Title page
Metformin ameliorates body mass gain and early metabolic changes in ovariectomized rats

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1. Abstract

Estradiol has been used to prevent metabolic diseases, bone loss and menopausal symptoms, even though it might raise the risk of cancer. Metformin is usually prescribed for type 2 diabetes mellitus and lowers food intake and body mass while improving insulin resistance and the lipid profile. Ovariectomized rats show increased body mass, insulin resistance and changes in the lipid profile. Thus, the aim of this work was to evaluate whether metformin could prevent the early metabolic dysfunction that occurs early after ovariectomy. Female Wistar rats were divided into the following groups: SHAM-operated (SHAM), ovariectomized (OVX), ovariectomized + estradiol (OVX + E2) and ovariectomized + metformin (OVX + M). Treatment with metformin diminished approximately 50% of the mass gain observed in ovariectomized animals and reduced both the serum and hepatic triglyceride levels. The hepatic levels of phosphorylated AMP-activated protein kinase (pAMPK) decreased after OVX, and the expression of the inactive form of hepatic acetyl-CoA carboxylase (ACC) was also reduced. Metformin was able to increase the levels of pAMPK in the liver of OVX animals, sustaining the balance between the inactive and total forms of ACC. Estradiol effects were similar to those of metformin but with different proportions. Our results suggest that metformin ameliorates the early alterations of metabolic parameters, and rescues hepatic AMPK phosphorylation and ACC inactivation observed in ovariectomized rats.
2. Introduction

Metabolic syndrome is defined as the presence of at least three of a set of features, such as abdominal obesity, high fasting glycemia, high blood pressure and dyslipidemia, that is characterized by one or more disorders as increased levels of serum triglycerides or reduced levels of high density lipoprotein (HDL). Postmenopausal women have an elevated risk of developing metabolic syndrome compared to premenopausal women (1). In menopause, there is a reduction of ovarian function that is linked to decreased estradiol production. In this context, estrogens are used for hormone therapy to prevent the development of metabolic disorders (2, 3). It has been shown that although estrogen hormone replacement therapy has many benefits, it also increases the risk of developing cancer (3–5). In this study, we used ovariectomized animals, a model in which animals have both ovaries removed and therefore mimics the estrogen deficiency that occurs during menopause. Ovariectomy is frequently used in literature for a better understanding of metabolic disorders of menopause (6, 7), and for testing pharmacological alternatives to prevent menopause disturbances (8, 9).

During recent decades, the biguanide metformin has become one of the most prescribed antidiabetic drugs worldwide. Although the mechanism of action of metformin is not fully understood (10), metformin is widely used for the treatment of metabolic disorders found in type 2 diabetes mellitus, polycystic ovary syndrome and metabolic syndrome (11). Metformin ameliorates glycemia levels, insulin sensitivity and body mass gain, which are some of the alterations detected in ovariectomized rodents (11–13).

The goal of this study was to evaluate the effects of metformin administration in the metabolism of short-term ovariectomized rats, comparing to estrogen replacement. Metformin was able to partially prevent body mass gain and food efficiency, and the drug also prevented the increase in food ingestion. Metformin treatment also decreased
hepatic and serum triglyceride levels, probably due to an increase of liver phosphorylated AMP-activated protein kinase (pAMPK) and inactivated acetyl-CoA carboxylase (ACC) levels. Taken together, our data suggest that metformin has metabolic effects similar to those observed with estradiol treatment and therefore has a potential role in the prevention of early alterations in metabolic parameters in ovariectomized rats.

3. Materials and methods

Animals

Female Wistar rats (Rattus norvegicus) were maintained in a 12 h light-dark cycle at a constant temperature of 22 ± 1°C. Water and food (NUVILAB-CR-1, from Quimtia, Parana, Brazil) were available ad libitum. Animal handling and euthanasia procedures were approved by the Institutional Animal Care and Use Committee (CEUA) of the Universidade Federal do Rio de Janeiro, Brazil (number 170/13).

