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

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Abstract

Objective: AMP-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, plasma leptin and adiponectin levels were measure in female rats after either Ovx or sham operations. These patterns were also observed after treatment with 17β-estradiol (E2), compound C and leptin in Ovx rats.

Results: Compared with control rats, Ovx led to increase body weight and food intake at 2 to 8 weeks after operation. Meanwhile, the plasma levels of leptin and adiponectin, and the hypothalamic pAMPKα expression were significantly increased after Ovx. Replacement of E2 significantly reversed these effects. Treatment with compound C, an AMPKα inhibitor, for one week marked reduction of food intake, body weight, and plasma leptin and adiponectin levels. Meanwhile, these effects were reverse when withdrawal of compound C. In addition, central injection of leptin also significantly reduced the body weight, food intake, plasma levels of leptin and

adiponectin and the expression of hypothalamic pAMPKα relative to that of the Ovx group.

Conclusion: The increased hypothalamic pAMPKα expression may contribute to the hyperphagia during the development of Ovx-induced obesity in rats.

Key Words: AMPK – Hypothalamus – Ovariectomy – Obesity – Leptin – Adiponectin.

INTRODUCTION

AMP-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 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] Previous study had 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 females.[4] Food intake in human females varies across the menstrual cycle with daily food intake

lowest during the periovulatory period when estrogen levels are at maximum.[5] Menopausal women tend to gain body fat, which appears 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, hypo-estrogenic states are associated with decreased activity and an increase in body weight in females.

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 undertaken, therefore, to test whether phosphorylated AMPKα in the hypothalamus is affected by Ovx and thus would 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 (NIH 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, i.p.) 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 (ICV) 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 bregma on the mid-line 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 prior to 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 DMSO and injected in a volume of 5 μL (ICV). The entire injection process lasted under 2 min, and the rats were returned to their cages with the minimum of 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 the three groups: (1) Sham group: rats had undergone sham operations (n = 12); (2) Ovx group: rats were ovariectomized bilaterally (n = 12); (3) Ovx+E2 group: Ovx rats were injected with E2 (50 μg/kg per day, IM, once daily; Sigma-Aldrich Corp., St. Louis, MO) for 1-7 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, 8 week 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 the six groups: (1) Sham group: rats had undergone sham operations (n = 6); (2) Sham + compound C group: rats 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 one week, beginning six weeks after sham operations (n = 6); (3) Ovx + DMSO group: Ovx rats were treated with 5 μL of dimethyl sulfoxide (DMSO; ICV, twice a week) for one week, beginning 6 weeks after Ovx (n = 6); (4) Ovx + compound C group: Ovx rats were treated with 5 μL of compound C (50 nmol/5 μL, ICV, twice a week) for one week, beginning 6 weeks after Ovx (n = 6); (5) Ovx + DC group: Ovx rats were treated with 5 μL of compound C (50 nmol/5 μL, ICV, twice a week) for one week, beginning 6 weeks after Ovx, and then was monitored for another two weeks (n = 6); (6) Ovx + leptin group: Ovx rats were treated with 5 μL of leptin (0.5 nmol/5 μL, ICV, twice a week; R&D systems, Inc) for one 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, 8 week after Ovx and centrifuged at 1500 × g for 10 min at 4℃. Plasma adiponectin (AdipoGen, Inc., Seoul, Korea) and leptin (Millipore, Temecula, CA), respectively, were measured by 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 azide, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 0.5 mM PMSF, 1 mM DTT 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 min 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 were separated by

SDS-PAGE, 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 x-ray film 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 ovariectomy (Ovx) and 17β-estradiol 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 weight (240.7 ± 1.5 g) and the sham group gained around 50 g during the experimental period and reaching a mean weight of 287 ± 3 g after the 8 weeks. The Ovx rats gained significantly more and after 8 weeks the mean weights in this group was 353 ± 6 g, whereas, the Ovx+E2 rats gained much less (final weight 272 ± 2 g) compared to the Ovx group.

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

The 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/rat/week at 8 weeks after surgery. The Ovx rats consumed significantly more (148 ± 2 g/rat/week) at 2 weeks after Ovx and reach maximum (163.9 ± 3 g/rat/week) at 8 weeks after surgery. Whereas, the Ovx+E2 rats consumed similar level (129 ± 2 g/rat/week) at 8 weeks after Ovx when compare with the control rats.

Time-course changes in hypothalamic phosphorylated AMPKα protein expression after Ovx

Protein expression in the hypothalamus of Ovx rats was studied by

immunoblotting analysis. The level of phosphrylated AMPKα (pAMPKα) proteins in sham-operated rats was not significantly different at the time points tested (Fig. 2). However, pAMPKα protein accumulated significantly at 1 - 4 weeks after Ovx when compared with the sham group ($p < 0.05$), and was significantly further elevated at 8 weeks. After E2 replacement, the accumulation of pAMPKα protein was significantly reversed and was negligibly expressed during the experimental period.

