Estradiol enhances sociosexual behavior and can have proliferative effects in ovariectomized rats

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Abstract Although estradiol (E₂) may have some beneficial effects as a treatment for menopause symptoms, E₂ also has trophic effects that can increase vulnerability to some cancers, such as breast cancer. In the present study, a model to investigate the concomitant behavioral and proliferative effects of E₂ was developed. First, the effects of different duration of chronic E₂ exposure (2 vs 6 months), or no such exposure, on proliferation (tumor incidence and weight, uterine weight) in adult, ovariectomized (OVX) rats

was determined. Second, the effects of different dosages of E₂ (0.03 or 0.09 mg/kg) compared to vehicle only on sexual behavior, and measures of proliferation of adult OVX rats treated with a chemical carcinogen (DMBA; 1.25, 12.50, or 25.00 mg), or inert vehicle, were investigated. Vehicle or E₂ was administered subcutaneously (SC) to OVX rats once per week for 14 weeks. Six months of continuous E₂ exposure increased tumor incidence, tumor weight, and uterine weight, compared to 2 months of E₂ or no E₂ exposure. Rats administered DMBA had increased incidence, number, and size of tumors compared to vehicle treatment, and this effect appeared to be augmented by E₂. Compared to vehicle, E₂ increased lordosis and uterine weight. Thus, E₂ may have the unfavorable effect of increasing proliferation when administered in chronic situations. Studies investigating the action of E₂ for these effects are ongoing.

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Introduction

The population is aging, and a trend towards better health care in industrialized nations has resulted in women, in particular, experiencing greater longevity. The life-expectancy for US women is now 80 years. The age of onset of menopause has remained stable. As such,

many women are living typically one-third to one-half of their lives in a 17β -estradiol (E_2)-deficient state (Minino et al. 2007). Ovarian cessation in E_2 production can be associated with physical symptoms and changes in quality-of-life/psychological measures (e.g., hot flashes, night sweats, genital dryness, cognition, anxiety, and mood changes) that can be relieved with E_2 -based therapies in some women. However, in recent years, following the publication of some of the negative findings from a large randomized controlled trial—the Women’s Health Initiative (WHI)—the benefits of the use of E_2 -based treatments in relation to their potential risks have been questioned and women have begun to reconsider their treatment options for alleviating menopausal symptoms (Rapp et al. 2003; Rossouw et al. 2002; Shumaker et al. 2003).

E_2 is a pleiotropic hormone that results in growth and differentiation. Acting as a mitogen it promotes breast and endometrial tumors, which account for ~40% of cancer incidence among women. The role of E_2 to increase risk for breast cancer is widely recognized, and greater breast cancer risk has been shown to be associated with high serum E_2 (Clemons and Goss, 2001; Toniolo et al. 1995; Thomas et al. 1997; Cauley et al. 1999; Hankinson et al. 1998) and administration of exogenous E_2 to postmenopausal women (Collaborative Group on Hormonal Factors in Breast Cancer, 1997; Magnusson et al. 1999; Colditz et al. 1995). A history of greater exposure to E_2 , such as occurs related to parity, early age of menarche, and late-onset menopause (Hulka 1996), is associated with an increased breast cancer risk (Hulka 1996; Lambe et al. 1996; Madigan et al. 1995; Ramon et al. 1996). Breast cancer risk also tends to be increased by other factors that increase circulating E_2 levels, such as obesity (Thomas et al. 1997). Furthermore, bilateral oophorectomy reduces endogenous E_2 levels and breast cancer risk in premenopausal women (Schairer et al. 1997; Meijer and van Lindert 1992). Although E_2 can be beneficial in reducing symptoms of menopause among some women, inconsistencies in these effects, and the association between E_2 and breast cancer risk remains a concern for postmenopausal women, ultimately reducing the enthusiasm for their use as treatment options.