Animal Procedure

Female rats weighing between 180 and 220 g underwent ovariectomy surgery. The bilateral ovariectomy was performed by surgical procedure, as previously described with few modifications (14, 15). In brief, just prior the surgery, animals were weighted to calculate the amount of anesthetic required for general anesthesia. A combination of 50 mg/kg ketamine and 5 mg/kg xylazine was administrated by intraperitoneal injection. Abdominal area was cleaned with ethanol. To access peritoneal cavity. A surgical scalpel was used to make a small transverse peritoneal incision on the middle
part of the lateral abdomen at both sides of the female rats. Ovaries were identified and removed (OVX), or exposed and reinserted into the peritoneal cavity (SHAM). After the procedure, animals were divided into four groups: sham-operated rats treated with corn oil subcutaneously (vehicle) (SHAM); ovariectomized rats treated with vehicle subcutaneously (OVX); ovariectomized rats treated with 7 µg/kg of body mass of estradiol benzoate (Sigma-Aldrich, St. Louis, USA) subcutaneously with an injected volume of approximately 180-250 µL (OVX + E2), and ovariectomized rats treated with vehicle subcutaneously and 5 g/L of 1,1-dimethylbiguanide hydrochloride (metformin) in drinking water (purified from Sigma-Aldrich or from commercial MERCK, Darmstadt, Germany, drugs used after filtration and validation that the biological actions were the same) (OVX + M). The dose of estradiol benzoate was the same as used by Pantaleão et al. 2010 (16). Based on Johns Hopkins manual of animal care and use committee (http://web.jhu.edu/animalcare/procedures/rat.html; retrieved August 28, 2019) and the results described by Castro et al. 2014 (17), Wistar rats drink approximately 10 mL/100 g of body mass per day. This allows us to calculate a daily ingestion of approximately 500 mg metformin/kg/day, which is a dose used found in the literature that is able to ameliorate some cardiovascular parameters, protect against mitochondria damage, decrease insulin levels, food intake, body weight gain, serum triglycerides, increase glucose tolerance and insulin sensitivity (18–23). Animals were housed individually, and daily food intake was monitored. Rats were weighed daily, and the percentage of mass gain was calculated by setting the mass immediately after surgery as 100%. Food efficiency was calculated by the difference between final (at 20 days postsurgery) and initial (immediately after surgery) body mass, and dividing by the total food intake in grams during the twenty days of treatment of each animal.
Twenty days after ovariectomy, the rats were fasted for 14 h and euthanized by decapitation at 21 days postsurgery. Blood was collected from the trunk, centrifuged at 1,500 g for 20 min at room temperature, and the serum was stored at -80°C. The liver, white gastrocnemius (W. Gastrocnemius) and soleus muscles, uterus, interscapular brown adipose tissue (BAT) and white adipose tissue depots (retroperitoneal, inguinal and gonadal) were excised, weighed and normalized to the rat tibia length.

**Serum analyses**

The levels of serum total cholesterol, total triglycerides and high-density lipoprotein (HDL) were analyzed with commercial kits (K083-2, K117-2 and K071-1, respectively from Bioclin, Belo Horizonte, Brazil). Insulin and 17β-estradiol levels were measured by radioimmunoassay kits from MP Biomedicals (Santa Ana, USA). The quantitative insulin sensitivity check index (QUICKI) was calculated as previously described:

\[
\text{QUICKI} = \frac{1}{\text{LOG fasting glycemia (mg/dL)} + \text{LOG fasting insulin (µU/mL)}}
\]

(24).

**Hepatic cholesterol and triglyceride levels**

Protocol based on Bucolo & David 1973 and Allain *et al.* 1974 (25, 26) with minor modifications, as described by Nigro *et al.* 2014 (27). Briefly, thirty milligrams of liver was homogenized in 350 µL of PBS and 350 µL of 0.25% deoxycholic acid, and 10 µL of the homogenate was incubated for 5 min at 37°C. Two hundred microliters of cholesterol or triglyceride (TG) reagent from a Bioclin (Belo Horizonte, Brazil) kit was added and incubated for more than 10 min at 37°C. The absorbance was read at 500 nm according to the manufacturer’s instructions.
RNA isolation and quantification

Approximately 50 mg of liver or 100 mg of hypothalamus was homogenized in TRI Reagent (Sigma-Aldrich, St. Louis, USA) according to the manufacturer’s instructions. Samples were treated with 0.04 IU/μL of DNase (Thermo Fisher Scientific, Waltham, USA) for 20 min at room temperature. After TRI Reagent extraction, cDNA was synthesized with minor alterations using 1 μL of oligo dT primers and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA). For real-time PCR, the manufacturer’s protocol from a Power SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, USA) was used. The primers used are described in Table 1. Messenger RNA (mRNA) expression of the genes of interest was calculated using the relative standard curve method and normalized to the mRNA expression of the housekeeping gene peptidylprolyl isomerase A, Ppia.

Western blotting analysis

Approximately 50 mg of liver was homogenized in 500 μL of buffer containing 20 mM Tris (pH 8.0), 150 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 0.5 mM Na₃VO₄, 1 μM phenylmethylsulfonyl fluoride, 10 mM NaF and 1x protease and phosphatase inhibitor cocktails (Sigma-Aldrich P8340 and P0044, respectively). After 10 min of centrifugation at 1,500 g at 4°C, the supernatant was collected. The protein concentration was determined as described by Smith and collaborators (28) using a bicinchoninic acid assay kit (Thermo Fisher Scientific). Electrophoresis was performed on polyacrylamide SDS-PAGE gels as described by Laemmli (29) using a 7.5% (w/v) gel for ACC and a 10% (w/v) gel for AMPK. Wet transfer to a polyvinylidene difluoride membrane was performed for 120 min at 110 V in a buffer containing 192 mM glycine, 25 mM Tris and 20% (v/v) methanol. The
membranes were blocked for 1 hour in 3% (w/v) BSA in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBS-T). Membranes were incubated overnight with the appropriate primary antibodies (Table 2). Then, the membranes were washed and incubated with the appropriate secondary antibody for 1 h. After detection of the immunocomplex using a Luminata Forte Western HRP Substrate (Millipore), the membranes were incubated with 0.01% (w/v) sodium azide in 3% (v/v) BSA in TBS-T to inhibit the signal from the secondary antibody. Then, immunodetection was also performed for α-tubulin as a loading control. Images were quantified using ImageJ 1.52a software (National Institutes of Health, NIH, Bethesda, USA) (30). Western blotting for phosphorylated and total ACC, and phosphorylated and total AMPK proteins, were done using different polyacrylamide gels and thus values of the same animal were normalized.

