected t-tests) multiple-range tests to compare three or more groups or Student's *t* test to compare two groups. Shapiro-Wilk test of normality was done for each data set before performing parametric analysis. All data had the probability > 0.05 and passed the normality test. All statistical analyses were done using GB-Stat software for the Macintosh (Dynamic Microsystems, Inc., Silver Spring, MD). $P < 0.05$ were considered to be significant.

Results

Experiment 1: Changes in PDE3B activity, p-Akt, and p-STAT3 levels in the hypothalamus of male mice following peripheral injection of insulin or leptin

To examine if insulin utilizes PDE3B pathway in transducing its action in the hypothalamus in regulation of energy homeostasis, we first examined if peripheral injection of insulin increases PDE3B activity in the hypothalamus. Because we have previously demonstrated that leptin stimulates PDE3B activity in the hypothalamus [44, 50], we also examined the effect of peripheral leptin on PDE3B activity in the hypothalamus as a positive control. As expected, peripheral injection of leptin significantly increased PDE3B activity ($p < 0.0001$) in the MBH within 30 min post-injection (Fig. I). Most importantly, as shown in Figure 1, peripheral injection of insulin also significantly increased ($p < 0.0001$) PDE3B activity in the MBH at 15 or 30 min post-injection, which was associated with increased phosphorylation of Akt at serine^{473} (p-Akt^{ser473}) in the MBH ($p < 0.01$ and $p < 0.05$ at 15 and 30 min, respectively, Supplementary Figs. S1A and S1B). In addition, like insulin, leptin increased p-Akt^{ser473} levels in the MBH ($p < 0.05$, Supplementary Figs. S1A and S1B). However, whereas leptin increased p-STAT3^{Tyr705} levels ($p < 0.01$) in the MBH, insulin failed to do so (Supplementary Figs. S1C and S1D). Notably, although glucose was not co-administered with insulin and hence the likelihood of provoking hypoglycemia and counter-

regulatory responses exist, these responses are not known to trigger phosphorylation of Akt and STAT3, or to activate PDE3B. This also appears to be unlikely because we obtained essentially similar results following central administration of insulin (see below the results of Experiment 2).

To address whether the changes in p-Akt^{Ser473} and p-STAT3^{Tyr705} levels seen in the MBH extract were confined to specific area(s) of the hypothalamus, we processed brain sections, at the level of the median eminence (ME)-arcuate region (ARC), for IHC with p-Akt^{Ser473} antibody or p-STAT3^{Tyr705} antibody to examine p-Akt^{Ser473}-positive and p-STAT3^{Tyr705}-positive cells, respectively. IHC confirmed the finding obtained by western blotting in that both insulin and leptin increased p-Akt^{Ser473}-positive cells, but only leptin increased p-STAT3^{Tyr705}-positive cells in the hypothalamus (Supplementary Fig. S2). In addition, whereas i.p. insulin injection increased the number of p-Akt^{Ser473}-positive cells only in the ARC ($p< 0.01$), i.p. leptin injection increased the number of p-Akt^{Ser473}-positive cells in the ARC ($p <0.05$), as well as in the VMN ($p <0.05$), and DMN ($p <0.05$) as compared to saline control (Supplementary Fig. S5A). Also the number of p-Akt^{Ser473}-positive cells in the ARC was significantly increased ($p<0.01$) in the insulin treated group as compared to the leptin treated group (Supplementary Fig. S5A).

Experiment 2: Changes in PDE3B activity, p-Akt, and p-STAT3 levels in the hypothalamus of male mice following central injection of insulin or leptin

