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Estrogen-induced Proliferation of Uterine Epithelial Cells Is Independent of Estrogen Receptor α Binding to Classical Estrogen Response Elements*

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Acting via the estrogen receptor (ER), estradiol exerts pleomorphic effects on the uterus, producing cyclical waves of cellular proliferation and differentiation in preparation for embryo implantation. In the classical pathway, the ER binds directly to an estrogen response element to activate or repress gene expression. However, emerging evidence supports the existence of nonclassical pathways in which the activated ER alters gene expression through protein-protein tethering with transcription factors such as c-Fos/c-Jun B (AP-1) and Sp1. In this report, we examined the relative roles of classical and nonclassical ER signaling *in vivo* by comparing the estrogen-dependent uterine response in mice that express wild-type ER α , a mutant ER α (E207A/G208A) that selectively lacks ERE binding, or ER α null. In the compound heterozygote (AA/–) female, the nonclassical allele (AA) was insufficient to mediate an acute uterotrophic response to 17 β -estradiol (E₂). The uterine epithelial proliferative response to E₂ and 4-hydroxytamoxifen was retained in the AA/– females, and uterine luminal epithelial height increased commensurate with the extent of ER α signaling. This proliferative response was confirmed by 5-bromo-2'-deoxyuridine incorporation. Microarray experiments identified cyclin-dependent kinase inhibitor 1A as a nonclassical pathway-responsive gene, and transient expression experiments using the cyclin-dependent kinase inhibitor 1A promoter confirmed transcriptional responses to the ER α (E207A/G208A) mutant. These results indicate that nonclassical ER α signaling is sufficient to restore luminal epithelial proliferation but not other estrogen-responsive events, such as fluid accumulation and hyperemia. We conclude that nonclassical pathway signaling via ER α plays a critical physiologic role in the uterine response to estrogen.

The uterus responds to cyclical changes in estrogen and progesterone levels in preparation for embryo implantation. Estro-

gen mediates the principal proliferative response of the uterus through two related but distinct estrogen receptors (ER α ² and ER β) (1–3). ER α is the predominant form in the murine uterus (4), but ER β transcripts are also detected at very low levels (4). After estrogens bind to the ligand domain of the ER, it undergoes conformational changes that allow interactions with coactivator molecules (5–7). In the classical pathway of estrogen action, the ER binds as a dimer to EREs in the promoter regions of target genes (8). However, it is now clear that the ER can regulate genes that lack a canonical ERE, suggesting additional pathways for estrogen action (9–11). For example, the ER may activate genes via protein-protein tethering with c-Fos/c-Jun B (AP-1) and Sp1 (12, 13) or suppress expression mediated through NF- κ B (14). This alternate response mechanism has been referred to as the tethered or nonclassical pathway.

We previously generated an ER α mutant that selectively functions via the nonclassical pathway. Substitution of two amino acids in the DNA recognition sequence of the first zinc finger eliminated ER α binding to EREs but preserved ligand binding, dimerization, and receptor interactions with other transcription factors (*i.e.* AP-1) and co-factors (*i.e.* SRC-1) (11, 15). These alanine mutations were “knocked in” to the murine germ line to generate the nonclassical ER knock-in (NERKI) mouse (11). Heterozygote NERKI females (AA/+) possessing one AA and one wild-type ER α allele are infertile and have grossly enlarged uteri with cystic hyperplasia (11). This phenotype was unexpected, given that one normal wild-type ER α allele remains and the uterus in heterozygote ER α null (+/–) mice is developmentally and physiologically normal (16). In contrast, the uteri of complete ER α null (–/–) mice demonstrate no mitogenic response to stimulation with estradiol or growth factors (*i.e.* epidermal growth factor or insulin-like growth factor-1) (16). This comparison suggests that nonclassical signaling participates in the uterine response to hormones through as yet unknown mechanisms. To gain further insight into the different uterine signaling pathways, we generated a mouse model with the exclusive expression of nonclassical ER α

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² The abbreviations used are: ER, estrogen receptor; ERE, estrogen response element; E₂, estradiol; 4HT, 4-hydroxytamoxifen; NERKI, nonclassical ER knock-in; BrdUrd, 5-bromo-2'-deoxyuridine; LEH, luminal epithelial height; PR, progesterone receptor; ERKO, estrogen receptor knockout; ANOVA, analysis of variance.

Nonclassical ER α Uterine Signaling

(AA/–) signaling by crossing heterozygote NERKI males (AA/+) with heterozygote ER α null females (+/–). The objective of this study was to use this *in vivo* model to evaluate the contribution of nonclassical ER α signaling, in the absence of ERE-mediated pathways, as a potential mediator of the uterine response to the ER ligands, estradiol and 4-hydroxytamoxifen.

EXPERIMENTAL PROCEDURES

Experimental Animals—All animal experiments were approved by the Northwestern University Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Animals. The generation of mutant mice containing the AA allele has previously been described (11). The mutant (NERKI) colony was maintained on a hybrid background of 129/SvJ and C57BL/6J strains by crossing heterozygote male NERKI mice (AA/+) and heterozygote female ER α null mice (+/–). The α ERKO colony was maintained on pure C57BL/6J (17). The AA allele was transmitted by heterozygote male NERKI mice, because the heterozygote NERKI females are infertile (11).

Mice were maintained for 12 weeks before ovariectomy via a single ventral incision following an intraperitoneal injection of 50 μ l/mouse of a mixture containing 25 mg/ml ketamine (80 mg/kg; Ketaset; American Home Product Corp., Madison, NJ), 1.27 mg/ml xylazine (10 mg/kg; Rompun; Bayer Corp., Pittsburgh, PA), and 0.25 mg/ml acetopromazine (American Home Product Corp.). At least eight animals of each genotype were used per treatment. After allowing 2 weeks for recovery, mice were injected with sesame oil (100 μ l; 6-, 24-, and 72-h groups), 17 β -estradiol (E₂; 100 ng/mouse; 6-, 24-, and 72-h groups), or 4-hydroxytamoxifen (4HT; 50 μ g/mouse; 72-h group) and sacrificed at 6 or 24 h after the last injection. The sesame oil vehicle and all hormones were obtained from Sigma. All treatments were prepared in sesame oil and injected (100 μ l/mouse) subcutaneously. Two hours before sacrifice, 5-bromo-2'-deoxyuridine (BrdUrd) labeling reagent (Sigma) was administered intraperitoneally at a dose of 5 mg/100 g of body weight dissolved in 100 μ l of saline.

To assess stromal cell proliferation, ovariectomized mice were primed by a subcutaneous injection of 100 ng of E₂ in 100 μ l of sesame oil for 2 days (18). Four days later, mice were injected subcutaneously with 1 mg of progesterone dissolved in 100 μ l of sesame oil daily for 4 consecutive days. On the fourth day, 100 ng of E₂ was injected subcutaneously following the last progesterone injection in a different location. The animals were sacrificed 18 h after the last injection.

Serum Hormone Measurements—Serum estradiol and progesterone were measured by radioimmunoassay at the University of Virginia Ligand Assay and Core Laboratory. All measurements were performed in groups of at least 10 animals. The interassay coefficient of variation was <5% for estrogen and <6% for progesterone measurements.

