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Title: The role of receptor-interacting protein 140 in the accumulation of fat in ovariectomised rats

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Abstract: Background Receptor-interacting protein 140 (RIP140) is a corepressor for nuclear receptors with an important role in the inhibition of energy expenditure. Postmenopausal women have increased white adipose tissue (WAT), and excessive accumulation of adipose tissue (obesity) implies a health risk. The aim of the present work was to investigate the time-course of RIP140 expression in WAT during the development of ovariectomy (OVX)-induced obesity in rats.
Methods OVX was performed in female Sprague-Dawley (SD) rats 8 weeks old. Body weight and food intake were determined once a week. WAT of sham-operated, OVX, and OVX plus 17β-estradiol therapy (OVX/E2) female SD rats was weighed and used to analyse RIP140 and uncoupling protein 1 (UCP-1) expression by Western blot.
Results Food intake and body weight were significantly increased during the 2-8 weeks after OVX. Even though body weight increased until sacrifice, food intake progressively decreased from 9 to 16 weeks after ovariectomy in rats. Meanwhile, increased WAT mass and decreased RIP140 expression in WAT were observed in OVX rats. In contrast, the expression of UCP-1, a key target gene of RIP140, in WAT of OVX rats was significantly higher than in sham-operated rats. All of these alterations caused by OVX were mostly reversed by the replacement of 17β-estradiol.
Conclusions The down-regulation of RIP140 in WAT may play a compensatory role in OVX-induced obesity in rat.

Response to Reviewers: We appreciate the comments of the Reviewer and believe that our manuscript has been improved by attention to them. The followings are our responses to the specific issues raised:
Reviewer #1
1.This is a very interesting paper that suggests that weight gain in ovariectomised rats can be reversed by estrogen and that compensatory mechanisms can be measured at a molecular level. Specifically RIP140 an inhibitor of UCP-1 falls in the setting of weight gain allowing increased thermogenesis. As such this is an interesting paper and is worthy of submission it has no therapeutic implications. I am curious as to why the authors used Western Blot vs RT PCR. Second there needs to be more discussion as to how the WAT was collected. I have difficulty understanding how this can be adequately quantities without MR. Please comment
Response: Thanks for your constructive comments.

1.The western blot is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract. Thus, it was used to investigate the expression of RIP140 and UCP-1 proteins in white adipose tissue in this study and others (Debevec et al., 2007).
2.Total body MRI which offers the most accurate currently available method of assessing body composition components, however, the amount of adipose tissue can be shown by the weight of adipose tissue in many studies (Remesar et al., 2002; Meli et al., 2004). In this study the white adipose tissue was collected as follow: The rats were anaesthetised with Zoletil (15 mg/kg, i.p.) and blood was collected by intra-abdominal aortic artery. Rats were then skinned, and the main adipose tissue masses were carefully dissected, weighed, frozen and stored for further analyses (del Mar Grasa et al., 2001). The subcutaneous white adipose tissue was obtained from the inguinal areas of each rat. Please see the Methods section.
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Reviewer #2
A well written paper with sound methodology. I am however not sure why such a very basic science paper is submitted to an obesity surgery journal.
Minor comments
Please omit any extrapolations in your discussions to human application. You have no basis for this. This remains a rat study, elegant as it my be. You must also take great caution to try and relate this to human female menopause-in humans this is a very gradual process that encompasses the perimenopause in times of time frame. What you created in your rats is "iatrogenic" or instant surgical menopause.
Response: Thanks for your constructive comments. We have deleted the extrapolations in our discussion to human. In addition, we also agree with the reviewer's opinion. In clinical practice, menopause is diagnosed retrospectively when the absence of regular menses lasts for at least 12 months (Lund, 2008). It is due to the aging of the ovary resulting from the exhaustion of ovarian follicles (Wise et al., 1996) causing infertility and a progressive loss of its hormonal activity (Greendale and Sowers, 1997). Loss of ovarian estrogen production is the key pathophysiological event responsible for the consequences of menopause. The incidence of obesity is higher in post-menopausal women than in age-matched pre-menopausal women; this increase is thought to be associated with the decline of estrogen levels after menopause. In fact, estrogen replacement therapy is an effective treatment to reduce body weight and fat accumulation in post-menopausal women (Svendsen et al. 1995). In rats, estrogen treatment reduces energy intake (Donohoe and Stevens 1983), and it has been demonstrated that ovariectomy (OVX) induces an increase in body weight, which can be reversed with estrogen replacement treatment (Wade et al. 1985). Thus, estrogen insufficiency in human can be modeled using ovariectomised rats. This model is characterized by mild obesity and is useful to study how hypoestrogenism alters adiposity in many studies (Ainslie et al., 2001 ; Meli et al., 2004).
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The role of receptor-interacting protein 140 in the accumulation of fat in ovariectomised rats

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* Short title: The receptor-interacting protein 140 in ovariectomised rats

Abstract

Background Receptor-interacting protein 140 (RIP140) is a corepressor for nuclear receptors with an important role in the inhibition of energy expenditure. Postmenopausal women have increased white adipose tissue (WAT), and excessive accumulation of adipose tissue (obesity) implies a health risk. The aim of the present work was to investigate the time-course of RIP140 expression in WAT during the development of ovariectomy (OVX)-induced obesity in rats.

