A nude mouse model of obesity to study the mechanisms of resistance to aromatase inhibitors

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Abstract

Obesity is a risk factor for breast cancer progression. Breast cancer patients who are overweight or obese or have excess abdominal fat have an increased risk of local or distant recurrence and cancer-related death. Hormone depletion therapies can also cause weight gain, exacerbating the risk for these patients. To understand the effect of obesity on hormone-dependent human breast cancer tumors, we fed ovariectomized athymic nude mice a diet containing 45% kcal fat and 17% kcal sucrose (high fat sucrose diet (HFSD)), 10% kcal fat (low fat diet (LFD)), or a standard chow diet (chow). The mice fed the HFSD developed metabolic abnormalities consistent with the development of obesity such as weight gain, high fasting blood glucose, and impaired glucose tolerance. These mice also developed hyperinsulinemia and insulin resistance. The obese mice also had a higher tumor growth rate compared to the lean mice. Furthermore, the obese mice showed a significantly reduced responsiveness to letrozole. To understand the role of obesity in this reduced responsiveness, we examined the effect of insulin on the growth of MCF-7Ca cells in response to estrogen or letrozole. The presence of insulin rendered MCF-7Ca cells less responsive to estrogen or letrozole. The presence of insulin rendered MCF-7Ca cells less responsive to estrogen and letrozole. Exogenous insulin treatment of MCF-7Ca cells also resulted in increased p-Akt as well as ligand-independent phosphorylation of ERα. These findings suggest that diet-induced obesity may result in reduced responsiveness of tumors to letrozole due to the development of hyperinsulinemia. We conclude that obesity influences the response and resistance of breast cancer tumors to aromatase inhibitor treatment.

Key Words
- aromatase inhibitors
- diet-induced-obesity
- MCF-7Ca xenografts
- breast cancer

Introduction

Obesity (~30% Americans) and breast cancer (one in eight lifetime risk) together affect a significant proportion of the female population. Therefore, the role of obesity in breast cancer is an important question. Obesity is characterized by not only increased adipose burden but also changes in metabolism and adipose biology. Elevated serum insulin levels (hyperinsulinemia), insulin resistance, and the interaction with cancer-associated adipocytes can provide cancer cells with a survival benefit not observed in a leaner body. Obesity leads to altered expression of hormones, growth factors, and inflammatory markers, such as cytokines and adipokines. These together promote cancer cell survival, angiogenesis, and metastasis and reduce apoptosis. Breast adipose tissue of obese breast cancer
patients shows distinct crown-like structures (CLS), which are macrophage infiltrates (Morris et al. 2011). These macrophages produce a variety of pro-inflammatory factors that can promote tumor growth and invasion. Studies have suggested that obesity and the resulting chronic inflammatory state increase aromatase expression and estrogen synthesis, which can result in tumor growth (Morris et al. 2011, Subbaramaiah et al. 2011). These findings may also explain why the aromatase inhibitor (AI) anastrozole was less effective in obese women as compared to women of normal weight (Sestak et al. 2010). Moreover, breast cancer patients treated with hormone depletion therapy such as AIs gain weight during treatment (Redig & Munshi 2010, Champ et al. 2012). In a mouse model, high doses of letrozole have been shown to induce the enlargement of adipocytes and insulin insensitivity (Maliqueo et al. 2013). Supporting these findings, studies in the aromatase knockout (ArKO) mouse show that by 1 year of age mice have significantly higher levels of insulin as well as leptin, along with two to four times more visceral adiposity; the increase in adiposity was not accompanied by weight gain, but rather by loss of lean body mass, i.e., skeletal muscle mass (Jones et al. 2000, Simpson et al. 2005). Furthermore, humans with aromatase deficiency also have marked increases in glucose and insulin levels (Maffeï et al. 2004, Simpson et al. 2005).

Despite all of these known phenotypic differences in obese patients, cancer treatment regimens do not take into account the modulatory effects of obesity on tumor growth and drug responsiveness. We postulate that breast cancers in obese patients are phenotypically different from those of leaner patients and, as such, employ different mechanisms of resistance to treatment, resulting in relapse and progression. An understanding of the differences in the adaptive mechanisms that cancer cells utilize under the influence of obesity is an important step in better tailoring secondary therapies to treat recurrent breast cancers.

About 75% of the patients diagnosed with breast cancer are hormone sensitive and responsive to tamoxifen and AIs. Although AIs have significantly improved the outcome of hormone-sensitive postmenopausal breast cancer, not all patients respond to AI despite the presence of estrogen receptors in their tumors, and those that do respond eventually become resistant. Therefore, understanding the mechanisms of resistance to AIs is crucial for advances in treatment. Several mechanisms and pathways have been implicated in the development of resistance to AIs, and newer pathway inhibitors are being developed to overcome resistance (Johnston 2005, 2006). However, it is unknown which pathway is activated in a particular subset of patients, and clinical trials have also emphasized the importance of patient selection (Johnston et al. 2008). Heterogeneity in response and resistance to AIs is not completely understood and we hypothesize that the presence of obesity (and its underlying molecular phenotype) is one of the responsible factors. Because breast cancer is exposed to a dramatically different hormonal milieu on the development of obesity, understanding the influence of obesity on the resistance to AIs and response to secondary therapies is important. In the current study, we have developed and characterized a mouse model of obesity to study the influence of obesity on the growth of human tumors and their response to treatment with the AI letrozole.