Uterine Morphometry—Intact 12-week-old females of each genotype were selected. Using vaginal cytology, the cycle stage was monitored to select wild-type animals in proestrus, when circulating estradiol levels are highest. The AA/– and ER α (–/–) females do not exhibit a regular cycle. Whole uteri from each genotype were fixed in 10% neutral buffered paraformal-

dehyde for 24 h, transferred to 70% ethanol, embedded in paraffin, and sectioned at a thickness of 5 μ m. Slides were deparaffinized and rehydrated through a graded alcohol series. Hematoxylin and eosin staining was performed using standard protocols. The uterine radius was measured from the serosal side to the apical surface of the luminal epithelium. The muscle layer consisted of the inner circular layer. The luminal epithelial height was measured from the basement membrane to the apical surface. All measurements were performed using a Zeiss Axioskop microscope (Thornwood, NY) equipped with an Optronics MicroFire digital camera (Goleta, CA) and Image-Pro Express version 4.0 (Media Cybernetics, Inc., Silver Spring, MD). The measurements were taken from three different areas (proximal, middle, and distal) per longitudinal section, and five measurements per area were analyzed.

The number of positively stained BrdUrd cells in a random cross-section from the midportion of the uterus was counted. Approximately 500 epithelial cells were counted in four separate fields per section, and the average number of positive cells was calculated.

Immunohistochemistry—For immunohistochemistry, sections were deparaffinized in xylene and rehydrated in descending concentrations of ethanol followed by antigen retrieval in sodium citrate buffer (microwave 10 min at full power). Sections were blocked in 10% normal goat serum for 30 min and incubated with primary antibody at 4 °C overnight. The primary antibody was cyclin D2 (1:40; catalog number SC-593; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and secondary antibody was biotinylated goat anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA) for 30 min.

BrdUrd fluorescence was performed after deparaffinization and rehydration. Antigen retrieval was completed with acid treatment (0.01 N HCl) at room temperature for 3 min, followed by pepsin (1 mg/ml in 0.01 N HCl at 37 °C for 1 h) and DNase treatment (100 units/ml DNase in reaction buffer at 37 °C for 1 h). Nonspecific binding was blocked with 5% normal goat serum for 1 h. Slides were incubated using rat anti-BrdUrd/fluorescein isothiocyanate (catalog number MCA2060FT; Serotec Ltd., Oxford, UK) diluted to 1:20 at 4 °C overnight. After additional washes, sections were incubated with 0.1% Sudan Black in 70% ethanol for 10 min to block autofluorescence, washed with tap water, and mounted with Vectashield mounting medium (Vector Laboratories, Inc.).

Western Blot Analysis—Whole uterine protein extracts were prepared by homogenizing tissues in buffer containing protease inhibitor mixture (Roche Applied Science), 1 mM EDTA, and 1 mM EGTA. The protein concentration was determined by the DC protein assay (Bio-Rad). Fifty micrograms of total uterine protein were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Amersham Biosciences). Immunodetection was carried out using antibody AB-52 (1:1000; catalog number SC-810) to detect the progesterone receptor (PR) (Santa Cruz Biotechnology, Inc.) in 5% milk, Tris-buffered saline with 0.05% Tween 20. Proteins were visualized with an ECL Plus detection kit (Amersham Biosciences) according to the manufacturer's instructions.

Reverse Transcription-PCR—Wild-type ER α (+/+), AA/–, and ER α null (–/–) mice were ovariectomized, allowed to recover for 10–14 days, and then treated with sesame oil control (Sigma) or E₂ (1 μ g/mouse; Sigma). Uteri were collected 2 h after treatment and snap-frozen in liquid nitrogen. Three or four uteri from each treatment group were pooled, and RNA was prepared using Trizol reagent (Invitrogen) and the RNeasy clean up protocol (Qiagen, Valencia, CA). cDNA was synthesized and analyzed by real time PCR using SYBR green dye as previously described (19). Relative transcript levels were quantified in comparison with wild-type control and normalized to 18 S rRNA (20). Primer sequences were selected using Primer Express (Applied Biosystems, Foster City, CA) and were purchased from Sigma. Primer sequences were as follows: 18 S (forward, 5'-GAAACTGCGAATGGCTCA-TTAA (positions 966–987); reverse, 5'-GAATCACCACAGTTATCCAAGTAGGA (positions 1046–1021)); *cdkn1A* (*p21*) (forward, 5'-CAGCGACCATGTCCAATCC (positions 193–211); reverse, 5'-CGAAGAGACAACGGCAC-ACTT (positions 264–244)); and aquaporin 5 (*aqp5*; forward, 5'-ACAGGGCCTCTTTGGAATTAGG-3' (positions 1474–1495); reverse, 5'-TGAGCTTGCCTTCAC (positions 1549–1530)).

Cell Culture and Transfection—An Ishikawa ER α negative endometrial cell line was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were plated in 12-well culture dishes containing phenol red-free Dulbecco's modified Eagle's medium supplemented with 5% dextran charcoal-stripped fetal bovine serum 24 h before transfection. Cells were cotransfected with 10 ng/well of wild-type mouse ER α or mutant nonclassical ER^{E207A/G208A} (AA) expression vector and 500 ng/well of the indicated reporter constructs (AP-1-luciferase or *p21*-luciferase) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The 2.4-kb full-length human *p21* reporter was kindly provided by Dr. Xiao-Fan Wang (Duke University) (21, 22). The ER expression and AP-1 reporter constructs have been previously described (11, 15). One day later, cells were treated with vehicle (0.1% EtOH), 1 nM E₂, or 100 nM ICI 182,780 for 24 h. 17 β -Estradiol was obtained from Sigma, and ICI 182,780 was from Tocris (Ellisville, MO). Duplicate wells were harvested, and cell extracts were prepared for luciferase activity determinations using the Luciferase assay system kit (Promega, Madison, WI) and a Microplate Clarity luminometer (Bio-Tek Inc., Winooski, VT). Relative luciferase units were normalized to total cellular protein measured by the BCA assay (Pierce).

Statistical Analysis—All reported values represent the mean \pm S.E. Differences were considered significant at $p < 0.05$ using factorial analysis of variance (ANOVA) with appropriate *post hoc* tests (SPSS version 14.0).

RESULTS

Morphology of the Uterus in the Nonclassical NERKI Mouse—The primary genotypes compared are 1) wild-type ER α (+/+), 2) nonclassical NERKI (AA/–), and 3) ER α null (–/–), since these allow the effect of the NERKI (AA) allele to be compared relative to the wild-type (+) and knock-out (–) alleles. Steroid

TABLE 1

Serum estrogen and progesterone concentrations in adult females

Values shown are mean \pm S.E.

| Hormone | ER α (–/–) (S.E.) | AA/– \pm S.E. | Wild type (+/+) \pm S.E. |
|----------------------|--------------------------|-----------------------------|-----------------------------|
| Estradiol (pg/ml) | 69.9 \pm 5.9 | 40.2 \pm 1.7 ^a | 33.3 \pm 1.4 ^b |
| Progesterone (ng/ml) | 3.6 \pm 0.75 | 3.7 \pm 0.65 | 4.6 \pm 0.73 |

^a ANOVA with Bonferroni procedure, AA/– versus ER α (–/–), $p < 0.01$.

^b ANOVA with Bonferroni procedure, wild type (+/+) versus ER α (–/–), $p < 0.01$.

