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Enhanced susceptibility of ovaries from obese mice to 7,12-dimethylbenz[a]anthracene-induced DNA damage

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ABSTRACT

7,12-Dimethylbenz[a]anthracene (DMBA) depletes ovarian follicles and induces DNA damage in extra-ovarian tissues, thus, we investigated ovarian DMBA-induced DNA damage. Additionally, since obesity is associated with increased offspring birth defect incidence, we hypothesized that a DMBA-induced DNA damage response (DDR) is compromised in ovaries from obese females. Wild type (lean) non agouti (a/a) and KK.Cg-Ay/J heterozygote (obese) mice were dosed with sesame oil or DMBA (1 mg/kg; intraperitoneal injection) at 18 weeks of age, for 14 days. Total ovarian RNA and protein were isolated and abundance of Ataxia telangiectasia mutated (*Atm*), X-ray repair complementing defective repair in Chinese hamster cells 6 (*Xrcc6*), breast cancer type 1 (*Brca1*), Rad 51 homolog (*Rad51*), poly [ADP-ribose] polymerase 1 (*Parp1*) and protein kinase, DNA-activated, catalytic polypeptide (*Prkdc*) were quantified by RT-PCR or Western blot. Phosphorylated histone H2AX (γH2AX) level was determined by Western blotting. Obesity decreased ($P < 0.05$) basal protein abundance of PRKDC and BRCA1 proteins but increased ($P < 0.05$) γH2AX and PARP1 proteins. Ovarian ATM, XRCC6, PRKDC, RAD51 and PARP1 proteins were increased ($P < 0.05$) by DMBA exposure in lean mice. A blunted DMBA-induced increase ($P < 0.05$) in XRCC6, PRKDC, RAD51 and BRCA1 was observed in ovaries from obese mice, relative to lean counterparts. Taken together, DMBA exposure induced γH2AX as well as the ovarian DDR, supporting that DMBA causes ovarian DNA damage. Additionally, ovarian DDR was partially attenuated in obese females raising concern that obesity may be an additive factor during chemical-induced ovotoxicity.

Introduction

7,12-Dimethylbenz[a]anthracene (DMBA) is a polycyclic aromatic hydrocarbon which destroys all follicle types leading to ovarian failure in mice and rats (Mattison and Schulman, 1980) and is one component liberated through cigarette smoke and car exhaust fumes (Gelboin, 1980). It is recognized that female smokers experience ovarian senescence at an earlier age than their non-smoking counterparts (Jick and Porter, 1977), and low level DMBA exposure caused follicle depletion in neonatal culture rat ovaries, raising concern that ovotoxicity can be induced at levels which may be representative of passive DMBA exposure (Madden et al., 2014).

DMBA requires the action of ovarian enzymes cytochromes p450 (Cyp) isoforms 1A1 and 1B1 (Shimada et al., 2003) and microsomal epoxide hydrolase (mEH) (Rajapaksa et al., 2007; Igawa et al., 2009; Madden et al., 2014) for biotransformation to the ovotoxic metabolite, DMBA 3,4-diol, 1,2-epoxide, which are both carcinogenic and has the potential to form DNA adducts (Miyata et al., 1999). DMBA exposure also induces the DNA damage response (DDR) in cultured neonatal rat ovaries, indicating that DNA damage is a potential mechanism by which DMBA induces its ovotoxic effects (Ganesan et al., 2013).

Double-strand breaks (DSBs) in DNA are cytotoxic lesions, generated by ionizing radiation and man-made chemicals (van Gent et al., 2001). DSBs can be sensed by a PI3K family member Ataxia telangiectasia mutated (ATM) protein (Norbury and Hickson, 2001; Yang et al., 2003; Giunta et al., 2010). ATM phosphorylates histone H2AX (γH2AX) which leads, within seconds, to recruitment of DNA repair molecules to the site of DSBs (Sedelnikova et al., 2002; Svetlova et al., 2010), thus γH2AX has become a gold standard marker for localizing DSBs. DNA DSBs pose a serious threat to both cell viability and genome stability if left unrepaired or repaired incorrectly, and could potentially lead to permanent damage with resulting negative consequences for gamete health (Petrillo et al., 2011; Summers et al., 2011). Two major pathways can repair DNA DSBs: Non-Homologous End Joining (NHEJ) (Chiruvella et al., 2012) and Homologous Recombination (HR) (Scully et al., 1997).

