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PRECLINICAL STUDY

Effects of estradiol with micronized progesterone or medroxyprogesterone acetate on risk markers for breast cancer in postmenopausal monkeys

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Abstract The addition of the synthetic progestin medroxyprogesterone acetate (MPA) to postmenopausal estrogen therapy significantly increases breast cancer risk. Whether this adverse effect is specific to MPA or characteristic of all progestogens is not known. The goal of this study was to compare the effects of oral estradiol (E2) given with either MPA or micronized progesterone (P4) on risk biomarkers for breast cancer in a postmenopausal primate model. For this randomized crossover trial, twenty-six ovariectomized adult female cynomolgus macaques were divided into social groups and rotated randomly through the following treatments (expressed as equivalent doses for women): (1) placebo; (2) E2 (1 mg/day); (3) E2 + P4 (200 mg/day); and (4) E2 + MPA (2.5 mg/day). Hormones were administered orally, and all animals were individually dosed. Treatments lasted two months and were separated by a one-month washout period. The main outcome measure was breast epithelial proliferation, as measured by Ki67 expression. Compared to placebo, E2 + MPA resulted in significantly greater breast proliferation in lobular ($P < 0.01$) and ductal ($P < 0.01$) epithelium, while E2 + P4 did not. Intramammary gene expression of the proliferation markers Ki67 and cyclin B1 was also higher after treatment with E2 + MPA ($P < 0.01$) but not E2 + P4. Both progestogens significantly attenuated E2 effects on body weight, endometrium, and the TFF1 marker of estrogen receptor activity in the breast. These findings suggest that oral micronized progesterone has a more favorable effect on risk biomarkers for postmenopausal breast cancer than medroxyprogesterone acetate.

Keywords Hormone Therapy · Breast Cancer · Progesterone · Medroxyprogesterone Acetate · Estrogen

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Introduction

Approximately 5,000 women a day in the U.S. enter the menopausal period [1]. Until recently, almost half of these women took some form of hormone therapy (HT) [2]. The most common type of combination estrogen plus progestin HT is conjugated equine estrogens (CEE) and medroxyprogesterone acetate (MPA). Prescriptions for CEE + MPA approached 20 million in 2001 but have since declined dramatically following release of results from the Women's Health Initiative (WHI) Trial [2]. Data from this large randomized clinical trial indicated that adding MPA to CEE significantly increased the risk of breast cancer [3–5], confirming results from numerous observational studies [6–8]. This evidence has contributed to widespread concern regarding progestin use and increased interest in alternative hormone therapies for postmenopausal women.

A major question regarding the WHI results is whether the adverse effects attributed to MPA can be

generalized to other progestogens [9], which may vary considerably in biologic activities [9–11]. Recently, micronized progesterone (P4) has attracted attention as a ''bioidentical'' hormone alternative to synthetic progestins [12]. Progesterone has not been used in traditional HT regimens due largely to low bioavailability when taken orally. This problem has been overcome through micronization [13], and oral micronized P4 is now approved for use in the U.S. [14]. Limited observational data show potentially favorable effects of P4 on breast cancer risk relative to synthetic progestins [15], and cell culture studies point to less proliferative and more pro-apoptotic activity compared to MPA [16]. Nevertheless, the direct effects of oral micronized P4 in the breast are currently unknown. To address this, we evaluated the breast effects of MPA and micronized P4 when given with estradiol (E2) in a postmenopausal primate model.

Materials and methods

Animal subjects

Twenty-six adult female cynomolgus monkeys (*Macaca fascicularis*) were obtained from Primate Products (Miami, FL) at an average age of 6.5 ± 0.3 years. The animals were randomly divided into social groups of 3–5 animals and maintained for three months to allow social acclimation. Animals were then ovariectomized and maintained for a 6-month baseline period for oral dose training and for physiologic adjustment to a postmenopausal state. One animal was removed from analysis due to residual ovarian activity (based on high serum progesterone values during washout periods). Throughout the experiment the animals were fed a standard control diet based on the typical North American diet.

Female cynomolgus macaques have distinctive reproductive similarities to women, including a 28-day menstrual cycle, comparable ovarian hormone patterns, and natural ovarian senescence [17, 18]. Macaques have >95% overall genetic coding sequence identity to humans [19], including key genes involved in breast cancer susceptibility [20]. Human and macaque mammary glands are similar in terms of microanatomy and development [21, 22], sex steroid receptor expression [23], responses to exogenous hormones [24], and the development of a heterogeneous spectrum of hyperplastic and neoplastic lesions in older animals [25].