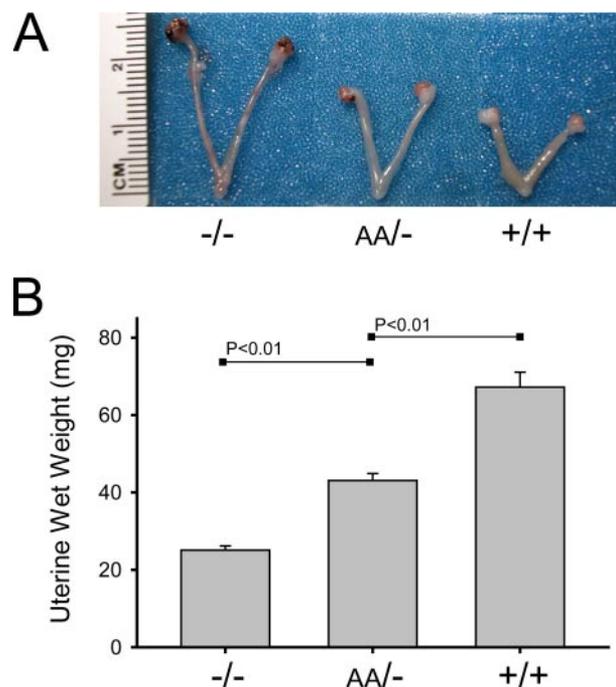


FIGURE 1. Uterine morphology in mice with different ER α genotypes. A, ovaries and genital tracts of 12-week-old ER α (–/–), AA/–, and wild-type (+/+) females. B, comparison of the uterine weight wet of 12-week-old intact mice. ($p < 0.01$, ANOVA followed by Bonferroni multiple-comparison procedure).

hormone levels were determined for ER α (–/–), AA/–, and wild-type females at 10–12 weeks of age (Table 1). Serum estradiol levels were greater in the ER α (–/–) mice than in the AA/– or wild-type mice, consistent with the absence of negative feedback in the ER α null mice. However, estradiol levels were not significantly different between AA/– and wild-type females. Serum progesterone levels were similar among all three genotypes (Table 1). Although the steroid hormone levels were similar between the AA/– and wild-type females, none of the 15 AA/– females became pregnant when bred to either wild-type or AA/– proven males. Fertility was confirmed in parallel matings between wild-type female littermates and AA/– males. The AA/– females had no evidence of corpus luteum formation on ovarian histology, and vaginal smears confirmed that the AA/– females do not cycle and are in constant diestrus.

The gross morphology of the AA/– uterus appeared normal and exhibited a substantial increase in the thickness of the uterine horns compared with ER α (–/–) (Fig. 1A). Consistent with the gross morphology, the wet weight of the AA/– uteri was 1.7-fold greater than that of ER α (–/–) animals (Fig. 1B). The mean uterine wet weight of intact 12-week-old animals was 43 \pm 2 mg in AA/– animals compared with 25 \pm 1 mg for ER α (–/–) and 67 \pm 4 mg in wild-type mice ($p < 0.01$). The activity

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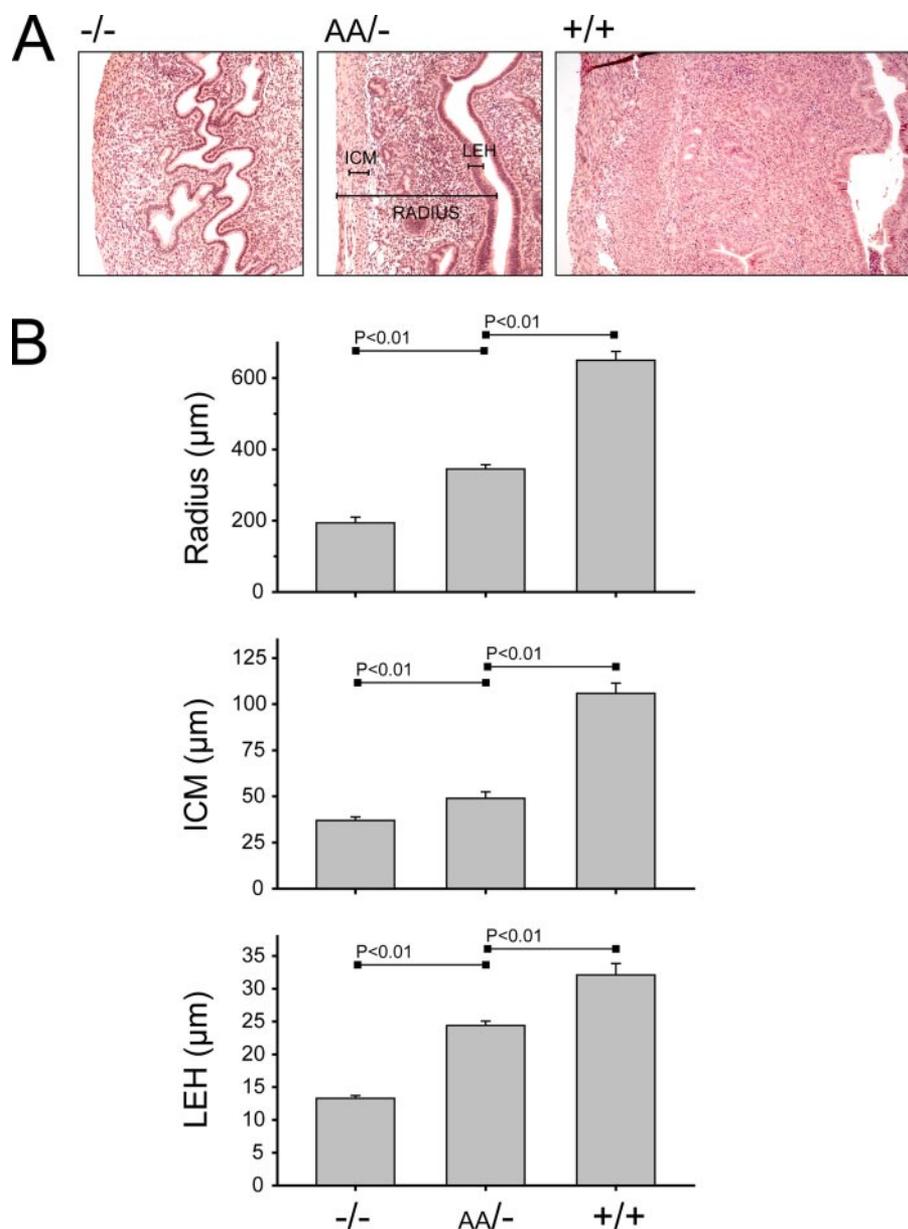


FIGURE 2. Uterine histology in mice with different ER α genotypes. *A*, representative longitudinal sections of the uterus stained with hematoxylin and eosin. All images are the same magnification. *ICM*, inner circular muscle; *R*, uterine radius. *B*, quantitative analysis of uterine morphometry ($p < 0.01$, ANOVA followed by Bonferroni multiple-comparison procedure).

of the uterine ER α signaling pathways was confirmed by the response to ovariectomy. After ovariectomy, the mean uterine wet weight did not differ between the ER α (-/-), AA/-, and wild-type animals (18.0 ± 1 , 21.4 ± 1 , and 23.1 ± 2 mg, respectively) (see Fig. 3).

Similar to ER α (-/-) and wild-type animals, the three major anatomical compartments of the uterus, the outer muscle layer, the inner stroma, and the epithelium (glandular and luminal), are all present in the AA/- uterus (Fig. 2*A*). When compared with the ER α (-/-) mice, the AA/- uterus contains more endometrial glands and a higher cellular density within the stroma (data not shown).

Quantitative comparison of longitudinal uterine sections revealed that the presence of a single AA allele increased the uterine radius, the inner circular muscle layer, and the luminal

epithelial height compared with ER α (-/-) (Fig. 2*B*). In AA/- animals, the mean uterine radius was $345 \pm 12 \mu\text{m}$ compared with $194 \pm 16 \mu\text{m}$ in ER α (-/-) ($p < 0.01$). The inner circular muscle increased to $49 \pm 4 \mu\text{m}$ in AA/- mice compared with $37 \pm 2 \mu\text{m}$ in ER α (-/-) mice ($p < 0.01$). The luminal epithelial height was greater in the AA/- mice compared with ER α (-/-) (24 ± 1 versus $13 \pm 1 \mu\text{m}$, $p < 0.01$). Despite the significant increase in size of all three major anatomical compartments of the uterus, estrogen signaling by the AA allele did not fully restore the uterus to the size of wild-type mice, and each functional compartment of the uterus was significantly larger in wild-type mice when compared with the AA/- but increased compared with ER α (-/-) animals.

