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The role of hypothalamic AMP-activated protein kinase in ovariectomy-induced obesity in rats

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Abstract

Objective: Adenosine monophosphate-activated protein kinase (AMPK) acts as a cellular energy sensor, being activated during states of low energy charge. Hypothalamic AMPK is altered by hormonal and metabolic signals and mediates the feeding response. The aims of this study were to examine whether the phosphorylation of AMPKα in the hypothalamus is affected by ovariectomy (Ovx) and thus would be involved in the development of obesity in rats.

Methods: Body weight, food intake, hypothalamic phosphorylated AMPKα (pAMPKα) protein expression, and plasma leptin and adiponectin levels were measured in female rats after either Ovx or sham operations. These patterns were also observed after treatment with 17β-estradiol, compound C, and leptin in Ovx rats.

Results: Compared with control rats, Ovx led to increased body weight and food intake at 2 to 8 weeks after operation. Meanwhile, plasma leptin and adiponectin levels and hypothalamic pAMPKα expression were significantly increased after Ovx. Replacement of estradiol significantly reversed these effects. Treatment with compound C, an AMPKα inhibitor, for 1 week produced a reduction in food intake, body weight, and plasma leptin and adiponectin levels. Meanwhile, these effects were reversed upon withdrawal of compound C. In addition, central injection of leptin also significantly reduced body weight, food intake, plasma leptin and adiponectin levels, and hypothalamic pAMPKα expression relative to those of the Ovx group.

Conclusions: Increased hypothalamic pAMPKα expression may contribute to hyperphagia during the development of Ovx-induced obesity in rats.

Key Words: AMP-activated protein kinase – Hypothalamus – Ovariectomy – Obesity – Leptin – Adiponectin.

Adenosine monophosphate-activated protein kinase (AMPK) is a heterotrimeric protein consisting of a catalytic subunit (α) and two regulatory subunits (β and γ). AMPK is a fuel-sensing enzyme activated by physiological and pathological stresses that deplete cellular adenosine triphosphate (ATP), including hypoxia, ischemia, and glucose deprivation, uncouplers of oxidative phosphorylation, exercise, and muscle contraction. Activation of AMPK represses ATP-consuming anabolic pathways and induces ATP-producing catabolic pathways. This is accomplished, at least in part, by regulating gene expression and the activities of key metabolic enzymes in fatty acid, cholesterol, and glucose metabolism and protein synthesis, as well as other metabolic pathways.[1] In addition to its role in the periphery, AMPK in the hypothalamus also regulates food intake.[2] A previous study has shown that leptin, glucose, melanocortin receptor agonist and antagonist, and fasting/refeeding change AMPK activity in several hypothalamic nuclei.[3] The alteration of hypothalamic AMPK activity was sufficient to change food intake and body weight. Other orexigenic and anorexigenic molecules for food intake regulation were also demonstrated to change hypothalamic AMPK activity.[2]

It is well known that estrogen is involved in the regulation of appetite, energy expenditure, body weight, and adipose tissue deposition/distribution in women.[4] Food intake in women varies across the menstrual cycle, with daily food intake lowest during the periovulatory period, when estrogen levels are maximal.[5] Postmenopausal women tend to gain body fat, which seems to be a consequence of the decline in endogenous estrogens.[6,7] In animal models, ovariectomy (Ovx) induces an increase in food intake and decreases ambulatory and wheel running activities, which is reversed with estrogen replacement.[8-10] Therefore, hypoestrogenic states

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are associated with decreased activity and an increase in body weight in women.

A number of studies demonstrated that menopause is associated with increased visceral adiposity and related metabolic pathologies, including insulin resistance, type 2 diabetes, and cardiovascular disease.[2,3,11,12] These observations highlight the importance of understanding the molecular and physiological mechanisms that underlie menopause-associated obesity and metabolic dysregulation. Currently, however, these mechanisms remain unclear. Rodent Ovx is one approach to modeling human menopause and studying the metabolic consequences of loss of ovarian function. The present study was therefore undertaken to test whether phosphorylated AMPKα (pAMPKα) in the hypothalamus is affected by Ovx and would thus be involved in the development of obesity in rats.