Effect of ovariectomy (Ovx) and 17β-estradiol substitution on plasma level of leptin

As shown in Figure 3A, the plasma leptin level of the sham group did not differ at the time points tested. After Ovx, the level of plasma leptin was significantly higher than those of the sham group at 2-8 weeks ($P < 0.05$). Replacement of E2 in Ovx rats markedly reduced leptin level at 2-8 weeks after Ovx when compared with the Ovx group ($P < 0.05$).

Effect of ovariectomy (Ovx) and 17β-estradiol substitution on plasma level of adiponectin

As shown in Figure 3B, the plasma adiponectin level of the sham group did not differ at the time points tested. After Ovx, the level of plasma adiponectin was significantly increased and was maintained at high levels during the experimental period, and was significantly higher than those of the sham group at 1-8 weeks ($P <$

0.05). Replacement of E2 in Ovx rats markedly reduced adiponectin level at 2-8 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 the Ovx-induced obesity, we evaluate the effects of AMPK inhibitor (compound C) on the body weight and food intake. As shown in Figure 4, after treatment with compound C (ICV) for one week, the body weight and food intake were significantly reduced when determined at week 7 after Ovx relative to those of the Ovx group ($P < 0.05$). Withdrawal of compound C, continued to monitor two weeks, the body weight and food intake were rebound to the similar level of Ovx group (at week 9).

Effect of the AMPK inhibitor, compound C, on hypothalamic phosphorylated AMPKα protein expression

As shown in Figure 5A, after treatment with compound C for one week, the expression of pAMPKα were significantly reduced when determined at week 7 after Ovx relative to that of the Ovx group ($P < 0.05$). And then, continued to monitor another two weeks, the expression of pAMPKα were reversed.

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

Plasma leptin and adiponectin level after compound C treatment are presented in

Figures 5B and 5C. Compound C was ICV injected (twice a week) for one week and the treatment obviously reduced the plasma levels of leptin and adiponectin. In addition, continued to monitor another two weeks without compound C treatment, the levels of leptin and adiponectin were rebound to the similar level of Ovx group.

Effects of intracerebroventricular (ICV) leptin on body weight and food intake

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

Effects of intracerebroventricular (ICV) leptin on hypothalamic phosphorylated AMPKα protein expression and plasma levels of leptin and adiponectin

As shown in Figure 5A, after treatment with leptin for one week, the expression of pAMPKα was significantly reduced when determined at week 7 after Ovx relative to that of the Ovx group ($P < 0.05$). Meanwhile, the plasma level of leptin and adiponectin was 1856.4 ± 233.7 pg/mL and 14297.4 ± 2665.8 ng/mL, respectively, which is statistically significantly lower than that of the Ovx group (4781.4 ± 716.9 pg/mL and 31845.2 ± 1319.8 ng/mL, respectively)($P < 0.05$) (Fig. 5B and 5C).

DISCUSSION

This is the first in vivo study demonstrating time-course change phosphorylation state of AMPKα (pAMPKα) expression in hypothalamus of Ovx-induced obesity rats. It is well known that hypothalamic pAMPKα play a role in the regulation of food intake. However, the relationship between pAMPKα and obesity induced by Ovx was not evaluated. Results demonstrated that a marked reduction in estrogen synthesis led to increase food intake, body weight and the hypothalamic pAMPKα expression. Meanwhile, the plasma level of leptin and adiponectin were increased after Ovx. Replacement of E2 significantly reversed these effects. Treatment with compound C, an AMPKα inhibitor, for one week marked reduction of food intake, body weight, and plasma leptin and adiponectin levels. Withdrawal of compound C trigged hyperphagia. Results suggested that increased hypothalamic pAMPKα may play an important role in the development of hyperphagia in the Ovx-induced obesity rats.

