Ovariectomy is associated with metabolic impairments and enhanced mammary tumor growth in MKR mice

Sarit Ben-Shmuel1,*, Eyal J Scheinman1,*, Rola Rashed1, Zila Shen Orr1, Emily J Gallagher2, Derek LeRoith1,2 and Ran Rostoker1

1Clinical Research Institute at Rambam (CRIR), Diabetes and Metabolism Clinical Research Center of Excellence, Rambam Medical Center, PO Box 9602, Haifa 31096, Israel
2Division of Endocrinology, Diabetes and Bone Diseases, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, PO Box 1055, Atran 4-36, New York, New York 10029, USA
*(S Ben-Shmuel and E J Scheinman contributed equally to this work)

Correspondence should be addressed to D LeRoith
Email
d_leroith@rambam.health.gov.il

Abstract

Obesity and type 2 diabetes (T2D) are associated with an increased risk of breast cancer incidence and mortality. Common features of obesity and T2D are insulin resistance and hyperinsulinemia. A mammary tumor promoting effect of insulin resistance and hyperinsulinemia was demonstrated in the transgenic female MKR mouse model of pre-diabetes inoculated with mammary cancer cells. Interestingly, in MKR mice, as well as in other diabetic mouse models, males exhibit severe hyperglycemia, while females display insulin resistance and hyperinsulinemia with only a mild increase in blood glucose levels. This gender-specific protection from hyperglycemia may be attributed to estradiol, a key player in the regulation of the metabolic state, including obesity, glucose homeostasis, insulin resistance, and lipid profile. The aim of this study was to investigate the effects of ovariectomy (including the removal of endogenous estradiol) on the metabolic state of MKR female mice and subsequently on the growth of Mvt-1 mammary cancer cells, inoculated into the mammary fat pad of ovariectomized mice, compared with sham-operated mice. The results showed an increase in body weight, accompanied by increased fat mass, elevated blood glucose levels, and hypercholesterolemia, in ovariectomized MKR mice. In addition, mammary tumor growth was significantly higher in these mice. The results suggest that ovarian hormone deficiency may promote impaired metabolic homeostasis in the hyperinsulinemic MKR female mice, which in turn is associated with an increased growth of mammary tumors.

Key Words

- insulin resistance
- hyperinsulinemia
- ovariectomy
- obesity
- breast cancer

Introduction

Numerous studies and meta-analyses confirm the association between type 2 diabetes (T2D) mellitus and an increased risk of breast cancer incidence and mortality in postmenopausal women (Michels et al. 2003, Coughlin et al. 2004, Larsson et al. 2007). Hyperglycemia, insulin resistance, and subsequently hyperinsulinemia, key features of T2D, have been suggested as potential molecular mechanisms underlying this association (Novosyadlyy & LeRoith 2010, Xu et al. 2014). Novosyadlyy et al. (2010) demonstrated the mammary tumor promoting effect of

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hyperinsulinemia in the transgenic female MKR mouse model of T2D inoculated with mammary cancer cells. Interestingly, female MKR mice exhibit marked hyperinsulinemia and insulin resistance, but only a mild increase in blood glucose levels, compared with male MKR mice, which manifest severe diabetes (Fernandez et al. 2001). This gender-specific phenomenon was observed in Zucker diabetic fatty rats (Clark et al. 1983), as well as in obese Agouti yellow mice (Klebig et al. 1995). In streptozotocin (STZ)-induced diabetes, male mice were susceptible to STZ, while female mice were resistant, unless they were ovariectomized (Paik et al. 2001). This gender-specific phenomenon was associated with increased mammary tumor growth.

Material and methods

Animals

MKR mice were used in this study. The generation of MKR transgenic mice (on the FVB/N background), carrying a dominant-negative insulin-like growth factor 1 (IGF1) receptor (IGF1r) specifically targeted to the skeletal muscle, has been described previously (Fernandez et al. 2001). Mice were fed standard chow and water and allowed to feed ad libitum and were kept on a 12 h light:12 h darkness cycle. Mice studies were performed according to the protocol approved by the Technion Animal Inspection Committee. Technion holds a National Institutes of Health (NIH) animal approval (license number A5026-01). MKR mice were subjected to sham surgery (MKR-Sham) or ovariectomy (MKR-OVX) at 6 weeks of age. Briefly, mice were anesthetized with isoflurane, and buprenorphine (50 µl of 0.03 mg/ml solution) was administered s.c. as an analgesic. A short dorsal midline incision was made, and ovaries and oviducts were exteriorized through muscle wall incisions bilateral to the spine. The oviduct was ligated and the ovary was removed by a single cut. Remaining tissue was replaced into the peritoneal cavity and skin was sutured.