All procedures in this study were conducted in compliance with State and Federal laws, standards of the U.S. Department of Health and Human Services, and guidelines established by the Wake Forest University Animal Care and Use Committee. The facilities and laboratory animal program of Wake Forest University are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

Study design and treatments

This study was a randomized, placebo-controlled, crossover trial in which all animals received each of the following four experimental treatments (expressed as equivalent hormone doses for women): (1) placebo; (2) micronized 17β-estradiol (E2), 1 mg/day (Estrace; Mylan Pharmaceuticals, Morgantown, WV, and Bristol-Myers Squibb, New York, NY); (3) E2 + micronized progesterone (P4), 200 mg/day (Prometrium; Solvay Pharmaceuticals, Marietta, GA); and (4) E2 + medroxyprogesterone acetate (MPA), 2.5 mg/day (Provera; Barr Laboratories, Pomona, NY). Absolute doses were as follows (in mg/kg body weight): 0.05 for E2, 11.1 for P4, and 0.139 for MPA. Hormone doses were designed to represent clinically-used regimens approved for postmenopausal women. Dose scaling was based on caloric intake rather than body weight to better account for species differences in metabolism. Treatments were administered orally for two months, followed by a one-month washout period during which all animals were dosed with a placebo. Breast biopsies, serum samples, and other measures were taken at the end of each treatment period.

The animals were dosed each morning between 8:00 and 10:00 am. Oral doses of estradiol and MPA were administered within a fruit punch (Crystal Lite®) vehicle, while the progesterone (P4) dose was injected into a small marshmallow or piece of fruit (banana or tangerine). Micronized P4 is formulated in a peanut oil-based vehicle, and the marshmallow or fruit dosing of P4 was used to avoid immediate parenteral absorption. Control animals received a placebo fruit punch, and all non-P4 animals were given a placebo marshmallow or fruit piece. For dosing, all animals were trained to enter a catch cage, drink the fruit punch from a syringe, and then eat the marshmallow or fruit. Distinctive shave patterns and tattoos allowed for easy identification of individual animals within each social group. The amount of fruit punch intake was recorded daily for each animal as follows: A = all, B = all but a few drops, M = most, S = some, or N = none. Dosing compliance was high, with 96.3% of all dosings falling in the A, B, or M categories. Individual drug doses were calculated based on body

weight at the start of each dosing phase. Body weights were then monitored at 2 weeks and 4–6 weeks into each treatment period. Doses were recalibrated for a particular individual animal if a change in body weight ≥0.5 kg was found. This change occurred in a total of 6 animals across the study.

Serum estrogens and progesterone

Serum estradiol (E2), estrone (E1), and progesterone (P4) concentrations were measured from samples collected 2–3 h and 24–28 h after dosing. Blood was collected by femoral venipuncture following sedation with ketamine and stored at –70°C. Estrogen and P4 concentrations were quantitated by radioimmunoassay using commercially available kits and protocols from Diagnostic Systems Laboratories (E2, DSL–4800 ultrasensitive; E1, DSL-8700; P4, Coat-A-Count; Webster, TX, USA). For E2 and P4 assays, serum was extracted with ethyl ether using standard procedures. Intra- and inter-assay coefficients of variation (CV) were $<10\%$ for all serum assays except for P4, which had an inter-assay CV of 11%.

Breast biopsies

At the end of each treatment period, the animals were anesthetized with ketamine and buprenorphine for breast biopsy, blood collection, uterine ultrasound, vaginal cytology, and body weight measurement. For the breast biopsy, a 1.5-cm incision was made in a preselected breast quadrant and a small (~0.4 gram) sample of mammary gland was removed. Biopsies were performed by an experienced veterinary surgeon (CJL). The incision was sutured, and the animals were monitored and given analgesia during recovery following ACUC-approved clinical procedures. The biopsy site was tattooed to prevent later resampling near the same site. Half of the biopsy sample was frozen; the other half was fixed at 4°C in 4% paraformaldehyde for 24 h, transferred to 70% ethanol, and then processed for histology using standard procedures.

Immunohistochemistry

Immunostaining procedures were performed on fixed, paraffin-embedded mammary gland tissues using commercially-available primary monoclonal antibodies for the proliferation marker Ki67 (Ki67/MIB1) (Dako, Carpinteria, CA), the apoptosis marker cleaved caspase 3 (CC3) (Cell Signaling Technologies, Beverly, MA), and the sex steroid receptors estrogen receptor alpha (ERα) (NCL-ER-6F11, Novocastra, Newcastle-upon-Tyne, UK) and progesterone receptor (PGR) (NCL-PGR, Novocastra). Staining methods included antigen-retrieval with citrate buffer (pH 6.0), biotinylated rabbit anti-mouse F_c antibody as a linking reagent, alkaline phosphatase-conjugated streptavidin as the label, and Vector Red as the chromogen (Vector Laboratories, Burlingame, CA). Cell staining was quantified by a computer-assisted counting technique, using a grid filter to select cells for counting and our modified procedure of cell selection [23, 24]. Numbers of positively stained cells were measured as a percentage of the total number examined (100 cells). All measurements were made blinded to treatment group.

Endometrial area

Endometrial area was determined by trans-abdominal ultrasound using a Sonosite 180 portable ultrasound machine with a 5.0 MHz linear transducer (Sonosite, Bothell, WA). Maximal transverse cross-sectional area was measured on static representative digital images using public domain software (NIH ImageJ 1.33j, available at http://rsbweb.nih.gov/ij/).