Acute Uterine Response to Estradiol Is Deficient in Nonclassical NERKI Mice—To examine whether nonclassical signaling influences the acute uterotrophic response to E₂, ovariectomized mice from each genotype received a single subcutaneous injection of E₂ (100 ng/mouse) and were sacrificed 6 h later. The presence of a single AA allele did not facilitate the acute uterotrophic response, which is characterized by hyperemia and water imbibition (Fig. 3). After ovariectomy alone, there was no difference in the uterine wet weight based on the extent of ER α signaling. Six hours after estradiol, the uterine wet weight of AA/- animals was greater than in ER α (-/-) mice (22.7 versus

11.3 mg, $p < 0.01$) but did not differ from the AA/- ovariectomized mice. In contrast, the uterine wet weight of wild-type animals increased significantly after a single injection of E₂ ($p < 0.01$). No increase in uterine wet weight was seen in the ER α (-/-) or AA/- mice, even after extended treatment with E₂ (72 h) or 4HT (72 h, data not shown) (Fig. 3).

Epithelial Cell Responses to Estradiol and 4-Hydroxytamoxifen Are Preserved in Nonclassical NERKI Mice—Based on histology, a proliferative response of the uterine epithelium was observed after 24 h of E₂ and 72 h of 4HT in both AA/- and wild-type animals (Fig. 4). There was no evidence of luminal epithelial proliferation in response to the control injection of sesame oil for any of the genotypes. The ER α (-/-) mice did not respond to either E₂ or 4HT. The treatment of AA/- and wild-type mice with E₂ for 24 h resulted in moderate hypertro-

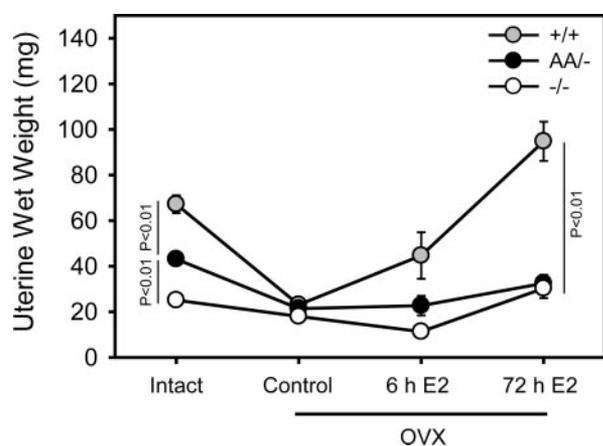


FIGURE 3. Uterine weight responses to acute and prolonged estrogen treatment. Ovariectomized (OVX) 12-week-old ER α (-/-), AA/-, and wild-type (+/+) mice were treated with sesame oil or E₂ (100 ng/mouse) and sacrificed 6 or 24 h after the last injection ($p < 0.01$, ANOVA followed by Bonferroni multiple-comparison procedure).

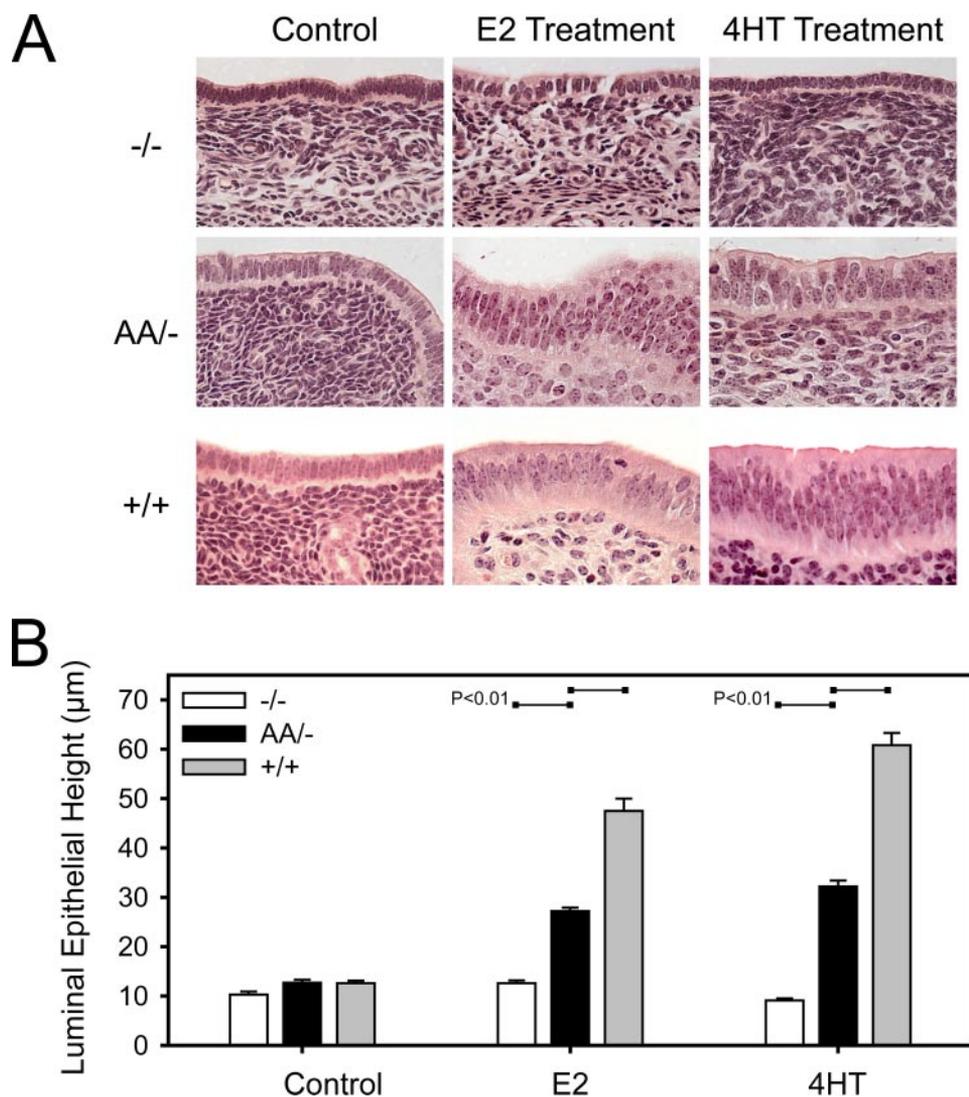


FIGURE 4. Luminal epithelial responses to estrogen and 4-hydroxytamoxifen. *A*, longitudinal uterine sections were examined for the luminal epithelial response to E₂ and 4HT using the same magnification. Adult ovariectomized mice were given a single subcutaneous injection of E₂ (100 ng/mouse) or three daily injections of 4HT (50 μg/mouse) and sacrificed 24 h after the last injection. Mice injected with sesame oil were sacrificed after 24 and 72 h and served as controls. The experiments were repeated three times with similar results. *B*, quantitation of luminal epithelial height ($p < 0.01$, ANOVA followed by Bonferroni multiple-comparison procedure).

phy of the luminal epithelium (Fig. 4*A*, middle column). In wild-type animals treated with 4HT for 72 h, there was marked hyperplasia and stratification of the luminal epithelium. In the AA/- mice, the proliferative response to 4HT was accompanied by greater nuclear enlargement and cytoplasmic clearing (Fig. 4*A*, right column).