Methods OVX was performed in female Sprague-Dawley (SD) rats 8 weeks old. Body weight and food intake were determined once a week. WAT of sham-operated, OVX, and OVX plus 17β-estradiol therapy (OVX/E2) female SD rats was weighed and used to analyse RIP140 and uncoupling protein 1 (UCP-1) expression by Western blot.

Results Food intake and body weight were significantly increased during the 2-8 weeks after OVX. Even though body weight increased until sacrifice, food intake progressively decreased from 9 to 16 weeks after ovariectomy in rats. Meanwhile, increased WAT mass and decreased RIP140 expression in WAT were observed in OVX rats. In contrast, the expression of UCP-1, a key target gene of RIP140, in WAT of OVX rats was significantly higher than in sham-operated rats. All of these alterations caused by OVX were mostly reversed by the replacement of 17β-estradiol.

Conclusions The down-regulation of RIP140 in WAT may play a compensatory role in OVX-induced obesity in rat.

Introduction

Energy homeostasis is a highly regulated process that requires precise control of food intake and energy expenditure [1]. The major and most efficient storage of energy occurs in the form of triglycerides in white adipose tissue (WAT), and it is now clear that the adipocyte itself is an endocrine cell; therefore, altered adipocyte function can alter systemic energy balance [2, 3]. Evidence from both human and animal experiments suggests that oestrogen plays an important role in the regulation of WAT. Postmenopausal women have increased WAT, and oestrogen therapy decreases WAT levels compared with untreated postmenopausal women [4, 5]. Animal experiments have also shown that ovariectomy (OVX) of rodents increases WAT and that oestrogen replacement decreases WAT [6, 7]. Excessive accumulation of adipose tissue (obesity) implies a health risk. It leads to various chronic morbidities, including insulin resistance, type II diabetes, and cardiovascular disease [8-10]. 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.

Receptor-interacting protein 140 (RIP140) is a nuclear protein of 1158 amino acids [11]. The RIP140 gene is expressed in many tissues; it is localised in specific cell types and subject to developmental control. The highest levels of expression are

detected in metabolic tissues, including adipose tissue, liver and muscle, and in the ovary [12]. The expression of RIP140 is regulated by a number of hormones, including oestrogen [13] and vitamin D [14]. It is also regulated during adipogenesis, as exemplified in 3T3-L1 cells, where there is a progressive increase in expression during differentiation in response to oestrogen-related receptor signalling [12, 15]. The physiological importance of RIP140 was evaluated using mice devoid of the RIP140 gene (RIPKO). RIP140-null mice accumulate markedly less fat in their adipose tissue, are resistant to high-fat diet-induced obesity and exhibit improved glucose tolerance and insulin sensitivity [12, 16, 17]. These changes are associated with increased expression of metabolic genes in WAT and muscle, resulting in an increased metabolic rate and energy expenditure [12, 18]. Along these lines, in the absence of RIP140, the expression of uncoupling protein 1 (UCP-1) is elevated in adipocytes derived from WAT, with increased energy dissipation facilitating the process of thermogenesis as well as maintaining the redox balance and reducing the generation of reactive oxygen species [16, 19, 20]. Therefore, the aim of this study was to analyse the influence of menopause-associated obesity on RIP140 protein expression in the WAT of OVX rats.

Material and methods

Animal preparation

Female Sprague-Dawley (SD) rats (8 weeks old) 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. Rats were anaesthetised with Zoletil (15 mg/kg, i.p.) and underwent bilateral ovariectomy at 8 weeks of age. Small incisions were made bilaterally on the sides of the back 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 anaesthesia, visualisation of the ovaries via incisions into the abdominal cavity, and closure of the wounds.