Food was weighed every 3–4 days. Food intake is presented as daily intake (g) per 100 g mice.

Cell culture

Mvt-1 mouse mammary cancer cells were derived from MMTV-c-Myc/Vegf transgenic mice tumor explants, as previously described (Pei et al. 2004). Cells were cultured in DMEM (Biological Industries, Beit Haemek, Israel) supplemented with 10% v/v fetal bovine serum (Biological Industries) and 1% v/v penicillin:streptomycin (Biological Industries). Cells were maintained at 37 °C in a humidified atmosphere consisting of 5% CO2 and 95% air.

Syngeneic orthotopic tumor models

Mvt-1 cells were detached with trypsin solution (Biological Industries) into single cells and suspended in PBS at a

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concentration of $0.5 \times 10^6$ cells/ml; 100 $\mu$l (50 000 cells) were then injected into the left inguinal mammary fat pads (no. 4) of MKR-Sham or MKR-OVX mice, 35 days after ovariectomy (11 weeks old).

Tumor volume was monitored once a week using calipers and calculated in $\text{mm}^3$ by the formula: $\text{width}^2 \times \text{length} \times 0.5$. At sacrifice, tumors were removed and weighed.

**Blood glucose measurement**

Blood glucose levels were measured weekly in a non-fasting state. Blood samples were taken from the tail vein and glucose levels were determined using an automated glucometer (Accu-Check Preforma System, Roche Diagnostics, Mannheim, Germany).

**Body composition**

Body composition was measured every 2 weeks and was determined in non-anesthetized mice using the NMR analyzer (Minispec LF50/mq7.5 Analyzer, Bruker Optic GmbH, Germany). Results are presented as the ratio between fat mass and lean mass.

At sacrifice, inguinal fat was collected and weighed. Results are presented as inguinal fat mass percent of total body weight.

**Glucose-tolerance test and insulin-tolerance test**

A glucose-tolerance test (GTT) and insulin-tolerance test (ITT) were performed during daylight time, after 4 h fasting. Glucose (2 g/kg body weight) or insulin (12 IU/kg, NovoRapid Vial, Novo Nordisk, Bagsvaerd, Denmark) were administered i.p. Blood glucose levels were measured at the indicated time points.

**Serum analysis**

At sacrifice, blood samples were taken from the heart of anesthetized mice. Blood was allowed to clot and was then centrifuged for 10 min at 6000 $\times g$ at 4 $^\circ C$. Supernatant (serum) was collected into a fresh tube and maintained in $-80 ^\circ C$ for analysis. Serum insulin, leptin, and adiponectin levels were measured with Rat/Mouse Insulin 96-Well Plate Assay Kit, Mouse Leptin 96-Well Plate Assay Kit, and Mouse Adiponectin 96-Well Plate Assay Kit, respectively (Millipore, Billerica, MA, USA) according to manufacturer’s instructions. Data acquisition was performed using the PowerWave XS2 (BioTek, Winooski, VT, USA) and Gen5 Software. Cholesterol and triglycerides were measured by Siemens Dimension analyzer (Siemens Healthcare, Erlangen, Germany).

**Protein extraction and western blot analysis**

Tumors were homogenized in lysis buffer (10 mmol/l Tris-HCl pH 7.5, 150 mmol/l sodium chloride, 1 mmol/l sodium pyrophosphate, 1 mmol/l $\beta$-glycerolphosphate, 1 mmol/l sodium orthovanadate, 50 mmol/l sodium fluoride, 1.25% v/v CHAPS (3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate), and Complete Protease Inhibitor Cocktail (Roche Diagnostics). Lysates were rotated at 4 $^\circ C$ for 1 h and centrifuged at 16000 $g$ for 10 min. Supernatants were collected and protein concentrations were determined with Protein Assay Kit (Bio-Rad). Protein (12 $\mu$g) was electrophoresed through a 10% polyacrilamide gel and transferred to a nitrocellulose membrane. The membranes were immunoblotted with the desired antibody, followed by an appropriate secondary antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Immunoreactivity was detected by ECL (WesternBright Quantum Western blotting detection kit, Advansta, Melano Park, CA, USA), using luminescent image analyzer LAS-4000 (Fujifilm, Tokyo, Japan). Densitometry analysis was performed using ImageQuant Software (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA).