Abbreviations: DMBA, 7,12-dimethylbenz[a]anthracene; DDR, DNA damage response; DSBs, double-strand breaks; NHEJ, Non-Homologous End Joining; HR, Homologous Recombination; TLR, Toll like receptor; Cyp, cytochromes p450; IGF, insulin growth factor; PI3K, phosphatidylinositol-3 kinase, Akt, protein kinase B; ATM, Ataxia telangiectasia mutated; XRCC6, X-ray repair complementing defective repair in Chinese hamster cells 6; BRCA1, breast cancer type 1; RAD 51, Rad 51 homolog; PARP1, poly [ADP-ribose] polymerase 1; PRKDC, protein kinase, DNA-activated, catalytic polypeptide; γH2AX, phosphorylated histone H2AX; mEH, microsomal epoxide hydrolase; TEMED, N′N′N′N′-tetramethylethylenediamine.

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Key signaling molecules involved in NHEJ repair include the X-ray repair complementing defective repair in Chinese hamster cells 6 and 5 (XRCC6 and XRCC5) heterodimer which recognizes and binds to the DSB, recruiting protein kinase DNA-activated, catalytic polypeptide (PRKDC) to the DSB ends (Calsou et al., 2003; Dobbs et al., 2010; Chiruvella et al., 2012). During HR, breast cancer type 1 (BRCA1) can be phosphorylated by ATM and co-localizes with RAD51 recombinase (RAD51) at the site of DNA damage to induce DSB repair (Scully et al., 1997).

Approximately 65% of women in the United States are overweight or obese (Flegal et al., 2010) and the associated reproductive complications include menstrual cycle disturbances, ovulatory dysfunction, infertility, decreased conception, early pregnancy loss and congenital abnormalities in offspring (Cardozo et al., 2012; Sauber-Schatz et al., 2012). Obesity has been associated with altered insulin and insulin growth factor (IGF) signaling (Qatanani and Lazar, 2007), likely due to obese females being typically hyperinsulinemic with concomitant insulin resistance (Kashyap and Defronzo, 2007; Choi and Kim, 2010). Insulin serves as a regulator of hepatic enzymes involved in the metabolism of xenobiotics (Woodcroft and Novak, 1999), mediated, at least partly, through activation of phosphatidylinositol-3 kinase (PI3K) and variety of downstream effectors including protein kinase B (Akt) (Niswender et al., 2003; Kim and Novak, 2007). Insulin increases mEH protein expression in primary cultured rat hepatocytes (Kim et al., 2003). Also, increased ovarian PI3K signaling and mEH have been demonstrated in mice fed a high fat diet until obese (Nteeba et al., 2013). The obese lethal yellow mouse was also found to have both higher basal levels of mEH and greater mEH induction in response to DMBA exposure (Nteeba et al., 2014). These data suggest that ovarian tissue from obese females could have potentially greater exposure to the ovotoxic metabolite of DMBA, due to higher levels of ovarian mEH, and thus greater DMBA bioactivation to a metabolite that interacts with DNA (Miyata et al., 1999). The objective of this study was therefore to investigate ovarian DMBA-induced DNA damage as evidenced by γH2AX appearance and induction of the DDR in both lean and obese mice.

Methods and materials

Reagents

7,12-Dimethylbenz[a]anthracene (DMBA; CAS # 57-97-6), sesame oil (CAS # 8008-74-0), 2-β-mercaptoethanol, 30% acrylamide/0.8% bisacrylamide, ammonium persulfate, glycerol, N′N′N′N′-tetramethylethylenediamine (TEMED), Tris base, Tris HCL, sodium chloride, Tween-20 were purchased from Sigma-Aldrich Inc. (St Louis, MO). RNeasy Mini kit, QIA shredder kit, RNeasy Min Elute kit, and Quantitect TM SYBR Green PCR kit were purchased from Qiagen Inc (Valencia, CA). All primers were purchased from the Iowa State University DNA facility. All primary antibodies were purchased from Abcam (Cambridge, MA) with the exception of the BRCA1 (C-20) primary antibody which was from Santa Cruz Biotechnology (Santa Cruz, CA). RNA later was obtained from Ambion Inc. (Austin, TX). Goat anti-mouse and anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Ponceau S was from Fisher Scientific. ECL plus chemical luminescence detection kit was obtained from GE Healthcare, Amersham (Buckinghamshire, UK).