The uterine luminal epithelial height (LEH) increased with the restoration of estrogen signaling in the nonclassical NERKI and wild-type females but not the ER α (-/-) animals (Fig. 4*B*). The presence of a single nonclassical allele in the AA/- animals resulted in a mean LEH of $27.2 \pm 0.7 \mu\text{m}$ after E₂ and $32.2 \pm 1.2 \mu\text{m}$ with 4HT compared with a control LEH of $12.7 \pm 0.6 \mu\text{m}$. The increase in LEH was greater for wild-type mice after each hormone treatment ($47.5 \pm 2.5 \mu\text{m}$ after estradiol and $60.8 \pm 2.5 \mu\text{m}$ post-4HT). The LEH response to E₂ and 4HT differed significantly between ER α (-/-), AA/-, and wild-type animals ($p < 0.01$).

Uterine BrdUrd Incorporation Is Preserved in Nonclassical NERKI Mice—The proliferative response of the AA/- uterine epithelial cells was confirmed by the presence of BrdUrd incorporation (Fig. 5*A*). The percentage of BrdUrd-positive luminal epithelial cells was markedly greater in the AA/- and wild-type uteri compared with the ER α (-/-) animals. In response to 24 h of E₂, the AA/- and wild-type mice had a similar pattern of proliferation with ~80 and 82% of the luminal epithelial cells positive, respectively, for BrdUrd incorporation versus 2% in ER α (-/-) ($p < 0.01$). The pattern of epithelial proliferation was similar but diminished in intensity after 72 h of E₂ (data not shown). Collectively, these results suggest that a proliferative response of uterine epithelial cells to estrogenic hormones in AA/- animals is preserved and does not require classical ER α signaling.

The uterine response to 72 h of 4HT provided additional evidence of a distinct nonclassical response. The ER α (-/-) animals had almost no BrdUrd-positive cells. In the AA/- uterus, the number of positive epithelial cells was 27%. In contrast, the percentage of BrdUrd-positive epithelial cells was only 5% in the wild-type mice ($p < 0.01$ compared with AA/-). At the 72-h time point, the diminished BrdUrd positivity in the wild-type mice suggests that continued proliferation is minimal despite the pronounced

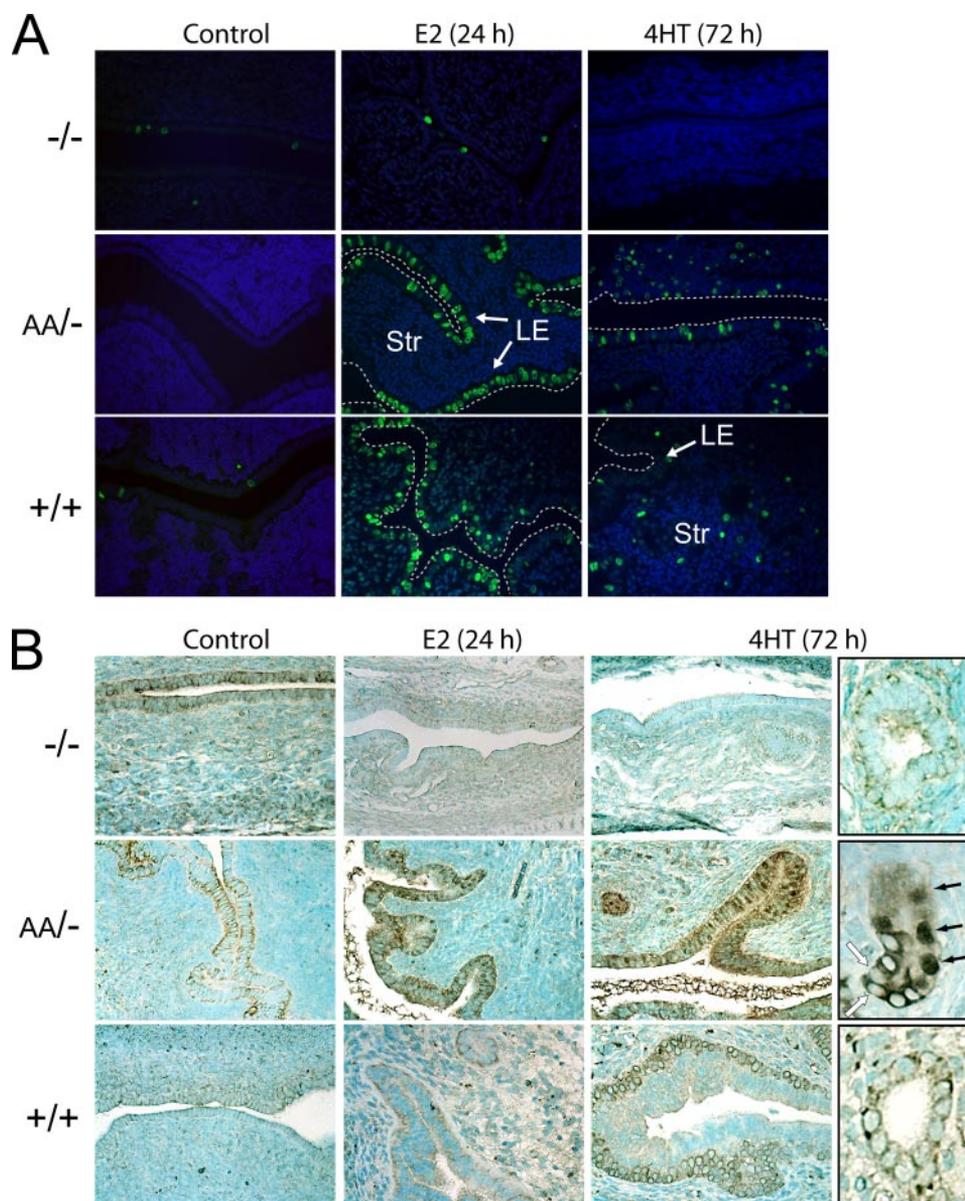


FIGURE 5. Cellular proliferation in response to estrogen and 4-hydroxytamoxifen. *A*, uterine cross-sections were examined for BrdUrd incorporation as an index of cellular proliferation (green labeling). LE, luminal epithelium; Str, stroma. Approximately 500 epithelial cells were counted in four separate fields per cross-section, and the average number of positive cells was calculated. *B*, uterine cross-sections were examined for cyclin D2 immunostaining. The dark brown deposits indicate the sites of positive staining. Examples of cyclin D2 mobilized from the cytoplasm to the nucleus are depicted by arrows. The last column includes images taken at increased magnification ($\times 400$).

increase in the luminal epithelial height (Fig. 4A). In addition to luminal epithelial proliferation, there was evidence of scattered stromal cell proliferation after 72 h of 4HT in both the AA/– and wild-type uteri. This response may be regulated by a nonclassical ER α -dependent mechanism, or it may be secondary to the prolonged exposure to 4HT. The absence of stromal proliferation after 24 h of 4HT suggests that the prolonged exposure to 4HT may influence the stromal response (23).

Cyclin D2 Translocation Is Mediated by the Nonclassical Pathway—In previous studies, estrogen-induced proliferation of the luminal and glandular epithelium has been associated with the nuclear accumulation of cyclin D1 and D2 (18, 24). To further explore the role of cyclin D1 and D2 in nonclassical

signaling, we analyzed the pattern of epithelial localization of cyclin D1 and D2 after E₂ and 4HT stimulation. Although there was accumulation of cyclin D1 in the nucleus of wild-type uterine luminal epithelial cells after both E₂ and 4HT, there was no evidence of cyclin D1 immunostaining in AA/– or ER α (–/–) mice after either hormone treatment (data not shown).