Vaginal maturation

Vaginal keratinocytes were collected with a cotton swab, rolled onto a glass slide, and fixed using a cytologic fixative (Spray-cyte, Surgipath Medical Industries, Richmond, IL). Slides were stained using a modified Papanicolau method. Maturation value (MV) was calculated using the following formula: MV = (0.2 × % parabasal cells) + (0.6 × % intermediate cells) + (% superficial cells).

Intramammary gene expression

Expression of key genes associated with breast proliferation (Ki67, cyclin B1), apoptosis/cell survival (BAD, survivin), and estrogen receptor expression / activity (ERα, PGR, TFF1) were measured in breast samples using quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR). Macaque-specific qRT-PCR primer-probe sets for internal control genes (GAPDH, β-Actin) and other targets (BAD, survivin, ERα, TFF1) were generated through the Applied Biosystems (ABI) Taqman Assay-by-Design service (Foster City, CA), while human Taqman assays were used for Ki67, cyclin B1, and PGR. Target macaque cDNA sequences were determined either from published sequences or from PCR product sequences generated using human primer sets. GenBank

accession numbers for macaque sequences are as follows: GAPDH, DQ464111; β-Actin, DQ464112; BAD, AB220436; survivin, AB168802; ERα, DQ469336; and TFF1, DQ464113. To eliminate genomic DNA contamination, all probes were designed to span an exon–exon junction. Breast RNA was extracted, purified, quantitated, qualitatively evaluated for intactness, and reverse transcribed using techniques described previously [26]. Quantitative RT-PCR reactions (20 μl volume) were performed on an ABI Prism 7000 Sequence Detection System using standard Taqman reagents. The thermocycling protocol has been described elsewhere [26]. Relative expression was determined using the ΔΔ*Ct* method described in ABI User Bulletin #2 (available online at http://www.ukl.uni-freiburg.de/core-facility/taqman/user_bulletin_2.pdf). The *Ct* values for the control genes GAPDH and β-Actin were averaged for use in internal calibration, while reference breast tissue RNA was run in parallel for plate-to-plate calibration. Calculations were performed utilizing ABI Relative Quantification SDS Software v1.1.

Histomorphometry

Breast morphology was quantified by histomorphometry as described previously [24]. Briefly, H&E-stained slides were digitized using a Hitachi VK-C370 camera and video capture board (Scion LG-3; Scion, Frederick, MD), and measurements were taken with public domain software (NIH ImageJ 1.33j). Three microscopic fields were randomly selected and examined at 20× magnification. Epithelial area was determined by manual tracing of lobuloalveolar units and expressed as a percentage of the total area examined. All tissues were also examined for histologic changes by a board-certified veterinary pathologist (JMC).

Statistics

A mixed general linear model with repeated measures was used to determine means and to test for treatment effects. Control, E2, E2 + P4, and E2 + MPA treatments were modeled as fixed effect variables. To evaluate any potential carryover effects, phase and treatment in the previous phase were incorporated as fixed effect covariates for all tissue endpoints. Variables were also screened in the initial model for phase-by-treatment interactions. All variables were evaluated for their distribution and equality of variances among diets, and $\log_{10}$ transformations were performed where appropriate to improve homogeneity of variance. Data are reported either as mean (± standard error) for untransformed data or mean (90% confidence interval) for retransformed data. A subset of mammary gland samples (n = 1–4 per group) did not have epithelial tissue on histologic sectioning and were excluded from immunohistochemical analyses. Endometrial area was unmeasurable in a subset of ultrasound images (n = 1–7 per group). Two RNA samples were excluded due to poor intactness. Data were analyzed using the SAS statistical package (version 8; SAS Institute, Cary, NC). A two-tailed significance level of 0.05 was chosen for all comparisons.

Results

Body weight

Body weight decreased significantly during E2 treatment compared to control (P = 0.002) (Table 1). This effect was attenuated by the addition of P4 (P = 0.08 vs. control) and MPA (P = 0.33 vs. control). Percent change in BW during treatment was + 2.4% for control, –8.7% for E2, –2.4% for E2 + P4, and –0.8% for E2 + MPA. Weight gain during the washout periods was higher following hormone treatments than after placebo (P < 0.05 for all).

