Impact of obesity on ovotoxicity induced by 7,12-dimethylbenz[a]anthracene in mice

Jackson Nteeba  
_Iowa State University_

Shanthi Ganesan  
_Iowa State University_, shanthig@iastate.edu

Aileen F. Keating  
_Iowa State University_, akeating@iastate.edu

Follow this and additional works at: https://lib.dr.iastate.edu/ans_pubs

Part of the Animal Experimentation and Research Commons, Animal Sciences Commons, Cellular and Molecular Physiology Commons, Genetics Commons, and the Molecular Biology Commons

The complete bibliographic information for this item can be found at https://lib.dr.iastate.edu/ans_pubs/486. For information on how to cite this item, please visit http://lib.dr.iastate.edu/howtocite.html.
Impact of obesity on ovotoxicity induced by 7,12-dimethylbenz[a]anthracene in mice

Abstract
Insulin, elevated during obesity, regulates xenobiotic biotransformation enzymes, potentially through phosphatidylinositol 3-kinase (PI3K) signaling, in extraovarian tissues. PI3K regulates oocyte viability, follicular activation, and ovarian chemical biotransformation. 7,12-Dimethylbenz[a]anthracene (DMBA), a carcinogen and ovotoxicant, destroys all stages of follicles, leading to premature ovarian failure. Obesity has been reported to promote DMBA-induced tumors, but it remains unknown whether obesity affects ovarian xenobiotic metabolism. Therefore, we investigated ovarian expression of xenobiotic metabolism genes—microsomal epoxide hydrolase (Ephx1), glutathione S-transferase (GST) class Pi (Gstp1) and class mu 1 (Gstm1), and PI3K-signaling members (protein kinase B [AKT] alpha [Akt1], beta [Akt2], and the forkhead transcription factor subfamily 3 [Foxo3])—in lean and obese female mice after DMBA exposure (1 mg/kg; intraperitoneal injection for 14 days). Relative to lean, obese mice had decreased (P < 0.05) healthy primordial and primary follicle numbers but increased (P < 0.05) secondary and preovulatory follicle numbers. Obesity increased (P < 0.05) Akt1, Akt2, Gstm1, and Ephx1 mRNA and pAKTSer473/Thr308, GSTM1, GSTP1, and EPHX1 protein levels. DMBA decreased (P < 0.05) ovarian weight in lean and obese mice, however, obese DMBA-treated females had a greater reduction (P < 0.05) in ovarian weight. In both lean and obese mice, DMBA decreased (P < 0.05) all stages of healthy follicle numbers, increased Gstp1 and Ephx1 mRNA as well as GSTM1, GSTP1, and EPHX1 protein levels, and decreased Akt1 and Akt2 mRNA as well as pAKTSer473 or pAKTThr308, FOXO3, and pFOXO3Ser253 protein expression. There was an additive effect between obesity and DMBA exposure for increased Gstm1 and Ephx1 mRNA as well as GSTM1 and EPHX1 protein expression.

Keywords
7, 12-dimethylbenz[a]anthracene, environmental contaminants and toxicants, obesity, ovary, ovotoxicity

Disciplines
Animal Experimentation and Research | Animal Sciences | Cellular and Molecular Physiology | Genetics | Molecular Biology

Comments
This is a manuscript of an article published as Nteeba, Jackson, Shanthi Ganesan, and Aileen F. Keating. "Impact of obesity on ovotoxicity induced by 7, 12-dimethylbenz [a] anthracene in mice." Biology of reproduction 90, no. 3 (2014): 68. doi: 10.1095/biolreprod.113.114215. Posted with permission.

This article is available at Iowa State University Digital Repository: https://lib.dr.iastate.edu/ans_pubs/486
Impact of Obesity on Ovotoxicity Induced by 7,12-dimethylbenz[a]anthracene in Mice

Running Title: OBESITY ACCELERATES DMBA-INDUCED OVOTOXICITY

Jackson Nteeba, Shanthi Ganesan and Aileen F. Keating
Department of Animal Science, Iowa State University, Ames, Iowa

1Supported by award number R00ES016818 to AFK. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Environmental Health Sciences or the National Institutes of Health. Presented in part at the 46th Annual Meeting of the Society for the Study of Reproduction, 22-26 July, Montreal, Canada.

2Correspondence: Aileen F. Keating, 2356J Kildee Hall, Department of Animal Science, Iowa State University, Ames, IA 50011. E-mail: akeating@iastate.edu; telephone 515-294-3849, fax 515-294-4471.