In contrast to the lack of cyclin D1 immunostaining, cyclin D2 mobilized from the cytoplasm to the nucleus exclusively in the uterine luminal and glandular epithelial cells in the AA/– mice after 24 and 72 h (data not shown) of E₂ treatment (Fig. 5B). Nuclear localization was also seen after 72 h of 4HT. There was no evidence of nuclear localization of cyclin D2 after any of the control treatments. We did not observe nuclear accumulation of cyclin D2 following the control, E₂, or 4HT treatments in the ER α (–/–) or wild-type animals. However, the luminal epithelial cells adjacent to the basement membrane in wild-type mice revealed intense cytoplasmic localization of cyclin D2 after 72 h of 4HT.

aqp5 and p21 Regulate Estrogen-mediated Responses—To examine whether the mutant ER α allele (AA) mediates estrogen responses of endogenous uterine genes, two transcripts were selected from ER α -dependent uterine genes identified in previous microarray studies (19, 25). The *aqp5* gene was chosen for analysis, since the lack of water imbibition and hyperemia in the AA/– uterus suggested a possible role for Aqp proteins, which are

proteins that form membrane water channels (26). The *aqp5* transcript was increased 40-fold after a 2-h E₂ treatment in wild-type samples, but this increase was absent in both AA/– and ER α (–/–) samples (Fig. 6A), indicating that this response requires ER-ERE interaction. The cell cycle regulator, *cdkn1A* (*p21*), was also selected for further analysis, since the retention of the epithelial proliferation response in the AA/– would be consistent with the previously documented ER α -dependent increase in *cdkn1A* (*p21*) transcript levels (19, 25). In contrast to *aqp5*, reverse transcription-PCR analysis indicated that the 20-fold *cdkn1A* (*p21*) increase in response to E₂ was preserved in the AA/– uterus (Fig. 6B), suggesting that this gene is regulated via the tethered nonclassical mechanism.

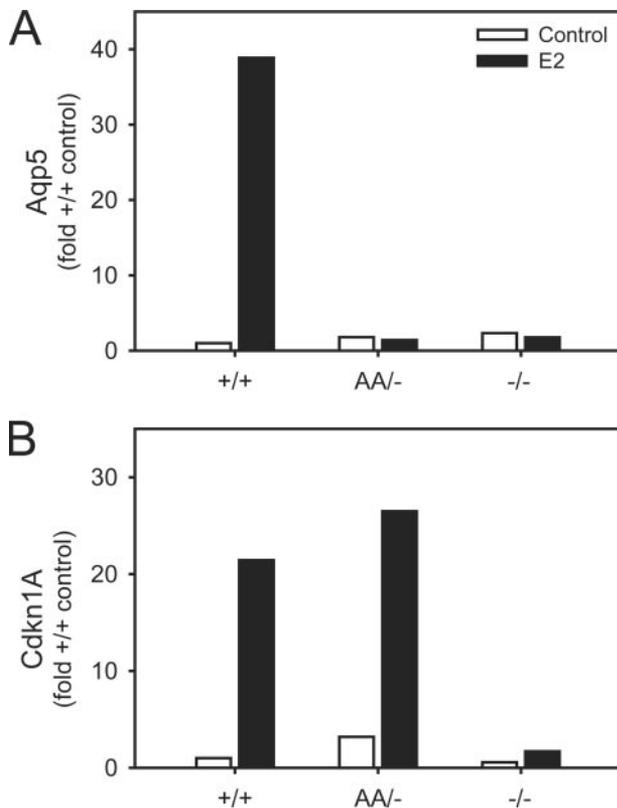


FIGURE 6. Verification of estrogen-regulated responses by reverse transcription-PCR. A and B, *aqp5* and *cdkn1A* (*p21*) were analyzed by reverse transcription-PCR. Wild-type (+/+), AA/–, or ER α (–/–) samples were prepared from a pool of three or four uteri after mice were treated with sesame oil control or E₂ for 2 h.

Nonclassical Pathway Regulation of the *cdkn1A* (*p21*) Promoter in Uterine Endometrial Cells—To further elucidate the molecular mechanism responsible for regulating *cdkn1A* (*p21*) activity, an ER α -negative endometrial cell line was cotransfected with wild-type ER α or mutant ER^{E207A/G208A} (AA) expression vector along with the *p21*-luciferase reporter construct (Fig. 7). The *p21*-luciferase reporter contains several Sp1 binding sites that play an important role in transcriptional regulation (22). As a positive control for the nonclassical pathway, the ER expression constructs were also cotransfected with the AP-1-luciferase reporter. E₂ treatment did not significantly alter the transcriptional activity of either AP-1 or *p21* reporters in the presence of wild-type or mutant ER. However, the antiestrogen ICI 182,780 significantly enhanced both AP-1 and *p21* promoter activity in the presence of both wild-type and mutant ER (AA). These effects were specific, since no response was observed using an empty vector backbone (data not shown). When ER was cotransfected with an ERE reporter, estradiol activated and the antiestrogen ICI 182,780 repressed transcription, indicating that these cells retain the ability to signal via the classical pathway (data not shown). Taken together, these results indicate that *cdkn1A* (*p21*) is regulated *in vitro* by the nonclassical pathway.

Progesterone Receptor Expression in the Nonclassical NERKI Uterus—The uterine expression of the PR is compartment-specific and primarily regulated by the classical pathway involving EREs (27–30). To determine whether the nonclassical allele

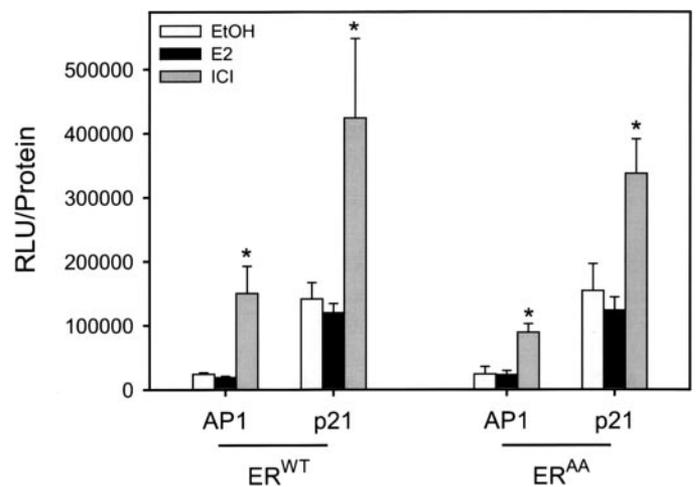


FIGURE 7. ICI 182,780 stimulates AP-1 and *cdkn1A* (*p21*) nonclassical reporters. Shown is transient ER α -negative Ishikawa endometrial cells with wild-type ER α or ER^{E207A/G208A} (AA) expression vectors and indicated reporters (AP1, AP-1-luciferase; *p21*, *p21*-luciferase). Cells were treated with vehicle (EtOH), E₂, or ICI 182,780 and subsequently harvested for measurement of the luciferase activity assay, which was normalized to protein content. Data are expressed as mean \pm S.E. from three separate experiments. The asterisks indicate that ICI treatment is statistically different from EtOH and E₂ for each group ($p < 0.05$, ANOVA followed by Dunnett's multiple-comparison procedure).

altered the expression of PR, we performed Western blot analysis of whole uterine protein extracts from intact animals. The data suggested that the relative expression of progesterone receptor isoforms, PR A and PR B, differs by genotype (Fig. 8A). Both AA/– and ER α (–/–) animals have reduced levels of total PR A and PR B compared with wild-type mice ($p < 0.01$). Notably, the expression of PR A appears to be differentially regulated in the AA/– females. The level of PR A is significantly increased in the AA/– mice compared with the ER α (–/–) animals ($p < 0.01$). The relative level of PR B expression is similar in the AA/– and ER α (–/–) animals. These results indicate that nonclassical signaling does not restore the expression of either PR isoform to wild-type levels.