The following antibodies were used: Phospho-Akt (Thr308), total-Akt, and $\beta$-actin (Cell Signaling Technology, Danvers, MA, USA).

**Statistical analysis**

Results are presented as mean $\pm$ S.E.M. Statistical analyses were performed using GraphPad Prism (Version 5; GraphPad Software, Inc., La Jolla, CA, USA). The Student’s $t$-test and two-way ANOVA, followed by Bonferroni multiple comparison post-test, were used to determine the statistical significance of differences between groups. $P<0.05$ was considered statistically significant.

**Results**

Sham surgery and ovariectomy of MKR mice (MKR-Sham and MKR-OVX respectively) were performed at 6 weeks of age. At the beginning of the experiment, body weights of all mice were similar (Fig. 1A). However, 2 weeks after surgery an increased weight gain was observed in MKR-OVX group compared with MKR-Sham mice. Maximum
Figure 1
Ovariectomy leads to increased body weight and fat mass in MKR mice. (A) Body weight was followed on a weekly basis from before surgery (day 0) until sacrifice. Results are representative of three independent experiments and presented as mean ± S.E.M., n = 10. (B) Body composition was analyzed every 2 weeks. Results are representative of the three last analyses before sacrifice and presented as the ratio between fat mass and lean mass for each mouse + mean, n = 6. Panel: body composition results before surgery.

A difference in body weight of 3 g was reached 3 weeks after surgery and remained constant until the end of the experiment. Body composition results, presented as the ratio between fat mass and lean mass, were also similar between the two groups at the beginning of the experiment (Fig. 1B panel). However, at the end of the experiment, the fat-to-lean ratio of ovariectomized mice was significantly higher by 16% than the ratio calculated for sham-operated mice (Fig. 1B). To support this result, inguinal fat was weighed at sacrifice and presented as percent of total body weight (Fig. 1C). Indeed, the percentage of inguinal fat mass in MKR-OVX mice was higher by 1.5-fold than in MKR-Sham mice. Daily food intake was similar for both groups (Fig. 1D).

Blood glucose levels on the day of the surgery and 2 weeks after surgery were similar for both MKR groups (Fig. 2A). In parallel to the increase in body weight, blood glucose levels 3 weeks after surgery were significantly higher for MKR-OVX mice compared with MKR-Sham mice. Overall, glucose levels remained elevated by about 20% in the MKR-OVX group until the end of the experiment. Glucose tolerance and insulin sensitivity were also studied in MKR-OVX and MKR-Sham mice. Fasting glucose (blood glucose at time 0, before glucose load) was significantly higher in MKR-OVX mice compared with MKR-Sham groups (Fig. 2B). Blood glucose levels remained elevated for the MKR-OVX group throughout the assay. However, calculation of the area under the curve from the baseline of each group resulted in similar values (Fig. 2C). These data suggest that the elevated glucose levels observed after glucose load emanate from higher baseline glucose levels and that glucose tolerance in ovariectomized MKR mice is similar to sham-operated mice. Insulin resistance (Fig. 2D), as well

Figure 2
Ovariectomy leads to increased blood glucose levels under fasting and non-fasting conditions. (A) Blood glucose levels under non-fasting conditions were followed on a weekly basis from before surgery (day 0) until sacrifice. (B) GTT: glucose (2 g/kg) was administered i.p. to fasting mice, and blood glucose was measured at the indicated times. (C) Area under the curve for each group was calculated above the respective baseline using GraphPad Prism Software. (D) ITT: insulin (12 IU/kg) was administered i.p. to fasting mice, and blood glucose was measured at the indicated times. Results are representatives of three independent experiments and presented as mean ± S.E.M., n = 10. Two-way ANOVA followed by Bonferroni multiple comparison post-test (A, B and D) or Student’s t-test (C) were used to determine the statistical significance; *P < 0.05, **P < 0.01, ***P < 0.001 vs MKR-Sham.
as serum levels of insulin, triglycerides, and adiponectin (Table 1), was also similar between the two MKR groups. Serum cholesterol levels were significantly higher by 30% in MKR-OVX mice compared with the MKR-Sham group (Fig. 3A). Interestingly, MKR-OVX mice showed a twofold increase in serum leptin levels compared to MKR-Sham mice (Fig. 3B).