METHODS

Animal preparation

Female Sprague-Dawley rats were obtained from the National Laboratory Animal Breeding and Research Center of the National Science Council, Taiwan. Handling of the animals was in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (publication no. 85-23, revised 1996). This study was approved by the National Defense Medical Center Institutional Animal Care and Use Committee, Taiwan.

To produce the estrogen-deficient condition, young rats were anesthetized with sodium pentobarbital (50 mg/kg IP) and underwent bilateral Ovx at 8 weeks of age. Small incisions were made bilaterally on the sides of their backs to expose the ovaries retroperitoneally. The ovaries were clamped and removed, and the uterine tubes were ligated. The muscle and skin were then sutured. The sham procedure consisted of anesthesia, visualization of the ovaries through incisions into the abdominal cavity, and closure of the wounds.

Intracerebroventricular cannulation and injection

Animal surgical procedures and handling were carried out as described previously.[13,14] Five weeks after Ovx, the rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and placed in a Kopf stereotaxic frame. The third cerebral ventricle was cannulated with a permanent 22-gauge stainless-steel guide cannula (Plastics One Inc.) stereotactically placed 0.8 mm posterior to the bregma on the midline and implanted 6.5 mm below the outer surface of the skull. All animals used in the study were mock injected on two occasions to acclimatize them to the procedure before the first study day. Substances were administered via a stainless-steel injector placed in, and projecting 1 mm below, the tip of the guide cannulae. All compounds were dissolved in 0.9% saline or dimethyl sulfoxide (DMSO) and injected in a volume of 5 μL (intracerebroventricular [ICV]). The entire injection process lasted less than 2 minutes, and the rats were returned to their cages with minimum disruption. Correct placement of the cannula into the third cerebral ventricle was confirmed by injection of angiotensin II (150 ng) as described previously.[15] Animals not displaying a prompt and sustained drinking response were excluded from further study.

Experimental groups

In the first set of experiments, the rats were randomly divided into three groups—(1) sham group: rats that had undergone sham operations (n = 12); (2) Ovx group: rats that were ovariectomized bilaterally (n = 12); and (3) Ovx + estradiol (E_2) group: Ovx rats that were injected with E_2 (50 μg/kg/d IM, once daily; Sigma-Aldrich Corp., St Louis, MO) for 1 to 7 weeks, beginning 1 week after Ovx (n = 12).

To investigate the time courses of pAMPK expression in the hypothalamus, rats were reanesthetized with pentobarbital (60 mg/kg IP) and killed at 1, 2, 4, and 8 weeks after Ovx (n = 3 for each time point tested). Body weights were measured before the rats were killed at the end of the experiments. Blood samples were collected by abdominal aortic puncture.

In the second set of experiments, the rats were divided into six groups—(1) sham group: rats that had undergone sham operation (n = 6); (2) sham + compound C group: rats that were treated with 5 μL of compound C ((6-[4-(2-piperidin-1-yl-ethoxy)-phenyl])-3-pyridin-4-yl-pyyrazolo[1,5-a]pyrimidine; 50 nmol/5 μL, ICV, twice a week; Merck, Whitehouse Station, NJ), an inhibitor of AMPK,[16] for 1 week, beginning 6 weeks after sham operations (n = 6); (3) Ovx + DMSO group: Ovx rats that were treated with 5 μL of DMSO (ICV, twice a week) for 1 week, beginning 6 weeks after Ovx (n = 6); (4) Ovx + compound C group: Ovx rats that were treated with 5 μL of compound C (50 nmol/5 μL, ICV, twice a week) for 1 week, beginning 6 weeks after Ovx (n = 6); (5) Ovx + DC group: Ovx rats that were treated with 5 μL of compound C (50 nmol/5 μL, ICV, twice a week) for 1 week, beginning 6 weeks after Ovx, and then monitored for another 2 weeks (n = 6); and (6) Ovx + leptin group: Ovx rats that were treated with 5 μL of leptin (0.5 nmol/5 μL, ICV, twice a week; R&D Systems, Inc.) for 1 week, beginning 6 weeks after Ovx (n = 6).

Measurements of food intake and body weight

Each rat was housed in a single cage, and food intake was measured every week when replacing food. Food intake rate was determined by measuring the loss of food from the food container during the feeding period. Throughout the experiment period, body weight was monitored once a week.