Nonclassical NERKI Mice Lack the Proliferative Response to Progesterone Pretreatment—In adult ovariectomized mice, the uterine stromal cells do not proliferate in response to estradiol treatment alone. Previous studies have shown that progesterone pretreatment permits the uterine stromal cells to respond to estradiol with a round of cellular proliferation (18, 31). Progesterone pretreatment induces ~30–40% of the stromal cells to enter into DNA synthesis (18, 31). Our results confirmed that only the wild-type stromal cells responded to progesterone pretreatment with BrdUrd incorporation. The AA/– and ER α (–/–) animals failed to show any stromal cell proliferation (Fig. 8B). These results suggest that classical ER α signaling is required for stromal cell proliferation and are consistent with the observed lack of water imbibition and hyperemia in the AA/– and ER α (–/–) animals.

DISCUSSION

The major finding in this study is that estrogen-induced responses in the uterus can be divided into classical and nonclassical pathways by using genetic models that selectively discriminate between these mechanisms. Somewhat unexpected-

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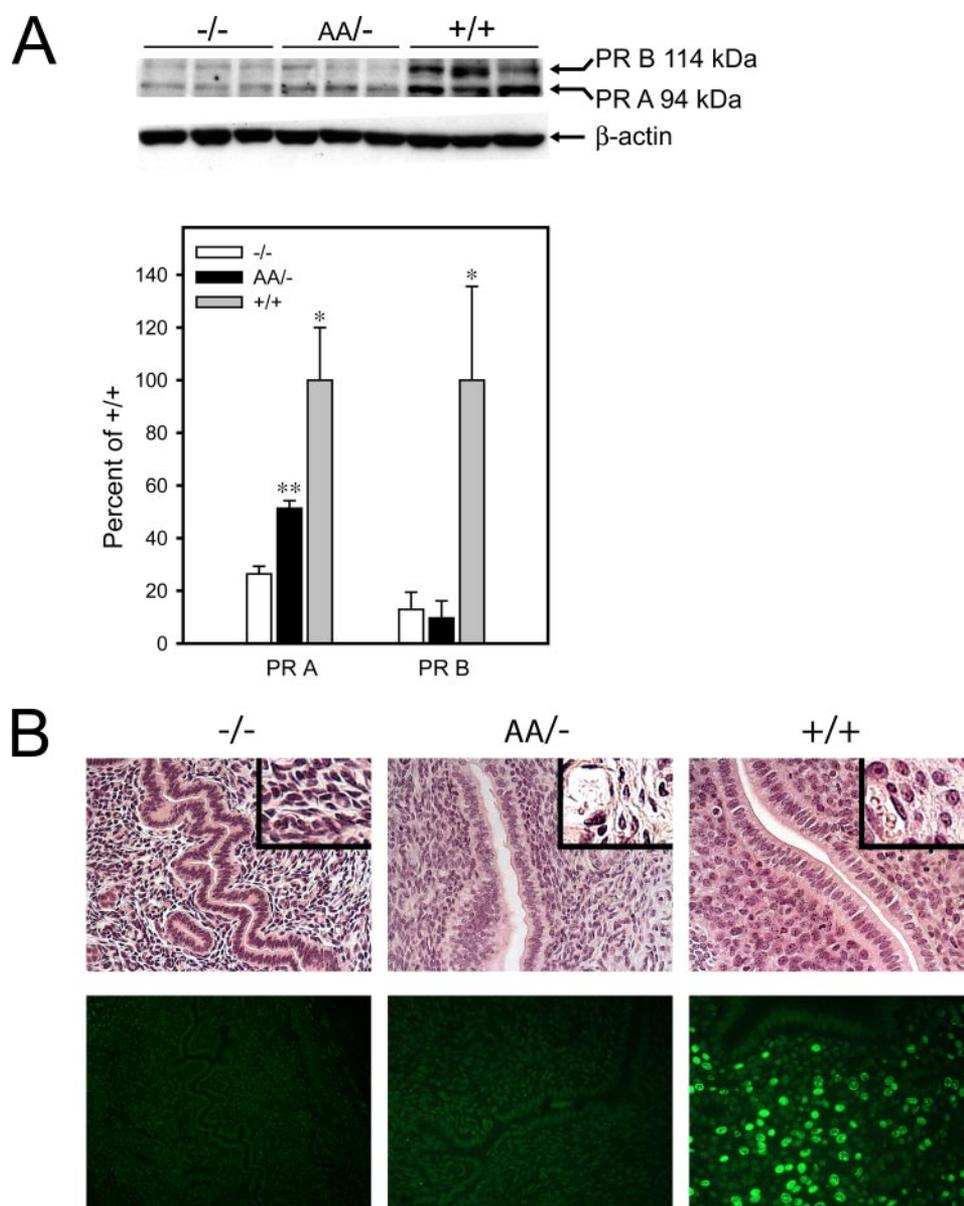


FIGURE 8. Progesterone receptor expression in the uteri of mice with different ER α genotypes. *A*, Western blot analysis of whole uterine extracts identifies both PR A and PR B isoforms. Actin is included as a control for protein recovery. Quantitation of PR A and PR B isoforms is expressed relative to wild type (+/+). Asterisks represent statistical difference between all groups ($p < 0.01$, ANOVA followed by Bonferroni multiple-comparison procedure). *B*, stromal response to progesterone pretreatment BrdUrd incorporation serves as an index of cellular proliferation (green labeling). The inset demonstrates the altered stromal response by genotype taken at $\times 400$.

edly, the nonclassical pathway, which is ERE-independent, mediates the estrogen-dependent epithelial cell proliferation and growth responses, whereas the classical pathway is required for other responses, such as hyperemia and water imbibition.

The generation of estrogen receptor knock-out (ERKO) mice has provided extensive information regarding the importance and specific functions of estrogen signaling in the uterus and other tissues (16, 32). The α ERKO (ER α null (-/-)) females are infertile, and the β ERKO females demonstrate varying degrees of ovarian subfertility (17, 33). The development of the uterus in the three different knock-out animals (α ERKO, β ERKO, and the compound $\alpha\beta$ ERKO) appears to be normal, suggesting that the ER is not required for the initial development of the three

definitive compartments: the myometrium, the stroma, and the epithelium (17, 33, 34). Consistent with these findings, the partial restoration of estrogen signaling with one nonclassical allele (AA/-) also does not alter the normal development of the murine uterus. Regardless of the degree of estrogen signaling, the uteri of the ER α (-/-), AA/-, and wild-type mice are structurally similar before the onset of reproductive cycles.

In the uterus, the physiologic and genomic responses to estrogen have been described as biphasic (25, 35). The early events occur within the first few hours after estrogen administration and include hyperemia and uterine fluid uptake (or water imbibition). The water imbibition results in a rapid increase in uterine wet weight. The mechanisms that result in water imbibition are poorly understood. The physiologic processes include the regulation of congestion and inflammation in many cell types (endothelial, stromal, and endometrial cells) (30). Studies have shown that the signaling factor vascular endothelial growth factor is critical to the regulation of uterine fluid accumulation (36, 37). Vascular endothelial growth factor is primarily expressed in the luminal epithelium of the murine uterus (37). The late phase response peaks after 24–72 h and includes a wave of epithelial cell proliferation and differentiation (16). The epithelial layer transforms into columnar secretory cells with abundant mitosis (38). The later phase also involves the induction of

target genes, such as lactoferrin (39). In the murine uterus, tamoxifen elicits a similar response to estrogen, since it mediates water imbibition and epithelial cell proliferation (40). However, the effects of tamoxifen may be longer lasting, particularly in the glandular epithelium (40).