Experimental groups

One week after the operation, the rats were randomly divided into three groups: (1) sham group, rats that had undergone sham operation (n=18); (2) OVX group, OVX rats injected intramuscularly (IM) with the vehicle for oestrogen for 15 weeks, beginning 1 week after ovariectomy (n=18); (3) OVX/E2 group, OVX rats injected

with 17β-estradiol (E2; 50 μg/kg/day, IM, once daily) for 15 weeks, beginning 1 week after ovariectomy (n=18). To investigate the time-courses of RIP140 and UCP-1 expression in WAT, rats were anaesthetised with Zoletil (15 mg/kg, i.p.) and killed at 4, 12 or 16 weeks after ovariectomy (n=6 for each time point tested). Blood samples were collected by intra-abdominal aortic puncture. Rats were then skinned, and subcutaneous WAT was carefully excised and weighted. The subcutaneous white adipose tissue was obtained from the inguinal areas of each rat [21]. Then the WAT was snap-frozen in liquid nitrogen and stored at -80°C for later analysis.

Measurements of food intake and body weight

Each rat was housed in a single cage. 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.

Western blot analysis

The adipose tissue (0.3 g) obtained from each animal was ground in a mortar containing liquid nitrogen. The powdered tissue was then suspended in 1 mL of lysis buffer [20 mM Tris-HCl (pH 7.5), 10 mM NaF, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulphonylfluoride (PMSF), 1 mM Na_3VO_4, leupeptin, and 10 μg/ml trypsin inhibitor] and agitated at 4°C for 1 h. After 1 h, cell lysates were obtained by

centrifugation at 100,000 × *g* for 15 min at 4°C. Protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA) according to the manufacture's instructions. For Western blot analysis, 100 μg protein of the tissue lysate was dissolved in Laemmli sample buffer, boiled for 5 min, and subjected to SDS-PAGE (10 or 8% polyacrylamide). The proteins were transferred from gel to nitrocellulose membrane at 250 mA for 2 h at room temperature. The membrane was then blocked with 1x TBST and 5% nonfat dried milk for 40 min at room temperature and probed with rabbit polyclonal RIP140 antibody (1:2000; Affinity Bioreagents, Golden, CO) or rabbit polyclonal UCP-1 antibody (1:1000; Chemicon International) in 1x TBST, 5% nonfat dried milk, and 0.1% Tween 20 at 4°C overnight. The secondary antibody (anti-rabbit or anti-goat IgG-horseradish peroxidase conjugate; 1:2000 dilution; Cell Signaling) was incubated for 1 h at room temperature. Subsequently, the blot was extensively washed with TBST, developed using an enhanced chemiluminescence kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions, and exposed to X-ray film (Kodak, Rochester, NY, USA). To verify that blots were loaded with equal amounts of protein lysates, they were also incubated in the presence of the antibody against β-actin (Sigma-Aldrich Corp.). The density of the respective bands was quantified by densitometric scanning of the blots using Image-Pro software (Media Cybermetrics, Inc.).

Statistical analysis

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 (E2) substitution on body weight

The mean body weights of the rats in the three groups are given in Fig. 1. At the time of surgery, all rats had similar body weight (240.7 ± 1.5 g). The control group gained ~60 g during the 16-week study period, reaching a mean weight of 296 ± 4 g after 16 weeks. The OVX rats gained significantly more, reaching 422.2 ± 15.9 g after 16 weeks, whereas the OVX/E2 rats gained much less (final weight 281.67 ± 6.5 g) than the OVX group ($P < 0.05$).

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

Food intake in all rats was measured once a week. As shown in Fig. 2, the food intake of rats within each group did not differ at the time of operation. The control rats ate 120 ± 3 g/rat/week 16 weeks after OVX. The OVX rats consumed significantly more (148 ± 2 g/rat/week) 2 weeks after OVX and reached a maximum (163.9 ± 3.9 g/rat/week) at 8 weeks after surgery, and then progressively decreased to the same level as the control group. During the entire experiment, the OVX/E2 rats consumed a similar level as the control rats.

Effects of ovariectomy and 17β-estradiol substitution on WAT mass

WAT mass was examined at the end of the study by dissection of the adipose tissue. As shown in Fig. 3, the amount of WAT was significantly increased in the

OVX rats compared to the OVX/E2 rats ($P < 0.05$). The 17β-estradiol–substituted OVX/E2 rats had significantly less WAT than the OVX rats ($P < 0.05$).

RIP140 protein expression in WAT

To evaluate whether accumulation of WAT after OVX was caused by the effect of RIP140, we measured the expression of RIP140 protein in WAT. As shown in Fig. 4, the expression of RIP140 protein in WAT compared with control rats was significantly lower in OVX rats ($P < 0.05$), whereas OVX/E2 rats expressed more RIP140 than the OVX rats ($P < 0.05$).