Some of these limitations in the clinical studies of E_2 for its functional effects have been explored using animal models. A typical approach taken to model menopause and the effects of E_2 is ovariectomy (OVX), the surgical removal of the main endogenous

source of E_2 —the ovaries. For example, compared to young proestrous rats (with high physiological E_2 levels), OVX increases anxiety and depressive behavior and decreases sexual responding of rats (Frye et al. 1998; reviewed in Walf and Frye 2006; 2005b). These effects can be reversed with E_2 administration, but the magnitude of the response is influenced by dosing and/or the E_2 regimen utilized (reviewed in Walf and Frye 2006). Given the role of E_2 for functional and trophic processes, it is important to design an animal model in which the effects of E_2 for behavior and proliferation can be characterized concomitantly, and to determine whether there are differences in the potency of E_2 ’s effects for these processes. In Experiment 1, the duration of chronic E_2 exposure (i.e., 0, 2, or 6 months) associated with increased proliferation was determined. In Experiment 2, the dose-dependent effects of E_2 , administered once weekly for 3.5 months, on sexual behavior and proliferation were determined. Specifically, in this experiment, OVX rats were administered different dosages of E_2 (0, 0.03, or 0.09 mg/kg) and/or the chemical carcinogen 7, 12-dimethylbenz(a) anthracene (DMBA; 1.25, 12.50, 25.00 mg) or an inert control substance. Other studies have demonstrated dose and/or duration-dependent effects of DMBA for mammary and ovarian hyperplasia (Stewart et al. 2004; Ting et al. 2007). Sexual responding and proliferation (incidence of tumors, tumor weight, and uterine weight) were determined 3.5 months post-DMBA. We hypothesized that there would be duration and/or dose-responsive effects of E_2 and/or DMBA on behavior and/or proliferation.

Materials and methods

All methods utilized have been approved by the Institutional Animal Care and Use Committee at University at Albany- SUNY, and were performed in accordance with accepted standards of humane animal use.

Animals and housing

Subjects ($n=66$) were adult (4–8 months of age) female rats that were obtained from our breeding colony (original stock from Taconic Farms; Germantown, NY). Rats were group-housed (3–5 per cage) in polycarbonate cages (45×24×21 cm), containing woodchip shavings for bedding, in a temperature-controlled room

(21 ± 1 °C) in the Laboratory Animal Care Facility of The Life Sciences Research Building at The University at Albany-SUNY. Rats were maintained on a 12/12-h reversed light cycle (lights off at 8:00 a.m.) with ad libitum access to Rodent Chow and tap water in their home cages.

Procedure

In Experiment 1, the duration of chronic E_2 exposure that would produce measurable proliferative effects was determined. The rationale for this study was that we have previously observed an increased likelihood of rats that are OVX at 2–3 months of age and then administered chronic E_2 implants for typically 6–12 months to develop tumors, compared to control rats (OVX and not E_2 -replaced). As such, in the present study, we used a similar approach to compare the incidence of tumors and uterine weights in OVX rats that had not been exposed to E_2 ($n=4$), exposed to E_2 via chronically implanted silastic capsules filled with E_2 for 2 months ($n=4$) or 6 months ($n=6$). Tissues were collected when rats were 4–8 months of age. In this experiment, the possible dose-dependent effects of E_2 on proliferation were not determined and this is of interest. However, the high incidence of tumors in rats that were chronically administered E_2 for 6 months substantiated using a shorter (3.5 months versus 6) and less chronic (weekly SC injections vs implants) E_2 exposure paradigm so that dose-dependent effects could be determined. Furthermore, in Experiment 2, rats were administered the chemical carcinogen DMBA, and we based the length of the present study on several published reports (Cheung et al. 2003; Russo et al. 1990; Russo and Russo 1996, 1998; Ting et al. 2007). As such, in Experiment 2, whether there were additive effects of E_2 and carcinogen exposure for proliferation (i.e., tumorigenesis, uterine weight) and socio-sexual behavior was determined. Rats were OVX at 2 months of age and randomly assigned to 1 of 12 experimental groups ($n=3$ –5/group). Rats were administered DMBA once and then E_2 weekly. In week 6 of the protocol, rats were tested for sexual receptivity on one occasion 44–48 h after E_2 -priming. At 3.5 months after administration of DMBA, or the inert control substance, tumors (if present) and uterine tissue of rats were collected and weighed. Details of the methodology utilized are included in the following sections.

Ovariectomy

All rats were OVX under xylazine (12 mg/kg; Bayer, Shawnee Mission, KS) and ketamine (60 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) anesthesia. Surgery occurred at least 1 week before initiation of experimental protocol.