Because increased PDE3B activity and associated changes in p-Akt^{ser473} and p-STAT3 levels in the MBH following peripheral insulin injection could be a direct or indirect effect of insulin, this study examined if the central injection of insulin increased PDE3B activity. As shown in Figure 2, intracerebroventricular injection of insulin increased PDE3B activity in the MBH by 2.5-fold ($p < 0.001$) as compared to that in the saline injected control. As a positive control, leptin increased PDE3B activity in the MBH as expected ($p < 0.01$, Fig. 2). Furthermore, as seen following peripheral injection, both insulin ($p < 0.01$) and leptin ($p < 0.05$) significantly increased p-Akt^{ser473} levels, but only leptin increased p-STAT3^{Tyr705} levels ($p < 0.01$) in the hypothalamus (Supplementary Fig. S3). In addition, IHC demonstrated increased p-Akt^{Ser473}-positive cells in the ARC, VMN and DMN following leptin or insulin injection (Supplementary Figs. S4B and S4C; Supplementary Fig. S5B) and only leptin, but not insulin, to increase p-STAT3^{Tyr705}-positive cells in the hypothalamus (Supplementary Figs. S4E and S4F). Also, circulating insulin levels following icv injection did not differ among the groups (saline: 0.199 ± 0.025, leptin: 0.214 ± 0.057, insulin: 0.210 ± 0.034; ng/ml; mean ± SEM; n = 7/group), suggesting that the effects of insulin following central administration are direct, local actions.

Experiment 3: Cilostamide, a specific PDE3 inhibitor, reverses the anorectic and body weight-reducing effects of central insulin

To address whether increased PBE3B activity has any physiological role in mediating insulin action on food intake and body weight regulation, this study examined whether PDE3B inhibition reverses the effect of insulin on these parameters. As shown in Figure 3A, central injection of insulin significantly decreased ($p < 0.01$) food intake at 12 or 24 hr post-injection, and this effect was blunted by prior injection of cilostamide. Similarly, central

injection of insulin significantly decreased ($p < 0.05$) 24-hr body weight and prior cilostamide injection blunted this effect of insulin (Fig. 3B).

Experiment 4: Cilostamide reverses the stimulatory effect of insulin on Pomc gene expression in the hypothalamus

Because insulin signaling in POMC neurons plays an important role in energy homeostasis [64–66], we examined whether cilostamide reverses the insulin-induced increase in *Pomc* gene expression in the MBH. In addition, since NPY/AgRP neurons in the hypothalamus are also the known targets of insulin [67], and NT plays an important role in energy homeostasis [68–71], we also examined *Npy*, *Agrp* and *Nt* gene expression. As shown in Figure 4, central injection of insulin significantly increased ($p < 0.01$) *Pomc* mRNA levels in the MBH as compared to that following saline injection, and importantly, this effect of insulin was completely reversed by prior administration of cilostamide. Although there was a tendency towards an increase in *Pomc* mRNA levels following cilostamide alone, it was not significant and the underlying cause is currently unknown. However, in contrast to its stimulatory effect on *Pomc* gene expression, insulin did not have any effect on *Nt*, *Npy* or *Agrp* mRNA levels at this time point, and cilostamide had also no effect on these gene expressions (Fig. 4). Notably, previous studies have also reported central insulin having no effect on *Npy* and *Agrp* gene expression at 2 hour post-injection [7, 21].

Discussion

The physiological role of the PDE3B pathway, if any, in mediating insulin action in the hypothalamus is completely unknown. In addition to providing first demonstration of the ability of peripherally or centrally administered insulin to stimulate PDE3B activity in the hypothalamus in mice, we demonstrate that a specific PDE3 inhibitor, cilostamide blunts the anorectic and body weight-reducing effects as well as the stimulatory effect of insulin on *Pomc* gene expression in the hypothalamus.

Cumulative evidence suggests that insulin action in the hypothalamus plays a significant role in energy homeostasis and in glucose and fat metabolism [1–4, 14–17]: yet, the intracellular signaling mechanisms mediating these actions of insulin are incompletely understood. Whereas the PI3K-Akt pathway has been established as the primary mechanism of insulin signaling in many tissues including the hypothalamus [22], there are several signaling molecules downstream of Akt –including mTOR/S6 kinase and Foxo1– have been implicated in insulin signaling in the hypothalamus and energy homeostasis [23–30]. Our laboratory is investigating the role of PDE3B in the hypothalamus in the regulation of energy homoeostasis and has demonstrated PDE3B signaling to play an important role in mediating leptin action in the hypothalamus [44,45]. Because PDE3B is localized in various hypothalamic sites which have been implicated in energy homeostasis and also reported to express insulin receptor (IR) [2, 34, 64–66], and because PDE3B pathway plays an important role in mediating insulin action in peripheral tissues, particularly in the adipose tissue and liver [31–33, 49,53], in the present study we sought to examine whether PDE3B plays any role in mediating insulin action in the hypothalamus in the regulation of energy homeostasis.