Biochemical assays

Blood samples were withdrawn from the abdominal aorta at 1, 2, 4, and 8 weeks after Ovx and centrifuged at 1,500*g* for 10 minutes at 4°C. Plasma adiponectin (AdipoGen, Inc., Seoul, Korea) and leptin (Millipore, Temecula, CA) were measured using enzyme-linked immunosorbent assay kits for rats.

Western blot analysis

Frozen hypothalamus samples were homogenized in 150 μL of lysis buffer at 4°C (1% Triton X-100, 50 mM Tris-HCl [pH 7.5], 250 mM mannitol, 1 mM EDTA, 1 mM sodium

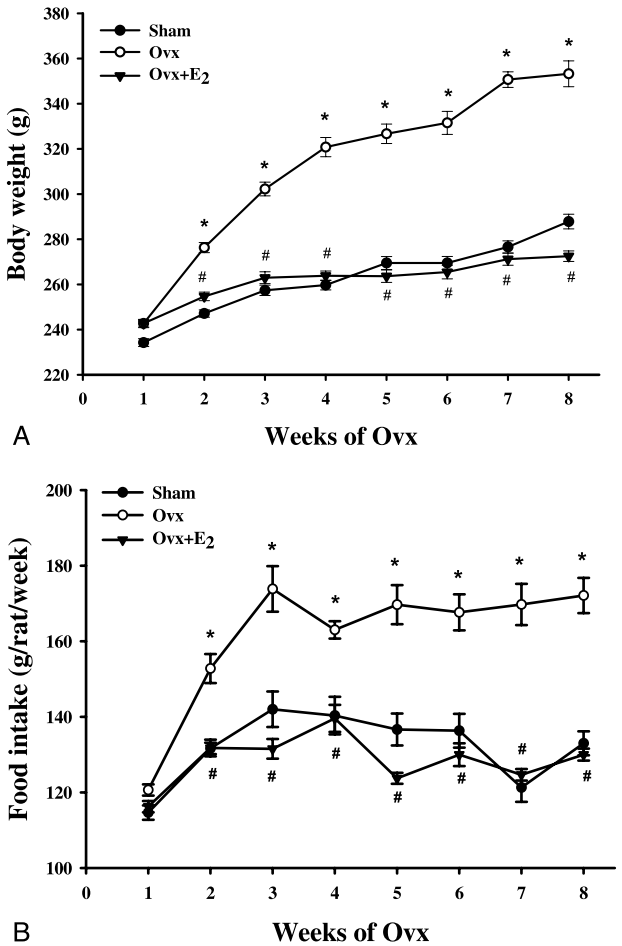

FIG. 1. Changes in body weight (**A**) and food intake (**B**) in sham, Ovx, and Ovx + E_2 rats. Values are means ± SEM. *$P < 0.05$ versus the sham group. #$P < 0.05$ versus the Ovx group. Ovx, ovariectomized; E_2, estradiol.

azide, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1.6 μL/mL final solution of antiproteases cocktail; Protein Inhibitor Cocktail Set III; Merck Calbiochem, Fontenay-sous-bois, France) by manual grinding. Insoluble material was removed by centrifugation for 20 minutes at 14,000*g* at 4°C. The protein concentration of the supernatant was determined by the BCA kit (Pierce, Rockford, IL). One-hundred micrograms of protein extract obtained from each tissue was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and blotted with anti-phospho-AMPKα2 (Thr172; Cell Signaling Technology, Danvers, MA) and β-actin (Sigma-Aldrich Corp.) antibody. The protein bands on the radiograph were scanned and densitometrically analyzed with an Image-pro software, as described previously.[17]

Statistics

All measurements are expressed as group mean ± SEM. Statistical evaluation was performed with one-factor analysis of variance followed by the Tukey-Kramer test. A *P* value of less than 0.05 was deemed statistically significant.

RESULTS

Effect of Ovx and 17β-E_2 substitution on body weight

The mean body weights of the rats in the three groups are outlined in Figure 1A. At the time of operation, all rats had similar body weights (240.7 ± 1.5 g); the sham group gained around 50 g during the experimental period and reached a mean weight of 287 ± 3 g after 8 weeks. The Ovx rats gained significantly more, and after 8 weeks, the mean weight in this group was 353 ± 6 g, whereas the Ovx + E_2 rats gained much less (final weight, 272 ± 2 g) compared with the Ovx group.