ABSTRACT
Insulin, elevated during obesity, regulates xenobiotic biotransformation enzymes, potentially through phosphatidylinositol 3-kinase (PI3K) signaling, in extra-ovarian tissues. PI3K regulates oocyte viability, follicular activation and ovarian chemical biotransformation. 7,12-dimethylbenz[a]anthracene (DMBA), a carcinogen and ovotoxicant, destroys all stage follicles, leading to premature ovarian failure. Obesity has been reported to promote DMBA-induced tumors, but it remains unknown whether obesity impacts ovarian xenobiotic metabolism. Therefore, we investigated ovarian expression of xenobiotic metabolism genes (microsomal epoxide hydrolase (Ephx1), Glutathione S-transferase (GST) class Pi (Gstpi) and class mu 1 (Gstm1)) and PI3K signaling members (protein kinase B (AKT) alpha (Akt1), beta (Akt2), and the forkhead transcription factor subfamily 3 (Foxo3)) in lean and obese female mice after DMBA exposure (1 mg/kg; intraperitoneal injection for 14 days). Relative to lean, obese mice had decreased ($P < 0.05$) healthy primordial and primary follicle numbers but increased ($P < 0.05$) secondary and pre-ovulatory follicles numbers. Obesity increased ($P < 0.05$) Akt1, Akt2, Ephx1 mRNA and pAKT$^{Ser473/Thr308}$, GSTM1, GSTP1 and EPHX1 protein levels. DMBA decreased ($P < 0.05$) ovarian weight in lean and obese mice, however, obese DMBA-treated females had a greater reduction ($P < 0.05$) in ovarian weight. In both lean and obese mice, DMBA decreased ($P < 0.05$) all stage healthy follicle numbers, increased Gstpi, Ephx1 mRNA and GSTM1, GSTP1 and EPHX1 protein levels and decreased Akt1, Akt2 mRNA, pAKT$^{Ser473}$ or pAKT$^{Thr308}$, FOXO3 and pFOXO3$^{Ser253}$ protein expression. There was an additive effect between obesity and DMBA exposure for increased Gstpi, Ephx1 mRNA, and GSTM1 and EPHX1 protein expression.

Summary sentence: Ovaries from obese mice have altered capacity for chemical biotransformation and increased DMBA-induced ovotoxicity.

Keywords: Ovary, obesity, 7,12-dimethylbenz[a]anthracene, ovotoxicity
INTRODUCTION

Obesity is positively correlated with a number of health hazards some of which, including diabetes [1-5], cardiovascular disease [6-9] and cancer [10-14], are the leading causes of preventable death. The prevalence of obesity and obesity-related health complications, such as type 2 diabetes, which were previously considered “adult diseases”, are now also rising dramatically in children [8, 15, 16] leading to premature death and infertility. In recent years, a strong association between increased body mass index with enhanced incidence in reproductive health impairments has been reported [12, 17-20]. Obese women have an increased likelihood to display signs of polycystic ovarian syndrome (PCOS), reduced fecundity and poor quality oocytes [19, 21]. There has also been a strong link between obesity and an increased risk of birth defects, premature and still-birth [22-28] and gestational diabetes [29, 30]. Although, there is a strong association between obesity and compromised reproductive health, the molecular mechanisms involved remain ill-defined. Additionally, despite the alarming prevalence of obesity and obesity associated maladies, little is known of how this epidemic may influence ovarian xenobiotic metabolism.

Ovaries are important for supplying the germ cell necessary for perpetuation of species and production of hormones essential for female growth and development [31-33]. At birth, females are born with a limited number of primordial follicles, which once depleted, cannot be replenished [33-35]. It is accepted that the process of folliculogenesis is an irreversible process; once follicles are recruited from the resting pool to the growing pool they will undergo atresia if not selected for further growth to ovulation [36-38]. Unlike the cyclic maturation of follicles to ovulation, initial primordial follicular activation has been identified to be regulated, independent of the pituitary gonadotropins, largely by local ovarian factors including the phosphatidylinositol 3-kinase (PI3K) pathway [38-41]. A balance between dormancy, activation and atresia of primordial follicles is critical for the female reproductive lifespan [37, 38, 42]. Any environmental factor(s) that could accelerate the rate of primordial follicle activation and the process of atresia, would greatly threaten the reproductive potential of the female.

Obesity can alter insulin sensitivity in a number of target tissues including muscle, liver, adipose tissue and the ovary [2, 43-46]. Insulin hormone binds to its receptor resulting in auto-phosphorylation and recruitment of the insulin receptor substrate proteins (Irs) [47-49], which in turn regulate numerous downstream insulin-mediated signaling pathways including PI3K signaling [2, 43]. PI3K signaling events are largely mediated through Protein kinase B (AKT), a subfamily comprising of three mammalian isoforms Akt1, Akt2 and Akt3 [50]. Upon PI3K activation, it recruits AKT to the membrane where AKT is phosphorylated (pAKT). pAKT dissociates from the membrane and shuttles to the cell nucleus where it has the ability to phosphorylate and inactivate several targets including forkhead transcription factor subfamily 3 (FOXO3).

A number of environmental chemicals can target the ovary and destroy the primordial follicles as well as other follicle types, leading to premature ovarian failure, infertility and other health impairments [31, 32, 51-53], making ovarian xenobiotic metabolism critical for protection of the female germ cell. The polycyclic aromatic hydrocarbon, 7,12-dimethylbenz[a]anthracene (DMBA), is an environmental carcinogen [54-58] as well as ovotoxicant [51, 52, 59, 60]. Human exposure to DMBA is mainly through smoke or fumes from burning of organic substances such as coal, car exhaust and cigarette smoke [59, 61]. Relative to non-smokers, women who are cigarette smokers undergo early onset of menopause and suffer infertility [62-64]. Studies from animal models have demonstrated that DMBA exposure can destroy follicles of all types resulting in accelerated premature ovarian insufficiency and other reproductive complications [32, 51, 59, 65, 66]. In the liver, the parent compound DMBA is bioactivated to its more toxic metabolite, DMBA-3,4-diol-1,2-epoxide by cytochrome P450 isoforms 1B1 (CYP1B1) and 1A1 (CYP1A1) [59, 61, 67, 68] in conjunction with microsomal epoxide hydrolase (EPHX1) [69, 70]. Several studies have also demonstrated the ovary’s capacity to metabolize DMBA to its more ovotoxic metabolite, DMBA-3,4-diol-1,2-epoxide via the action of EPHX1 [51, 59, 61, 68, 70-72].
Insulin can regulate the expression xenobiotic biotransformation genes products such as the CYP’s, Glutathione S-transferases (GST) and EPHX1 through PI3K/AKT signaling [73, 74]. Inhibition of PI3K has also been reported to alter the expression of EPHX1, GSTP1 and GSTM1 genes in postnatal day 4 cultured rat ovaries [75-77]. GSTP1 and GSTM1 are members of the GST superfamily of proteins involved in phase II metabolism of xenobiotic compounds. These enzymes function in the detoxification of electrophilic compounds, including carcinogens, therapeutic drugs, environmental toxins and products of oxidative stress, by conjugation with glutathione [78]. Though the effects of obesity on DMBA-induced ovotoxicity have not been previously explored, regulation of EPHX1 by insulin in non-ovarian tissues has been reported [73, 74]. Since obesity can alter insulin action to its target tissues including the ovary, we hypothesized that obesity-induced increased insulin could increase PI3K signaling and alter xenobiotic gene expression leading to accelerated DMBA-induced ovotoxicity.