To establish the role of PDE3B in mediating insulin action, it is imperative to demonstrate if insulin stimulates PDE3B activity in the hypothalamus. Our demonstration that peripheral insulin administration increased PDE3B activity in the hypothalamus suggested for the first time the possibility of PDE3B to play a role in mediating hypothalamic action of insulin. We also confirmed the activation of PDE3B in the hypothalamus by peripheral leptin [50]. Furthermore, increased p-Akt^{Ser473} and p-STAT3 levels by insulin and leptin, respectively, confirmed the action of these peripherally administered hormones in the hypothalamus [22, 72].

Whereas increased PDE3B activity following peripheral insulin administration could be due to its direct and/or indirect effects in the hypothalamus, our finding that icv administration of insulin increased PDE3B activity in the hypothalamus clearly suggests that insulin directly acts in the hypothalamus to induce PDE3B activity. Increased PDE3B activity by central leptin not only confirms our previous finding in rats [44], it also validates the results with central insulin administration. Together the results obtained following peripheral and central administration of insulin clearly demonstrate that insulin induces PDE3B activation in the hypothalamus, an important first step toward establishing PDE3B as a target of insulin action in this part of the brain.

To establish the physiological significance of insulin-induced increase in hypothalamic PDE3B activity, we next examined if PDE3 inhibition could reverse the effects of central insulin on food intake and body weight. To this end, we used cilostamide that has been widely used as a specific PDE3 inhibitor [31, 46–48, 60–62]. Although there are some controversies whether central insulin reduces food intake or not [73, 74], our study shows that central injection of insulin significantly decreased food intake and body weight in our experimental system. This is in accord with the previous finding in mice [7]. Importantly, the finding that prior treatment with cilostamide blunted the anorectic and body weight reducing effects of insulin strongly suggests a role for the PDE3B pathway in mediating hypothalamic action of this hormone in the regulation of food intake and body weight.

To begin to examine the target neurons that mediate PDE3B pathway of insulin signaling in the hypothalamus in the regulation of energy homeostasis, in the present study we mainly focused on POMC, NT and NPY/AgRP neurons that have been implicated to play an obligatory role in energy homeostasis [41, 64, 67, 68–71]. Furthermore, POMC and NPY/AgRP neurons not only express both Lepr-b and IR [64, 66], they also express PDE3B [34]; and insulin is known to induce *Pomc* gene expression [21, 65] and inhibit *Npy* gene expression [67]. Whereas hypothalamic NT neurons are the targets of leptin signaling and have been implicated in energy homeostasis [68–71, 75, 76], and insulin increases *Nt* gene expression in a hypothalamic neuronal cell line [77], whether insulin also acts on these neurons *in vivo* is unknown. Therefore, we tested if cilostamide would modify the effect of insulin on *Pomc, Nt* and *Npy/Agrp* gene expression, if it does then it will provide the evidence in support of a role for the PDE3B pathway in mediating insulin action on these hypothalamic neurons. Our finding of cilostamide reversing the insulin-induced *Pomc* gene expression indeed suggests a role for PDE3B in mediating insulin action in POMC neurons, which is likely to mediate, at least partly, the hypothalamic action of insulin in regulating energy homeostasis. Notably, although almost all POMC neurons express PDE3B [34], it