Reproductive tract measures

The primary indication for progestin use by postmenopausal women is protection of the endometrium from

Table 1 Treatment effects on body weight and reproductive tract measures[1, 2]

	Control	E2	E2 + P4	E2 + MPA
Body weight (kg)				
Pre-treatment	4.17 ± 0.16^{a,b}	4.24 ± 0.16^{a}	4.20 ± 0.16^{a}	4.03 ± 0.16^{b}
Post-treatment	4.25 ± 0.16^{a}	3.84 ± 0.16^{b}	4.07 ± 0.16^{c}	4.00 ± 0.16^{c}
Change during treatment	0.10 ± 0.06^{a}	–0.40 ± 0.05^{b}	–0.13 ± 0.06^{c}	–0.03 ± 0.05^{a}
Change during washout	0.06 ± 0.02^{a}	0.14 ± 0.02^{b}	0.16 ± 0.03^{b}	0.18 ± 0.02^{b}
Endometrial area (cm^2)	0.16 ± 0.02^{a}	0.34 ± 0.02^{b}	0.23 ± 0.04^{a}	0.21 ± 0.03^{a}
Vaginal maturation index	57.1 ± 3.2^{a}	88.7 ± 4.5^{b}	81.1 ± 4.8^{b}	76.6 ± 4.0^{b}

[1] Control = Placebo; E2 = 17β-Estradiol; P4 = Micronized progesterone; MPA = Medroxyprogesterone acetate

[2] Values represent mean ± standard error. Different letters indicate significant group differences (P < 0.05)

Table 2 Treatment effects on serum estrogen and progesterone concentrations[1–4]

		Control	E2	E2 + P4	E2 + MPA
Estradiol (pg/ml)					
2 week:	2–3 h PD	–	323.2^{a} (290.4, 359.8)	218.6^{b} (195.9, 243.8)	310.7^{a} (279.1, 345.8)
2 month:	24–28 h PD	$<5^{a}$	23.9^{b} (21.0, 27.3)	12.1^{c} (10.6, 13.8)	22.0^{b} (19.4, 25.1)
Estrone (pg/ml)					
2 month:	24–28 h PD	51.0^{a} (46.6, 55.8)	444.0^{b} (405.8, 485.8)	300.1^{c} (273.4, 329.5)	402.0^{b} (367.4, 439.8)
Progesterone (ng/ml)					
2 week:	2–3 h PD	–	0.82^{a}(0.63, 1.03)	13.69^{b}(12.09, 15.49)	0.71^{a} (0.53, 0.91)
2 month:	24–28 h PD	0.68^{a} (0.57, 0.79)	0.99^{a,b} (0.86, 1.12)	2.51^{c} (2.28, 2.76)	1.08^{b} (0.95, 1.22)

[1] Control = Placebo; E2 = 17β-Estradiol; P4 = Micronized progesterone; MPA = Medroxyprogesterone acetate

[2] Serum was collected 2–3 hours post-dosing (PD) 2 weeks into each treatment period and 24–28 h post-dosing at the end of each 2-month treatment period

[3] For conversion to SI units, multiply by the following conversion factors: 3.70 for estrone (pmol/l), 3.67 for estradiol (pmol/l), and 3.18 for progesterone (nmol/l)

[4] Values represent means (90% CI). Different letters indicate significant group differences ($P < 0.05$)

stimulation by unopposed estrogen. To confirm this effect, endometrial area was evaluated using transabdominal ultrasound. Endometrial area following E2 treatment was significantly greater compared to other treatments ($P < 0.05$ for all), while E2 + P4 and E2 + MPA were not significantly different from control or from each other (Table 1). Vaginal maturation index, which is a sensitive measure of systemic estrogen exposure, was significantly higher in all hormone treatment groups compared to control ($P < 0.001$ for all) (Table 1).

Serum estrogens and progesterone

The range of serum estrogen and progesterone concentrations were similar to those reported for women taking comparable oral hormone doses [13, 27] (Table 2). Serum estradiol concentrations were significantly higher in all hormone treatment groups compared to control ($P < 0.0001$ for all) at the 2-month lag timepoint. However, serum E2 was significantly lower after E2 + P4 treatment compared to E2 and E2 + MPA ($P < 0.001$) at both acute and lag timepoints. A similar pattern was noted for estrone measured at the 2-month lag timepoint. Progesterone concentrations were significantly higher following E2 + P4 treatment at both timepoints compared to all groups ($P < 0.0001$). Serum P4 was also higher after E2 + MPA in the lag ($P = 0.02$) but not acute samples ($P = 0.67$), likely due to metabolism of MPA to a cross-reactive species. Serum estradiol and progesterone values from a subset of washout samples were <5 pg/ml (<18 pmol/l) and 0.56 ± 0.07 ng/ml (1.78 nmol/l), respectively.