UCP-1 protein expression in WAT

To clarify whether the biological effect of RIP140 remained throughout the experimental period, we evaluated the expression of UCP-1, a key target gene of RIP140, in WAT. As shown in Fig. 5, the expression of UCP-1 in the WAT of OVX rats was significantly higher than in controls ($P < 0.05$), whereas OVX/E2 rats exhibited similar UCP-1 expression to control rats ($P < 0.05$).

Discussion

This is the first in vivo study demonstrating the time-course of changes of RIP140 protein expression in WAT of OVX-induced obese rats. RIP140 plays an important role in the inhibition of energy expenditure. In the OVX group, a marked reduction in oestrogen level led to increased body weight and WAT mass and decreased RIP140 protein expression in WAT, accompanied by the up-regulation of UCP-1 protein expression. Replacement of 17β-estradiol significantly reversed these alterations. These results imply that the abnormal lipid accumulation of subcutaneous WAT cannot be accounted for by the up-regulation of RIP140 protein in WAT in OVX-induced obesity. On the contrary, the reduced RIP140 protein expression under OVX suggests a compensatory mechanism to accelerate energy expenditure and reduce fat accumulation in obese states.

Many pieces of evidence suggest that body fat content is controlled, at least partially, by the metabolism of adipose tissue itself. First, most of the candidate genes for obesity have important roles in adipocytes [22]. Second, many mouse models that are prone or resistant to obesity are created by transgenic modification of adipose tissue [23]. In these transgenic models, metabolic changes in white but not in brown adipose tissue are mostly responsible for the altered accretion of body fat, highlighting the importance of lipid metabolism in adipocytes of the white fat. In this

study, ovariectomised rats had increased adiposity compared with rats with intact ovaries (Fig. 3). Meanwhile, 17β-estradiol treatment in ovariectomised rats significantly reduced white adipose mass. These data confirm the report by D'Eon et al. [24] and suggest that oestrogen plays important roles in adipose tissue biology and in the prevention of obesity, possibly via decreased expression of lipogenic genes in adipose tissue.

The evidence emerging from both in vivo studies and cellular systems has identified a role for RIP140 as an important regulatory factor in many metabolic processes. RIP140-knockout mice are leaner than their control littermates, even when challenged by high-fat feeding [12]. Interestingly, this phenotype is not linked to a defect in adipogenesis. Meanwhile, the WAT of these animals expresses high levels of UCP-1 [12], a protein normally confined to the brown adipose tissue (BAT), where it contributes to adaptive thermogenesis. In addition to repressing the uncoupling of respiration, RIP140 inhibits other aspects of energy expenditure in the adipose tissue by repressing genes implicated in fatty acid oxidation, mitochondrial biogenesis and oxidative phosphorylation [17]. In primary adipocytes from RIP140-null mice, increased metabolic rate and release of chemical energy in the form of heat have been observed [16]. However, no studies have analysed the expression of RIP140 in obesity induced by ovarectomy in rats. We observed a down-regulation of RIP140

protein levels in OVX-induced obese rats compared to sham rats (Fig. 4). Supplementation with 17β-estradiol reversed this alteration. These findings suggest that the down-regulation of RIP140 may be a compensatory effect to counteract obesity. This speculation is supported by a report maintaining that both reduced and increased body weight are associated with compensatory changes in energy expenditure [25].

UCP-1 is a proton transporter that uncouples oxidative metabolism from ATP synthesis and dissipates energy through heat generation [26]. UCP-1 was once thought to be expressed only in BAT; recently, it was reported that UCP-1 mRNA and protein are also detectable in the WAT of mouse and human [27, 28]. A number of studies have demonstrated that UCP-1 expression is regulated by both nuclear receptors and alterations in the levels of intracellular cAMP, with coregulators such as PGC-1α acting as key factors required for the integration of different signalling pathways [29, 30]. Recent studies have shown that UCP-1 is a key target gene of RIP140 in adipocytes [12, 16]. In the present study, we demonstrated that the expression of UCP-1 was significantly increased in WAT from OVX rats, compared with that from sham rats (Fig. 5). These changes were reversed by supplementation with 17β-estradiol. These findings further corroborate the notion that the down-regulation of RIP140 may be a compensatory mechanism to counteract fat

accumulation via subsequent up-regulation of UCP-1 expression, increasing the energy expenditure in WAT.

