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Abstract: Background: We recently reported that phospholipase A2 (PLA2)-mediated production of prostaglandins within the ventromedial hypothalamus (VMH) plays a critical role in systemic glucose homeostasis. However, the role of PLA2 in the VMH in regulating food intake is still unclear. Here, we attempted to investigate the role of PLA2 in regulating food intake and body weight. Methods: We injected an adeno-associated virus encoding short hairpin RNA (AAV-shRNA) targeting cytosolic phospholipase A2 (shPla2g4a) into the VMH. We assessed food intake, body weight, oxygen consumption, glucose tolerance, and insulin sensitivity. Results: Three weeks after the AAV injection, the shPla2g4a group increased food intake and body weight gain compared to controls (shSCRM). Energy expenditure, oxygen consumption, and respiratory quotient were comparable between groups. Conclusion: Our findings suggest that the cPLA2-mediated pathway in the VMH is critical for feeding behavior and maintaining energy homeostasis. Further investigation is needed to elucidate the underlying mechanisms.

Keywords: cPLA2; Hypothalamus; prostaglandins; obesity; VMH

1. Introduction

Prostaglandins (PGs) are widely distributed in the central nervous system (CNS) and play diverse physiological functions. [1]. PGs regulate not only inflammation, but also appetite, sleep-awake, and emotion [1–6]. Cytosolic phospholipase A2 (cPLA2) catalyzes phospholipids containing arachidonic acid, as a precursor for PG synthesis [7,8]. Recently, we reported that cPLA2-knockdown (KD) in the ventromedial hypothalamus (VMH) steroidogenic factor-1 (SF-1) neuron of mice fed a regular chew diet (RCD) leads to decreased insulin sensitivity in skeletal muscle [9]. This finding suggests that cPLA2 enhances systemic insulin sensitivity by activation of dmVMH glucose-excited (GE) neurons. Additionally, another study demonstrated that inhibition of PG synthesis by cyclooxygenase-1/2 (COX-1/2) inhibitors impairs the counter-regulatory response by suppressing glucagon sensitivity [10]. In cPLA2-KD mice, cFos expression is specifically suppressed in the dorsomedial VMH (dmVMH). In contrast, in wild-type mice, intraperitoneal (i.p.) glucose administration increases cFos expression in the VMH and the arcuate nucleus (ARC) [9]. These studies suggest that ARC neurons are regulated by PG, and thus, it is required to evaluate the function of PG-regulated neuronal activity in the hypothalamus on energy homeostasis.

The regulation of the CNS in the termination of feeding after a meal is complex and involves multiple mechanisms. The extracellular signal-regulated kinase (ERK) in the hypothalamus mediates the anorexigenic and thermogenic effects of leptin, suppressing food intake and increasing

sympathetic outflow to brown adipose tissue [11]. ERK in the nucleus tractus solitarius (NTS), activated by the gut-derived satiety signal cholecystokinin (CCK), also plays a crucial role in suppressing food intake [12]. The melanocortin receptor (MC4R) agonist MTII also increases p-ERK in the NTS, suppressing food intake [13]. Cyclic AMP (cAMP)- response element binding protein (CREB) is a transcription factor that is regulated by ERK. In the NTS, cAMP-ERK-CREB cascade may serve as a molecular integrator for converging satiety signals from the gut and adiposity signals from the hypothalamus [14]. CREB targets COX-2 expression, which is crucial for prostaglandin (PG) synthesis [15]. However, the relationship between insulin-induced ERK signaling and PG synthesis, particularly in regulating hyperphagia, remains unclear. This study aimed to investigate the role of hypothalamic PG in the development of hyperphagia. We found that cPLA2-KD in the hypothalamus of RCD-fed wild-type mice resulted in a significant increase in food intake, leading to a body weight gain of approximately 5 g within three weeks. Our findings suggest that cPLA2 in the hypothalamus is necessary for terminating feeding by regulating hypothalamic neuronal activity in response to insulin-ERK signaling.