E_2 administration

In Experiment 1, OVX rats were chronically implanted with two silastic capsules filled with 17β - E_2 (Steraloids, Newport, RI). In Experiment 2, a more physiological regimen of E_2 was utilized to mimic endogenous fluctuations over the estrous cycle of rats and determine potential dose-dependent effects of E_2 . OVX rats were administered a vegetable oil vehicle (0.2 cc) or a low (0.03 mg/kg) or moderate (0.09 mg/kg) dosage of E_2 , once weekly for 3.5 months. This injection regimen was based upon our previous investigation of dose-dependent effects of E_2 . We found that 0.06–0.09 mg/kg E_2 SC to young, OVX rats 44–48 h before behavioral testing is effective at producing physiological levels of E_2 in plasma, akin to those observed when rats are naturally sexually receptive, and enhancing affective and sexual responding of rats (Frye et al. 1998; Walf and Frye 2005b). Sexual behavior testing occurred during week 6 of the protocol.

DMBA administration

In Experiment 2, rats were administered an inert control substance (i.e., vegetable oil) or the chemical carcinogen DMBA (Sigma, St. Louis, MO). DMBA was dissolved in vegetable oil to the following concentrations: 1.25, 12.50, or 25.00 mg (based upon Constantinou et al. 2001). DMBA was administered by gavage, using a needle that was 16-gauge, curved, 3 inches long, and had a 3 mm diameter ball on the end. Rats received a single administration of DMBA or vehicle.

Behavioral testing

In Experiment 2, rats were tested once to assess sexual behavior during week 6 of the protocol. Rats were tested for sexual behavior in a Plexiglas chamber (50×25×30 cm) using previously described methods (Frye et al. 1998; Walf and Frye 2005b). The frequency of lordosis (lordosis quotient; LQ) exhibited by experimental

female rats was recorded for 10 mounts or 10 min, whichever occurred first.

Proliferation measures and tissue collection

Proliferation measures were based upon tumorigenesis (incidence of tumor per group, number of tumors, mean wet weight of tumors) and uterine weight. Rats were euthanized by rapid decapitation. Rats were palpated and visually inspected during necropsy to determine presence of tumors. If tumors were present, they were dissected out, counted, and weighed. Uteri (not including fallopian tubes) of each rat were dissected out and weighed.

Statistical analyses

In Experiment 1, one-way analyses of variance tests (ANOVAs) were utilized to determine effects of the E_2 condition on uterine weights. In Experiment 2, two-way ANOVAs were utilized to determine the effects of E_2 dosage and DMBA dosage on all endpoints. If main effects were found, group differences were determined by Fisher's post hoc tests. A P -value of ≤ 0.05 was considered significant and ≤ 0.10 a tendency.

Results

Experiment 1

There was an increased incidence of tumors among rats that were administered E_2 for 6 months compared to those administered E_2 for 2 months or vehicle (Fig. 1). Among rats administered E_2 for 6 months with tumors, the average weight of these was 0.54 grams (± 0.31 SEM). Rats administered E_2 for 6 months had increased uterine weights compared to those administered short term E_2 or vehicle [$F(2,11)=8.19$, $P<0.01$] (Fig. 1).

Experiment 2

The incidence of rats developing tumors based upon E_2 and DMBA dosage is depicted in Fig. 2. There was a significant main effect of DMBA administration to increase mean number of tumors [$F(3,40)=6.30$, $P<0.01$] and tumor weight [$F(3,40)=6.80$, $P<$

Fig. 1 Tumor incidence (top) and uterine weight (bottom; means in grams $\pm$ SEM) of ovariectomized (OVX) rats administered estradiol (E_2) for 0, 2, or 6 months. * Significant ($P\leq 0.05$) effect of E_2 regimen vs other conditions

0.01] (Table 1). Compared to an inert substance, all dosages of DMBA increased the average number of tumors. The 1.25 mg dosage of DMBA significantly increased tumor weight compared to administration of vehicle or other dosages of DMBA. There was a significant main effect of E_2 to increase mean number of tumors [$F(2,40)=7.04$, $P<0.01$] and tumor weight [$F(2,40)=3.64$, $P<0.04$] (Table 1). Compared to vehicle, 0.03 or 0.09 mg/kg E_2 increased the average number of tumors and tumor weight.

There were significant main effects of E_2 [$F(2,40)=13.58$, $P<0.01$] and DMBA [$F(3,40)=3.10$, $P<0.04$] to increase uterine weight (Fig. 2). Compared to vehicle, 0.03 or 0.09 mg/kg E_2 increased uterine weights to a similar extent as DMBA administration.

Lordosis quotients of rats were altered by E_2 , but not DMBA administration (Fig. 3). There was a significant main effect of E_2 administration to increase [$F(2,40)=27.38$, $P<0.01$] LQ. Compared to vehicle, 0.03 or 0.09 mg/kg E_2 increased LQ.