In summary, oestrogen regulates the expression and activity of many genes involved in the control of energy homeostasis. A number of factors may be involved in the OVX-induced changes in RIP140 expression. In our study, body weight and WAT mass were profoundly increased in OVX rats. RIP140 inhibits energy expenditure in adipose tissue by repressing metabolic genes. In OVX rats, the expression of RIP140 was decreased in WAT, accompanied by increased UCP-1. Therefore, the down-regulation of RIP140 protein in WAT seems to rule out increased RIP140 as a causative role in OVX-induced obesity. It is possible that the gene expression changes we observed reflect a compensatory mechanism to accelerate energy expenditure and reduce fat accumulation in OVX-induced obesity. Further studies are needed to clarify the possible molecular mechanism of the decrease of RIP140 expression in WAT of OVX rats.

Acknowledgements

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Disclosure Statement

The authors have nothing to disclose.

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kinase in adipocytes: cAMP induction of the uncoupling protein 1 (UCP-1) gene requires p38 MAP kinase. J Biol Chem. 2001;276:27077-82.

Legends

Figure 1 Body weight of the rats in the three different groups was monitored weekly. Sham, sham operated; OVX, ovariectomised without oestrogen treatment; OVX/E2, ovariectomised with 17β-estradiol (E2) supplementation. Data are expressed as mean ± SEM (n = 6 in each). $^{*}P < 0.05$ for OVX vs. sham group. $^{\#}P < 0.05$ for OVX/E2 vs. OVX group.

Figure 2 Food intake of the rats in the three different groups was monitored weekly. Sham, sham operated; OVX, ovariectomised without oestrogen treatment; OVX/E2, ovariectomised with 17β-estradiol (E2) supplementation. Data are expressed as mean ± SEM (n = 6 in each). $^{*}P < 0.05$ for OVX vs. sham group. $^{\#}P < 0.05$ for OVX/E2 vs. OVX group.

Figure 3 Weight of white adipose tissue in 16-week-old rats in the sham, OVX, and OVX/E2 groups. The amount of white adipose tissue is expressed as percent of body weight. Data are expressed as mean ± SEM (n = 6 in each). $^{*}P < 0.05$ for OVX vs. sham group. $^{\#}P < 0.05$ for OVX/E2 vs. OVX group.

Figure 4 Time-course of changes of RIP140 protein expression in white adipose tissue

of the sham, OVX, and OVX/E2 groups. Depicted are a typical display of RIP140 protein expression (upper panel) and the statistical analysis of the changes of RIP140 levels (lower panel). Data are expressed as mean ± SEM (n = 6 in each). *$P < 0.05$ for OVX vs. sham group. $^{\#}P < 0.05$ for OVX/E_2 vs. OVX group.

Figure 5 Time-course of changes of UCP-1 protein expression in white adipose tissue of the sham, OVX, and OVX/E2 groups. Depicted are a typical display of UCP-1 protein expression (upper panel) and the statistical analysis of the changes of UCP-1 levels (lower panel). Data are expressed as mean ± SEM (n = 6 in each). *P < 0.05 for OVX vs. sham group. $^{\#}P < 0.05$ for OVX/E_2 vs. OVX group.

Figure 1

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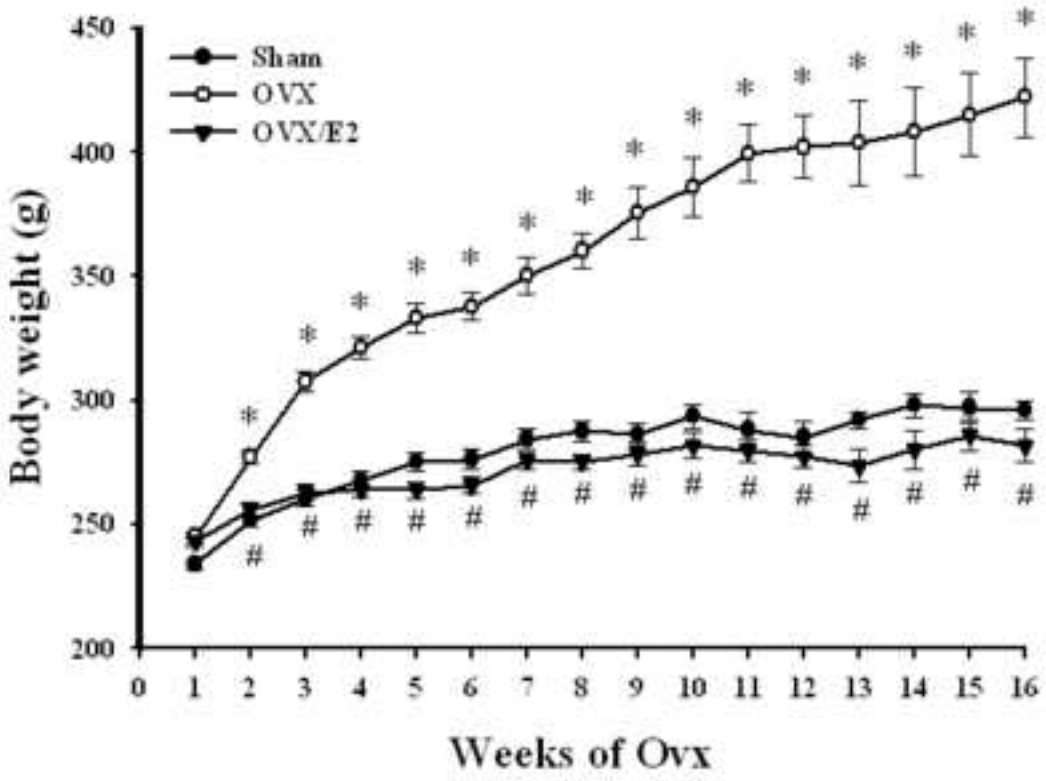
Figure 1