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 estradiol (E2), 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 E2 to ovariectomised mice and rats could prevent these

changes in feeding behavior.[23] Similarly to these reports, in the present study, withdrawal of ovarian hormones in rats increased food intake and body mass (Fig. 1). Meanwhile, previous study had shown that the loss of ovarian hormones did not affect resting energy expenditure or fuel utilization during the experimental period (35 days after Ovx).[24] Thus the rapid mass gain after Ovx appears 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 mainly related 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] orexigenic factors (*e.g.* ghrelin)[32] activate hypothalamic AMPK, whereas leptin and other anorexigenic agents suppress AMPK activity in the hypothalamus. In our present study, Ovx lead to a significantly increased the hypothalamic AMPKα phosphorylation (Fig. 2). Meanwhile, treatment with compound C, an AMPKα inhibitor, for one week marked reduction of food intake and body weight (Fig. 4). Withdrawal of compound C trigged hyperphagia, indicating that AMPK activity remained throughout the duration of experiment (Fig. 5A). These results suggested 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 the AMPK, and inhibition of AMPK in discrete hypothalamic regions is also critical for the anorexigenic effects of leptin.[3] Consisting with previous results,[34,35] in our current study, the plasma level of leptin of Ovx rats was significantly higher than that of sham rats (Fig. 3A). However, we did not see the reduction of hypothalamic pAMPKα and food intake in Ovx rats, despite significantly increased plasma leptin level. It may be explained by leptin resistance in Ovx-induced obesity is decreased leptin transport across the blood-brain barrier or dysfunction of leptin receptor in hypothalamus. However, previous study had shown that the analysis of the hypothalamus revealed no modulation of leptin receptor (Ob-Rb) expression at 7 week after Ovx, whereas in a long-term study (22 week) the hypothalamic Ob-Rb expression 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 trigged hyperphagia, indicating that leptin biology remained throughout the duration of the chronic treatment.[24] Moreover, intracerebroventricular (ICV) leptin administration decreased food intake, body weight, and hypothalamic pAMPKα at week 7 after Ovx (Fig. 5). Taken together, these data suggested that Ovx increased body weight by producing hyperphagia may be not a consequence of leptin resistance. However, these data does 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 estradiol 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 one week after Ovx and was maintain at high level during the experimental period (Fig. 3B). Meanwhile, recent study had shown that injection of adiponectin significantly stimulates activates AMPK in the hypothalamus, leading to stimulation of food intake and decreases energy expenditure.[40] Therefore, the elevated adiponectin may be a major contributor to the increase of 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 the

Ovx-induced changed in hypothalamic pAMPKα expression. In our study, plasma leptin and adiponectin concentration was profoundly increased in Ovx rats. Both of leptin and adiponectin modulated the activity of hypothalamic pAMPKα. Moreover, ICV administration of compound C inhibited the increased in hypothalamic AMPK activity and food intake. Thus, the increased of hypothalamic pAMPKα may be a major contributor to the hyperphagia in Ovx rats. Further studies are needed to clarify how Ovx produces the increased of 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 cause increased hypothalamic AMPKα phosphorylation may contribute to the development of hyperphagia in rats. Meanwhile, the phosphorylation of hypothalamic AMPKα may associated with the increased of adiponectin in Ovx rats. Thus, Modification of hypothalamic AMPKα phosphorylation by chemicals may have preventive potential for the development of obesity after menopause.

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Figure Legends

Figure 1 Changes in body weight (A) and food intake (B) in sham, ovariectomized (Ovx) rats and Ovx + E2 rats. Values are means ± SEM. *$P < 0.05$ vs. sham group. # $P < 0.05$ vs. Ovx group.

Figure 2 Time course of changes in AMPKα phosphorylation in hypothalamus from sham, Ovx and Ovx+E2 rats. Data are given as mean ± SEM. (n=3 for each time point) *$P < 0.05$ vs. sham group. # $P < 0.05$ vs. Ovx group.

Figure 3 Time course of changes on plasma leptin (A) and adiponectin (B) levels in sham, Ovx and Ovx+E2 rats. Data are given as mean ± SEM. (n=3 for each time point) *$P < 0.05$ vs. sham group. # $P < 0.05$ vs. Ovx group.

Figure 4 The effects of compound C, an AMPK inhibitor, on body weight (A) and food intake (B) in ovariectomized (Ovx) rats. Ovx rats were treated with compound C for one week, beginning 6 weeks after Ovx. Values are means ± SEM. *$P < 0.05$ vs. sham group. # $P < 0.05$ vs. Ovx group.

Figure 5 The effects of leptin and compound C, an AMPK inhibitor, on hypothalamic AMPK phosphosrylation (A), plasma leptin (B), and plasma adiponectin (C) in Ovx

rats. Ovx+DC as Ovx rats were treated with compound C for one week, beginning 6 weeks after Ovx, and then was monitored for another two weeks. Data are given as mean ± SEM. (n=6) *$P < 0.05$ vs. sham group. # $P < 0.05$ vs. Ovx group.

A summary to the Table of Contents:

Estrogen deficiency cause increased hypothalamic AMPKα phosphorylation contributes to the development of hyperphagia in rats. Thus, AMPK may serves as a target for the development of a new anti-obesity agent.