2. Results

To knock-down PLA2g4a gene expression, we injected AAV9-RFP-U6-m-PLA2G4A-shRNA or AAV9-U6-shRNA (SCRM)-EF1a-GFP bilaterally in the VMH of C57BL mice (Figure 1a). mCherry or GFP derived from viral vectors were found in both the VMH and ARC, suggesting that AAV successfully infected the hypothalamus (Figure b). Body weight of shPLA2 mice was comparable to that of shSCRM mice at two weeks after AAV injection, but it tended to increase at three weeks post-AAV injection (Figure 1c). Importantly, when we calculate weight gain, shPLA2 mice increased weight gain compared to shSCRM mice at three weeks (Figure 1d). Furthermore, food intake was significantly higher in shPLA2 mice at two weeks post-AAV injection (Figure 1e-g).
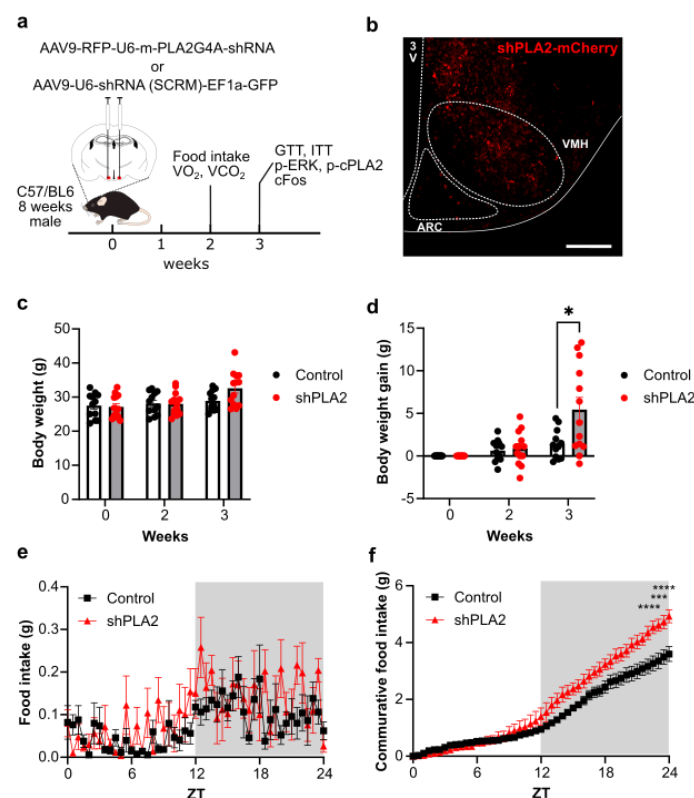
Figure 1. shRNA against cPLA2 in the hypothalamus increased body weight and food intake
a, A schematic illustrating the experimental timeline of AAV injection into the VMH, food intake, oxygen consumption, tolerance tests, and immunohistochemistry. **b**, Representative image of mCherry expression after AAV injection. **c**, Body weight in 0, 2, and 3 weeks after the injection of shRNA against cPLA2 into the VMH. **d**, Body weight gain in 0, 2 and 3 weeks after the shPLA2 injection. **e**, food intake in 2 weeks after the shPLA2 injection. **f**, Cumulative food intake (ZT=0-24). All data represent the mean ± SEM; *$p<0.05$; ***$p<0.001$; ****$p<0.0001$.

Figure 1. shRNA against cPLA2 in the hypothalamus increased body weight and food intake.