Statistical analysis

Data are expressed as the mean ± S.E.M. and were analyzed using GraphPad Prism 6 Software. Daily body mass gain was analyzed by two-way ANOVA followed by Bonferroni’s post-test. All the other data were analyzed by one-way ANOVA with Tukey’s post-test to detect differences between all groups. Statistical significance was inferred when \( p < 0.05 \). Symbols were used to describe \( p \) values smaller than 0.05.
4. Results

Effects of estradiol and metformin on the body mass gain and food intake of ovariectomized rats

Based on the various well-described benefits of metformin, we decided to investigate the effect of this drug on the phenotype observed in ovariectomized rats. As documented previously (27), sham-operated (SHAM) rats showed a progressive elevation of their body masses from day 1 to the end of day 20 after surgery (data not shown).

In agreement with the literature (6, 9, 13), the ovariectomized animals gained significantly more body mass than the SHAM animals (Fig. 1, black and white squares, respectively). Estradiol replacement completely prevented body mass gain in the OVX animals (OVX + E2, light gray squares), and metformin administration (5 g/L in drinking water) was also able to attenuate body mass gain in the OVX group (OVX + M, dark gray squares); while the OVX rats presented a 16% body mass gain, the OVX + M group gained only 7% more body mass than SHAM group (Table 3). The prevention of body mass gain was statistically significant from the 13th day after surgery onward, although the trend was already observed in the first days of metformin administration.

To further understand the change in body mass, we weighed several tissues (Table 3). We did not observe any changes in the W. Gastrocnemius portion, soleus or BAT mass. Twenty-one days after OVX, an increased inguinal white adipose tissue content was observed in OVX rats compared to SHAM group ($p=0.09$). This effect was prevented by estradiol treatment, and metformin promotes a decrease ($p=0.11$) comparing to OVX. There was a trend to increase retroperitoneal adipose tissue mass in OVX rats ($p=0.12$) that was abolished in the metformin and estradiol treatment groups comparing to SHAM. We also observed an increase in the liver mass of OVX + E2 rats, although
their liver triglyceride content was reduced (Fig. 3). This increment might be an effect of estradiol increasing cell proliferation, as already documented (31).

Although OVX animals showed a significant increase in food intake (Table 4), it was not solely responsible for the observed increase in body mass since food efficiency (body mass gain/food intake) was also increased in OVX rats. Metformin treatment prevented the increase in food intake but did not completely normalize food efficiency, since it was smaller in the OVX + M than in the OVX group, but was higher in OVX+M than in the SHAM group. Estradiol treatment completely prevented the increase in both food intake and food efficiency.

To better understand the alterations in food intake observed in OVX rats, we analyzed the hypothalamic mRNA levels of orexigenic and anorexigenic markers (Fig. 2), but the levels were unaltered.

**Serum hormone levels**

Despite the slight increase in insulin levels in the OVX + E2 group comparing to SHAM group \( (p=0.06) \), and the significant increase comparing OVX and OVX + M groups, glycemia did not differ among the groups. However, metformin treatment slightly increased QUICKI index when comparing to OVX + E2 (Table 4). Although minor changes were detected in OVX + E2 group, we observed that short-term ovariectomy did not significantly alter these parameters.

Although serum 17β-estradiol levels were not significantly decreased in the OVX and OVX + M groups, these groups showed a significant decrease in uterus mass (Table 3 and 4), which indicates atrophy of this tissue. The OVX + E2 group showed increase in both 17β-estradiol serum levels and uterus mass.
Serum and hepatic lipid profiles

The OVX group showed a statistically significant increase in serum cholesterol levels, with no statistical changes in the OVX + E2 and OVX + M groups (Fig. 3A). Despite this observation, there was a decrease in the hepatic cholesterol levels in OVX + E2 in comparison to OVX and OVX + M groups (Fig. 2B).

Triglyceride levels were reduced in the serum of OVX rats but did not change in the liver. The OVX + E2 group had an opposite profile, with no changes in serum TG levels compared to SHAM and lower hepatic TG levels compared to SHAM and OVX. In contrast, metformin treatment reduced both the serum and hepatic triglyceride content comparing to SHAM and OVX group, respectively.

Hepatic AMPK and ACC levels

Since it is known that metformin is a potent activator of AMPK and to better understand the reduction in the serum and hepatic triglyceride levels observed in the OVX + M rats, we investigated the protein levels of AMPK and ACC in our model. AMPK plays a key role in metabolic homeostasis in the cell. Activated AMPK (pAMPK) phosphorylates ACC, leading to the inhibition of ACC and the blockade of lipid synthesis (32).