MATERIALS AND METHODS

Reagents
7,12-dimethylbenz[a]anthracene (>98% DMBA), 2-β-mercaptoethanol, 30% acrylamide/0.8% bis-acrylamide, ammonium persulfate, glycerol, N,N’,N„N’-Tetramethyl-ethylenediamine (TEMED), Tris base, Tris HCl, sodium chloride, Tween-20, bovine serum albumin (BSA), ascorbic acid (Vitamin C), phosphatase inhibitor, protease inhibitor and transferrin were purchased from Sigma-Aldrich Inc. (St. Louis, MO). RNA later was obtained from Ambion Inc (Austin, TX). Hanks' Balanced Salt Solution (without CaCl2, MgCl2, or MgSO4) and superscript III one-step RT-PCR System were obtained from Invitrogen Co. (Carlsbad, CA). RNeasy Mini kit, QIAshredder kit, RNeasy MinElute kit and QuantitectTM SYBR Green PCR kit were purchased from Qiagen Inc. (Valencia, CA). Custom designed primers were obtained from the DNA facility of the Office of Biotechnology at Iowa State University. Ponceau S was purchased from Fisher Scientific (Waltham, MA, USA). SignalFire ECL Reagent and Anti-pAKT Ser473 antibody were from Cell Signaling Technology (Danvers, MA, USA). Anti-FOXO3, anti-pFOXO3 Ser253, anti-alpha Tubulin (TUBA), anti-GSTP1 and anti-GSTM1 antibodies were purchased from Millipore (Temecula, CA, USA). Anti-pAKT Thr308 antibody was purchased from Abcam (Cambridge, MA, USA). Goat anti-rabbit and donkey anti-goat secondary antibodies were purchased from Pierce Biotechnology (Rockford, IL). Restore PLUS Western Blot Stripping Buffer was purchased from Thermo SCIENTIFIC (Rockford, IL, USA).

Animals
Four week old female wild type normal non-agouti (a/a; designated lean; n = 15) and agouti lethal yellow (KK.Cg-Ay/J; designated obese; n = 15) mice were obtained from the Jackson Laboratory (Bar Harbor, Maine) and housed at the animal facility at Iowa State University. All experimental protocols and procedures were approved by the Iowa State University Animal Care Committee (IACUC). Animals were maintained under controlled lighting (12 hr light/12 hr dark) and temperature (21-22°C) conditions. Food and water were provided ad libitum. At 6 weeks of age, non-agouti and agouti lethal yellow mice (n = 5 genotype) were sacrificed. At 18 weeks of age, glucose tolerance testing confirmed that obese mice were less glucose tolerant than their lean littermates and had higher systemic basal glucose level. Both lean and obese mice (n = 10 genotype), were intraperitoneally (i.p.) dosed with sesame oil or DMBA (> 98%; 1mg/kg/day) for 14 days. This dose was chosen based on destruction of approximately 50% of primary and secondary follicles, with a higher loss of primordial follicles [52].

Tissue Collection
Mice were euthanized at 6 weeks of age or 3 days after the end of dosing (approximately 20 weeks of age) during the pro-estrus stage of cyclicity and body weight was recorded. Ovaries were collected, trimmed of excess fat and weighed. One ovary was fixed in 4% paraformaldehyde for histological analysis while the other ovary was stored in RNA later at -80°C for RNA and protein expression studies.
Histology and Follicle Counting

The histology work was performed at the Iowa State University Veterinary Medicine Histopathology laboratory. Briefly, one ovary from each animal was fixed in 4% paraformaldehyde overnight, transferred to 70% ethanol, dehydrated, embedded in paraffin blocks, serially sectioned (5 µM) and every 6th section (4-6 sections/slide) was mounted (15–20 slides per animal), and stained with hematoxylin and eosin (H & E). Digital images were acquired with a Leica DMI300B Fluorescent Microscope. Numbers of healthy follicles (oocyte-containing follicles showing a distinct oocyte nucleus) were classified and counted in every 12th section according to the procedures as previously described [79, 80]. Briefly, primordial follicles contained an oocyte surrounded with a single layer of squamous-shaped granulosa cells, primary follicles contained an oocyte surrounded by a single layer of cuboidal-shaped granulosa cells, secondary follicles contained an oocyte surrounded by multiple layers of granulosa cells and antral follicles contained an oocyte surrounded by at least two layers of granulosa cells and a fluid-filled antral space.