We next examined oxygen consumption, carbon dioxide production, and the respiratory quotient (RQ) using an in vivo calorimetry system. However, these parameters did not differ between the shPLA2 and control groups (Figure 2a-f). In addition, hypothalamic shPLA2 did not affect glucose tolerance or insulin sensitivity at three weeks post-AAV injection (Figure 3a, b).
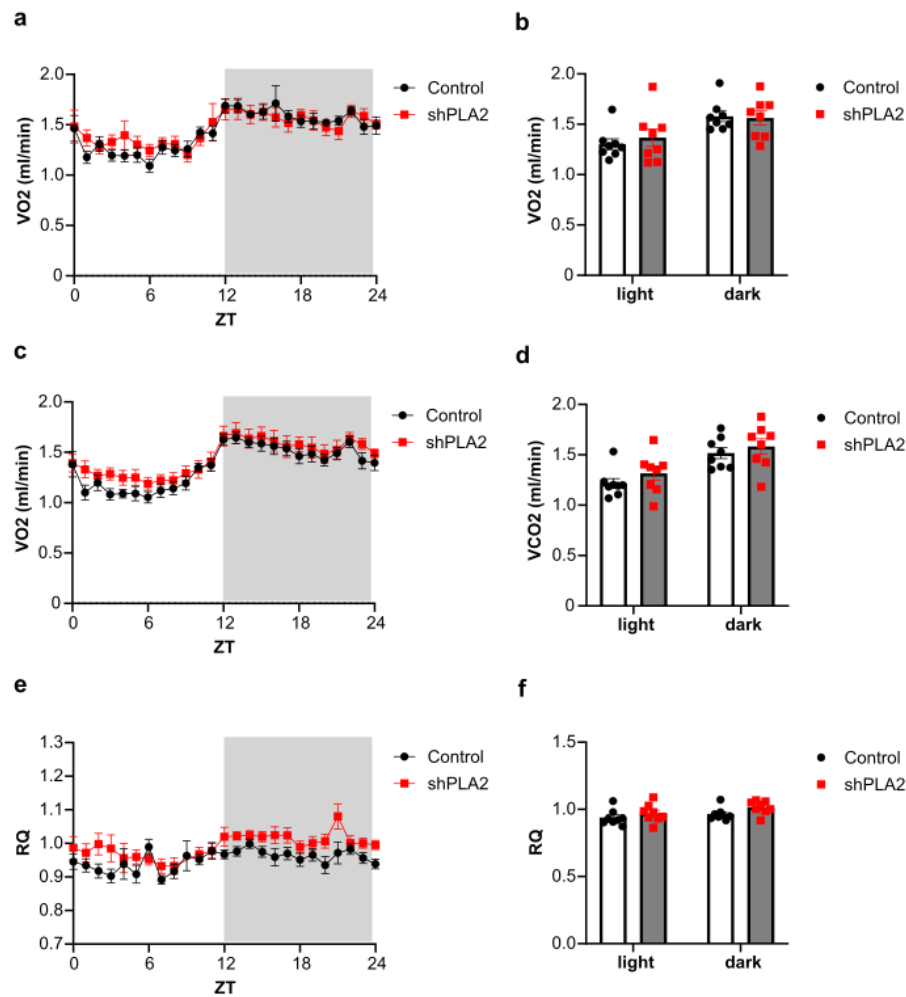
Figure 2. shRNA against cPLA2 in the hypothalamus did not affect oxygen consumption
a, Volume of oxygen consuption (VO2). **b**, Average VO2 in light (ZT=0-12) and dark (ZT=12-24) period.
c, Volume of carbon dioxide production (VCO2). **d**, Average VCO2 in light and dark period. **e**, Respiratory quotient (RQ). **f**, Average RQ in light and dark period. All data represent the mean ± SEM; no significance in $p<0.05$.