has been reported that the POMC neurons that respond to insulin are largely distinct from the ones that respond to leptin [78]. Thus, it is likely that the stimulatory effect of insulin on hypothalamic *Pomc* gene expression represent a direct action on insulin-sensitive POMC neurons. Whereas the mechanism(s) by which PDE3B mediates insulin action on POMC gene expression was not addressed in this study, insulin is known to increase POMC transcription by repressing FOXO1, which reduces POMC promoter activity [29]. In addition, insulin-stimulated PI3K—Akt activation is required for FOXO1 phosphorylation, which would translocate FOXO1 from the nucleus to cytoplasm resulting in removal of FOXO1 repression on POMC promoter activity and an increase in POMC gene expression [79, 80]. Presently, we do not know whether cilostamide inhibits insulin-stimulated phosphorylation of FOXO1 or whether activation of FOXO1 requires PDE3B activation. It is also unknown if the *Pomc* gene requires two signals, one from the phosphorylation and exclusion of FOXO1 from the nucleus and the other from activation of PDE3B. We speculate that FOXO1 and PDE3B are most likely two independent downstream signaling components of the PI3K-Akt pathway of insulin signaling in POMC neurons, which will be the subject of future investigation. Although central insulin administration had no effect on *Npy, Agrp* or *Nt* gene expression at 2 hour post-injection and it may require examining this response at a later time point [77] and therefore the effect of cilostamide could not be documented, because these neurons, particularly NPY/AgRP neurons, express PDE3B as well as insulin receptors [34, 66], involvement of these neurons in mediating insulin action through the PDE3B pathway can not be completely ruled out, which requires further investigation. Additionally, it is unknown if the effect of cilostamide is also mediated through a different class of neurons that may not be insulin-sensitive but indirectly modify the activity of insulin-responsive neurons. This possibility exists because, besides the ARC, PDEB is highly expressed in various other hypothalamic sites including the VMN, DMN, PVN and ventral premammillary nucleus regions that do not express POMC, NPY/AgRP or NT [34]. Moreover, whereas our previous study has shown PDE4 not to mediate leptin action in the hypothalamus [44], it remains to be determined if PDE4, particularly PDE4B that is highly expressed in the hypothalamus [81, 82], is involved in transducing insulin action in the hypothalamus.

Our finding that whereas insulin increased only p-Akt and not p-STAT3 levels, but leptin increased both p-Akt and p-STAT3 levels in the mouse hypothalamus deserves some additional comments. Particularly, based on the finding that PI3K inhibitor reversed the stimulatory effect of leptin on PDE3B activity and that leptin failed to increase p-Akt, we previously concluded that in rats, PI3K but not Akt is upstream of the PDE3B pathway of leptin signaling in the hypothalamus [51]. Although whether PI3K-Akt is upstream of the PDE3B pathway of insulin/leptin signaling was not specifically addressed in this study, the finding of increased p-Akt by insulin and leptin in association with increased PDE3B activity suggest the possibility that Akt may be upstream of the PDE3B pathway of both insulin and leptin signaling in the mouse hypothalamus. In addition, this study with biochemical and IHC approaches confirms previous reports of insulin not having any effect on p-STAT3 levels in the hypothalamus [51, 83]. It is noteworthy that p-Akt response to peripheral and central insulin was somewhat different in various hypothalamic regions. Specifically, peripheral insulin administration increased the number of p-Akt^{Ser473}-positive

cells mainly in the ARC but not in the VMN or DMN, whereas central insulin administration increased the number of p-Akt^{Ser473}-positive cells in the ARC as well as in the VMN and DMN. Furthermore, the number of p-Akt^{Ser473}-positive cells was significantly higher in the ARC following peripheral insulin as compared to central insulin administration. Although reason behind this discrepancy is not clear, a shorter duration of insulin treatment could be necessary to obtain optimal p-Akt activation in the hypothalamic neurons following central injection. However, both central and peripheral leptin treatment increased the number of p-Akt^{Ser473}-positive cells in the ARC, VMN and DMN.

Whereas PI3K has been established to mediate both leptin and insulin signaling in the hypothalamus [22, 44, 84], whether PDE3B could be another signaling component in the hypothalamus that also mediates the action of both these hormones has not been addressed previously. Because both insulin and leptin resistance occur in the hypothalamus during the development of DIO, determining whether the PDE3B pathway integrates these two obligatory signals that regulate energy and glucose homeostasis by acting on hypothalamic neurons would be quite significant. It is particularly important because *Pde3b*-null mice show altered energy and glucose homeostasis [85] and DIO is associated with impaired PI3K-PDE3B pathway of leptin signaling in the hypothalamus [50, 55]. Thus, our present finding in mice of PDE3B activation in the hypothalamus by both insulin and leptin and reversal of anorectic and body weight reducing effect as well as stimulatory effect of insulin on POMC gene expression by PDE3B inhibitor cilostamide suggest the possibility that PDE3B could be a key signaling node mediating both leptin and insulin action in the hypothalamus in regulation of energy homeostasis. This position is also supported by our previous demonstration of cilostamide reversing anorectic and body weight reducing effects and stimulatory effect of leptin on POMC gene expression in the rat hypothalamus [44, 61].