RNA Isolation

Total RNA was isolated using Qiagen RNeasy Mini Kit (n = 3 ovaries per treatment) according to the manufacturer’s protocol. Briefly, ovaries were lysed and homogenized using a hand-held homogenizer followed by applying the homogenate to a QIAshredder column with subsequent centrifugation at 16100 RCF for 2 minutes at room temperature. The resulting supernatant was applied to an RNeasy Mini column, allowing RNA to bind to the filter cartridge. Following washing, RNA was eluted from the filter and concentrated using an RNeasy MinElute Kit according to the manufacturer’s protocol. The final total concentrated RNA was eluted using 14 µl of RNase-free water and RNA concentration determined using an ND-1000 Spectrophotometer (λ = 260/280 nm; NanoDrop technologies, Inc., Wilmington, DE).

First Strand cDNA Synthesis and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA (0.5 µg) was reverse transcribed into cDNA utilizing the Invitrogen Superscript III Reverse Transcriptase as detailed in the manufacturer’s procedures. Two microliters of diluted cDNA (1:25) was amplified on an Eppendorf Mastercycler using a Quantitect SYBR Green PCR kit and primers specific for mouse Gapdh, Ephx1, Gstp1, Gstm1, Akt1 (sequences as indicated in Nteeba et al [81]) and Akt2 (forward primer: 5’- TGG ACC ACA GTC ATC GAG AG-3’; reverse primer: 5’-CTT GTA ATC CAT GGC GTC CT-3’). The PCR cycling program consisted of a 15 min hold at 95°C and 45 cycles of: denaturing at 95°C for 15 sec, annealing at 58°C for 15 sec, and extension at 72°C for 20 sec at which point data was acquired. Product melt conditions were determined using a temperature gradient from 72°C to 99°C with a 1°C increase at each step. Three replicates of each sample (n = 3 wells per treatment) were included. Statistical analysis was performed on the normalized ΔCT for each sample. There was no difference in ovarian Gapdh mRNA level between sesame control and DMBA-treated ovaries in lean or obese mice. Therefore, for each sample, relative mRNA expression of each of the above genes was normalized using Gapdh as a housekeeping gene and relative fold change calculated using the $2^{-\Delta\Delta CT}$ method. The results are presented as mean fold change ± standard error relative to the sesame control group.

Protein Isolation and Western Blot Analysis

Total ovarian protein was isolated and immunoblots performed according to the procedure of Nteeba et al [81]. Briefly, ovaries (n = 3 per treatment) were homogenized in 300 µl of ice-cold tissue lysis buffer and protein quantified using a standard BCA protocol on a 96-well assay plate. Total protein (15 µg) was separated on a 10% SDS-PAGE and subsequently transferred to nitrocellulose membranes. Following blocking, membranes were incubated with specific primary antibodies [Rabbit Anti-GSTP1 (1:250); Rabbit Anti-GSTM1 (1:200), Goat Anti-EPHX1 (1:500), Rabbit Anti-pAKT$^{Ser473}$ (1:500), Rabbit Anti-pAKT$^{Thr308}$ (1:500), Rabbit Anti-FOXO3 (1:500), Rabbit Anti-pFOXO3$^{Ser253}$ (1:1000)] in 5% BSA in TTBS for 15-20 h at 4°C. HRP-conjugated secondary antibodies (1:2000 – 1:5000) were added for 1h at room temperature, and then membrane-bound HRP were washed three times for 5 minutes in TTBS. Autoradiograms were visualized on X-ray films in a dark room following 7 min incubation of membranes.
with 1X SignalFire ECL reagent. Equal protein loading was confirmed by Ponceau S staining of nitrocellulose membranes prior to antibody incubation. Additionally, blots were stripped and probed with an anti-TUBA or anti-GAPDH antibody. Densitometry of the appropriate sized bands was measured using Carestream molecular imaging software version 5.0 (Carestream Health Inc., Rochester, NY) which eliminates background noise. Values were normalized to the appropriate loading control (TUBA, GAPDH, or Ponceau S staining).

Statistical Analysis
Statistical analyses were performed using either the Two-Way ANOVA followed by Bonferroni multiple comparisons to assess interaction between strain and drug or the One-Way ANOVA followed by Tukey’s multiple pairwise comparison function of GraphPad Prism 5.5 software with a statistical significance level set at \( P < 0.05 \). Bars represent means ± SEM. Different letters indicate significant difference from respective pairs; \( n = 3 \) per treatment.

RESULTS

**Obesity Decreases the Number of Healthy Primordial Follicles in Murine Ovaries**
We determined the effect of DMBA exposure on ovarian weight and the number of healthy follicles in lean and obese mice (Fig. 1). There was no effect of DMBA on body weight (Fig. 1A); however, compared to sesame oil, DMBA decreased ovarian weight in both lean (\( P < 0.05 \)) and obese (\( P < 0.001 \)) treated groups (Fig. 1B). There was no difference between ovarian weight of lean and obese sesame treated groups; however, DMBA treatment further reduced (\( P < 0.05 \)) ovarian weight in the obese compared to lean mice (Fig. 1B). In both lean and obese females, DMBA treatment significantly decreased (\( P < 0.0001 \)) the number of healthy follicles compared to sesame-treated females (Fig. 1C-1F). Interestingly, ovaries from obese mice had both decreased (\( P < 0.0001 \)) number of healthy primordial (Fig. 1C) and primary (Fig. 1D) follicles but increased (\( P < 0.001 \)) secondary (Fig. 1E) and pre-ovulatory (Fig. 1F) follicles compared to ovaries from lean sesame-treated mice. No impact of obesity or DMBA on corpora lutea numbers were observed.