Figure 2. shRNA against cPLA2 in the hypothalamus did not affect oxygen consumption.
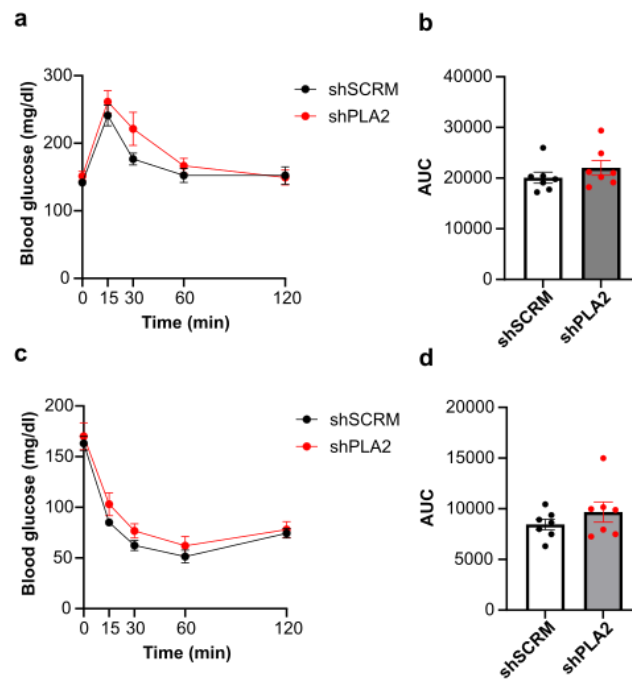
Figure 3. Glucose tolerance test and insulin tolerance test in shSCRM or shPLA2 injected mice
a, Blood glucose levels (0-120 minutes) during i.p. glucose tolerance test (GTT) three weeks after the injection of shSCRM or shPLA2 into the VMH. **b**, Area under the curve (AUC) of blood glucose levels during i.p. GTT. **c**, Blood glucose levels (0-120 minutes) during i.p. 0.75U/kg insulin tolerance test (ITT) three weeks after the injection of shSCRM or shPLA2 into the VMH. **d**, Area under the curve (AUC) of blood glucose levels during i.p. ITT. All data represent the mean ± SEM; no significance in $p < 0.05$.

Figure 3. Glucose tolerance test and insulin tolerance test in shSCRM or shPLA2 injected mice.

To evaluate the effect of insulin on the neuronal activation modulated by ERK signaling, we performed immunohistochemical staining for phosphorylated p44/42 MAPK (p-ERK1/2) in the hypothalamus following insulin injection (Figure 4a). Sixty minutes after insulin i.p. administration, p-Erk expression in the VMH was significantly higher than in the saline-treated control group (Figure 4b). In contrast, while p-Erk expression in the ARC showed a trend toward an increase, the difference was not statistically significant (Figure 4c). Given that insulin administration enhances PG production in the hypothalamus in our recent finding *[10]*, we also performed immunohistochemical staining for phosphorylated cPLA2 (p-cPLA2) in the hypothalamus of these mice (Figure 4d). Although the mean intensity of p-cPLA2 staining tended to increase after insulin injection in the VMH and ARC, no significant differences were observed between groups (Figure 4e, f).
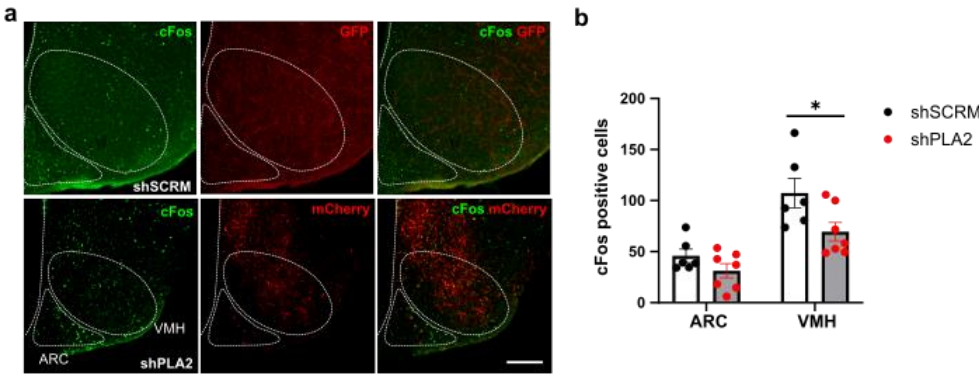
Figure.4
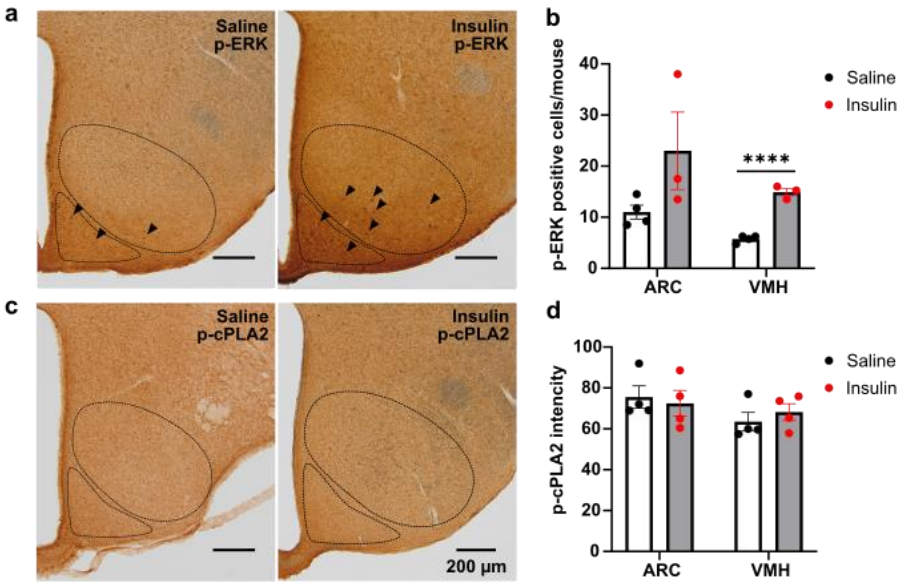
Figure 4. shRNA against cPLA2 in the hypothalamus increased insulin-induced phospho-ERK expression but not phospho-cPLA2 immunohistochemistry