Breast epithelial proliferation

The primary outcome for this study was breast epithelial proliferation, as measured by expression of the Ki67 (MIB1) nuclear antigen. Ki67 expression is an important prognostic indicator in human breast cancer [28] which has been used extensively in our model to predict risk associated with hormonal agents [24, 29–31]. Compared to placebo treatment, lobular proliferation was 99% greater after E2 ($P = 0.09$), 58% greater after E2 + P4 ($P = 0.47$), and 194% greater after E2 + MPA ($P = 0.009$) (Fig. 1A). Ductal proliferation was also significantly higher after E2 + MPA treatment (+544%, $P = 0.006$ vs. placebo) but not E2 (–38%, $P = 0.80$) or E2 + P4 (+75%, $P = 0.72$). Intramammary expression of Ki67 mRNA followed a pattern similar to that seen by immunostaining, with the greatest expression occurring after E2 + MPA treatment (4.9-fold increase, $P = 0.007$ vs. control) compared to E2 (3.0-fold increase, $P = 0.03$ vs. control) and E2 + P4 (2.5-fold increase, $P = 0.22$ vs. control) (Fig. 1B). We also measured transcript levels of cyclin B1, a key molecule in cell cycle progression specific to the G2 to M phase transition. Cyclin B1 mRNA was significantly increased after E2 + MPA treatment (4.3-fold increase, $P = 0.002$ vs. control) but not E2 (0.4-fold increase, $P = 0.47$ vs. control) or E2 + P4 (1.5-fold increase, $P = 0.14$ vs. control) (Fig. 1D). We should note that for Ki67 and cyclin B1 gene expression, a significant phase × treatment interaction was detected ($P < 0.01$).

Breast epithelial apoptosis

Prior studies have reported differences among progestogens in their ability to induce apoptosis [16] and maintain cell survival [32]. To investigate this idea we first measured expression of the terminal apoptosis marker cleaved caspase 3 (CC3), a central molecule in the induction of apoptosis [33]. Breast immunostaining for this marker was generally low compared to Ki67, with <5% of cells staining overall and no significant

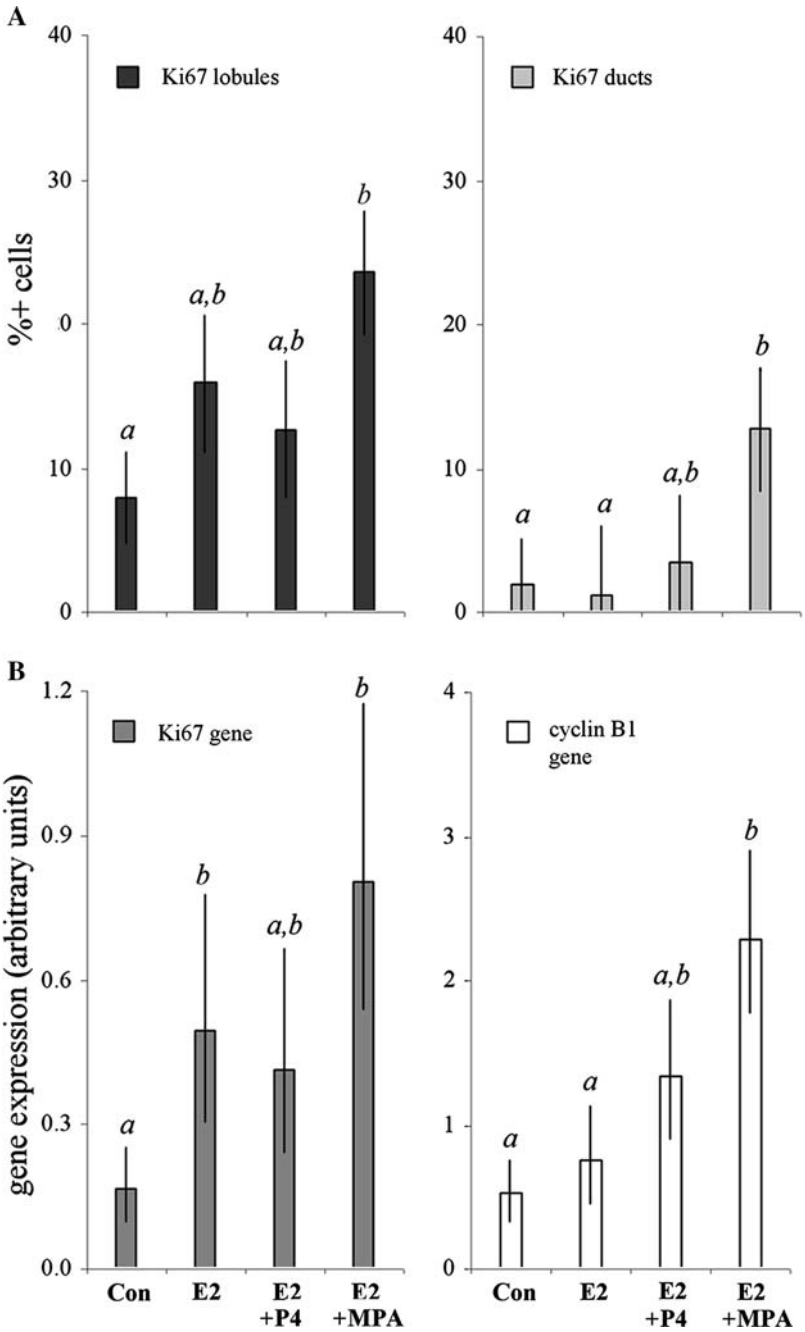

Fig. 1 Hormone effects on cellular proliferation in the breast. (**A**) Immunostaining for the proliferation marker Ki67 in lobular and ductal epithelium. (**B**) Intramammary expression of mRNA transcripts for Ki67 and cyclin B1, as determined by quantitative RT-PCR. Treatment groups were placebo (Con), estradiol (E2), estradiol + micronized progesterone (E2 + P4), and estradiol + medroxyprogesterone acetate (E2 + MPA). Different letters indicate significantly different values between groups ($P < 0.05$). Vertical lines indicate standard error