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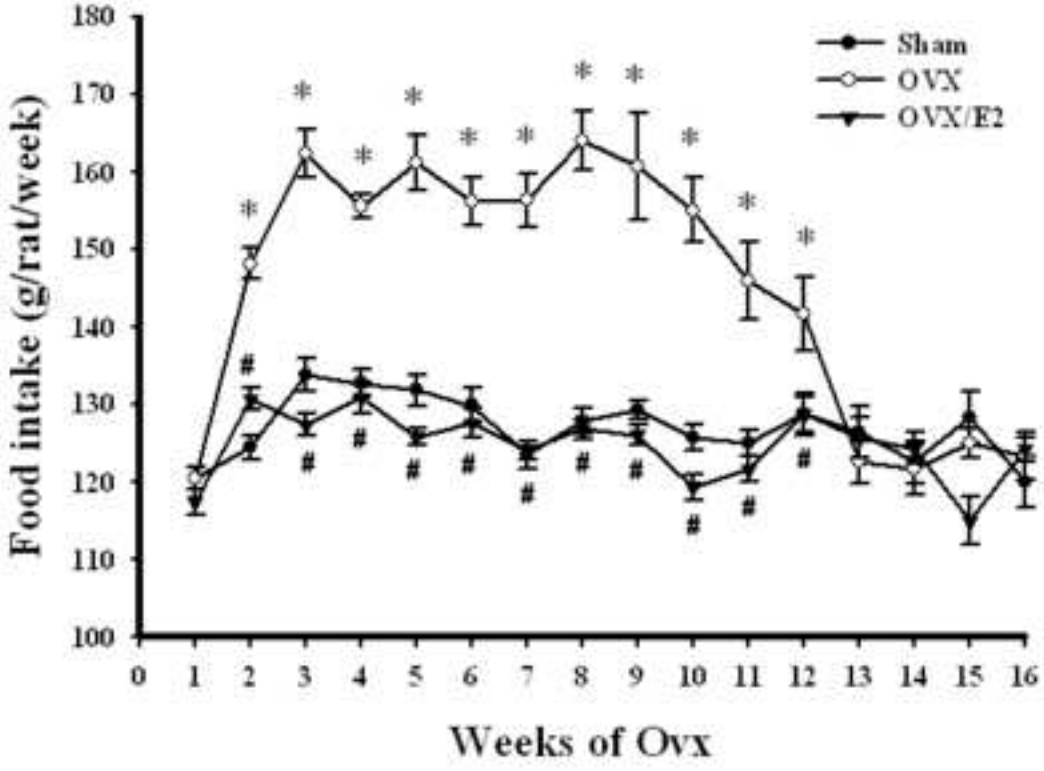