**Materials and methods**

**Materials**

DMEM, penicillin/streptomycin solution (10 000 IU each), 0.25% trypsin–1 mM EDTA solution, Dulbecco's PBS (DPBS), and G418 were obtained from Life Technologies. Human insulin and Matrigel were obtained from Sigma Chemical Company. Antibodies against phospho-ERα (S118 and S167) were purchased from Millipore (Billerica, MA, USA). Antibodies against p-Akt and Akt were purchased from Cell Signaling Technology (Beverly, MA, USA). The antibody against ERα was purchased from Santa Cruz Biotechnology. Androstenedione ($\Delta^4$A) was purchased from Steraloids (Newport, RI, USA).

MCF-7 human breast cancer cells that are stably transfected with the human placental aromatase gene (MCF-7Ca) were kindly provided by Dr S Chen (City of Hope, Duarte, CA, USA) (Zhou et al. 1990). Letrozole was kindly provided by Novartis Pharma.

**Cell culture**

MCF-7Ca cells were routinely cultured in DMEM supplemented with 5% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 700 μg/ml G418 (Zhou et al. 1990, Sabnis et al. 2005). Cell proliferation assays were performed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay as described earlier (Sabnis et al. 2005). The results were expressed as a percentage of the vehicle-treated control.

**Cell line authentication**

The cell line used in this study was authenticated using CellCheck service (microsatellite marker short tandem
repeat (STR analysis) provided by Idexx Radil (Columbia, MO, USA). The cell lines were found to be identical to the genetic profile reported for the MCF-7 cell line (ATCC# HTB-22). The authenticated cells were inoculated in mice at passage 10 and used for in vitro studies at passage number <20.

Nude mouse model of diet-induced obesity

All animal studies were performed according to the guidelines and approval of the Animal Care and Use Committee of the University of Maryland, Baltimore. Female ovariectomized (OVX) athymic nude mice 4–6 weeks of age were obtained from the National Cancer Institute – Frederick Cancer Research and Development Center (Frederick, MD, USA). The mice were housed in a pathogen-free environment under controlled conditions of light and humidity in a temperature-controlled (22 °C) facility with a strict 12 h light:12 h darkness cycle and received food and water ad libitum. These mice were fed a 45% kcal fat (Research Diets, New Brunswick, NJ, USA) diet containing primarily lard with 17% sucrose to produce diet-induced obesity (DIO) (high fat sucrose diet (HFSD)) or a 10% low fat control with 0% sucrose (low fat diet (LFD)) diet for an indicated time. A standard chow-based diet was used as a comparison to previous results (n = 10/group).

Fasting glucose and insulin levels

On week 6 after initiating diets, fasting glucose and i.p. glucose tolerance test (IP-GTT) measurements were performed. To measure fasting plasma glucose levels, mice were fasted for 6 h during the day (Andrikopoulos et al. 2008). Food was removed from the cages but mice were allowed free access to water. Mice were weighed and baseline blood glucose levels were determined using a glucometer (TRUEResult Blood Glucose Kit, Nipro Diagnostics, Fort Lauderdale, FL, USA) and glucose test strips according to the manufacturer’s instructions. Blood was acquired by a tail-vein prick using a lancet. For insulin measurements, blood was collected using retro-orbital eye bleeding and then quantified with serum C-peptide ELISA. Mice were given fluid replacement at the end of the blood collection period. For the i.p. insulin tolerance test (IP-ITT), regular recombinant human insulin (Sigma) was injected at 0.5 IU/kg and blood glucose was measured using the glucometer before injection (time 0) and at 5, 10, 15, 30, 60, 90, and 120 min after injection. For insulin measurement, blood was collected (at 0, 15, 30, and 90 min) using retro-orbital eye bleeding and then quantified with serum C-peptide ELISA. Mice were given fluid replacement at the end of the blood collection period. For the i.p. insulin tolerance test (IP-ITT), regular recombinant human insulin (Sigma) was injected at 0.5 IU/kg and blood glucose was measured using the glucometer before injection (time 0) and at 5, 10, 15, 30, 60, 90, and 120 min after the injection.