main treatment effect in either lobular ($P = 0.46$) or ductal ($P = 0.97$) epithelium (Fig. 2A). We next measured intramammary expression of the pro-apoptotic marker BAD (BCL2-antagonist of cell death) and the cell survival marker survivin. No significant main treatment effects were seen for either BAD ($P = 0.36$) or survivin ($P = 0.62$) (Fig. 2B).

Breast epithelial area

Breast epithelial area was quantified as a surrogate marker of mammary gland density. For this measure, epithelial regions in a section of breast tissue were traced by digital morphometry and expressed as a percent of total tissue area. Calculated epithelial area was 2.6% for control, 5.0% for E2, 1.9% for E2 + P4, and 6.5% for E2 + MPA, although the main treatment effect was not significant ($P = 0.15$). Adjusting for body weight or body mass index had negligible effects on this outcome.

Breast estrogen receptor expression and activity

We next measured breast expression of the two major sex steroid receptors mediating estrogen and progestin

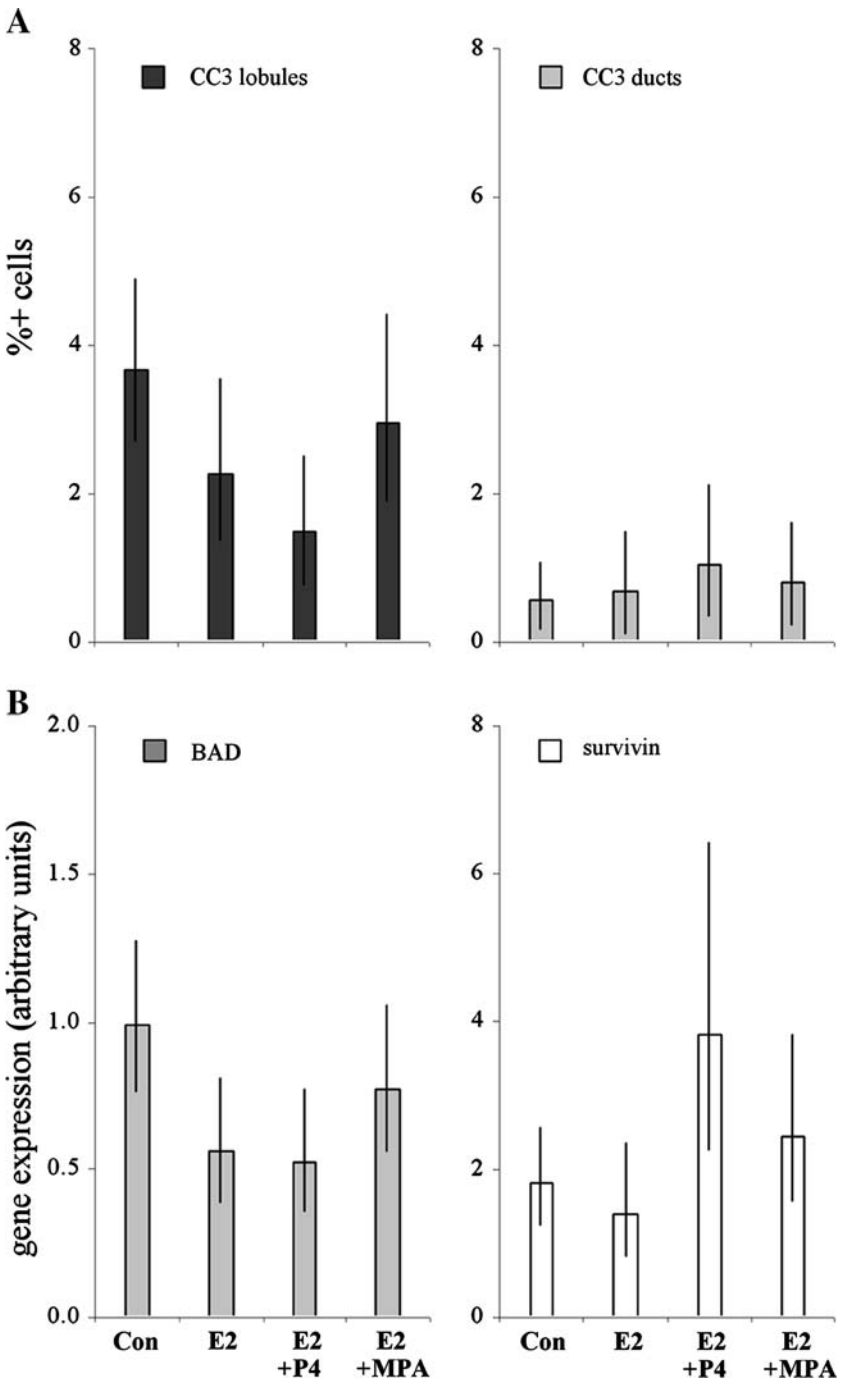

Fig. 2 Hormone effects on apoptosis in the breast. (**A**) Immunostaining for the apoptosis marker cleaved caspase 3 (CC3) in lobular and ductal epithelium. (**B**) Intramammary expression of mRNA transcripts for apoptotic (BAD) and cell survival (survivin) markers, as determined by quantitative RT-PCR. Treatment groups were placebo (Con), estradiol (E2), estradiol + micronized progesterone (E2 + P4), and estradiol + medroxyprogesterone acetate (E2 + MPA). No significant main treatment effects were found for any of these measures. Vertical lines indicate standard error

effects, estrogen receptor alpha (ERα) and progesterone receptor (PGR). No main treatment effects were seen for ERα expression in either lobular ($P = 0.20$) or ductal ($P = 0.11$) epithelial cells on immunohistochemistry (Fig. 3A) or for intramammary ERα gene expression ($P = 0.28$) (data not shown). All hormone treatments resulted in higher PGR protein expression in lobular and ductal epithelium; however, a significant carryover effect was detected for PGR immunostaining ($P = 0.001$ in lobules, $P = 0.05$ in ducts), excluding this data from further analysis. No such carryover was noted for PGR gene expression ($P = 0.88$), which was significantly higher after all hormone treatments ($P < 0.0001$ for E2, $P = 0.02$ for E2 + P4, and $P = 0.001$ for E2 + MPA vs. control) (Fig. 3B).

Despite minimal effects on ERα expression, a striking treatment effect was seen for ERα activation, as determined by gene expression of the marker trefoil factor 1 (TFF1, also known as pS2). This gene is driven by a near-consensus estrogen response element (ERE)