Interestingly, despite an apparent role for PDE3B in the appetite-suppressing effects of both leptin [44] and insulin (present study), the *Pde3b-null* mice do not develop obesity and with less gonadal adipose tissue mass [85]. One of the reasons would be the development of compensations in the *null* mice. Also, because PDE3B is expressed in variety of tissues including the hypothalamus, liver and adipose tissues, etc., that play obligatory role in energy homeostasis, whole body knock-out may not produce the expected effect. It is also possible that reduced adipose tissue seen in the *null* mice, is most likely due to deletion of *Pde3b* in the adipocytes, presumably leading unopposed cAMP-dependent lipolysis. Nevertheless, the *Pde3b-null* mice exhibited all other metabolic phenotypes such as insulin resistance and altered glucose homeostasis seen during obesity [85], suggesting a critical role for PDE3B in energy homeostasis. In addition, it is unknown whether the altered metabolic phenotypes seen in the *null* mice were due to deletion of *Pde3b* in specific hypothalamic neurons. Answer to these critical questions would require studies in mice with tissue/neuron-specific knock-out of *Pde3b* as well as knock-down of PDE3B in adult hypothalamus, which would be the subject of future investigations.

In summary, we have demonstrated insulin induction of PDE3B activity in the mouse hypothalamus. This finding along with the reversal of anorectic and body weight-reducing effects as well as the stimulatory effect of insulin on *Pomc* mRNA levels by a specific PDE3 inhibitor have identified PDE3B pathway as a novel intracellular signaling mechanism of

insulin action in the hypothalamus. Future studies are warranted towards understanding the physiological role of the PDE3B pathway in mediating insulin action in the hypothalamus in the regulation of energy homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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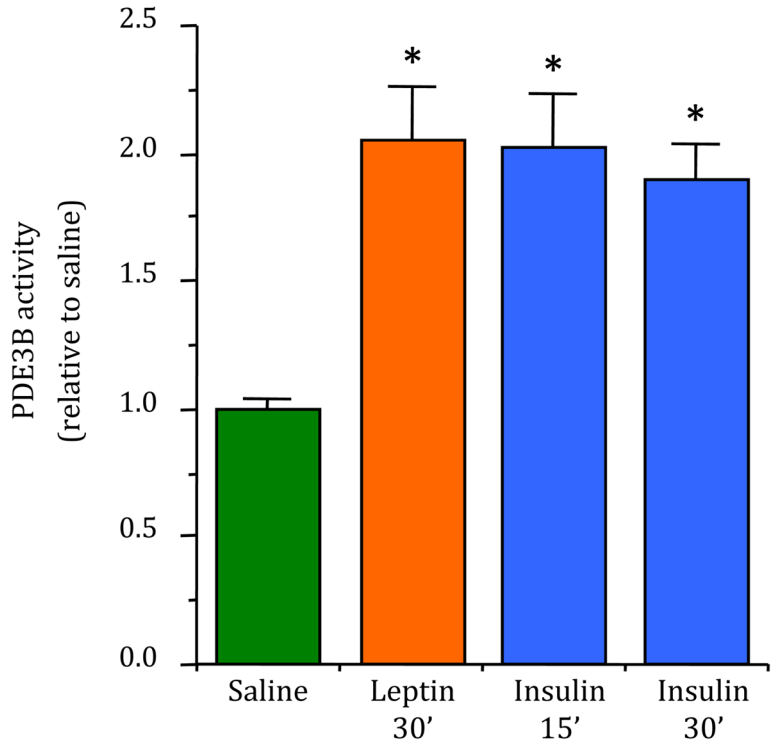

Fig. 1.