Tumor growth in OVX female athymic nude mice

The mice were fed the diets described above for 6 weeks and the body weight of each mouse was measured every week. On week 6, the mice were inoculated with MCF-7Ca cells. The tumor xenographs of MCF-7Ca cells were grown in the mice as previously described (Yue et al. 1994, Sabnis et al. 2008, 2009, 2010, 2013a,b). Briefly, each mouse received s.c. inoculations in one site per flank with 100 μl of cell suspension containing ~2.5 × 10⁶ cells in Matrigel. The mice were injected daily with supplemental Δ⁴A (100 μg/day). Weekly tumor measurements began 3 weeks after cell inoculations and treatments began when the tumors reached ~250 mm³. Tumors were measured with calipers and volumes were calculated using the formula 4/3πr²r (r₁ < r₂). Mice were assigned to groups for treatment so that there was no statistically significant difference in tumor volume among the groups at the beginning of treatment (n = 5). Letrozole and Δ⁴A for injection were prepared using 0.3% weight per volume (w/v) hydroxypropyl cellulose (HPC) in 0.9% w/v NaCl solution and injected s.c. five times weekly. The doses of letrozole (10 μg/day) and Δ⁴A (100 μg/day) used are as previously determined and reported (Sabnis et al. 2008, 2010).

Tissue collection

At necropsy, mice were fasted for 6 h and blood was collected by cardiac puncture. Tumors and adipose tissue were collected, flash frozen, and stored at −80 °C. Adipose tissue was collected from ovarian, perirenal, mesenteric, subcutaneous/mammary, and brown fat pads. Uterine wet weight was also measured.

Radiometric ³H₂O release assay for aromatase activity measurement

For measuring aromatase activity, the tissues were homogenized in ice-cold DPBS containing protease and
phosphatase inhibitors. The resulting homogenate was used for the aromatase activity assay. The radiometric \(^3\)H\(_2\)O release assay was performed as described previously (Long et al. 1998, Sabnis et al. 2008, 2009). The tissue homogenate was incubated for 30 min with 1 \(\mu\)Ci/ml of [\(^{1}\)H] \(\Delta^4\)A as substrate in the presence of molecular oxygen and an NADPH generating system. The activity of the enzyme was calculated from the \(\Delta^3\)H\(_2\)O released during the incubation and corrected for protein concentration in the tissue homogenates.

**RNA extraction and RT and PCR**

RNA was extracted and purified using the RNeasy Lipid Tissue Mini Kit (Qiagen) as per manufacturer’s protocol. Total RNA concentration and purity were determined using Qubit 2.0 Fluorometer (Life Technologies) using the manufacturer’s protocol. RNA was diluted with DNase/RNase free water to 0.08 \(\mu\)g/\(\mu\)l and 0.64 \(\mu\)g of RNA was reverse transcribed with iScript cDNA Synthesis Kit (Bio-Rad) per the manufacturer’s protocol. Analysis of mRNA expression was carried out by real-time qRT-PCR as described earlier with GAPDH serving as housekeeping expression. The reverse transcribed material was used for RT-PCR using murine primers were used for RT-PCR: Aromatase Forward: 5’-AAG CTC TGA CGG GCC CTG GT-3’, Reverse: 5’-ACG TAG CCC GAG GTG TCG GT-3’; Cox-2 Forward: 5’-ATT CTT TGC CCA GCA CTT CA-3’, Reverse: 5’-GGG ATA CAC CTC TCC ACC AA-3’ (Subbaramaiah et al. 2012).

**Western blotting**

Cellular protein extracts were prepared as described earlier (Sabnis et al. 2009). A total of 25 \(\mu\)g protein from each sample was analyzed by western blotting using SDS–PAGE as described previously (Sabnis et al. 2009). Bands were quantitated by densitometry using ImageJ. The densitometric values are corrected for \(\beta\)-actin loading control.

**Statistical analysis**

One-Way ANOVA with the Tukey Kramer post-test or Student’s t-test was used for in vitro studies. For in vivo studies, mixed-effects models were used. The tumor volumes were analyzed with S-PLUS (7.0, Insightful Corp., Tibco, Palo Alto, CA, USA) to estimate and compare an exponential parameter \((\beta)\) controlling the growth rate for each treatment group. The original values for tumor volumes were log transformed. The graphs are represented as mean \(\pm\) S.E.M. All \(P\) values <0.05 were considered statistically significant.