Fig. 2 Tumor incidence (*top*) and uterine weight (*bottom*; means in grams $\pm$ SEM) of OVX rats administered estradiol (E_2) for 3.5 months and/or the chemical carcinogen, DMBA. * Significant ($P \leq 0.05$) effect of E_2 regimen vs other conditions

Discussion

The results of the present study partially supported our hypothesis that duration and dosing of E_2 exposure would alter proliferation, and that E_2 would increase sexual behavior of OVX rats. Exposure of OVX rats to E_2 for 6 months increased tumor incidence and uterine

weights compared to E_2 for 2 months or no such exposure. E_2 had similar effects when administered for 3.5 months in Experiment 2. Specifically, both dosages of E_2 enhanced sexual receptivity (i.e., lordosis in response to mounting by a male rat) compared to vehicle. Compared to no carcinogen exposure, rats administered DMBA had increased incidence, number,

Table 1 Number of tumors and tumor weight (mean $\pm$ SEN) of variectomized (OVX) rats administered E_2 and/or DMBA

DMBA condition	E_2 condition	Number of tumors	Tumor weight (g)
0	Vehicle	0	0
	0.03 mg/kg	0.4 $\pm$ 0.4	0.03 $\pm$ 0.03
	0.09 mg/kg	0.4 $\pm$ 0.2	0.02 $\pm$ 0.01
1.25 mg	Vehicle	1.0 $\pm$ 0.6 ^a	0.06 $\pm$ 0.05 ^a
	0.03 mg/kg	1.4 $\pm$ 0.4 ^{a,b}	0.15 $\pm$ 0.06 ^{a,b}
	0.09 mg/kg	1.2 $\pm$ 0.4 ^{a,b}	0.14 $\pm$ 0.04 ^{a,b}
12.50 mg	Vehicle	0.6 $\pm$ 0.4 ^a	0.01 $\pm$ 0.01 ^a
	0.03 mg/kg	1.6 $\pm$ 0.2 ^{a,b}	0.07 $\pm$ 0.02 ^{a,b}
	0.09 mg/kg	2.0 $\pm$ 0.0 ^{a,b}	0.05 $\pm$ 0.01 ^{a,b}
25.00 mg	Vehicle	0.3 $\pm$ 0.3 ^a	0.02 $\pm$ 0.00 ^a
	0.03 mg/kg	1.7 $\pm$ 0.3 ^{a,b}	0.05 $\pm$ 0.02 ^{a,b}
	0.09 mg/kg	1.7 $\pm$ 0.3 ^{a,b}	0.06 $\pm$ 0.01 ^{a,b}

^aSignificant main effect of DMBA vs no carcinogen exposure

^bSignificant main effect of E_2 vs vehicle administration. There was no interaction between these variables for these measures

Fig. 3 Mean ($\pm$ SEM) lordosis quotients (LQ) of OVX rats administered estradiol (E_2) and/or the chemical carcinogen DMBA. * Significant ($P \leq 0.05$) effect of E_2 vs vehicle

and weight of tumors. Irrespective of dose, E_2 increased tumor number and weight compared to vehicle. Compared to vehicle, both dosages of E_2 similarly increased uterine weight, irrespective of DMBA dosage. Taken together, these data suggest that E_2 has effects to increase sexual behavior of OVX rats as well as having detrimental trophic effects.

The current findings confirm previous data on the effects of E_2 on tumorigenic processes in animal models. Consistent with our results, administration of high dosages of E_2 benzoate via chronic SC implants produces mammary gland proliferation at 13 weeks and palpable tumors at 20 weeks following initiation of E_2 treatment in OVX Noble rats (Leung et al. 2003). In the present study, we found that tumor incidence was increased in OVX Long-Evans rats administered chronic E_2 for 6 months, compared to 2 months or no such E_2 exposure. In the follow-up experiment, we found that E_2 increased tumor burden among OVX rats, and, although not significant, there were apparent modest effects of E_2 on augmenting the effects of DMBA with respect to tumor burden. There was no clear interaction between E_2 and DMBA dosing because E_2 similarly increased tumorigenesis at each level of DMBA. DMBA itself increased tumorigenesis and uterine weights. Although the lowest dosage of DMBA produced tumors with the highest weights, other measures of tumorigenesis, such as incidence of tumors per group and number of tumors, demonstrated the more expected pattern of higher dosing of DMBA producing more robust effects. Other studies have demonstrated that DMBA reliably produces tumors, which are mostly adenocarcinomas and hormone-dependent (Cheung et al. 2003; Russo et al. 1990; Russo and Russo 1996, 1998). Dietary exposure of E_2 to OVX Big Blue transgenic rats that were exposed to DMBA increased