**DMBA and Obesity Have an Additive Effect on Ovarian Ephx1 and Gstm1 Gene Expression**
Relative to lean counterparts, ovaries from obese mice had increased ovarian Gstm1 (Fig. 2A; \( P < 0.01 \)) and Ephx1 (Fig. 2C; \( P < 0.05 \)) mRNA levels. However, obesity did not affect Gstp1 (Fig. 2B) mRNA levels in murine ovaries. In lean ovaries, DMBA did not alter Gstm1 mRNA expression. Gstp1 and Ephx1 mRNA levels were increased (\( P < 0.05 \)) in ovaries of both lean and obese treated groups compared to those of their respective control groups (Fig. 2A – 2C). Compared to lean DMBA-treated females, ovaries from obese DMBA-treated females displayed increased (\( P < 0.01 \)) Gstm1 and a strong tendency for increased (\( P = 0.06 \)) Ephx1 but no difference in Gstp1 mRNA levels (Fig. 2A – 2C). Lack of any impact of genotype on Gstm1, Gstp1 or Ephx1 mRNA level was confirmed in ovaries from 6 week old mice (pre-obese) (Fig. 2D).

**DMBA Increases Ovarian GSTM1, GSTP1, and EPHX1 Protein Levels in Obese but Not in Lean Mice**
Ovaries from obese mice had increased (\( P < 0.05 \)) basal ovarian GSTM1 protein levels relative to their lean counterparts (Fig. 3A). DMBA exposure did not impact GSTM1 protein level in lean ovaries however there was increased (\( P < 0.05 \)) GSTM1 protein levels in ovaries of obese treated groups compared to their respective control groups (Fig. 3A). Relative to lean counterparts, ovaries from obese mice had increased basal ovarian GSTP1 (\( P < 0.001 \)) protein levels (Fig. 3B). Interestingly, DMBA increased (\( P < 0.05 \)) GSTP1 protein level in ovaries of lean mice, but decreased (\( P < 0.05 \)) ovarian GSTP1 protein level in obese treated groups compared to those of their respective control groups (Fig. 3B). Similar to mRNA results, ovaries from obese mice had increased (\( P < 0.05 \)) basal ovarian EPHX1 protein levels relative to their lean counterparts (Fig. 3C). DMBA increased (\( P < 0.05 \)) EPHX1 protein levels...
levels in ovaries of both lean and obese treated groups compared to their respective control groups (Fig. 3C). Relative to lean DMBA treated mouse ovaries; obesity resulted in a greater increase ($P < 0.01$) in EPHX1 protein levels, relative to the obese sesame oil-treated mouse ovaries (Fig. 3C).

**Obesity and DMBA Have Opposing Effects on Ovarian Akt1/2 mRNA Expression**

Ovaries from obese females displayed higher ($P < 0.05$) Akt1 (Fig. 4A) and Akt2 (Fig. 4B) mRNA levels than lean females; however, DMBA exposure significantly reduced ovarian Akt1/2 mRNA levels in both lean ($P < 0.001$) and obese ($P < 0.05$) treated groups (Fig. 4). Akt1 and Akt2 mRNA levels were not found to differ between genotype in ovaries from 6 week old mice (pre-obese) (Fig. 4C).

**Obesity Increases Ovarian Phosphorylated AKT Without Affecting Phosphorylated FOXO3 Protein Levels**

DMBA decreased ($P < 0.05$) ovarian $\text{pAKT}^{\text{Ser473}}$ protein in lean mice, while obesity increased ($P < 0.05$) basal $\text{pAKT}^{\text{Ser473}}$ levels and the reduction observed in the lean mice was absent from ovaries of obese mice. In lean mice, there was no impact of DMBA on $\text{pAKT}^{\text{Thr308}}$ protein, however obesity increased ($P < 0.05$) basal ovarian $\text{pAKT}^{\text{Thr308}}$ protein levels (Fig. 5). In contrast to lean ovaries, those from obese mice had a decrease in $\text{pAKT}^{\text{Thr308}}$ when exposed to DMBA. Lean and obese females did not differ in FOXO3 protein level, and although there was a visible reduction in DMBA-treated lean mice, this was not significant due to variation. $\text{pFOXO3}^{\text{Ser253}}$ protein expression was reduced by DMBA in both lean and obese ovaries, and there was a lower level basally ($P < 0.05$) of $\text{pFOXO3}^{\text{Ser253}}$ due to obesity.