**Results**

**Obesogenic diet induces obesity and glucose tolerance in athymic nude mice**

To study the effect of obesity on tumor growth and treatment resistance, we developed and characterized a mouse model of DIO. Other studies have used high fat diets to induce obesity in C57B/6j mice (Bell et al. 2008, Subbaramaiah et al. 2011). In our study, we used immune suppressed athymic nude mice to study obesity and its effects on human mammary tumors. Mice were fed an obesogenic HFSD, with 45% of the calories from saturated fat (lard) and 17% of the calories from sucrose. As a control diet, mice were fed a LFD that had 10% kcal fat. We also included a group of mice fed a chow diet to compare the results with our previously published work. As shown in Fig. 1A, mice fed an HFSD gained a significant amount of weight compared to mice fed the LFD \((P=0.0036)\) or chow diet \((P=0.0003)\) by week 12 (Supplementary Figure 1, see section on supplementary data given at the end of this article). There was no statistically significant difference in weight between the mice fed the low fat and chow diets \((P=0.907)\). The rate of weight gain was also significantly higher in mice fed the HFSD compared to mice fed low fat \((P=0.0055)\) or chow \((P=0.0026)\) diets. The rate of weight gain was calculated from a linear regression line. Food intake of mice on both LFD and HFSD was not significantly \((P=0.78)\) different (data not shown). However, obesity is not merely weight gain. The underlying metabolic phenotype includes changes in levels of several hormones, growth factors, and pro-inflammatory cytokines as well as changes in the fat distribution. To study the underlying phenotype of DIO, we first examined fasting blood glucose levels and glucose tolerance with IP-GTT. The mice were fasted for 6 h and then blood glucose was measured at time 0 and 5, 10, 15, 30, 60, 90, and 120 min after injecting 2 g/kg of D-glucose i.p. The fasting blood glucose level of mice fed the obesogenic diet was significantly higher than the mice fed the lean \((P=0.0003)\) or chow diet \((P<0.0001)\) (Fig. 1B). Furthermore, the IP-GTT showed that mice fed the HFSD had significantly \((P<0.0001)\) impaired glucose tolerance compared to the mice on LF or chow diets (Fig. 1C and D). The blood glucose levels of mice fed the obesogenic diet rose to a higher level in response to i.p. D-glucose and
Obesogenic diet induces hyperinsulinemia and insulin resistance

As the mice exhibited impaired glucose tolerance, we measured the levels of insulin C-peptide and remained higher throughout the course of the experiment compared to the lean groups. As such, the area under the curve $\text{AUC}_{\text{glucose}}$ for obese mice was significantly higher than that of the lean mice. We next evaluated the effect of OVX on glucose tolerance. Mice fed the HFS diet (Fig. 2A and B) or LFD (Fig. 2C and D) had worse glucose tolerance if they were OVX, suggesting that OVX may exacerbate the effect of DIO on glucose tolerance. This effect was more pronounced in mice fed the obesogenic diet, suggesting obesity and OVX may act synergistically in a positive feedback manner to reduce glucose tolerance.

Effect of ovariectomy on glucose tolerance in mice fed the (A and B) and C). The HFS diet obese mice showed higher $\text{AUC}_{\text{C-peptide}}$ compared to the mice on low fat ($P=0.0002$) or chow diet ($P=0.0001$) diets. However, the rate of increase in the insulin levels was less than in obese mice (~100% increase in lean or chow fed mice vs 12.5% increase in obese mice). This suggests reduced insulin secretion on glucose stimulus in obese mice. Next, we examined the effect of insulin on the tissue responsiveness, using IP-ITT (Fig. 3D). Recombinant human insulin was given i.p. (0.5 U/kg) and for 6 h during the day and fasting blood glucose was measured using a glucometer. The fasting blood glucose level of the mice fed the HFS diet was significantly higher than the mice fed the LF diet ($P=0.0036$) or chow diet ($P=0.0003$) by week 12. (B) Mice were fasted via retro-orbital bleeding. Serum insulin levels were measured using rat/mouse C-peptide ELISA. Mice fed the obesogenic diet had significantly higher fasting insulin levels compared to mice fed the low fat ($P=0.0006$) or chow ($P<0.0001$) diets (Fig. 3A). The mice were also subjected to IP-GTT, and collected serum was subjected to C-peptide ELISA (Fig. 3B and C). The HFS diet mice fed the HFS diet have a significantly ($P<0.0001$) higher $\text{AUC}_{\text{glucose}}$ than the mice fed the LF or chow diet.

Figure 1
Effect of obesogenic diet on (A) weight gain (B) fasting blood glucose, and (C) glucose tolerance. Ovariectomized athymic nude mice were fed a diet containing 45% fat (primarily lard) and 17% sucrose (HFS) to induce obesity. A diet with 10% fat was used as a lean control diet (LF) and chow diet was used as a comparison. (A) Mice were weighed weekly and the percent change in weight was recorded. The mice fed the HFS diet gained a significant amount of weight compared to the mice fed the LF diet ($P=0.0006$) or chow diet ($P=0.0003$) (Fig. 3A). The mice were also subjected to IP-ITT (Fig. 3D). The HFSD obese mice showed higher AUCC-peptide compared to the mice on low fat ($P=0.0002$) or chow diet ($P=0.0001$) diets. However, the rate of increase in the insulin levels was less than in obese mice (~100% increase in lean or chow fed mice vs 12.5% increase in obese mice). This suggests reduced insulin secretion on glucose stimulus in obese mice. Next, we examined the effect of insulin on the tissue responsiveness, using IP-ITT (Fig. 3D). Recombinant human insulin was given i.p. (0.5 U/kg) and for 6 h during the day and fasting blood glucose was measured using a glucometer. The fasting blood glucose level of the mice fed the HFS diet was significantly higher than the mice fed the LF diet ($P=0.0003$) or chow diet ($P<0.0001$). (C) For the glucose tolerance test, blood glucose was measured at time 0 and 5, 10, 15, 30, 60, 90, and 120 min after injecting 2 g/kg of $\alpha$-glucose intraperitoneally in fasting mice. (D) The area under the curve (AUC) was calculated and plotted. Mice fed the HFS diet have a significantly ($P<0.0001$) higher $\text{AUC}_{\text{glucose}}$ than the mice fed the LF or chow diet.