Animals

Ovarian tissue was obtained as part of a larger study by our group (Nteeba et al., 2014). Briefly, four week old female wild type normal non-agouti (a/a; designated lean; n = 10) and agouti lethal yellow (KK.Cg-Ay/J; designated obese; n = 10) were purchased from Jackson Laboratories (Bar Harbor, ME 002468). All animals were housed in cages under a 12 h light/dark photoperiod with the temperature between 70 and 73°F and humidity approximately 20–30%. The animals were provided with a standard diet (Teklad 2014 global 14% protein rodent maintenance diet) with *ad libitum* access to food and water until 18 weeks of age. All animal experimental procedures were approved by the Iowa State University Animal Care and Use Committee.

In vivo DMBA exposure

Both lean and obese mice were intraperitoneally (i.p.) dosed with sesame oil (SO) or DMBA (95%; 1 mg/kg per day) for 14 days. This dose was chosen based on a report that it caused follicular loss in the ovary (Mattison and Thorgeirsson, 1979). Mice were euthanized 3 days after the end of dosing in their pro-estrus phase. One ovary from each mouse was fixed in 4% paraformaldehyde and one ovary was preserved at −80 °C for RNA and protein isolation. This ovary was powdered and half used to isolate RNA and protein was isolated from the other half. As a note, one ovary from an obese DMBA-treated female could not be localized therefore the final number in this group was n = 4, with n = 5 for all other treatments. No difference in body weights due to DMBA exposure was observed although the lethal yellow mice had higher body weights (Nteeba et al., 2014). DMBA reduced ovarian weight and volume in both lean and obese mice, relative to vehicle treated mice, and ovarian weight and volume were lower in obese DMBA-treated relative to lean DMBA-treated mice (Nteeba et al., 2014).

RNA was isolated from all ovaries (n = 4–5) using an RNeasy Mini kit (Qiagen) and the concentration was determined using an ND-1000 spectrophotometer (λ = 260/280 nm; NanoDrop technologies, Inc., Wilmington, DE) (n = 3). Total RNA (200 ng) was reverse transcribed to cDNA utilizing the Superscript III One-Step RT-PCR (Qiagen). Three randomly chosen cDNA samples per treatment were diluted (1:20) in RNase-free water and amplified on an Eppendorf PCR Master cycler using Quantitect SYBR Green PCR kit (Qiagen). Primers for *Atm, Brca1, Prkdc, Parp1, Rad51, Xrcc6* and *Gapdh* were designed by Primer 3 Input Version (0.4.0) and are listed in Table 1. The regular cycling program consisted of a 15 min hold at 95 °C and 45 cycles of denaturing at 95 °C for 15 s, annealing at 58 °C for 15 s, and extension at 72 °C for 20 s at which point data were acquired. There was no difference in *Gapdh* mRNA expression between treatments, thus each sample was normalized to *Gapdh* before quantification. Quantification of fold-change in gene expression was performed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Pfaffl, 2001).

Protein isolation and Western blotting

Protein was isolated from whole ovaries (n = 4–5) that had been homogenized in tissue lysis buffer containing protease and phosphatase inhibitors as previously described (Thompson et al., 2005). Briefly, homogenized samples were placed on ice for 30 min, followed by two rounds of centrifugation at 10,000 rpm for 15 min. The protein concentration was measured using a standard BCA protocol. SDS-PAGE was used to separate three randomly chosen protein homogenates per treatment which were then transferred to a nitrocellulose membrane. Membranes were blocked for 1 h in 5% milk in Tris-buffered saline containing Tween 20, followed by incubation with one of: anti-rabbit PARP1 antibody (1:200), anti-rabbit phosphorylated H2AX antibody (γH2AX; 1:100), anti-mouse ATM antibody (1:100), anti-mouse RAD51 antibody (1:500), anti-mouse XRCC6 antibody (1:100), anti-

Table 1
Primer sequences used in this study.

Gene	Forward primer	Reverse primer
Atm	TCAGCAGCACCTCTGATTCTT	AGACAGACATGCTGCCTCCT
Brca1	CCCTCTTAGTCTGCTGAGCT	CCTTTGGGTGGCTGTACTGA
Parp1	AAGTGCCAGTGTCAAGGAGA	ACAGGGAGCAAAAGGGAAGA
Prkdc	GCCACAGACCCCAATATCCT	TATCTGACCATCTCGCCAGC
Rad51	ATCCCTGCATGCTTGTTCTC	CTGCAGCTGACCATAACGAA
Xrcc6	GATCTGACACTGCCCAAGGT	TGCTTCTTCGGTCCACTCTT