a, Representative images of phospho-ERK (p-ERK) immunohistochemistry in the hypothalamus 60 minutes after i.p. injection of saline or 0.75U/kg insulin. **b**, Quantification of p-ERK expression in the hypothalamus; ARC: arcuate nucleus; VMH: ventromedial hypothalamus. **c**, Representative images of phospho-cPLA2 (p-cPLA2) immunohistochemistry in the hypothalamus 60 minutes after i.p. injection of saline or 0.75U/kg insulin. **d**, Quantification of p-cPLA2 expression in the hypothalamus. Scale bars: 200 µm. All data represent the mean ± SEM; ****$p<0.0001$.

Figure 4. shRNA against cPLA2 in the hypothalamus increased insulin-induced phospho-ERK expression but not phospho-cPLA2 immunohistochemistry

We also analyzed the effect of cPLA2-KD on the insulin-induced cFos expression in the hypothalamic nuclei. cFos immunohistochemical staining was performed in the hypothalamus 60 minutes after insulin administration in both shPLA2 and shSCRM groups (Figure 5a). cFos expression was significantly reduced in the VMH of shPLA2 mice (Figure 5b). cFos expression in the ARC tended to decrease in shPLA2 mice, but it was not significant (Figure 5b).

Figure 5. shRNA against cPLA2 in the hypothalamus decreased cFos expression after the insulin injection
a, Representative images of cFos immunohistochemistry in the hypothalamus 60 min after i.p. injection of 0.75U/kg insulin. GFP was replaced with red using ImageJ. **b**, Quantification of cFos expression in the hypothalamus of shSCRM or shPLA2 injected mice. Scale bar: 200 µm. All data represent the mean ± SEM; *$p<0.05$.

Figure 5. shRNA against cPLA2 in the hypothalamus decreased cFos expression after the insulin injection.