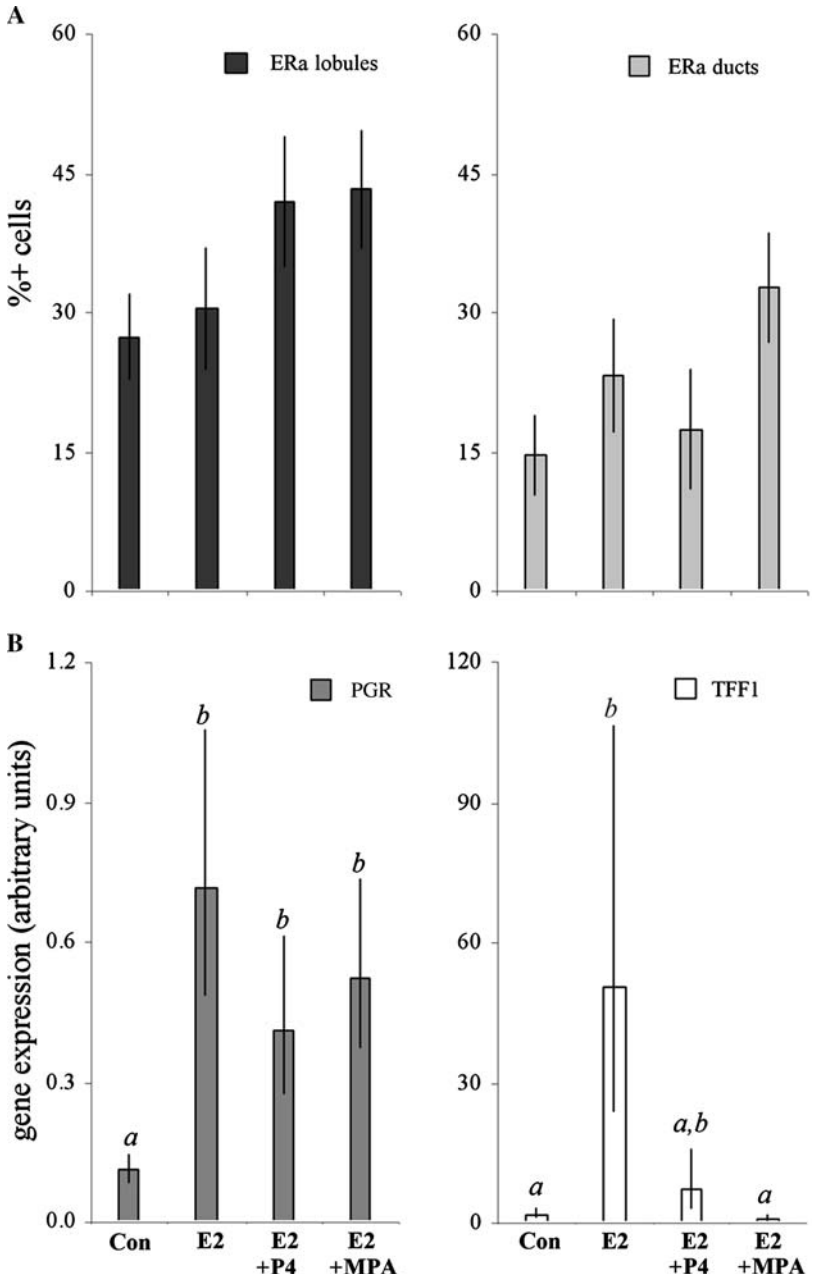

Fig. 3 Hormone effects on estrogen receptor alpha (ERα) and gene markers of estrogen receptor activity in the breast. (**A**) Immunostaining for ERα protein in lobular and ductal epithelium. (**B**) Intramammary expression of mRNA transcripts for progesterone receptor (PGR) and trefoil factor 1 (TFF1). Treatment groups were placebo (Con), estradiol (E2), estradiol + micronized progesterone (E2 + P4), and estradiol + medroxyprogesterone acetate (E2 + MPA). Different letters indicate significant differences between groups. Vertical lines indicate 90% CI

in the promoter region, and TFF1 expression serves as a sensitive indicator of ligand-dependent ER activity [34]. Expression of TFF1 was markedly higher after E2 treatment (+2656%, $P < 0.0001$ vs. control), an effect significantly diminished by the addition of P4 (+381%, $P = 0.22$ vs. control) or MPA (–51%, $P = 0.42$ vs. control) (Fig. 3B).

Discussion

The purpose of this study was to compare the effects of micronized progesterone and the synthetic progestin MPA on risk markers in the postmenopausal breast. Using an established primate model we found that progesterone resulted in less breast epithelial proliferation than MPA. No treatment effects were seen on measures of breast apoptosis or cell survival, while progesterone and MPA both attenuated estradiol effects on body weight, endometrium, and estrogen receptor activity in the breast.

Findings from the WHI [3–5] and several large observational studies [6–8] indicate that the addition of MPA to CEE may increase breast cancer risk in postmenopausal women by at least 30%. Consistent with this evidence, several previous studies in our

model have reported that adding MPA to CEE enhances overall breast epithelial proliferation by 30–50% [24, 29–31]. In the current study we again found increased proliferation with the addition of MPA to an estrogen, in this case 17β-estradiol. A corresponding effect was not seen with micronized progesterone. These results support a small body of prior evidence suggesting that progesterone may have a safer risk profile in the breast compared with synthetic progestins. Most recently, a cohort study of over 50,000 postmenopausal women in France reported significantly greater breast cancer risk in postmenopausal women using estrogen with a synthetic progestin but not those using an estrogen with oral micronized P4. The difference in risk between HT regimens with synthetic progestins and micronized P4 was highly significant ($P < 0.001$) [15]. In the Postmenopausal Estrogen/Progestin Interventions (PEPI) clinical trial, both CEE + MPA and cyclic CEE + P4 