Effect of Ovx and 17β-E_2 substitution on food intake

Food intake in all rats was measured once a week. As shown in Figure 1B, the food intake of rats within each group did not differ at the time of operation. The sham rats ate 133 ± 3 g/week per rat at 8 weeks after the surgical procedure. The Ovx rats consumed significantly more (148 ± 2 g/wk per rat) at 2 weeks after Ovx and reached maximum (163.9 ± 3 g/wk per rat) at 8 weeks after the surgical

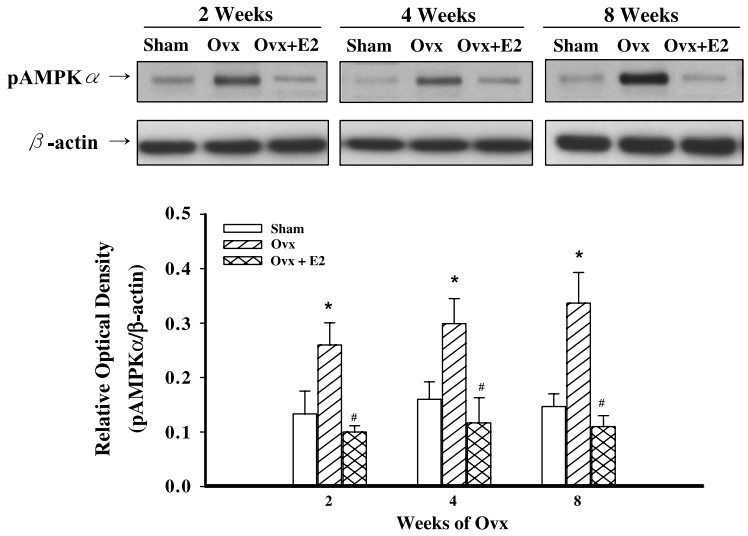

FIG. 2. Time-course changes in AMPKα phosphorylation in the hypothalamus from sham, Ovx, and Ovx + E_2 rats. Data are given as mean ± SEM (n = 3 for each time point). *$P < 0.05$ versus the sham group. #$P < 0.05$ versus the Ovx group. AMPKα, AMP-activated protein kinase α; Ovx, ovariectomized; E_2, estradiol; pAMPKα, phosphorylated AMPKα.

operation. The Ovx + E_2 rats consumed a similar level (129 ± 2 g/wk per rat) at 8 weeks after Ovx when compared with the control rats.

Time-course changes in hypothalamic pAMPKα protein expression after Ovx

Protein expression in the hypothalamus of Ovx rats was studied by immunoblotting analysis. The level of pAMPKα proteins in sham-operated rats was not significantly different at the time points tested (Fig. 2). However, pAMPKα protein accumulated significantly at 1 to 4 weeks after operation in the Ovx group when compared with the sham group ($P < 0.05$) and was significantly further increased at 8 weeks. After E_2 replacement, the accumulation of pAMPKα protein was significantly reversed and was negligibly expressed during the experimental period.

Effect of Ovx and 17β-E_2 substitution on plasma leptin levels

As shown in Figure 3A, the plasma leptin level of the sham group did not differ at the time points tested. Plasma leptin level was significantly higher in the Ovx group after operation than in the sham group at 2 to 8 weeks ($P < 0.05$). Replacement of E_2 in Ovx rats markedly reduced leptin level at 2 to 8 weeks after Ovx when compared with the Ovx group ($P < 0.05$).