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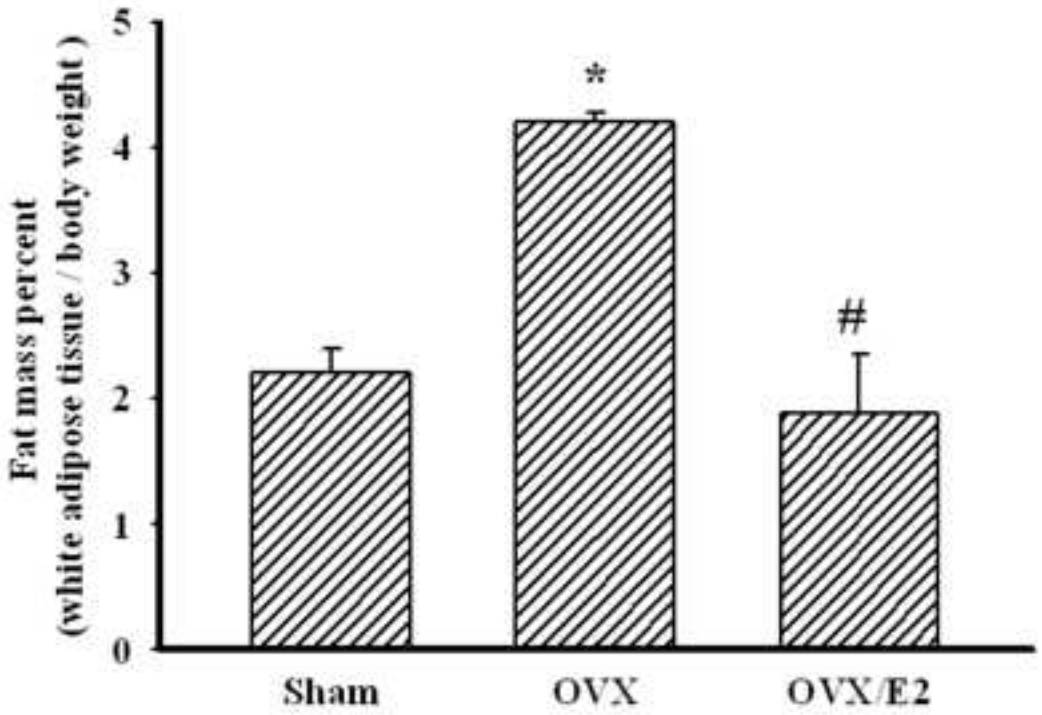

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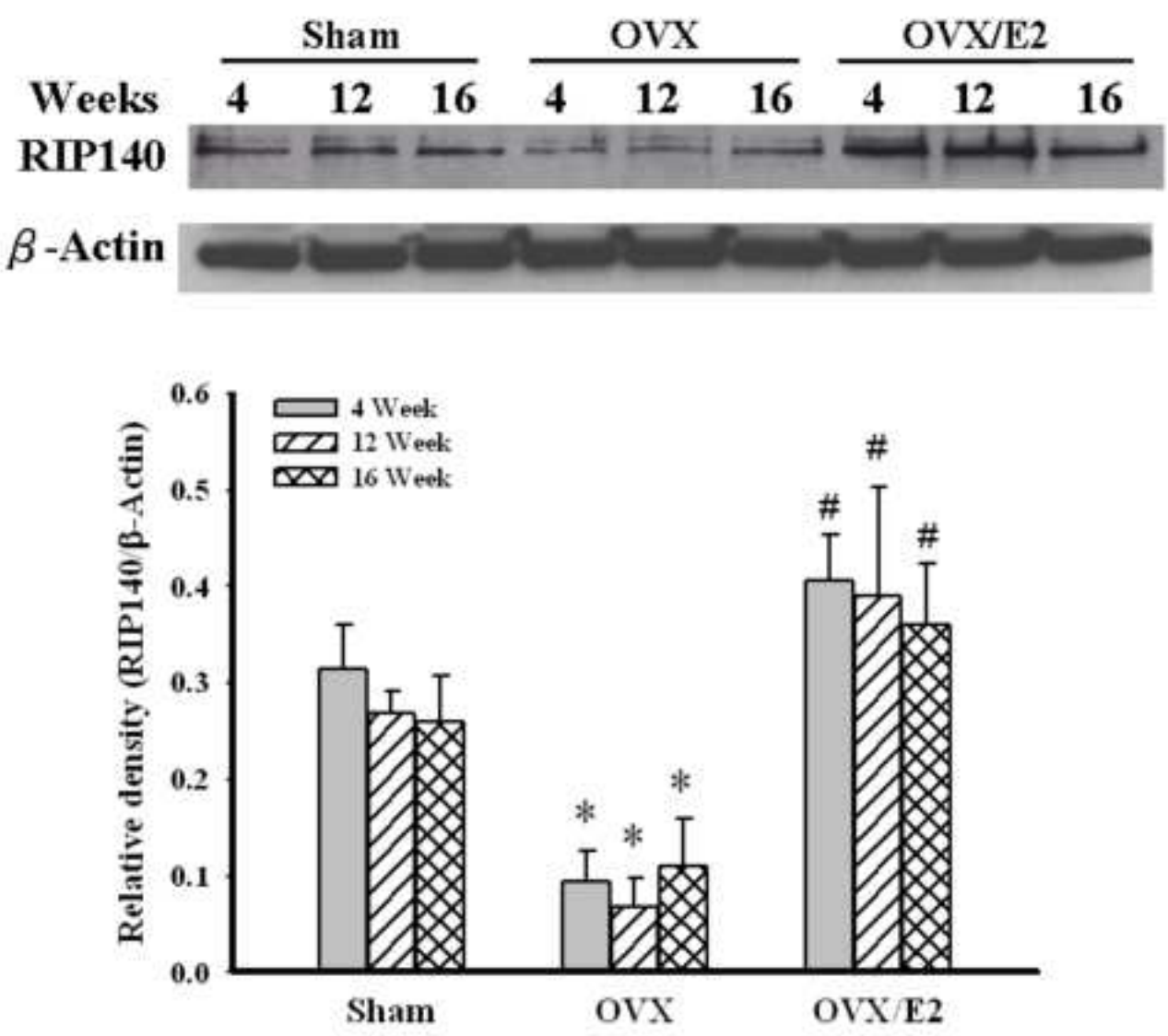