Hepatic pAMPK (Thr-172) levels were analyzed by Western blotting (Fig. 4A), and OVX diminished pAMPK levels, but this decrease was prevented by the estradiol and metformin treatments. We did not observe any changes in total AMPK (Figure 4B). Although there were no statistical changes in pAMPK/tAMPK ratio (Figure 4C), we observed the same expression profile as seen in pAMPK. No alterations were seen in AMPK mRNAs expression: *protein kinase AMP-activated catalytic subunit alpha 1* (*Prkaa1*) and *alpha 2* (*Prkaa2*) (Fig. 4D).
As expected given the reduced levels of pAMPK, pACC levels were also reduced in the OVX rats, but interestingly, metformin treatment did not normalize pACC levels (Fig. 5A). However, we noticed that the total ACC levels tended to decrease in the OVX + M group comparing to SHAM (p=0.06) (Fig. 5B). The data from Fig. 5A and 5B allowed us to estimate the amount of ACC in the inactive state by using the ratio of pACC/total ACC. As shown in Fig. 5C, we observed decreased levels of ACC inhibition only in the ovariectomized rats, thus metformin treatment increased the amount of ACC inhibition, which was not significantly different from neither SHAM nor OVX groups. Acetyl-CoA carboxylase alpha (Acaca) mRNA levels, which encodes ACC, were higher in the OVX rats treated with estradiol (Fig. 5D), corroborating the rise in the ACC protein levels. Since metformin treatment did not change the ACC mRNA levels, our results suggest that metformin acts mainly by post-transcriptional regulation of the AMPK/ACC pathway in this ovariectomized rat model.

5. Discussion

Here we show that, similar to estradiol administration, metformin treatment improves metabolism in ovariectomized rats, although the treatments achieved different degrees of impact. We focused in metformin prevention of ovariectomy’s metabolic effects. To minimized the effects of obesity that naturally develops after ovariectomy in rats (8), we have chosen a short-term treatment of only 21 days with a normal diet. Metformin is widely known to reduce body mass gain in human (33), rats (34) and mice (35), but in ovariectomized rodents, the effect of metformin on metabolism has been poorly explored. However, there are a few studies that investigated the treatment with
metformin for 1 (36) or 4 weeks (37, 38) that confirmed our results of reduced body mass gain (Fig. 1), but these studies did not focus on metabolic pathways.

Ovariectomy is known to augment adipose tissue depots, but this is usually shown with a longer post-OVX time course or the ingestion of a high fat diet (13, 39). We observed a tendency to increase inguinal and retroperitoneal adipose tissue depot in this short-term post-OVX that was prevented by metformin and estradiol treatment (Table 3). This observation alone does not explain the differences observed in body mass gain. Further studies should be performed to investigate the observed effects on body mass gain. In agreement with previous works (27, 40), here we found a rise in food efficiency induced by ovariectomy in the rats. For the first time of our knowledge, we identified that metformin treatment was able to decrease food efficiency (Table 4), showing that this drug can attenuate the development of early alterations observed in metabolic parameters detected in ovariectomized rats. In the present study, we show that metformin administration, as well as estradiol treatment, normalizes the food intake of OVX rats; however, previous studies in mice treated with a lower dose of metformin did not find similar results (37).

It has been shown that estradiol increases anorexigenic Pomc and decreases orexigenic Npy mRNA expression (41); and that metformin reduced the mRNA levels of Npy in a diabetic animal model and in cultured hypothalamic neurons (42, 43). Here, we did not find any differences in Npy, Pomc, Agrp and Pmch expression (Fig. 3), probably due to the 14 h of fasting used in this study to allow the analysis of serum lipids. Perhaps, this prolonged fasting time had hyper stimulated the expression of orexigenic genes, and inhibited the expression of anorexigenic gene.

Fasting glycemia did not change in this short time after ovariectomy, and insulin levels were increased only by estradiol treatment, which might be due to the positive effect of
estradiol on insulin secretion (44). This led to a minor increase of insulin sensitivity, detected by QUICKI calculation by metformin treatment when to OVX+E2 group (Table 4). Natural menopause in rodents occurs with the arrest of regular ovarian cycling, followed by irregular cycling, and then an arrest in cycling that maintains a constant cycle phase without a reduction in the estradiol levels (45). We did not observe any significant changes in serum 17β-estradiol levels in the OVX rats at 21 days, although some previous studies have reported decreased serum estradiol in models of ovariectomy (7, 46). Vaginal smears showed that the OVX and OVX + M rats were in a constant diestrous phase (data not shown), and uterus mass was hypotrophic in these two groups, demonstrating a low estradiol effect. The dose of 17β-estradiol administered was based on previous works and has been reported to be enough to abolish the body mass gain in ovariectomized rats (16, 27).