uterine dysplasia and cell proliferation (Aidoo et al. 2005). Hemiovariectomized rats administered DMBA and E_2 pellets had mammary dysplasia 3 and 6 months following initiation of treatment, whereas dysplasia was only noted at 6 months following treatment with DMBA alone (Ting et al. 2007). Furthermore, the effect of E_2 to increase mammary tumors following DMBA administration is not observed in animals that do not develop a mature hypothalamic-pituitary-gonadal axis (i.e., those that are castrated before puberty; Callejo et al. 2005). This suggests that prior E_2 exposure is an important variable in this model. Together, these data confirm that E_2 has trophic effects in animal models of hormone-dependent cancer.

In addition to confirming previously published findings on the effects of E_2 , the current findings extend these results by concomitantly investigating behavioral and tumorigenic processes in a whole animal model. The present data confirm the well-known effects of E_2 in promoting sexual response of OVX female rats. Although other studies have separately investigated the effects of E_2 on psychological symptoms and trophic effects, it is becoming increasingly important to look at both of these processes. Indeed, expression of steroid receptors in mammary glands of women with breast cancer may be associated with psychological symptoms (Razavi et al. 1990). Furthermore, the role of ovarian steroids, such as progesterone and its metabolites, needs to be taken into consideration. This may be particularly important because E_2 increases formation of progesterone's metabolites, and thus can enhance its functional effects (Cheng and Karavolas 1973; Rhodes and Frye 2005; Vongher and Frye 1999). We and others have demonstrated the robust effects that E_2 as well as progesterone and its metabolites have on affective and socio-sexual behavior (reviewed in Walf and Frye 2006; reviewed in Frye et al. 2006). As such,

investigating concomitant changes in psychological/behavioral effects and trophic effects of E_2 and how these effects may be altered by other steroids is timely and clinically relevant. Of interest would be to further investigate these effects in our model using older, anestrus or OVX rats, or with clinically utilized E_2 -based therapies. In the present study, it was necessary to determine the dose-dependent effects of E_2 —a primary estrogen secreted by the ovaries of women and rodents. However, although the regimen utilized was physiological to rats, women are not prescribed E_2 and would typically be administered more chronic (not once weekly) dosing of E_2 -based therapies, such as conjugated equine estrogens. Although few studies have investigated the effects of conjugated equine estrogens in rodent models of breast cancer, mammary carcinogenesis was increased in OVX rats administered conjugated equine estrogens alone or with a commonly prescribed progestin therapy, medroxyprogesterone acetate (Sakamoto et al. 1997). Future studies will further investigate the role and mechanisms of E_2 and E_2 -based therapies for these effects on behavior and proliferation.

An important topic in investigation of the therapeutic efficacy of E_2 is related to the differential distribution and actions of its cognate receptors, $ER\alpha$ and $ER\beta$. Compared to $ER\alpha$, $ER\beta$ is not highly concentrated in peripheral tissues (uterus, breast) that are sensitive to the proliferative/carcinogenic effects of E_2 (as reviewed in Gustafsson 2003). Importantly, $ER\beta$ is more widely distributed in the limbic regions of the brain, such as the hippocampus, which may account for some of the beneficial effects associated with this receptor subtype for affective and cognitive behavior (Shughrue et al. 1997; 1998; reviewed in Walf and Frye 2006). $ER\beta$ expression may be associated with better prognosis among women with breast cancer (reviewed in Shupnik 2007; Sugiura et al. 2007). Furthermore, the interaction between $ER\alpha$ and $ER\beta$ and/or other “non-genomic” membrane actions (e.g., GPR30) and/or downstream signaling pathways (e.g., MNAR; reviewed in Cheskis et al. 2007; 2008) are of interest in terms of the diverse actions of E_2 on the heart (myocytes, anti-apoptotic actions, nitric oxide synthesis, Ca^{++} channels, fibroblasts, anti-proliferation), liver (increase HDL, decrease LDL, increase CRP), blood vessels (vascular tone, anti-oxidant, atherosclerosis, angiogenesis), breast and uterus (proliferative, angiogenic), bone (proliferative), and CNS (trophic, anti-

apoptotic). Indeed, it is critical to discern the receptor mechanisms that are important for beneficial vs unwanted proliferative effects of E_2 in future studies utilizing our model.

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