Figure 4

Figure 5
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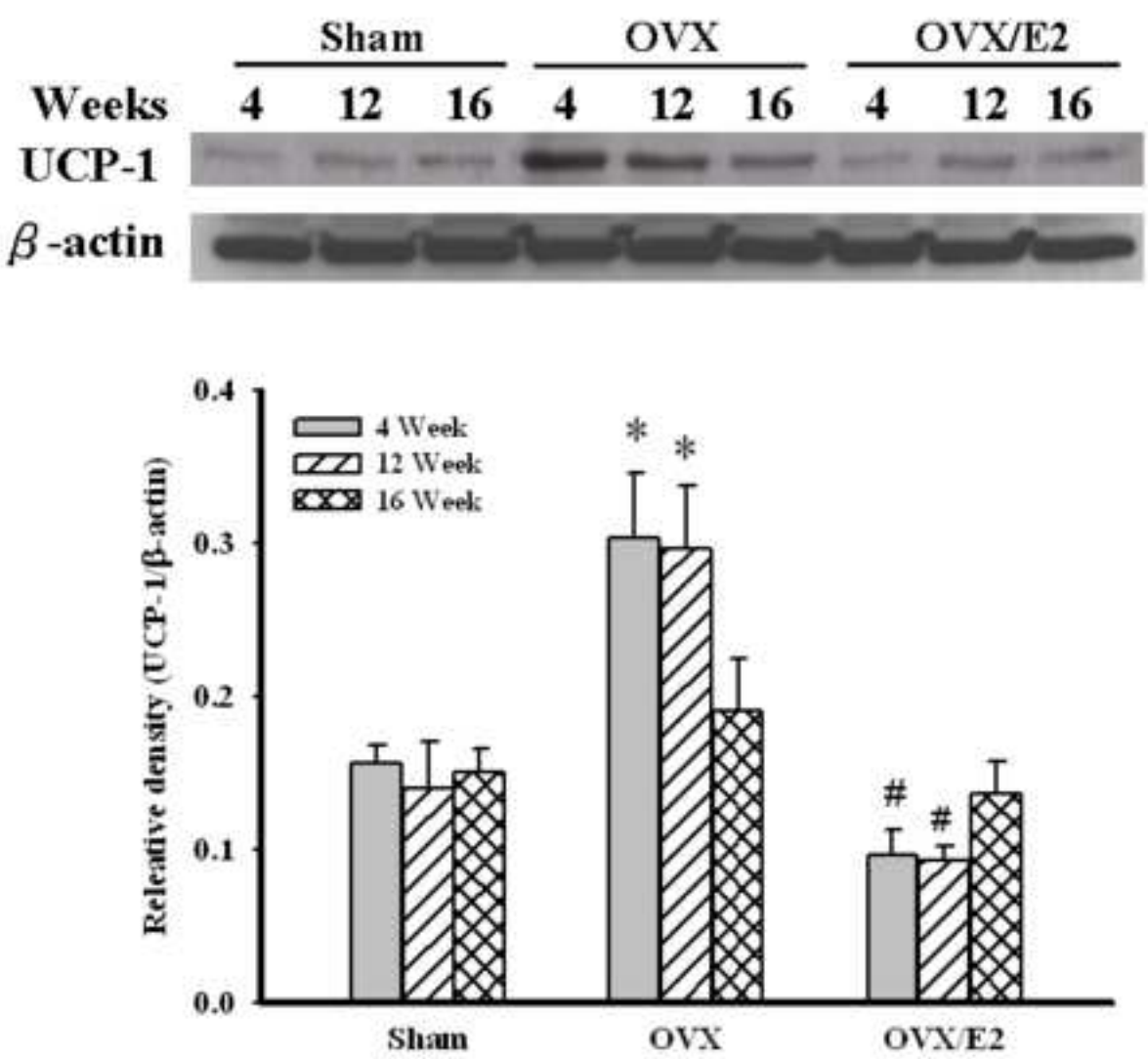

Figure 5