Pedram et al. 2013 (47) showed that estradiol activates AMPK, which diminishes the activation and mRNA levels of sterol regulatory element binding transcription factor 1 (Srebf1), which encodes sterol regulatory element-binding protein-1 (SREBP1), an important transcription factor of several lipogenic genes, and also decreases 3-hydroxy-3-methylglutaryl CoA reductase (Hmgcr), which encodes 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, a key enzyme of cholesterol synthesis. Although the role of estradiol in preventing the increase in serum cholesterol levels has already been described in a longer treatment protocol (48), we did not observe any significant changes in the serum cholesterol levels, and only a slight decrease in hepatic cholesterol in OVX+E2 comparing to OVX group (Fig. 2) or the Srebf1 mRNA levels (fold change relative to SHAM; SHAM: 1.00 ± 0.20; OVX: 0.76 ± 0.13; OVX+E2: 0.75 ± 0.16; OVX+M: 0.90 ± 0.17). We did not observe any significant effects of metformin on the
serum and hepatic cholesterol levels of OVX rats when comparing to SHAM or OVX groups, despite the findings of Sivasinprasad et al. 2016 (37), who observed a reduction in the serum cholesterol levels in 12 weeks post-OVX rats treated with metformin for more 4 weeks. Although the effects of metformin on cholesterol levels have already been demonstrated in other models (49, 50), the action of metformin in ovariectomized rats needs to be further studied.

Estradiol has been shown to play an important role in the expression of proteins involved in the hepatic production and formation of lipoproteins. This could explain the diminished levels of serum triglycerides observed in OVX rats, although we were not able to detect alterations in the hepatic TG content, as it has been seen in longer treatment models (9, 13, 51, 52). Geerling et al. 2014 (53) showed that metformin increases the mass and activity of BAT, draining serum triglycerides. We did not observe any alteration in BAT mass (Table 3) and in content of triglyceride per gram of tissue (data not shown). In fact, Nigro et al., 2014 (27) have shown, using the same experimental model, no alterations in BAT activity (oxygen consumption, ATP synthesis, and heat production) in ovariectomized rats. The authors have also found no differences in UCP1 protein levels and PGC1β mRNA expression in this tissue. So, BAT does not seem to be a metabolic target of changes in this short-term ovariectomy in rats, but further analysis is needed to exclude if metformin acts modulating BAT metabolism in this model. We saw a decrease in the levels of both serum and hepatic triglycerides, suggesting alterations in their production, utilization and/or absorption.

The precise mechanism by which metformin activates AMPK is still unknown, but it is widely accepted that this drug indirectly stimulates AMPK activation (10, 54, 55). We showed that the effect of metformin is preserved in the liver of OVX rats (Fig. 4). Our
study suggests that metformin is able to regulate the amount of total ACC, and increases the levels of the inactive state protein observed in this work (Fig. 5C).

AMPK is known as a metabolic sensor and has already been shown to be responsible for increased lipolysis, fatty acid β-oxidation and mitochondrial biogenesis and to induce decreases in fatty acid synthesis and gluconeogenesis, among other effects (32). All these functions are impaired after ovariectomy (13, 27, 56–59), providing evidence that AMPK may be a key protein involved in the metabolic dysfunction that occurs due to the estrogen deficiency induced by ovariectomy. ACC is a limiting enzyme for lipogenic synthesis. Fullerton et al. 2013 (60) showed in mice that a mutation in ACC that inhibits ACC phosphorylation by AMPK causes increased hepatic lipogenesis and decreased hepatic β-oxidation as well as insulin resistance and glucose intolerance. These features are also observed in ovariectomized models (13, 56, 58). Thus, AMPK and ACC may be implicated in the benefits promoted by metformin and estradiol in ovariectomized animals.

Here, we showed that metformin, by preventing increases in body mass and serum and hepatic triglyceride levels as well as avoiding the inactivation of hepatic AMPK/ACC, is a potential drug to prevent the early metabolic impairment observed in ovariectomized rats. Further studies should be performed to investigate whether metformin treatment would have the same effect in a long-term ovariectomy model.

6. Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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Figure Legends:

Figure 1 – Body mass gain in ovariectomized Wistar rats treated with estradiol or metformin. Body mass was measured during the 20 days postsurgery. The body mass percentage was calculated based on the body mass just after surgery and normalized to the SHAM mean of each experimental procedure for each day after surgery. The animal groups were SHAM-operated rats treated with vehicle (SHAM), ovariectomized rats treated with vehicle (OVX), ovariectomized rats treated with 7 µg/kg of body mass of estradiol benzoate (OVX + E2) and ovariectomized rats treated with vehicle and 5 g/L of metformin in the drinking water (OVX + M). Data are shown as the mean ± S.E.M. of 13-18 animals per experimental group. Two-way ANOVA was used to compare groups and followed by Bonferroni’s post-test to detect differences compared with the SHAM group. *p< 0.05.