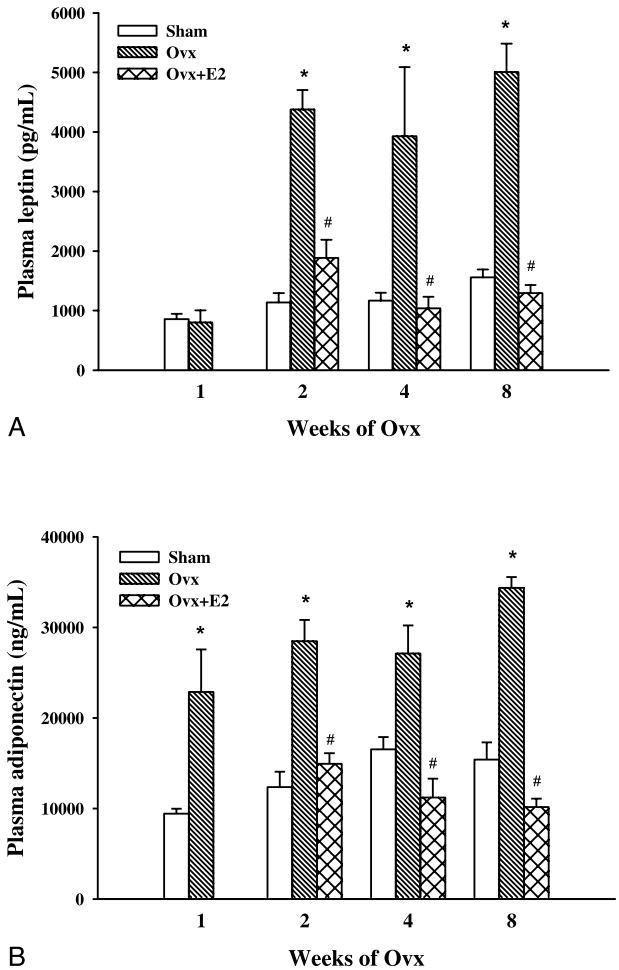

FIG. 3. Time-course changes in plasma leptin (**A**) and adiponectin (**B**) levels in sham, Ovx, and Ovx + E_2 rats. Data are given as mean ± SEM (n = 3 for each time point). *$P < 0.05$ versus the sham group. #$P < 0.05$ versus the Ovx group. Ovx, ovariectomized; E_2, estradiol.

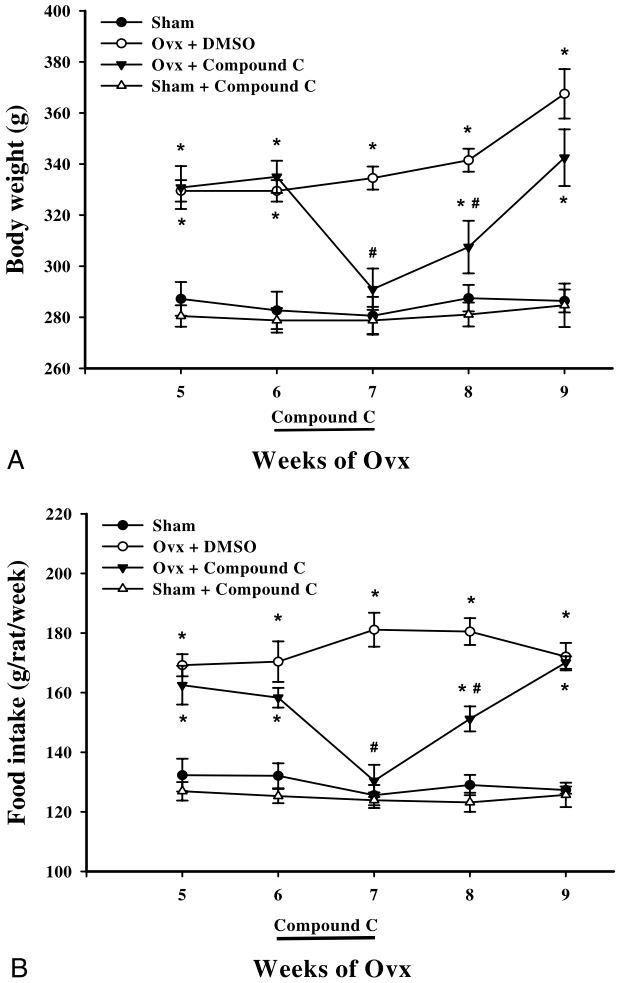

FIG. 4. Effects of compound C, an AMPK inhibitor, on body weight (**A**) and food intake (**B**) in Ovx rats. Ovx rats were treated with compound C for 1 week, beginning 6 weeks after Ovx. Values are means ± SEM. *$P < 0.05$ versus the sham group. #$P < 0.05$ versus the Ovx group. AMPK, AMP-activated protein kinase; Ovx, ovariectomized; DMSO, dimethyl sulfoxide.