Figure 2
Effect of ovariectomy on glucose tolerance in mice fed the (A and B) obesogenic or (C and D) lean diet. Athymic nude mice with or without ovaries (OVX or intact ovaries) were fed HFS or LF diet for 6 weeks. Mice were fasted for 6 h during the day and then blood glucose was measured at time 0 and 5, 10, 15, 30, 60, 90, and 120 min after injecting 2 g/kg of $\alpha$-glucose intraperitoneally. (B and D) The area under the curve (AUC) was calculated and plotted. The OVX mice fed the HFS or LF diets have significantly ($^*P<0.0001$) higher $\text{AUC}_{\text{glucose}}$ than the mice with intact ovaries fed the obesogenic or lean diets.
The mice fed the obesogenic diet had a more pronounced response to exogenous insulin than the mice fed the obesogenic diet, suggesting that the tissues of obese mice are less responsive to insulin.

**Insulin mediates letrozole resistance in vitro**

Obese mice exhibit significant hyperinsulinemia. Insulin can activate growth factor receptor pathways, which have been shown to be responsible for the acquisition of resistance to AIs. Hence, we evaluated the effect of insulin on the growth of MCF-7Ca cells in vitro. MCF-7Ca cells were plated in 24-well plates and serum starved for 40 h (0.1% charcoal treated serum). The cells were then treated with estradiol (E2) or letrozole (10⁻¹² M to 10⁻⁶ M) for 6 days in the presence or absence of insulin (2 μM) in otherwise serum-starved conditions. Cells were supplemented with Δ⁴A (25 nM) when being treated with letrozole. Cell viability was measured on day 7 using the MTT assay. In the absence of insulin, E2 produced a classical biphasic dose response curve with maximum stimulation observed at 1 nM. However, in the presence of 2 μM insulin, this stimulation was abrogated. However, a lower concentration of E2 (1 pM) showed the stimulation of cell growth when treated concomitantly with insulin (Fig. 4A). This suggests that insulin can induce hypersensitivity to E2. Furthermore, MCF-7Ca cells showed a significantly reduced antiproliferative response to letrozole in the presence of chronic insulin (Fig. 4B). This was more pronounced at lower doses of letrozole. Taken together, these results suggest that the continuous presence of high levels of insulin affects the response of breast cancer cells to estrogen and estrogen withdrawal. We previously reported activation of growth factor receptor pathways on the acquisition of letrozole resistance. Therefore, we next examined the effect of insulin on the activation of growth factor receptor pathways, which may mediate resistance to E2 and letrozole. Treatment with insulin (2 μM) for 3 h resulted in the activation of the insulin receptor (IR) as well as the IGF1 receptor (data not shown) and p-Akt in MCF-7Ca cells (Fig. 4C). Furthermore, ERα was also phosphorylated following insulin treatment on Serine 167, which is known to be activated by Akt. Serine 118 (MAPK target) was not phosphorylated following treatment with insulin. This suggests that insulin can activate ERα in a ligand independent manner through Akt and may render MCF-7Ca cells resistant to estrogen withdrawal that ensues on letrozole treatment.

**Obesogenic HFSD induces changes in adipose tissue mass**

Mice were euthanized after 12 weeks on the diets and adipose tissue depots were isolated, weighed, and stored at −80 °C for further analysis. As shown in Fig. 5A, mice fed the obesogenic diet had a significantly higher fat mass in all five sites (ovarian, mesenteric, perirenal, subcutaneous/mammary, and brown fat depots) than mice on lean or chow diets. The adipose tissue weights are normalized to total body weight. There was no significant difference in