Figure 2 – Hypothalamus mRNA expression. qRT-PCR for (A) Npy, neuropeptide Y, (B) Pomp, proopiomelanocortin, (C) Agrp, agouti-related neuropeptide, (D) Pmch, pro-melanin-concentrating hormone expression. The quantity of each target gene was normalized to that of Actb, actin, beta in the respective sample (n= 4-5). Data were normalized to the SHAM mean of each experimental procedure and are represented as the mean ± S.E.M. One-way ANOVA was used to compare groups and was followed by Tukey’s post-test to detect differences between groups. No statistically significant differences were detected.
Figure 3 – Lipid profile. (A) Serum lipid profile. Animals were fasted for 14 hours before being euthanized by decapitation, and their blood was collected and centrifuged as described in the Materials and Methods section. The cholesterol (white bars; SHAM mean 65.0 ± 2.7 mg/dL), triglyceride (black bars, SHAM mean 30.7 ± 2.6 mg/dL), and HDL (gray bars, SHAM mean 20.2 ± 1.0 mg/dL) levels were analyzed using commercial kits (n=7-14). (B) The hepatic cholesterol (white bars, SHAM mean 3.8 ± 0.3 mg/g) and triglyceride (black bars, SHAM mean 16.2 ± 0.6 mg/g) levels were analyzed using commercial kits (n=13-18). Data were normalized to the SHAM mean of each experimental procedure and are represented as the mean ± S.E.M. One-way ANOVA was used to compare groups and was followed by Tukey’s post-test to detect differences: *p < 0.05, **p < 0.01 vs. SHAM group; #p < 0.05, ##p < 0.01 vs. OVX group; &p < 0.05, &&p < 0.01 vs. OVX + E2 group.

Figure 4 – AMPK levels in the liver. (A) Western blotting for pAMPK (n=8-10) and (B) total AMPK (tAMPK) (n=5). Fifteen micrograms of total liver homogenate derived from the different experimental groups was loaded on the gel. The immunodetection was performed with an anti-pAMPK or anti-tAMPK antibody. After an overnight incubation in 0.01% sodium azide in 3% BSA, an anti-α-tubulin antibody was used as the loading control. Representative experiments for pAMPK, tAMPK and α-tubulin detection are shown. Each lane represents one animal. S-SHAM; O-OVX; E-OVX + E2; M-OVX + M. (C) The values of pAMPK/α-tubulin were normalized to those of tAMPK/α-tubulin in each sample (n=4-6). (D) qRT-PCR for Prkaa1 expression and Prkaa2 expression. The quantity of each target gene was normalized to that of Ppia in the respective sample (n= 8-10). Data were normalized to the SHAM mean of each
experimental procedure and are represented as the mean ± S.E.M. One-way ANOVA was used to compare groups and was followed by Tukey’s post-test to detect differences: *$p < 0.05$ vs. SHAM group.

**Figure 5 – Hepatic ACC levels.** (A) Western blotting for pACC (n=8-11) and (B) total ACC (tACC) (n=8-11). Fifteen micrograms of total liver homogenate derived from the different experimental groups was loaded on the gel. The immunodetection was performed with an anti-pACC or anti-tACC antibody. After an overnight incubation in 0.01% sodium azide in 3% BSA, an anti-α-tubulin antibody was used as the loading control. Representative experiments for pACC, tACC and α-tubulin detection, where each lane represents one animal, are shown. S-SHAM; O-OVX; E-OVX + E2; M-OVX + M. (C) The values of pACC/α-tubulin were normalized to those of tACC/α-tubulin in each sample (n=5-6). (D) qRT-PCR for Acaca expression. The quantity of the target gene was normalized to that of Ppia of in the sample (n= 9-10). Data were normalized to the SHAM mean of each experimental procedure and are represented as the mean ± SEM. One-way ANOVA was used to compare groups and was followed by Tukey’s post-test to detect differences: *$p < 0.05$, **$p < 0.01$ vs. SHAM group; ##$p < 0.01$ vs. OVX group; *$p < 0.05$, &$p < 0.01$ vs. OVX + E2 group.
| Gene   | Sequence F                  | NCBI reference | Fragment Size (bp) |
|--------|-----------------------------|----------------|-------------------|
| Prkaa1 | F: CAGTACACCGTCTGATATTTTCATG | NM_019142.2    | 142               |
|        | R: ACAATAGTCCACACCAGAAAGG    |                |                   |
| Prkaa2 | F: ACCGTCTATTGCCACTCTG       | NM_023991.1    | 126               |
|        | R: CTTCAGGAAGAGGTAACCTGGG    |                |                   |
| Acaca  | F: TCCGGCTTGCAACCTAGTAAA     | NM_022193.1    | 104               |
|        | R: CCCCCAAAACGAGTAACAA       |                |                   |
| Ppia   | F: GCTGTCTTGGGAACCTTGTCTG    | NM_017101.1    | 129               |
|        | R: CCGCTGTTCTCTTTTCGCC       |                |                   |
| Pome   | F: CCACCTGAGACATCTCTTCTCTC   | NM_139326.2    | 75                |
|        | R: GAATCTCGGCATCTTTCCAGG     |                |                   |
| Npy    | F: GACAGAGATATGGCAAGAGATCC   | NM_012614.2    | 148               |
|        | R: CTAGGAAAAAGTCAGGAGAAGCAAG |                |                   |
| Agrp   | F: CCATATAAGCTAGGGCACAAG     | NM_033650.1    | 93                |
|        | R: GACACAGCTCAGCAACATGG      |                |                   |
| Pmch   | F: ATTCTCCCCACATCTCTCTCG     | NM_012625.1    | 143               |
|        | R: CTACGTTCCTGATGGACCTTGG    |                |                   |
| Actb   | F: ACAACCTTCTTGAGCTCTCTC     | NM_031144.3    | 275               |
|        | R: GCCGTGTCAATGCGGTACT       |                |                   |