**DISCUSSION**

Obesity is associated with elevated blood glucose and insulin levels, altering insulin’s action on a number of organs including the ovary [2, 43-46]. Recently, it has been demonstrated that the ovary maintains insulin sensitivity during obesity even though other classical tissues including the liver, muscle and adipose tissue develop insulin resistance [43, 81]. Insulin has been previously shown to regulate hepatic xenobiotic metabolism [73, 74, 82, 83] and in the ovary insulin can activate the PI3K/AKT signaling, a pathway that has been demonstrated to play critical roles in metabolism and reproduction [84]. Previous work in rodent models has shown the involvement of PI3K signaling in insulin-mediated hepatic regulation of xenobiotic metabolism [83, 85]. Recently, the involvement of PI3K signaling in ovarian metabolism of chemicals including DMBA has been demonstrated [60, 75, 86]. Since obesity is associated with elevated plasma insulin levels, we hypothesized that obesity-induced increased insulin could increase PI3K signaling and alter xenobiotic gene expression leading to accelerated DMBA-induced ovotoxicity. To test this hypothesis, we used the lethal yellow mouse, an excellent model for human obesity [87], to investigate whether obese females have increased susceptibility to DMBA-induced ovotoxicity. We designed our experiments on the impact of obesity on chemical metabolism when mice were 18 weeks of age because elevated insulin has been shown in this mouse model at this timepoint [88]. In addition, we confirmed that the obese mice had elevated basal glucose and had a compromised response to a glucose tolerance test (data not shown) and that there was no impact of the lethal yellow genotype on follicle numbers at 6 weeks of age (unpublished data). Further, to eliminate any impact of genotype outside of the obese phenotype, we characterized gene expression in 6 week old mice; at this point there is no difference in body weight and the lethal yellow mice are considered pre-diabetic.

Relative to sesame oil, DMBA treatment did not impact body weight; however, compared to sesame oil, DMBA decreased ovarian weight in both lean and obese treated groups. Interestingly, we observed lower than expected primordial follicle numbers in ovaries of both lean and obese sesame oil treated animals, which could be due to their being 20 weeks of age, and potentially this is a time when follicle numbers are naturally beginning to decline. As expected, DMBA treatment significantly decreased the number of healthy follicles of all types in both lean and obese treated females compared to sesame-treated females. The observed decrease in ovarian weight and the number of healthy follicles in DMBA-treated lean and
obese females could be as a result of increased follicle loss induced by DMBA exposure as previously reported [59, 61]. Surprisingly, though we saw no difference between ovarian weight between lean and obese sesame oil-treated groups; there was a significant reduction in ovarian weight of obese females treated with DMBA compared to their lean DMBA-treated littermates, indicating that the obese ovaries suffered greater levels of DMBA-induced ovotoxicity, outside of follicle depletion. This data supports that the ovaries from obese, DMBA-treated mice had greater levels of ovotoxicity than their lean counterparts that received the same DMBA exposure. Despite lack of ovarian weight differences between lean and obese mice, ovaries from obese mice had both decreased number of healthy primordial and primary follicles but increased secondary and pre-ovulatory follicles compared to ovaries from lean sesame-treated mice. It was surprising that larger numbers of growing follicles did not impact ovarian weight, it has previously been shown that ovaries from mice lacking the Arylhydrocarbon receptor had lower numbers of all stage follicles without any difference in ovarian weight [89]. This difference in follicle populations could indicate increased activation of follicles from the primordial follicle pool.

DMBA has been demonstrated to have both carcinogenic and ovotoxic properties in animal models [54, 59, 61]. These two properties are mediated through DMBA’s metabolite 3,4-diol-1,2-epoxide which is formed during DMBA metabolism facilitated by CYP1B1, EPHX1 and CYP1B1/1A1 enzymes [59, 96-99]. Although the liver is the primary site for xenobiotic metabolism, the ovary has been demonstrated to have the capacity to metabolize xenobiotic compounds [60, 32 67, 68, 75, 76] including DMBA in absence of hepatic contributions [59, 96-99]. Several studies have also reported an inverse relation between BMI and estradiol levels in premenopausal women [92-94]. Decreased estradiol levels have been associated with increased activation of primordial follicle in mice [95]. So it is possible that obesity could trigger changes in the intrinsic ovarian signals responsible for initiation of follicle activation and recruitment, and the age of animal may be important when interpreting impacts of treatments on ovarian physiology.

Consistent with previous studies, ovaries from obese mice had increased ovarian Ephx1 mRNA [81] and EPHX1 protein [59, 61] levels relative to their lean counterparts. While DMBA treatment increased Ephx1 mRNA and EPHX1 protein levels in ovaries of both lean and obese treated groups compared to their respective control groups, ovaries from obese DMBA-treated females displayed a greater increase in Ephx1 mRNA and EPHX1 protein expression compared to lean DMBA-treated females. To the best of our knowledge, this is the first study to report a DMBA-obesity synergistic effect for increased ovarian Ephx1 mRNA and EPHX1 protein expression in mice. In extra-ovarian tissues, insulin has been demonstrated to increase hepatic EPHX1 expression [73, 74, 83] while glucagon to inhibit EPHX1 expression [83]. In rats, conditions of insulin deficiency induced by either Type 1 diabetes or starvation were associated with decreased EPHX1 enzyme activity yet on the other hand, addition of insulin or re-feeding restored EPHX1 activity [74]. Taken together, these data demonstrate that insulin plays a role in induction of EPHX1 expression and activity. Since obesity is associated with elevated levels of insulin, it is more likely that one of the mechanisms by which obesity accelerates ovarian EPHX1 expression is through hyperactivation of insulin-mediated EPHX1 induction signaling pathways such the PI3K pathway. In cultured primary rat hepatocytes, administration of insulin increased EPHX1 mRNA and protein expression in a time- and concentration-dependent manner [83], conversely, inhibition of PI3K by Wortmannin and LY294002 or the mTOR inhibitor, rapamycin, modulated the insulin-induced increase in EPHX1 [83], supporting the involvement of PI3K signaling in insulin-induced hepatic regulation of
EPHX1. Furthermore, Ki and Kim [85] confirmed the involvement of PI3K signaling in induction of EPHX1 through C/EBP transcription factors. With recent reports demonstrating the involvement of PI3K signaling in ovarian metabolism of DMBA [60, 75, 86], it is highly probable that the observed DMBA-obesity synergistic effect on ovarian Ephx1 mRNA and EPHX1 protein expression is mediated through insulin/PI3K signaling pathway.