3. Discussion

In the present study, the suppression of hypothalamic prostaglandin production by shRNA targeting cPLA2 resulted in short-term weight gain, accompanied by increased food intake. During this period, indirect calorimetry showed no differences in VO2 or VCO2, indicating that basal metabolism remained unchanged following hypothalamic shPLA2 injection. Additionally, RQ was unaffected, suggesting no alteration in metabolic substrate utilization. However, food intake becomes significantly higher in the shPLA2-injected group compared to controls two weeks after injection, suggesting that the neuronal activity regulating appetite may have changed.

In the VMH, i.p. insulin injection enhanced ERK phosphorylation but did not affect cPLA2 phosphorylation. Insulin has direct and indirect effects on the regulation of food intake. Insulin suppresses appetite directly via neuronal pathways [16]. Postprandial hyperinsulinemia also affects cerebrospinal fluid (CSF) insulin levels [12]. In rats, glucose infusion and refeeding resulted in elevated CSF insulin [12,17]. These findings suggest that peripheral insulin can reach the CNS and regulate food intake and body weight. Insulin treatment increases ERK1/2 expression in hypothalamic cells in vitro [18]. Short-term fasting also increases p-ERK1/2 levels in the hypothalamus, partially mediated by centrally produced insulin [19]. Enhanced ERK1/2 activity, achieved through deletion of its phosphatases DUSP6/8, leads to resistance to diet-induced obesity, improved glucose tolerance, and reduced serum triglycerides and lipid content in the liver and visceral adipose tissues [20]. In hypothalamic neurons, ERK1/2 mediates glucose-regulated POMC gene expression, influencing metabolic homeostasis [21]. These studies indicate that ERK activation by diet, blood glucose, and insulin is involved in both the improvement and worsening of metabolic-related diseases. Future research will need to examine hypothalamic p-ERK-positive neuron types to elucidate their relationship with feeding suppression.

According to previous reports, CREB regulates COX-2 activity [15,22]. A previous study has shown that SF1 neuron-specific deletion of CRTC1, a co-activator of CREB, leads to hyperphagia in mice fed a high-fat diet [23]. The authors recently reported that inhibition of the PG production pathway using the COX inhibitor ibuprofen suppressed insulin-induced cFos expression in the vlVMH [10]. In the present study, shPLA2 in the hypothalamus also reduced cFos-positive cells in the VMH after i.p. insulin injection. This result suggests that decreased arachidonic acid, a product of the ERK-CREB-COX pathway, may have suppressed PG-regulated neuronal activity in the hypothalamus. In this study, insulin injection did not change phosphorylation of cPLA2 in the hypothalamus. Thus, insulin may affect PG production via COX, but not cPLA2 activity. VMH neurons projecting to ARC-POMC neurons are essential for suppressing food intake [24]. In line with this, cFos in the ARC tended to decrease in shPLA2 mice, suggesting that POMC activity was decreased. Therefore, our findings suggest that cPLA2-regulated PG synthesis in the VMH is essential for feeding termination via POMC neurons, as its knockdown resulted in increased food intake and body weight gain.

PGs play a crucial role in metabolic control and appetite regulation. cPLA2 is crucial in generating arachidonic acid from phospholipids for PG synthesis [25]. In the CNS, PGs have diverse effects on food intake. Centrally administered PGE2 suppresses food intake via the EP4 receptor, whereas PGD2 increases food intake through the DP1 receptor, which is coupled to the Y1 receptor of neuropeptide Y [6]. Our previous study also highlighted that cPLA2-mediated hypothalamic phospholipid metabolism is critical for controlling systemic glucose metabolism under a regular chow diet [9].

Numerous studies have demonstrated the critical role of SF1 neurons in the VMH in regulating feeding behavior. The deletion of leptin receptors in VMH-SF1 neurons leads to diet-induced obesity [26]. The loss of insulin receptors in SF1 neurons suppresses weight gain under a high-fat diet by inhibiting phosphoinositide 3-kinase (PI3K) signaling, which is involved in insulin secretion [27]. Furthermore, the deletion of PI3 phosphatase in SF1 neurons induces excessive PI3K activation in the VMH, leading to hyperphagia and weight gain even under a regular-chow [27]. Additionally, chemogenetic inhibition of SF1 neurons using DREADDs enhances appetite and increases food intake

[28]. These studies collectively suggest that hyperphagia and subsequent weight gain result from changes in the activity of VMH SF1 neurons, which are regulated by humoral factors involved in postprandial feeding suppression.