Prkaa1 and Prkaa2, protein kinase AMP-activated catalytic subunit alpha 1 or 2 respectively; Acaca, acetyl-CoA carboxylase alpha; Srebf1, sterol regulatory element binding transcription factor 1; Ppia, peptidylprolyl isomerase A; Npy, Neuropeptide Y; Pome, proopiomelanocortin; Agrp, Agouti-related neuropeptide; Pmch, pro-melanin-concentrating hormone; Actb, actin, beta.

Table 1 Primers sequence for amplification of genes in liver or hypothalamus of Wistar rats via qRT-PCR
| **Table 2 Antibodies** | **Reference** | **Dilution** |
|------------------------|---------------|-------------|
| Phospho-AMPKα (Thr172) | #2531 (Cell signaling) | 1:2500 |
| AMPKα                  | #2532 (Cell signaling) | 1:2000 |
| Phospho-Acetyl-CoA Carboxylase (Ser79) | #3661 (Cell signaling) | 1:2500 |
| Acetyl-CoA Carboxylase | #3662 (Cell signaling) | 1:2500 |
| Monoclonal Anti-α-Tubulin | T5168 (Sigma) | 1:25000 |
| Anti-Rabbit IgG        | A0545 (Sigma) | 1:2500 |
| Anti-Mouse IgG         | A3673 (Sigma) | 1:2500 |
Table 3 Body mass gain and tissue mass/tibia length at the end of the 21 days of treatment

|                         | SHAM      | OVX       | OVX + E2   | OVX + M    |
|-------------------------|-----------|-----------|------------|------------|
| **Body mass gain**      | 1.00 ± 0.01 | 1.16 ± 0.01** | 0.99 ± 0.01## | 1.07 ± 0.01##**&|& |
| **Uterus**              | 1.00 ± 0.08 | 0.24 ± 0.01** | 1.25 ± 0.09##* | 0.25 ± 0.02##**&|& |
| **Liver**               | 1.00 ± 0.02 | 1.05 ± 0.02 | 1.13 ± 0.03** | 1.02 ± 0.03& | & | |
| **BAT**                 | 1.00 ± 0.05 | 1.07 ± 0.07 | 1.12 ± 0.06 | 1.16 ± 0.05 | & | |
| **Retroperitoneal**     | 1.00 ± 0.06 | 1.35 ± 0.18 | 0.97 ± 0.04 | 1.13 ± 0.09 | & | |
| **Inguinal**            | 1.00 ± 0.04 | 1.17 ± 0.05 | 0.97 ± 0.05# | 1.01 ± 0.06 | & | |
| **Gonadal**             | 1.00 ± 0.06 | 1.02 ± 0.01 | 0.94 ± 0.07 | 0.88 ± 0.06 | & | |
| **W. Gastrocnemius**    | 1.00 ± 0.05 | 1.05 ± 0.04 | 0.99 ± 0.03 | 0.99 ± 0.05 | & | |
| **Solear**              | 1.00 ± 0.03 | 1.02 ± 0.02 | 1.00 ± 0.03 | 1.02 ± 0.07 | & | |

White adipose tissue depots (retroperitoneal, inguinal and gonadal); BAT, interscapular brown adipose tissue; W. gastrocnemius, gastrocnemius-white portion. Values of SHAM means: body mass weight at 20 days postsurgery: 211.9 ± 3.4 g; uterus: 16.5 ± 1.8 mg/mm; liver: 180.9 ± 3.9 mg/mm; BAT: 5.9 ± 0.5 mg/mm; retroperitoneal: 33.5 ± 4.4 mg/mm; inguinal: 68.8 ± 5.8 mg/mm; gonadal: 52.9 ± 6.0 mg/mm; W. Gastrocnemius: 14.5 ± 1.8 mg/mm; solear: 5.0 ± 0.3 mg/mm. Data was normalized by sham mean of each experimental procedure and represented as mean ± S.E.M, n=10-11. One-way ANOVA was used to compare groups, followed by Tukey’s post-test to detect differences: *p < 0.05, **p < 0.01 vs. SHAM group; #p < 0.05, ##p < 0.01 vs. OVX group; &p < 0.05, &&p < 0.01 vs. OVX + E2 group.
Table 4 Animal parameters