Effect of Ovx and 17β-E_2 substitution on plasma adiponectin levels

As shown in Figure 3B, the plasma adiponectin level of the sham group did not differ at the time points tested. After Ovx, the plasma adiponectin level was significantly increased and was maintained at high levels during the experimental period and was significantly higher than those of the sham group at 1 to 8 weeks ($P < 0.05$). Replacement of E_2 in Ovx rats markedly reduced the adiponectin level at 2 to 8 weeks after Ovx when compared with the Ovx group ($P < 0.05$).

Effect of the AMPK inhibitor compound C on body weight and food intake

To address the role of pAMPKα in Ovx-induced obesity, we evaluated the effects of an AMPK inhibitor (compound C)

on body weight and food intake. As shown in Figure 4, when determined at week 7 after Ovx, body weight and food intake were significantly reduced in the group treated with compound C (ICV) for 1 week relative to those of the Ovx group ($P < 0.05$). In the group in which compound C was withdrawn and continued to be monitored for 2 weeks, body weight and food intake rebounded to levels similar to those of the Ovx group (at week 9).

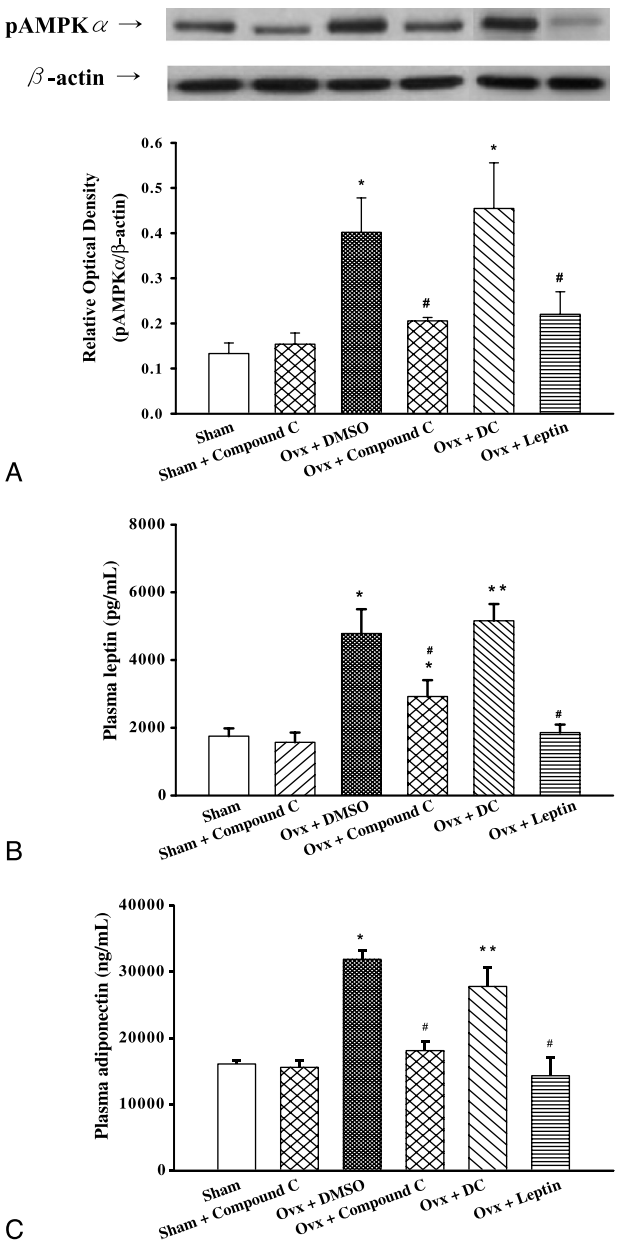

FIG. 5. Effects of leptin and compound C, an AMPK inhibitor, on hypothalamic AMPK phosphorylation (**A**), plasma leptin (**B**), and plasma adiponectin (**C**) in Ovx rats. Ovx + DC indicate Ovx rats treated with compound C for 1 week, beginning 6 weeks after Ovx, and then monitored for another 2 weeks. Data are given as mean ± SEM (n = 6). *$P < 0.05$ versus the sham group. #$P < 0.05$ versus the Ovx group. AMPK, AMP-activated protein kinase; Ovx, ovariectomized; pAMPKα, phosphorylated AMPKα; DMSO, dimethyl sulfoxide.