In this study, we demonstrated that hypothalamic cPLA2-KD induces obesity in a short period through increased food intake. During this process, cPLA2-KD suppressed the release of arachidonic acid, a substrate for PG production regulated by the ERK-CREB-COX pathway during postprandial hyperinsulinemia. Since obesity in this study resulted solely from increased food intake without peripheral metabolic changes, it is possible that feeding suppression, mediated by the activation of ARC-POMC neurons and regulated by PGs, was impaired. Although further studies are needed, this study advances knowledge in the regulation of systemic metabolism mediated by the PG synthesis pathway. This finding provides a novel therapeutic target for obesity treatment.

4. Materials and Methods

Animals

C57BL/6N male mice (Japan SLC, Shizuoka, Japan) were housed at room temperature with a 12-hour light and 12-hour dark cycle. The mice had free access to water and a regular chow diet (CLEA Japan, Tokyo, Japan). Experiments were conducted in the Experimental Animal Facility at Kumamoto University. Mice cages were changed once a week, and mouse care was performed according to the guidelines of the Animal Care and Use Committee at Kumamoto University.

Cannula Implantation and Adeno-Associated Virus (AAV) Injection

C57BL male mice were anesthetized with a combination of anesthetics (0.3 mg/kg medetomidine, 4.0 mg/kg midazolam, and 5.0 mg/kg butorphanol). The mice were placed on the stereotaxic instrument (Narishige, Tokyo, Japan). A hole in the skull was opened with a dental drill. A stainless-steel cannula (Plastics One, P1 Technologies, VA, USA) was inserted into the VMH using the following coordinates: an anterior-posterior (AP) direction; −1.5 (1.5 mm posterior to the bregma), lateral (L): ±0.4 (0.4 mm lateral to the bregma), dorsal-ventral (DV): −5.7 (5.7 mm below the bregma on the surface of the skull). For intracerebroventricular (i.c.v.) injection, the coordinates are as follows: AP: -0.3, L: 1.0, DV: −2.0. Cannulae were secured on the skulls with cyanoacrylic glue, and the exposed skulls were covered with dental cement. For AAV injection, the mice were injected in both sides of the VMH with a maximum of 0.3 μL AAV9-RFP-U6-m-PLA2G4A-shRNA (1.0×10^{12} GC/mL, shAAV-268768, Vector Biolabs, PA, USA) or AAV9-U6-shRNA (scrumble, SCRM)-EF1a-GFP (1.0×10^{12} GC/mL, SL100894, Signagen Laboratories, MD, USA) using the following coordinates: AP: − 1.5, L: ± 0.5, DV: − 5.7. Open wounds were sutured after the viral injection. The mice were allowed to recover for 5–7 days before the experiments began.

Body Weight Gain, Food Intake, and Oxygen Consumption

To evaluate the effects of hypothalamic cPLA2-KD on body weight and food intake, we compared food intake between the control and shPLA2 groups two weeks after AAV administration. Mice were placed in food intake measurement cages (cFDM-300AS, Melquest, Toyama, Japan) and habituated for 24 h, followed by food intake measurement from ZT 0 to 24. Indirect calorimetry data from mice injected with shPLA2 or shSCRM were measured using an indirect calorimetry system (MK-5000RQ/MS, Muromachi Kikai, Fukuoka, Japan). Mice were acclimated to the measurement chambers for one day prior to data collection.