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**Figure 3**

Effect of obesogenic diet on (A) serum insulin level and (B and C) insulin resistance to glucose in OVX athymic nude mice. Fasting insulin measurements were performed after 8 weeks on the diets. Mice were fasted for 6 h during daytime and blood was collected by retro-orbital collection. Serum C-peptide levels were measured by ELISA. (A) Mice fed the HFSD had significantly higher fasting serum C-peptide levels than mice fed the LF (P = 0.0006) or the chow (P < 0.0001) diets. The serum C-peptide was not significantly different between the mice fed the lean or chow diet (P = 0.4211). (B) Blood was collected in fasting mice at time 0 and 15, 30, and 90 min after injecting 2 g/kg of D-glucose intraperitoneally. Serum C-peptide levels were measured by ELISA. (C) The area under the curve (AUC) was calculated and plotted. Mice fed the HFSD had significantly higher AUC_C-peptide levels than mice fed the LF (P = 0.0002) or the chow (P = 0.0001) diets. AUC_C-peptide was not significantly different between the mice fed the LF or chow diet (P = 0.87). (D) Effect of obesogenic diet on glucose response to insulin in OVX athymic nude mice. OVX athymic nude mice with or without ovaries (OVX or intact ovaries) were fed an obesogenic HFSD for 8 weeks. Mice were fasted for 6 h during the day and then blood glucose was measured at time 0 and 5, 10, 15, 30, 60, 90, and 120 min after injecting 0.5 IU/kg of human insulin intraperitoneally.
the fat mass between mice fed the low fat vs chow diets. This suggests that weight gain induced by the obesogenic diet was due to increased adiposity. As such, this diet produced most of the comorbidities of obesity in nude mice as observed in the C57B/6 mice including weight gain and increased adiposity as well as impaired glucose tolerance, hyperinsulinemia, and insulin resistance.

**Obesogenic HFSD induces increased estrogen synthesis**

As the mouse uterus is extremely sensitive to estrogen stimulation, changes in uterine weight are useful in monitoring estrogenic effects in vivo (Langston & Robinson 1935, Owens & Ashby 2002). OVX mice not supplemented with estrogen or an estrogenic source but fed the obesogenic diet had a significantly higher uterine weight ($P = 0.036$) than mice on the LFD. This suggests that the obesogenic diet can lead to increased estrogen levels in this mouse model (Fig. 5B). Furthermore, mice that were supplemented with $D_4A$ and fed the HFSD also had an additional increase in uterine weight, which was significantly higher than in mice receiving LF ($P = 0.0013$) or chow ($P = 0.0026$) diets. These results suggest that increased adiposity in the obese mice serves as an additional source of aromatase that can convert the administered $D_4A$ to estrogen, which in turn can stimulate uterine growth. To corroborate our hypothesis, we examined aromatase activity (Fig. 6A) and aromatase mRNA (Fig. 6B) levels in the adipose tissue of obese and lean mice. The adipose tissue of the obese mice had significantly ($P < 0.0001$) higher levels of aromatase activity and aromatase mRNA expression than the adipose tissue of the lean mice in all adipose depots. Furthermore, visceral adipose depots such as mesenteric and perirenal had much higher levels of aromatase (mRNA and protein activity) than gonadal and subcutaneous fat depots. This suggests that visceral fat may have a greater impact than subcutaneous or gonadal fat on the production of estrogen through elevated aromatase expression. Increased production of PGE$_2$ is observed during inflammation through increased expression of COX-2. Prostaglandin PGE$_2$ is a known activator of the aromatase promoter (Zhao et al. 1996, Brodie et al. 2001, Karuppu et al. 2002). Therefore, we next examined the expression of COX-2 mRNA (Fig. 6C) in adipose tissues. We observed that the adipose tissue of obese mice had significantly ($P < 0.0001$) higher levels of COX-2 mRNA expression than the adipose tissue of lean mice in all adipose depots. Furthermore, visceral adipose depots such as perirenal had much higher levels of COX-2 mRNA than gonadal and subcutaneous fat depots.

**Figure 4**

Effect of insulin on the growth properties of MCF-7Ca cells. Hormone-dependent MCF-7Ca cells were serum starved for 40 h and then treated with (A) estradiol ($E_2$) or (B) letrozole at doses $10^{-12}$ to $10^{-6}$ M for 6 days in an otherwise serum-starved condition, and cell viability was measured by MTT assay. (C) Effect of insulin and $E_2$ on activation of growth factor pathways. MCF-7Ca cells were serum starved for 40 h and then treated with insulin (2 μM), $E_2$ (1 nM), the combination of $E_2$ and insulin, or a serum-containing medium for 3 h, and the cell lysates were analyzed by western blotting. Densitometric values (corrected for $\beta$-actin loading control) show fold change compared with the control (maintained in serum-containing medium).
Taken together, these results suggest that visceral adiposity may have a greater impact on breast cancer tumor growth through increased production of estrogen.

Obese mice are less responsive to letrozole than lean mice

Next, we evaluated the effect of obesity on the response of human hormone sensitive MCF-7Ca tumors to the AI letrozole. Mice were fed the obesogenic or lean diets for 6 weeks. Mice were inoculated with MCF-7Ca cells and tumors were allowed to form in the presence of the Δ4A supplement. Tumor measurements started 3 weeks after inoculation of the cells and treatment with letrozole started after the tumors reached ~250 mm³ (Fig. 7A). In light of the more rapid growth of the tumors in the mice fed the obesogenic diet, treatment with letrozole was started 4 weeks earlier than in the mice on either the low fat or chow diets. Mice received letrozole treatment until the tumors acquired resistance (double the initial volume). Tumor growth rates were calculated for the control group 3 weeks after inoculations and from the time treatment was started for mice in the letrozole group. As expected, the mice fed the obesogenic HFSD had significantly higher tumor growth rates (P=0.02) than the lean mice (Fig. 7B).