Effects of peripheral injection of insulin or leptin on PDE3B activity in the hypothalamus. The values for PDE3B activity are expressed as relative to saline (control) group. PDE3B activity (pmol cAMP hydrolyzed per min/μg protein) in the control group was 1.10 ± 0.16. Values represent the mean ± SEM for 6–8 animals per group. * $p < 0.0001$ vs saline control group.

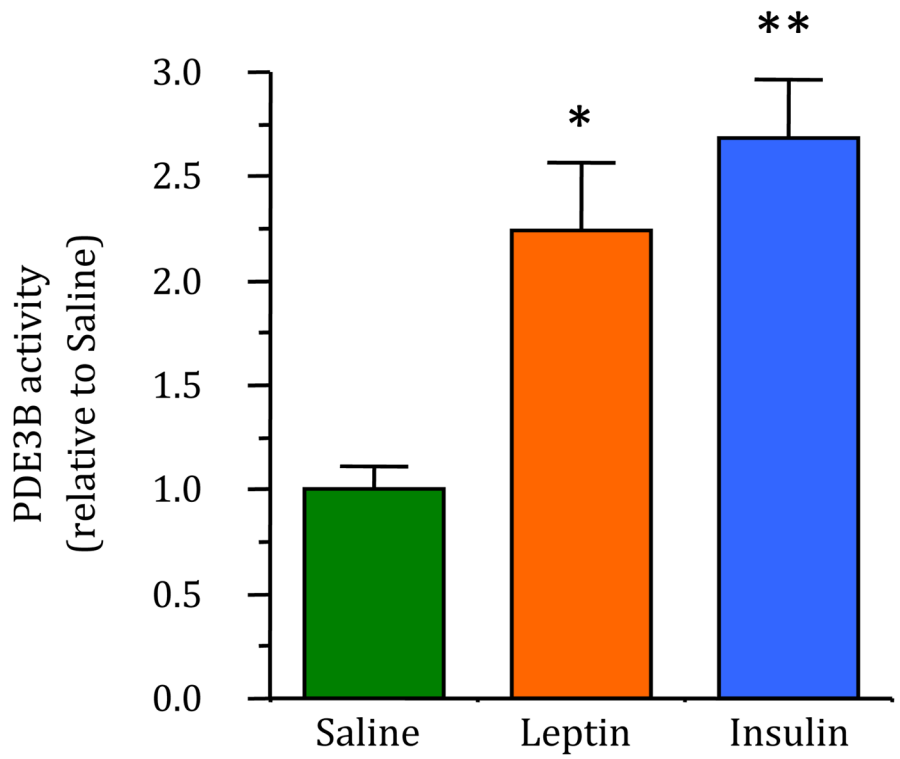

Fig. 2.

Effects of central injection of insulin or leptin on PDE3B activity in the hypothalamus. The values for PDE3B are expressed as relative to saline (control) group. PDE3B activity (pmol cAMP hydrolyzed per min/μg protein) in the control group was 0.90 ± 0.10. Values represent the mean ± SEM for 6–8 animals per group. * $p < 0.01$, ** $p < 0.0001$ vs saline control group.

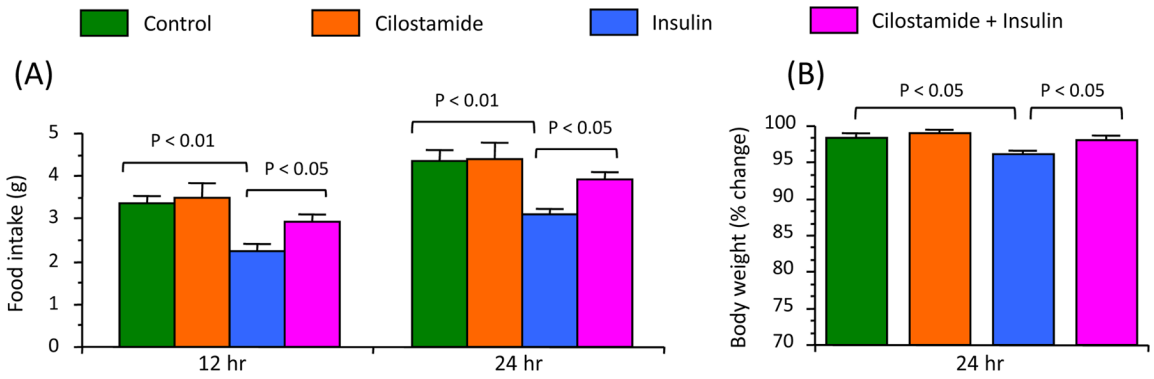

Fig. 3.