p-ERK and p-cPLA2 Immunohistochemistry

C57BL/6 male mice received an i.p. injection of saline or insulin (0.75 U/kg). The mice were deeply anesthetized with isoflurane and perfused with heparinized saline followed by 4% paraformaldehyde transcardially 60 minutes after the i.p. injection. Brain sections (50 μm each)

containing both VMH and ARC were collected. The floating sections were incubated in 1% H2O2 in 0.1 M phosphate buffer (PB) for 15 min at room temperature (RT) to inhibit endogenous peroxidase activity. After rising with PB, the sections were incubated with rabbit-anti-pErk antibody (1:1000, Cell Signaling Technology, #4370S, MA) or rabbit-anti-pCpla2 antibody (1:1000, Cell Signaling Technology, #2831S, MA) in blocking solution (0.1 M PB containing 4% normal horse serum, 0.1% glycine, and 0.2% Triton X-100) overweekend at room temperature. After rinsing with PB, the sections were incubated in a secondary antibody (1:500, Biotinylated goat anti-rabbit IgG, Vector lab, BA-1000, CA) for two hours at room temperature. After rinsing with PB, sections were incubated with ABC solution (VECTASTAIN Elite ABC kit, Peroxidase, Vector Laboratories, CA) for 2 hours at room temperature. The sections were rinsed with PB and incubated with DAB solution (DAB tablet, Wako, Japan) for 20 min at room temperature. The stained sections were rinsed with PB and mounted using Mount-Quick (Daido Sangyo, Japan). Cells were automatically counted using the ImageJ software plugin (Analyze Particles).

cFos Immunohistochemistry

C57BL/6 male mice received a shPLA2 or shSCRM injection 4 weeks before the i.p. injection of insulin (0.75 U/kg). The mice were deeply anesthetized with isoflurane and perfused with heparinized saline transcardially 60 minutes after insulin injection. Brain sections (50 μm each) containing both VMH and ARC were collected. After rising with PB, the sections were incubated with rabbit-anti-cFos antibody (1:1000, Cell Signaling Technology, #2250S, MA) in blocking solution (0.1 M PB containing 4% normal horse serum, 0.1% glycine, and 0.2% Triton X-100) overnight at room temperature. After rinsing with PB, the sections were incubated in a secondary antibody (1:500, Alexa 488 secondary antibody, Invitrogen, A11008, MA) or (1:500, Alexa 594 secondary antibody, Cell Signaling Technology, 8889S, MA) for two hours at room temperature. The stained sections were washed three times with PB and then mounted on glass slides using DAPI Fluoromount-G (Southern Biotech, Birmingham, AL). Cells were automatically counted using ImageJ.

Statistical Analysis

For repeated-measures analysis, a two-way ANOVA was used to analyze values over different time points, followed by the Sidak multiple comparisons test. For the statistical analysis of multiple independent groups, one-way ANOVA was followed by Tukey's multiple comparisons test. When only two groups were analyzed, statistical significance was determined by the unpaired Student's *t*-test (two-tailed *p*-value). Prism 10 software (GraphPad Software, San Diego, CA) was used for these statistical analyses. A value of $p < 0.05$ was considered statistically significant. All data are shown as mean ± SEM.

5. Conclusions

This study revealed that cPLA2-KD in the VMH induces obesity solely through increased food intake. cPLA2-KD suppressed the production of eicosanoids from membrane phospholipids and inhibited the synthesis of PGs during postprandial hyperinsulinemia, which may be essential for regulating neuronal activity in the VMH. Inhibition of VMH neurons resulted in hyperphagia, possibly via POMC neurons in the ARC.

Abbreviations

The following abbreviations are used in this manuscript:

cPLA2	cytosolic phospholipase A2
SCRM	scrumble
VMH	ventromedial hypothalamus
PGs	Prostaglandins
CNS	central nervous system
KD	knockdown
COX	cyclooxygenase
dm	dorsomedial
ARC	arcuate nucleus
ERK	extracellular signal-regulated kinase
NTS	nucleus tractus solitarius
CCK	cholecystokinin

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