were associated with significantly higher mammographic density, although the increase was ~35% less with CEE + P4 [35]. Lastly, two randomized clinical trials evaluating topical hormone administration before lumpectomy or cosmetic breast surgery both reported lower breast epithelial proliferation for E2 + P4 compared to E2 alone [36, 37]. While more data are clearly needed, these findings provide growing support for micronized progesterone as an alternative to MPA in postmenopausal hormone therapy regimens.

Mechanisms underlying the stimulatory effects of MPA in the breast are unknown. Data from this study demonstrate it is not due simply to global augmentation of ER-responses, as both progestogens inhibited expression of the ERE-driven TFF1 gene. This estrogen-antagonist type of effect is well-documented in the endometrium [38] but has not been noted previously in normal breast tissue. Estrogen-induced expression of PGR, which is upregulated by cross-talk between agonist-bound ER and AP–1 and Sp1 transcription factors [39], was not significantly altered by either progestagen, suggesting some type of specificity for progestagen-mediated ER effects. While these findings document clear interactions between progestogens and estrogen function in the breast, more research is needed to identify specific pathways responsible for any differential effects of P4 and MPA on breast proliferation.

The addition of progesterone to E2 resulted in significantly lower serum estradiol and estrone compared to E2 and E2 + MPA treatments. The cause of this unanticipated effect is not clear but may relate to a combination of factors, including greater estrogen metabolism, decreased gut absorption, and increased clearance. The presence of this effect in both acute and lag samples suggests that gut absorption and/or altered hepatic first pass metabolism may be primary contributors. While adjustment for serum E2 did not strongly affect intergroup differences, we cannot exclude the potential influence of altered serum E2 on the observed effects of P4 and MPA.

Data from this study indicate that important biologic differences may exist among different types of progestogens used for postmenopausal hormone therapy. Specifically, our results suggest that micronized progesterone may have a more favorable effect on risk biomarkers for breast cancer than MPA. Further studies are needed to confirm this finding and to identify cellular mechanisms for this difference. While preliminary, these findings represent an important step in characterizing the safety of oral micronized progesterone on the breast.

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