Mvt-1 mammary tumor cells were inoculated into the inguinal mammary fat pad of the MKR-OVX and MKR-Sham groups (50,000 cells per mouse). At the time of inoculation, the differences in body weight and glucose homeostasis between MKR-OVX and MKR-Sham mice had stabilized, indicating an existing effect of ovariectomy on the metabolic state of MKR mice. Tumor volume assessments commenced as tumors were detected and continued on a weekly basis until sacrifice. A gradual increase in the rate of tumor growth was observed in ovariectomized MKR-OVX mice compared with MKR-Sham mice (Fig. 4A). At the time of sacrifice (38 days after inoculation), the tumors of MKR-OVX mice were significantly larger (by approximately 40%) than MKR-Sham tumors. Also, tumor weights were higher in MKR-OVX mice compared with MKR-Sham mice (Fig. 4C and D).

Western blot analysis of protein extracted from tumors at the end of the experiment showed an increase in phospho-Akt (Thr308) levels by threefold in MKR-OVX mice compared with MKR-Sham mice (Fig. 4C and D), indicating that the tumorigenic outcome of ovarian hormone deficiency in MKR mice is mediated through activation of the PI3K/Akt pathway signaling pathway.

Table 1 Serum analysis of metabolic parameters. Data represent the mean ± s.e.m., n ≥ 13. Student’s t-test was used to determine the statistical significance.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MKR-Sham</th>
<th>MKR-OVX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (ng/ml)</td>
<td>5.35 ± 0.68</td>
<td>6.40 ± 0.57</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>314 ± 50</td>
<td>313 ± 55</td>
</tr>
<tr>
<td>Adiponectin (µg/ml)</td>
<td>3.74 ± 0.27</td>
<td>4.10 ± 0.26</td>
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Discussion

The results from this study suggest that ovarian hormones may have a protective effect on the metabolic state and consequently on mammary tumor development in MKR female mice. The effects of ovariectomy in these transgenic mice were evident by the increased body weight and fat mass, elevated blood glucose levels (in both fasting and non-fasting conditions), and increased mammary tumor growth in ovariectomized mice compared with sham-operated mice.

It is known that estradiol plays a role in the regulation of obesity and fat accumulation in both human and animal models. The prevalence of obesity markedly increases with the decline in estradiol levels following menopause (Carr 2003). Ovariectomized mice fed a high fat diet showed increased body weight and fat mass compared with sham-operated mice (Camporez et al. 2013). In the present study, we show increased body weight, which was accompanied by increased body fat mass, in ovariectomized hyperinsulinemic MKR female mice (fed regular chow) compared with sham-operated mice. The effects of estradiol on body weight are suggested to be mediated by ERα-expressing cells in the hypothalamus, which are involved in the regulation of energy intake and expenditure (Xu et al. 2011). In the present study, we hypothesize that the differences in body weight and fat mass between MKR-OVX and MKR-Sham mice were due to decreased energy expenditure of ovariectomized mice, because daily food intake was similar for both groups. Our hypothesis is supported by previous reports showing similar effects (Rogers et al. 2009, Camporez et al. 2013).

The MKR transgenic mouse model has been previously shown to have insulin resistance and elevated insulin levels in serum compared with WT mice (Fernandez et al. 2001). We verified these characteristics in both MKR-OVX and MKR-Sham mice compared with age-matched WT mice (data not shown). It has been shown that estradiol deficiency leads to hyperglycemia and insulin resistance in postmenopausal women and mouse models (Heine et al. 2000, Carr 2003, Yakar et al. 2006). In the present study, fasting and non-fasting blood glucose levels were elevated in ovariectomized MKR mice, suggesting that estradiol may be involved in glucose homeostasis in
Ovariectomy leads to increased mammary tumor growth in MKR mice and activated Akt signaling in inoculated tumor cells. Mvt-1 mammary tumor cells (50,000 cells) were inoculated into the mammary fat pad of ovariectomized and sham-operated MKR mice. (A) After detection of tumors, tumor volume was assessed at the indicated times. (B) Tumor weight was measured at sacrifice. (C) Protein was extracted from tumors and western blot analysis was performed using antibodies directed against phosphorylated Akt (Thr308, P-Akt), total Akt (T-Akt), and β-actin. (D) Densitometry analysis was performed using ImageQuant Software. Results are representative of two independent experiments and presented as mean ± s.e.m., n = 6. Two-way ANOVA followed by Bonferroni multiple comparison post-test (A) or Student's t-test (B and D) were used to determine the statistical significance; *P < 0.05, **P < 0.001 vs MKR-Sham.
Impairments induced by ovarian hormone deficiency - i.e., increased body weight, accumulation of fat mass, hyperglycemia, and hypercholesterolemia - are good candidates for mediating the tumorigenic outcome of ovariectomy, as they all favor tumor growth (as discussed below). Specifically, Mvt-1 cells do not express ER (data not shown), indicating that mammary tumor growth in our model is independent on the presence of estradiol, further supporting our suggestion.