The adult α ERKO uterus is hypoplastic and no longer responds to different ER agonists (*i.e.* estradiol, diethylstilbestrol, or hydroxytamoxifen) (17, 39, 41). The classic biphasic uterine response is absent in α ERKO females. The loss of estrogen responsiveness results in uterine weights that are approximately one-half normal size, and the animals fail to demonstrate a proliferative epithelial response (39, 42). In contrast, the uteri of adult β ERKO mice respond normally to ER ligands (33). The uterine responsiveness of the β ERKO mice suggests the

predominance of ER α in mediating the classic biphasic response. Similar to the ER α ($-/-$) females, the AA/ $-$ animals do not show an increase in uterine wet weight after an acute or prolonged exposure to estradiol or 4-hydroxytamoxifen. These findings suggest that classical ER α signaling is necessary to produce water imbibition. The AA/ $-$ uterus provides initial evidence that the classic biphasic response of estrogen signaling may be controlled by separate signaling mechanisms. The presence of a proliferative epithelial response in the absence of water imbibition has not been seen in other models (16, 17, 32).

The results shown here demonstrate that a partial uterotrophic response occurs with nonclassical signaling. The AA/ $-$ animals responded to both estradiol and 4-hydroxytamoxifen with hypertrophy and hyperplasia of the luminal and glandular epithelium. The proliferative response was confirmed by the incorporation of BrdUrd. The pattern and extent of BrdUrd localization in AA/ $-$ mice was nearly identical to wild-type animals. In contrast, the ER α ($-/-$) animals did not respond to these proliferative stimuli, as evidenced by the lack of epithelial proliferation and BrdUrd incorporation. The observed partial uterotrophic response, manifest as epithelial proliferation, raises the possibility of a gene dosage effect due to the presence of one functional ER α allele in the AA/ $-$. The lack of a similar phenotypic effect of the heterozygote ER α null ($+/-$) mice suggests that the AA/ $-$ response may represent distinct activities of nonclassical signaling (16).

Tissue recombination experiments have provided convincing evidence that uterine epithelial proliferation is regulated via a paracrine mechanism requiring the presence of ER α in the stroma (43). For example, only when the epithelium from α ERKO animals is recombined with wild-type stroma does epithelial proliferation occur in response to estradiol (44). The presence of the ER α receptor in the stroma is therefore obligatory for epithelial proliferation (43). Our findings suggest that classical ER α signaling in the stroma may not be involved in the pathways that mediate epithelial proliferation. Future recombination experiments with nonclassical AA/ $-$ stroma and α ERKO epithelium will be required to confirm that nonclassical ER α signaling is sufficient for epithelial proliferation.

Uterine epithelial cell proliferation is regulated by the D-type cyclins and the cyclin-dependent kinases (40, 45). Several studies have reported that cyclin D1 compartmentalization (cytoplasmic *versus* nuclear) provides one of the control mechanisms for cyclin D activity. In the uterus, both estradiol and tamoxifen have been shown to mobilize cyclin D1 from the cytoplasm to the nucleus prior to epithelial cell proliferation (18, 40). We observed a similar nuclear accumulation of cyclin D1 in the endometrial epithelial cells of wild-type but not ER α ($-/-$) or AA/ $-$ mice after treatment with estradiol and 4-hydroxytamoxifen.

However, cyclin D1-deficient female mice are fertile, suggesting that other D-type cyclins compensate for the loss of cyclin D1 (24). Estradiol has been shown to translocate cyclin D2 from the cytoplasm to the nucleus of luminal epithelial cells in cyclin D1-deficient mice (24). We observed a consistent and exclusive nuclear accumulation of cyclin D2 in the luminal and glandular epithelium of only AA/ $-$ mice in response to estradiol and 4-hydroxytamoxifen. The nuclear localization was

observed at 24 and 72 h of estradiol as well as after 72 h of 4-hydroxytamoxifen. These results are consistent with the observed nonclassical proliferative responses and suggest that cyclin D2 may be a mediator of epithelial proliferation in the nonclassical NERKI uterus. However, the molecular events and specifically the cell cycle kinetics remain to be studied in detail.

The loss of estrogen regulation of *aqp5* but not *cdkn1A* (*p21*) illustrates the utility of the AA/ $-$ model to identify pathways by which estrogen regulates distinct sets of genes. In this case, *cdkn1A* appears to utilize a tethered mechanism of interaction with ER α .

Estrogen has been shown to stimulate *cdkn1A* expression in the luminal epithelial cells of the mouse by immunohistochemistry (46). In addition, the administration of epidermal growth factor or insulin-like growth factor-1 to mice results in up-regulation of uterine *cdkn1A* independent of ER α (19), consistent with regulation of *cdkn1A* through AP-1 or other transcriptional regulators that are activated by growth factor signaling. Our results provide complementary evidence of the functional importance of *cdkn1A* in mediating the uterine epithelial proliferative response. *In vitro*, *p21* is transcriptionally activated by a nonclassical mechanism of ER α signaling.

The regulation of *cdkn1A* and *aqp5* is interesting in light of the uterine phenotypes observed in the AA/ $-$ mice. *cdkn1A*, which is increased rapidly by E₂, with a second peak at 12 h (25), is involved in regulation of S phase progression (47). Additionally, Cdkn1A protein is concentrated in the nuclei of uterine epithelial cells 12 h after E₂ treatment (25), suggesting a potential role in regulating the synchronous proliferative response of uterine epithelial cells (25, 38, 48). Thus, the preserved induction of this cell cycle regulator in the AA/ $-$ uterus is consistent with the observed retention of the proliferative response. Similarly, loss of E₂-mediated *aqp5* up-regulation in the AA/ $-$ uterus suggests that lack of this water channel protein may contribute to the phenotype of loss of water imbibition.

The progesterone signaling pathway plays a critical role in normal uterine physiology. The decrease in the overall expression of PR isoforms A and B in the intact AA/ $-$ and ER α ($-/-$) females provides further evidence that classical signaling through ER α is necessary to maintain the wild-type levels of PR expression. A previous study reported that PR levels in ER α ($-/-$) mice were \sim 60% of the wild type using a ligand binding assay (49). Our results suggest a greater reduction in PR levels in ER α ($-/-$) animals, perhaps because different methods of quantitation were used.

The relative expression of PR A to PR B appears to be increased specifically in the AA/ $-$ females. Our findings are consistent with the previous report indicating that PR A and PR B isoforms are present at similar levels in ER α ($-/-$) and wild-type females (49). Conneely and co-workers (50) reported that PR A and PR B are functionally distinct mediators of progesterone action *in vivo*, and thus the relative expression level of the two isoforms is likely to alter normal uterine responses. Very little is known about the mechanisms that control the relative levels of expression of PR A and PR B. Our results suggest that nonclassical ER α signaling may alter the relative levels of PR A and PR B isoforms in the AA/ $-$ uterus. It has been reported that a reduction in PR B expression might contribute to the observed progesterone resistance in

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women with endometriosis (51). Changes in the relative amounts of PR A and PR B may provide a partial explanation for nonclassical proliferative response in the AA/– animals compared with ER α (–/–). PR A can function as a dominant repressor of PR action and might directly or indirectly permit epithelial proliferation when PR B is diminished (52, 53).

Pretreatment with progesterone did not induce a wave of cellular proliferation in the underlying stromal cells of AA/– females in response to a single injection of estradiol. Only the wild-type females exhibited the expected uterine stromal cell proliferation. Our findings indicate that classical ER α signaling is necessary for a normal stromal proliferative response after progesterone pretreatment. The results confirm that the expression and function of the PR signaling pathway are altered in the nonclassical uterus. The results are not surprising, given the fact that the PR gene promoter contains EREs and has been shown to function through classical ER α signaling (27).

In summary, we have examined the uterine biphasic response to two classic uterine agonists. Our results indicate that the nonclassical ER α pathway is sufficient for uterine epithelial proliferation. These findings underscore the physiologic relevance of different ER α signaling pathways. The classical and nonclassical ER α pathways are also known to respond differentially to selective estrogen receptor modulators and therefore provide strategies for better understanding ligand-specific responses (54, 55).

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