| Parameter    | SHAM      | OVX       | OVX + E2   | OVX + M    |
|--------------|-----------|-----------|------------|------------|
| Food intake  | 1.00 ± 0.02 | 1.16 ± 0.03** | 1.02 ± 0.28## | 0.98 ± 0.02## |
| Food efficiency | 1.00 ± 0.14 | 3.33 ± 0.31** | 0.59 ± 0.20## | 2.02 ± 0.19##& & |
| Glycemia     | 1.00 ± 0.02 | 1.02 ± 0.04 | 1.01 ± 0.03 | 0.94 ± 0.03 |
| Insulin      | 1.00 ± 0.04 | 0.97 ± 0.04 | 1.20 ± 0.07## | 0.96 ± 0.05& |
| QUICKI       | 1.00 ± 0.01 | 1.00 ± 0.01 | 0.97 ± 0.01 | 1.01 ± 0.01& |
| 17β-estradiol| 1.00 ± 0.10 | 0.71 ± 0.06 | 3.24 ± 0.22##** | 0.93 ± 0.11&& |

Values of SHAM means: food intake: 378 ± 15 g; food efficiency: 0.042 ± 0.005 g/g; glycemia: 94.4 ± 3.2 mg/dL; insulin: 14.4 ± 0.8 µIU/mL; QUICKI: 0.321 ± 0.004; 17β-estradiol: 117.0 ± 20.89 pg/mL. Data was normalized by SHAM mean of each experimental procedure and represented as mean ± S.E.M, n=9-18. One-way ANOVA was used to compare groups, followed by Tukey’s post-test to detect differences: **p < 0.01 vs. SHAM group; #p < 0.05, ##p < 0.01 vs. OVX group; &p < 0.05, &&p < 0.01 vs. OVX + E2 group.
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Figure 3 – Lipid profile. (A) Serum lipid profile. Animals were fasted for 14 hours before being euthanized by decapitation, and their blood was collected and centrifuged as described in the Materials and Methods section. The cholesterol (white bars; SHAM mean 65.0 ± 2.7 mg/dL), triglyceride (black bars, SHAM mean 30.7 ± 2.6 mg/dL), and HDL (gray bars, SHAM mean 20.2 ± 1.0 mg/dL) levels were analyzed using commercial kits (n=7-14). (B) The hepatic cholesterol (white bars, SHAM mean 3.8 ± 0.3 mg/g) and triglyceride (black bars, SHAM mean 16.2 ± 0.6 mg/g) levels were analyzed using commercial kits (n=13-18). Data were normalized to the SHAM mean of each experimental procedure and are represented as the mean ± S.E.M. One-way ANOVA was used to compare groups and was followed by Tukey’s post-test to detect differences: *p < 0.05, **p < 0.01 vs. SHAM group; #p < 0.05, ##p < 0.01 vs. OVX group; &p < 0.05, &&p < 0.01 vs. OVX + E2 group.
Figure 4 – AMPK levels in the liver. (A) Western blotting for pAMPK (n=8-10) and (B) total AMPK (tAMPK) (n=5). Fifteen micrograms of total liver homogenate derived from the different experimental groups was loaded on the gel. The immunodetection was performed with an anti-pAMPK or anti-tAMPK antibody. After an overnight incubation in 0.01% sodium azide in 3% BSA, an anti-α-tubulin antibody was used as the loading control. Representative experiments for pAMPK, tAMPK and α-tubulin detection are shown. Each lane represents one animal. S-SHAM; O-OVX; E-OVX + E2; M-OVX + M. (C) The values of pAMPK/α-tubulin were normalized to those of tAMPK/α-tubulin in each sample (n=4-6). (D) qRT-PCR for Prkaa1 expression and Prkaa2 expression. The quantity of each target gene was normalized to that of Ppia in the respective sample (n= 8-10). Data were normalized to the SHAM mean of each experimental procedure and are represented as the mean ± S.E.M. One-way ANOVA was used to compare groups and was followed by Tukey’s post-test to detect differences: *p < 0.05 vs. SHAM group.
Figure 5 – Hepatic ACC levels. (A) Western blotting for pACC (n=8-11) and (B) total ACC (tACC) (n=8-11). Fifteen micrograms of total liver homogenate derived from the different experimental groups was loaded on the gel. The immunodetection was performed with an anti-pACC or anti-tACC antibody. After an overnight incubation in 0.01% sodium azide in 3% BSA, an anti-α-tubulin antibody was used as the loading control. Representative experiments for pACC, tACC and α-tubulin detection, where each lane represents one animal, are shown. S-SHAM; O-OVX; E-OVX + E2; M-OVX + M. (C) The values of pACC/α-tubulin were normalized to those of tACC/α-tubulin in each sample (n=5-6). (D) qRT-PCR for Acaca expression. The quantity of the target gene was normalized to that of Ppia of in the sample (n= 9-10). Data were normalized to the SHAM mean of each experimental procedure and are represented as the mean ± SEM. One-way ANOVA was used to compare groups and was followed by Tukey’s post-test to detect differences: *p < 0.05, **p < 0.01 vs. SHAM group; ††p < 0.01 vs. OVX group; ††p < 0.05, ‡‡p < 0.01 vs. OVX + E2 group.