Letrozole treatment of both the obese and lean mice resulted in significant (P=0.0436 and 0.025 respectively) reduction in the tumor growth rate. However, lean mice receiving letrozole treatment had a significantly lower tumor growth rate than the obese mice on the letrozole treatment (P=0.0263). The tumors grown in the obese mice did not regress to the same levels as tumors of the lean mice and exhibited a decreased lag period before resumption of growth. Thus, tumors growing in an obese environment exhibited diminished response to letrozole and acquired resistance (doubled in tumor volume as compared to starting volume) quicker than the tumors in the lean mice. This suggests that obesity influences the response and resistance of tumors to AIs such as letrozole.

Discussion

To study the effect of obesity on tumor response to AI treatment and resistance, we used a tumor xenograft model of DIO that mimics most aspects of human disease. Studies have shown that rodents fed a high sucrose, high fat diet have increased body weight (without any increase in food intake), abdominal fat deposition, hyperinsulinemia, and hyperglycemia and showed signs
of hepatic steatosis (Anstee & Goldin 2006), which we also observed in our model (Supplementary Figure 2, see section on supplementary data given at the end of this article). A diet high in saturated fats and carbohydrates mimics the human diet closely and is considered the best diet for studying DIO (Panchal & Brown 2011, Panchal et al. 2011). Obesity models that have been used previously in published obesity studies are genetically modified (such as ob/ob or db/db) models or DIO in C57B/6 mice. However, these models do not support the growth of human tumors, and thus, the interaction of the obesity phenotype with human tumors has not been completely understood. A few studies have used athymic nude mice for obesity studies and found that obesity induced with high caloric intake can increase the growth of MCF-7 tumors (Lamas et al. 2015) and T47D tumors (Nkhata et al. 2009). However, more studies to understand the effect of obesity on tumor response to treatment regimen are critical. In our model, lean/obese mice were inoculated with human breast cancer MCF-7Ca cells and treated with letrozole until the tumors became resistant. This new mouse model is based on our intra-tumoral aromatase xenograft model (Yue & Brodie 1993, Yue et al. 1994, Sabnis et al. 2008, 2009). This model mimics the postmenopausal breast cancer patient, as the source of estrogen is from non-ovarian tissue and is not under gonadotropin regulation. However, as the mouse has no significant production of peripherally formed estrogen, the MCF-7 human breast cancer cells stably transfected with the human aromatase gene (MCF-7Ca) (Zhou et al. 1990) serve as an autocrine source of estrogen to stimulate tumor growth in OVX, immune-suppressed mice. The resulting tumors synthesize estrogen from supplemented androstenedione (Δ^4A) and proliferate in response to estrogen acting via ERa. These tumors are therefore useful for studying and comparing the effects of both antiestrogens (AEs) and AIs in the same model system (Yue et al. 1994). Clinical trials (BIG 1-98, ATAC, MA-17) have now confirmed the results of our xenograft model (Baum et al. 2002, Goss et al. 2003, 2005, Thurlimann et al. 2005, Goss 2006). As found in

![Figure 6](http://erc.endocrinology-journals.org)

Changes in aromatase activity in mice fed the HFS, LF, or chow diet. OVX athymic nude mice were fed HFS, LF, or chow diets for 12 weeks. Mice were fasted for 6 h during the day and then euthanized. Adipose depots were collected, weighed, and stored at −80 °C for additional analysis. Indicated adipose tissue were homogenized and subjected to aromatase activity assay as described in Materials and Methods. Each adipose depot of the mice fed the HFSD had significantly higher aromatase activity than that of the mice fed the LF or chow diets. Furthermore, visceral adipose tissues such as mesenteric and perirenal had a significantly higher activity than the peripheral depots such as ovarian or subcutaneous in the obese mice. Changes in (B) aromatase and (C) Cox-2 mRNA in mice fed the HFS, LF, or chow diet. The indicated adipose tissue of mice fed the HFS, LF, or chow diets were homogenized and subjected to qRT-PCR as described in Materials and methods. Each adipose depot of the mice fed the HFSD had significantly higher aromatase mRNA than that of the mice fed the LF or chow diets. Furthermore, visceral adipose tissue of the mesenteric and perirenal depot had a significantly higher expression of aromatase and Cox-2 respectively than the peripheral depots such as ovarian or subcutaneous in the obese mice.
were inoculated. The mice were then supplemented with androstenedione, which was started 3 weeks after tumor cell inoculation. When the tumors reached 250 mm³, the mice were grouped to receive letrozole (10 mg/day) or control. (A) Tumors were measured weekly and volumes were calculated. (B) Growth rates of tumors were calculated using a linear regression model of the log transformed tumor volumes. Tumors of the obese mice have a significantly higher growth rate than the tumors of the lean mice (P=0.02). Although letrozole treatment significantly reduced the growth rate of tumors in both obese (P=0.0436) and lean (P=0.025) mice, letrozole-treated tumors of obese mice have a significantly higher growth rate than the letrozole-treated tumors of the lean mice (P=0.0263).