Effect of the AMPK inhibitor compound C on hypothalamic pAMPKα protein expression

As shown in Figure 5A, when determined at week 7 after Ovx, pAMPKα expression was significantly reduced in the group treated with compound C for 1 week relative to that of the Ovx group ($P < 0.05$). Upon continuation of monitoring for another 2 weeks, pAMPKα expression was reversed.

Effects of the AMPK inhibitor compound C on plasma leptin and adiponectin levels

Plasma leptin and adiponectin levels after compound C treatment are presented in Figure 5B and C. Compound C was ICV injected (twice a week) for 1 week, and the treatment obviously reduced the plasma leptin and adiponectin levels. In addition, upon continuation of monitoring for another 2 weeks without compound C treatment, the leptin and adiponectin levels rebounded to levels similar to those of the Ovx group.

Effects of ICV leptin on body weight and food intake

To clarify whether the biological effect of leptin remained throughout the experimental period, we evaluated the effect of leptin on body weight and food intake. After treatment with leptin for 1 week, body weight was significantly reduced (from 322.5 ± 3.6 to 245 ± 10.5 g) when determined at week 7 after Ovx. In addition, food intake was also reduced (from 160.8 ± 4 to 95 ± 5 g/wk per rat) after leptin treatment in Ovx rats.

Effects of ICV leptin on hypothalamic pAMPKα protein expression and plasma leptin and adiponectin levels

As shown in Figure 5A, when determined at week 7 after Ovx, pAMPKα expression was significantly reduced in the group treated with leptin for 1 week relative to that of the Ovx group ($P < 0.05$). Meanwhile, the plasma leptin and adiponectin levels were 1,856.4 ± 233.7 pg/mL and 14,297.4 ± 2,665.8 ng/mL, respectively, which were statistically significantly lower than those of the Ovx group (4,781.4 ± 716.9 pg/mL and 31,845.2 ± 1,319.8 ng/mL, respectively; $P < 0.05$; Fig. 5B and C).

DISCUSSION

This is the first in vivo study demonstrating time-course change phosphorylation state of AMPKα (pAMPKα) expression in the hypothalamus of rats with Ovx-induced obesity. It is well known that hypothalamic pAMPKα plays a role in the regulation of food intake. However, the relationship between pAMPKα and Ovx-induced obesity was not evaluated. Results demonstrate that a marked reduction in estrogen synthesis led to increased food intake, body weight, and hypothalamic pAMPKα expression. Meanwhile, plasma leptin and adiponectin levels were increased after Ovx. Replacement of E_2 significantly reversed these effects. Treatment with compound C, an AMPKα inhibitor, for 1 week produced a reduction in food intake, body weight, and plasma leptin and adiponectin levels. Withdrawal of compound C triggered hyperphagia. Results suggest that increased hypothalamic pAMPKα may

play an important role in the development of hyperphagia in rats with Ovx-induced obesity.

Feeding behavior is regulated by several neuropeptides and monoamine neurotransmitters in a complex manner.[18,19] In many species, estrogens are involved in food intake regulation. The concentration of plasma estrogens, especially E_2, inversely correlates with feeding during many physiological states.[20] In young mice and rats, Ovx induces weight gain mainly related to an increase in feeding.[21,22] Systemic administration of E_2 to Ovx mice and rats could prevent these changes in feeding behavior.[23] Similar to these reports, in the present study, withdrawal of ovarian hormones in rats increased food intake and body mass (Fig. 1). Meanwhile, a previous study had shown that the loss of ovarian hormones did not affect resting energy expenditure or fuel utilization during the experimental period (35 d after Ovx).[24] Thus, the rapid mass gain after Ovx seems to be the result of increased food intake.

AMPK is considered as a master regulator of food intake, and the regulation of AMPK activity is complex but related mainly to covalent modification of its α-subunit through phosphorylation of threonine-172 by upstream kinases.[25-27] As previously reported in many studies, changes in AMPK activity are paralleled by associated changes in [Thr172] AMPK phosphorylation.[28-31] Changes in hypothalamic AMPK activity can regulate food intake[3,28,29] and orexigenic factors (eg, ghrelin)[32] activate hypothalamic AMPK, whereas leptin and other anorexigenic agents suppress AMPK activity in the hypothalamus. In our present study, Ovx led to significantly increased hypothalamic AMPKα phosphorylation (Fig. 2). Meanwhile, treatment with compound C, an AMPKα inhibitor, for 1 week marked a reduction in food intake and body weight (Fig. 4). Withdrawal of compound C triggered hyperphagia, indicating that AMPK activity remained throughout the duration of experiment (Fig. 5A). These results suggest that the increased hypothalamic AMPKα phosphorylation observed in Ovx rats may account for the marked hyperphagia.