Just like EPHX1, regulation of GSTM1 and GSTP1 by insulin in non-ovarian tissues has been reported [73, 74, 101]. Insulin administration is known to increase GST gene expression through the PI3K/AKT/mTOR pathway while glucagon decreases such gene expression [101]. In alloxan- and streptozotocin-diabetic male Fischer-344 rats, diabetes decreased the activities of rat liver soluble GST’s, yet application of insulin to alloxan-diabetic individuals approximately restored the initial enzyme activities [74]. Similarly, starvation of Fischer-344 rats, resulted in reduced activities of GST enzymes, however, re-feeding restored their initial activities [74]. Taken together, these results suggest that insulin also regulates the hepatic activities of GST enzymes. Although in the current study we did not measure the activities of these enzymes, we observed increased Gstm1 mRNA, GSTM1 and GSTP1 protein levels in ovaries from obese sesame oil-treated females compared to lean mice, mimicking the trend observed during normal insulin activity. Taken together, these results could support the notion that despite insulin resistance in other tissues, the ovary maintains insulin sensitivity.

DMBA-induced increases in Gstp1 mRNA and GSTP1 protein levels in PND4 cultured rat ovaries have been reported [76], however the effect of DMBA on ovarian expressed GSTM1 has not been previously reported. In the present study, DMBA treatment had contrasting effects on GSTP1 and GSTM1 in both lean and obese treated mice. Interestingly, DMBA increased ovarian Gstm1 mRNA and GSTM1 protein levels in obese but not in lean mice yet on the other hand DMBA increased ovarian Gstp1 mRNA in both lean and obese females and GSTP1 protein levels in only lean but decreased GSTP1 protein expression in obese females. Furthermore, though there was no difference in ovarian Gstp1 mRNA and GSTP1 protein levels between lean DMBA- and obese DMBA-treated females, relative to lean DMBA-treated females; ovaries from obese females exhibited a greater increase in both Gstm1 mRNA and GSTP1 protein expression, following DMBA treatment. Given the divergent roles played by GST enzymes, depending on the physiological conditions and type of cells involved, changes in expression of genes encoding for these enzymes could be beneficial or have detrimental consequences for the cells. Although best known for their detoxification role in metabolism [53, 76, 102-105], GST enzymes have also been implicated in cell signaling, intracellular transport and isomerization of steroid hormones [105-107] as well as development of chemotherapeutic-drug resistance [104, 105, 108-111] and a variety of diseases including cancer, diabetes and inflammatory diseases [101, 104, 110, 112-114]. In particular, overexpression of GSTP1 has been associated with inactivation of cigarette smoke carcinogens [105] including detoxification of DMBA-induced toxicity [53, 76, 102], and development of drug resistance [105, 108, 110, 111, 114, 115], susceptibility to and poor prognosis of several cancers including breast, cervical and ovarian cancer [112]. This is could be due to its ability to selectively inhibit pro-apoptotic p38 and C-Jun N-Terminal Kinase 1 (JNK1) portions of the Mitogen-Activated Protein Kinase (MAPK) signaling cascades [53, 107, 108, 110, 114]. Like GSTP1, there is a growing body of evidence supporting the role of GSTM1 in regulating apoptotic pathways through direct protein-protein interactions, with Apoptosis Signal-regulating Kinase 1 (ASK1), which is upstream of JNK. GSTM1 sequesters ASK1 through complex formation and subsequent prevention of its induction of the pro-apoptotic p38 and JNK portions of the MAPK signaling cascade [101, 104, 108, 116]. It is important to note that increased expression of GSTs in obese females and during DMBA treatment in both lean and obese females did not avert DMBA-induced follicle loss. This would imply that the role of overexpressed GSTM1 and GSP1 could be not principally involved in detoxification but rather be involved in other cellular processes that are yet to be determined.
Insulin regulates xenobiotic metabolism through PI3K/AKT pathway [83, 85, 101]. Previous studies have also implicated PI3K/AKT/mTOR pathway in DMBA-induced ovotoxicity [86]. In the current study, obesity and DMBA had opposing effects on PI3K/AKT signaling pathway members’ gene expression. Ovaries from sesame oil-treated obese females displayed higher Akt1 and Akt2 mRNA levels concomitant with upregulated pAKT\textsuperscript{Ser473/Thr308} protein levels, without affecting total FOXO3, but reduced pFOXO3\textsuperscript{Ser253} protein expression compared to their lean counterparts. Surprisingly, DMBA treatment significantly reduced ovarian Akt1/2 mRNA levels in both lean and obese treated groups, mirrored by decreased pAKT\textsuperscript{Ser473} protein in lean but not obese ovaries. A decrease in pAKT\textsuperscript{Thr308} protein expression was observed in the ovaries from obese females. Interestingly, though pFOXO3\textsuperscript{Ser253} was decreased in both lean and obese DMBA-treated mice, FOXO3 protein expression was not impacted by either obesity or DMBA. These alterations to the PI3K pathway may be at least partially responsible for the altered dynamics of follicle activation observed in the obese mouse ovary.