Effects of cilostamide, a specific PDE3 inhibitor, on anorectic and body weight reducing effects of central insulin. A, Food intake at 12 and 24 hr after insulin injection. B, Body weight at 24 hr after insulin injection. Cilostamide (10 μg/μl DMSO) or DMSO (vehicle control) was injected into the lateral cerebroventricle 4 hours before insulin (4.4 mU/2 μl) or saline injection. Values represent the mean ± SEM for 9–10 animals per group.

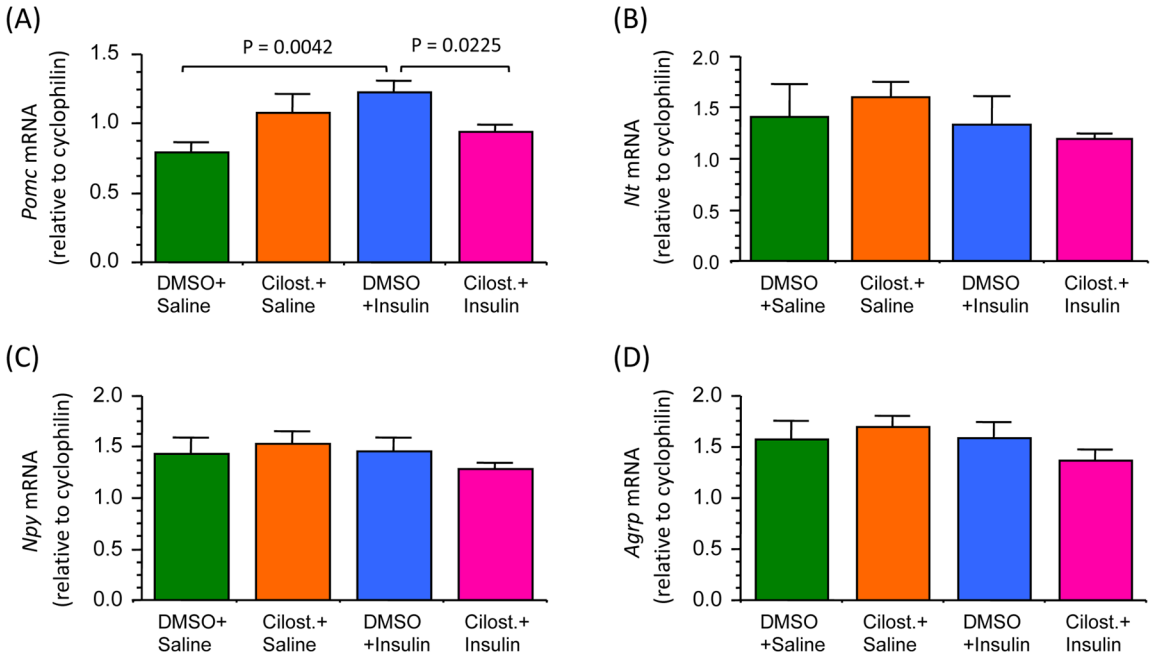

Fig. 4.

Changes in hypothalamic mRNA levels for POMC (A), NT (B), NPY (C) and AgRP (D) following DMSO + insulin or cilostamide + insulin. Mice fasted for 24 hours were injected with cilostamide (10 μg/μl) or DMSO (control), followed 40 min later, with icv insulin (4.4 mU/2 μl) or saline. Two hours later the medial basal hypothalamus was harvested to measure *Pomc, NTs, Npy* and *Agrp* mRNA levels by qPCR. Values represent the mean ± SEM for 5 animals per group.