Studies claim that obesity is a risk factor for breast cancer (Petrelli et al. 2002, Pierobon & Frankenfeld 2013, Robinson et al. 2014). Obesity may contribute to mammary tumor development through several mechanisms. Systemically, obesity is associated with insulin resistance and hyperinsulinemia and an increased bioavailability of several growth factors and adipokines, such as leptin, which is known to activate the PI3K/Akt signaling pathway and promote tumor growth, as reviewed by Hursting et al. (2012). In addition, obesity leads to subclinical inflammation in adipose tissue, which is characterized by macrophages surrounding necrotic adipocytes and forming crown-like structures (CLS) (Cinti et al. 2005). The local production of pro-inflammatory mediators in breast tissue, as evident by the increased numbers of CLS in mammary glands of obese mice and women (Morris et al. 2011, Subbaramaiah et al. 2011), is known to promote angiogenesis and to increase cancer progression (Sundaram et al. 2013). Hypercholesterolemia is also associated with the risk of breast cancer (Kitahara et al. 2011). ApoE knockout mice, which demonstrate high cholesterol and triglycerides levels in serum, were shown to have enhanced mammary tumor growth. Cholesterol-induced activation of the PI3K/Akt signaling pathway in tumor cells was suggested as the molecular mechanism linking hypercholesterolemia and tumor growth (Alikhani et al. 2013). Hyperglycemia can promote cancer growth by simply providing the energy necessary for cell proliferation and neoangiogenesis (Sciacca et al. 2013).

Although ovariectomy led to impairments in metabolic homeostasis in MKR female mice, the changes in metabolic parameters were moderate and did not reach the drastic levels of hyperglycemia and glucose intolerance reported previously for male MKR mice (Fernandez et al. 2001), suggesting that other factors, aside from estradiol, may play a role in metabolic control in the female MKR model. An important candidate may be progesterone, which has also been implicated in the regulation of metabolic homeostasis. However, while estradiol plays a protective role in metabolic control, progesterone promotes metabolic impairments, such as insulin resistance and hyperglycemia (Gonzalez et al. 2000; Picard et al. 2002). Thus, it is possible that the moderate metabolic impairments observed in MKR-OVX mice are the result of the interplay between estradiol and progesterone withdrawal caused by the removal of ovaries.

Insulin resistance and, subsequently, increased levels of insulin, with its cell proliferation effect, often stand at the basis of the association between T2D and breast cancer progression (Novosyadlyy et al. 2010). Interestingly, both ovariectomized and sham-operated mice in this study are insulin resistant and hyperinsulinemic. Nonetheless, the changes in metabolic parameters observed in ovariectomized MKR mice were able to drive mammary tumor growth at a higher rate than in sham-operated MKR mice. These results suggest that in the face of previously existing insulin resistance and hyperinsulinemia, other metabolic impairments (i.e., obesity, hyperglycemia and hypercholesterolemia) may promote the growth of mammary tumors.

Taken together, we suggest a protective role of ovarian hormones on the metabolic state of the female MKR transgenic mouse model for T2D. Ovariectomy leads to impaired metabolic homeostasis in MKR mice, manifested by increased body weight and fat mass, as well as hypercholesterolemia and hyperglycemia, which in turn is associated with an increased growth of mammary tumors in the face of insulin resistance and hyperinsulinemia in MKR mice. These results highlight the importance of closely monitoring and maintaining balanced body weight, as well as glucose and cholesterol levels, specifically in diabetic postmenopausal women, as impairments in these parameters may favor breast cancer outcome and/or worsening of breast cancer prognosis.

**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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