Several neuropeptides are involved in the regulation of feeding behavior.[18,19] Leptin, a hormone secreted by the adipocyte in proportion to fat stores, plays a major role in regulating energy homeostasis by decreasing food intake and increasing energy expenditure.[33] Rodents with diet-induced obesity and most obese humans are resistant to the effects of leptin.[34,35] Leptin resistance is defined as decreased sensitivity to the anorexigenic or weight loss effects of leptin. A hallmark of leptin-resistant states is hyperleptinemia. Leptin also modulates the activity of AMPK, and the inhibition of AMPK in discrete hypothalamic regions is also critical for the anorexigenic effects of leptin.[3] Consistent with previous results,[34,35] in our current study, the plasma leptin level of Ovx rats was significantly higher than that of sham rats (Fig. 3A). However, we did not see a reduction in hypothalamic pAMPKα and food intake in Ovx rats despite a significantly increased plasma leptin level. This may be explained by the fact that leptin resistance in Ovx-induced obesity decreased leptin transport across the blood-brain barrier or dysfunction of leptin receptors in the hypothalamus. However, the previous study has shown that the analysis of the hypothalamus revealed no modulation of leptin receptor (Ob-Rb) expression at 7 weeks after Ovx, whereas in a long-term study (22 wk), hypothalamic Ob-Rb expression was strongly decreased in Ovx rats.[36] Chronic leptin administration to Ovx rats reduced fat mass by decreasing energy intake and increasing lipid oxidation. Withdrawal of leptin triggered hyperphagia, indicating that leptin biology remained throughout the duration of the chronic treatment.[24] Moreover, ICV leptin administration decreased food intake, body weight, and hypothalamic pAMPKα at week 7 after Ovx (Fig. 5). Taken together, these data suggest that Ovx increased body weight by producing hyperphagia and the hyperphagia was not a result from leptin resistance. However, these data do not rule out the possibility that other neuropeptides (such as adiponectin) also play a role.

Adiponectin is exclusively produced by mature adipocytes.[37] It has been shown that circulating adiponectin concentration was increased by a decrease in serum E_2 concentration and was reduced by estrogen treatment in mice and humans.[38,39] In the present study, we also found similar results in that the plasma adiponectin level was dramatically increased at 1 week after Ovx and was maintained at high level during the experimental period (Fig. 3B). Meanwhile, a recent study has shown that injection of adiponectin significantly stimulates and activates AMPK in the hypothalamus, leading to stimulation of food intake and decreases in energy expenditure.[40] Therefore, the elevated adiponectin may be a major contributor to the increase in hypothalamic pAMPKα observed in Ovx rats.

Estrogen regulates the expression and activity of many of the genes involved in the control of energy homeostasis. A number of factors may be involved in Ovx-induced changes in hypothalamic pAMPKα expression. In our study, plasma leptin and adiponectin concentrations were profoundly increased in Ovx rats. Both leptin and adiponectin modulated the activity of hypothalamic pAMPKα. Moreover, ICV administration of compound C inhibited the increase in hypothalamic AMPK activity and food intake. Thus, the increased hypothalamic pAMPKα may be a major contributor to the hyperphagia in Ovx rats. Further studies are needed to clarify how Ovx produces increased hypothalamic pAMPKα expression.

CONCLUSIONS

In this study, the time course of AMPKα phosphorylation was evaluated in the hypothalamus of Ovx rats in vivo, together with food intake, body mass, and plasma adipocytokines. Ovx increased body mass by hyperphagia. Estrogen deficiency caused increased hypothalamic AMPKα phosphorylation and may contribute to the development of hyperphagia in rats. Meanwhile, the phosphorylation of hypothalamic AMPKα may be associated with the increased adiponectin in Ovx rats. Thus, modification of hypothalamic AMPKα phosphorylation by chemicals may have a preventive potential for the development of obesity after menopause.

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