Figure 7
Effect of HFSD on the growth of MCF-7Ca xenografts. Mice were fed HFS or LF diets for 6 weeks and then MCF-7Ca cells (2.5 x 10⁶ cells in Matrigel/site) were inoculated. The mice were then supplemented with androstenedione (100 μg/day) to support tumor growth. The measurement of the tumors was started 3 weeks after tumor cell inoculation. When the tumors reached ~250 mm³, the mice were grouped to receive letrozole (10 μg/day). (A) Tumors were measured weekly and volumes were calculated. (B) Growth rates of tumors were calculated using a linear regression model of the log transformed tumor volumes. Tumors of the obese mice have a significantly higher growth rate than the tumors of the lean mice (P=0.02). Although letrozole treatment significantly reduced the growth rate of tumors in both obese (P=0.0436) and lean (P=0.025) mice, letrozole-treated tumors of obese mice have a significantly higher growth rate than the letrozole-treated tumors of the lean mice (P=0.0263).

In the current study, we evaluated the effect of the obesogenic diet on the development of metabolic symptoms of obesity in athymic nude mice. We observed that athymic nude mice gain significant weight on a high fat and sugar diet. Higher fasting glucose and insulin levels, impaired glucose tolerance, and increased adiposity accompanied the weight gain. Next, we examined the effect of obesity on the growth of human breast cancer tumors and their response to AI letrozole. We observed that obese mice exhibited greater tumor growth rates and diminished response to letrozole compared to lean mice. This reduced responsiveness could be attributed to higher aromatase expression in the adipose tissue and an abundance of adipose tissue resulting in inadequate inhibition at the given dose of letrozole. Heterogeneity in response and resistance to AIs is not completely understood and we hypothesize that the presence of obesity (and its underlying molecular phenotype) is one of the responsible factors. The tumors may also develop letrozole resistance faster due to higher serum levels of insulin, which stimulates tumor growth. The results of this study suggest that the breast cancer tumors growing in an

these studies, AIs were significantly better than tamoxifen in controlling tumor growth in our xenograft model. However, anastrozole was found to be less effective than tamoxifen in overweight and obese patients (Sestak et al. 2010). On the other hand, letrozole was found to be as equally effective as tamoxifen in the BIG 1-98 trial (Ewertz et al. 2012). However, both studies confirmed that obesity increased hazard ratios for disease-free survival and overall survival. The discrepancy in these studies could be due to inadequate inhibition of aromatase by anastrozole compared to letrozole, which further emphasizes that one fixed dose of an AI may not be sufficient in treating patients with varying BMIs.

Previously published studies to investigate obesity have shown that high fat diets together with OVX can lead to an increase in pro-inflammatory cytokines including prostaglandin PGE₂, which can increase the expression of aromatase (Subbaramaiah et al. 2011). In this study, we used immune compromised athymic nude mice and observed increases in both COX-2 (an enzyme involved in PGE₂ production) and aromatase. As these mice do not have intact immune systems, all of the markers of inflammation may not be expressed. However, athymic nude mice are not completely devoid of an immune system (Shouval et al. 1983, Zhang et al. 2002), and many of the cytokines that are affected by obesity are expressed in nude mice. Our preliminary data showed that several cytokines and adipokines were expressed at comparable levels in nude mice and nonimmune suppressed C57B/6 mice (data not shown). In addition, DIO has been shown to affect immune modulators in athymic nude mice (Lamas et al. 2015). Based on these findings, we propose that the nude mouse model can be useful for studying the effect of DIO on the growth of human tumors and their response to therapy. Furthermore, the interaction of obese cancer-associated adipocytes with human tumors can be studied using this model.

In the current study, we evaluated the effect of the obesogenic diet on the development of metabolic symptoms of obesity in athymic nude mice. We observed that athymic nude mice gain significant weight on a high fat and sugar diet. Higher fasting glucose and insulin levels, impaired glucose tolerance, and increased adiposity accompanied the weight gain. Next, we examined the effect of obesity on the growth of human breast cancer tumors and their response to AI letrozole. We observed that obese mice exhibited greater tumor growth rates and diminished response to letrozole compared to lean mice. This reduced responsiveness could be attributed to higher aromatase expression in the adipose tissue and an abundance of adipose tissue resulting in inadequate inhibition at the given dose of letrozole. Heterogeneity in response and resistance to AIs is not completely understood and we hypothesize that the presence of obesity (and its underlying molecular phenotype) is one of the responsible factors. The tumors may also develop letrozole resistance faster due to higher serum levels of insulin, which stimulates tumor growth. The results of this study suggest that the breast cancer tumors growing in an
Obese microenvironment respond to treatments differently than the tumors growing in a lean microenvironment and therefore should be treated differently. However, currently presence of obesity is not taken into account when selecting a treatment regimen.

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-15-0168.

**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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