In conclusion, the ovary contains a finite number of primordial follicles, which once depleted, cannot be replaced; therefore ovarian xenobiotic metabolism of chemical compounds like DMBA which can deplete the primordial follicle pool is critical for protection of the female germ cell. Since insulin regulates hepatic expressed xenobiotic metabolism enzymes and obesity alters insulin sensitivity in a number of target tissues, understanding how obesity might influence the ovary’s capacity to metabolize chemicals is critical. Our data is in agreement with previous studies which demonstrated that insulin induces the hepatic activities of EPHX1 and GST enzymes [73, 74, 101]. We have shown that obesity increased mRNA and protein levels of pAKT, EPHX1, GSTM1 and GSTP1 compared to lean mice, mimicking the trend observed during insulin administration or re-feeding. Therefore, our data support the notion that despite insulin resistance in other tissues, the ovary seems to maintain insulin sensitivity and that obesity-induced increased insulin could increase PI3K signaling and alter xenobiotic gene expression leading to accelerated DMBA-induced ovotoxicity.

REFERENCES
18. Barber TM, Franks S. The link between polycystic ovary syndrome and both Type 1 and Type 2 diabetes mellitus: what do we know today? Womens Health (Lond Engl) 2012; 8:147-154.
FIGURE LEGENDS

Figure 1: Effect of DMBA on ovarian weight and follicle numbers in lean and obese mice
At 18 weeks of age lean and obese mice were intraperitoneally (i.p) dosed with sesame oil or DMBA (95%; 1mg/kg) for 14 days. Mice were euthanized 3 days after the end of dosing; body weight measured (A), ovaries collected, trimmed of excess fat and weighed (B). One ovary was fixed in 4% paraformaldehyde and complete serial sections were prepared and subjected to histological examination for the number of healthy follicles in both lean and obese treated groups (C-F); (C) healthy primordial follicle number; (D) healthy primary follicle number; (E) healthy secondary follicle number; (F) healthy pre-ovulatory follicle number. Bars represent mean follicle number per ovary ± SEM. Different letters indicate significant ($P < 0.05; n = 3$) difference from respective pairs.

Figure 2: Effects of DMBA on ovarian metabolism gene expression in lean and obese mice
18 weeks old lean and obese female mice were treated with sesame oil or DMBA (95%; 1mg/kg; i.p) for 14 days. Three days after dosing, ovaries were collected, trimmed of excess fat and total ovarian RNA isolated. Using quantitative (q) RT-PCR, relative mRNA levels of (A) *Gstm1*, (B) *Gstp1* and (C) *Ephx1* were evaluated after normalization to the housekeeping gene, *Gapdh*. (D) Ovarian *Gstm1*, *Gstp1*, *Ephx1* mRNA levels in 6 week old lean and obese mice. Values represent relative fold-change means ± SEM. Different letters indicate significant difference from respective pairs, $P < 0.05; n = 3$.

Figure 3: Effect of DMBA on ovarian GSTM1 protein in lean and obese mice
18 weeks old lean and obese female mice were treated with sesame oil or DMBA (95%; 1mg/kg; i.p) for 14 days. Three days after dosing, ovaries were collected, trimmed of excess fat and total ovarian protein was isolated ($n = 3$ per treatment group) from lean control, (LC), lean DMBA (LD), obese Control (OC) and obese DMBA (OD). (A) GSTM1, (B) GSTP1 or (C) EPHX1 proteins were quantified by Western blotting, followed by densitometric quantification of the protein band using Carestream molecular imaging software version 5.0. Bars represent means ± SEM. Different letters indicate significant difference from respective pairs; $P < 0.05; n = 3$.

Figure 4: Effects of DMBA on ovarian Akt1/2 expression in lean and obese mice
At 18 weeks of age lean and obese mice were intraperitoneally dosed with sesame oil or DMBA (95%; 1mg/kg) for 14 days. Ovaries were collected 3 days after the end of dosing, trimmed of excess fat, and total ovarian RNA isolated ($n = 3$ per treatment group). Relative mRNA expressions of (A) *Akt1* and (B) *Akt2* were normalized using *Gapdh* as a housekeeping gene and quantified using qRT-PCR. (C) Ovarian *Akt1* and *Akt2*, mRNA levels in 6 week old lean and obese mice. Bars represent means ± SEM. Different letters indicate significant difference from respective pairs, $p < 0.05; n = 3$.

Figure 5: Effect of DMBA on ovarian PI3K proteins in lean and obese mice
18 weeks old lean and obese female mice were treated with sesame oil or DMBA (95%; 1mg/kg; i.p) for 14 days. Three days after dosing, ovaries were collected, trimmed of excess fat and total ovarian protein was isolated ($n = 3$ per treatment group) from lean control, (LC), lean DMBA (LD), obese Control (OC) and obese DMBA (OD). Western blotting was performed to measure (A) pAKT$^{Ser473}$, (B) pAKT$^{Thr308}$, (C) FOXO3, and (D) pFOXO3$^{Ser253}$ levels. Densitometry of the appropriate bands was performed using Carestream molecular imaging software version 5.0. Bars represent means ± SEM. Different letters indicate significant difference from respective pairs; $P < 0.05; n = 3$. 
A

GSTM1 Protein Level

Lean CT  Lean DMBA  Obese CT  Obese DMBA

B

GSTP1 Protein Level

Lean CT  Lean DMBA  Obese CT  Obese DMBA

C

EPHX1 Protein Level

Lean CT  Lean DMBA  Obese CT  Obese DMBA