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Preclinical report

Strain differences in tamoxifen sensitivity of Sprague-Dawley and Fischer 344 rats

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Why some women are at increased risk for the development of endometrial carcinoma while taking the antiestrogen tamoxifen (Tam) for breast cancer treatment or prevention is unknown. Various strains of rodents display differences in sensitivity to compounds with estrogenic activity, but whether differences in Tam sensitivity exist in rodent strains has not been investigated. In the present study, we investigated whether rat strain differences in reproductive tract sensitivity to Tam and estrogen exist between Fischer 344 (F344) and Sprague Dawley (SD) rats. Immature (21–23 day; $n=6$/group), ovariectomized F344 and SD rats were treated with vehicle (control), 17β-estradiol (E_2) [1×10^{-6} to 1.0 μg/kg body weight (BW)] or 4-OH tamoxifen (4-OHT) (1×10^{-4} to 10 mg/kg BW) for 2 days and then sacrificed on day 3. Reproductive tracts were collected, weighed, and examined for changes in histomorphology and expression of ERα and nuclear receptor co-regulators (SRC1, p300, CARM1, GRIP1, SPA, REA and Uba3). Treatment with E_2 (1×10^{-5} μg/kg BW) increased ($p<0.05$) uterine epithelial cell height in F344 but not SD rats, demonstrating increased sensitivity of the F344 strain to E_2. Conversely, treatment with 1×10^{-3} mg/kg BW 4-OHT increased ($p<0.05$) uterine weight and epithelial cell height in SD but not F344 rats, demonstrating that the SD strain is more sensitive to the antiestrogen. Northern and Western blot and immunohistochemical analysis revealed that ERα expression levels in the SD and F344 uterus were not different. Expression of receptor co-regulators was higher in the uterus compared to the vagina regardless of strain and higher CARM1 expression was seen in SD uterus compared to F344 rats. Understanding differences in Tam sensitivity may help us to better understand why some women develop endometrial cancer while taking Tam and be beneficial in treatment decisions for breast cancer patients. [© 2002 Lippincott Williams & Wilkins.]

Key words: Estradiol, estrogen receptor, rat strain, tamoxifen, uterus.

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Introduction

Tamoxifen (Tam) is widely used for adjuvant therapy for breast cancer and the only compound known to prevent breast cancer incidence in healthy women.[1] The antiestrogen clearly is an important drug in breast cancer treatment and prevention; however, Tam produces some undesirable side effects, the most significant being an increased risk of endometrial carcinoma.[1–3] Tam-stimulated endometrial hyperplasia has been shown to be a plausible cause in uterine cancer development,[4] but why some women are at risk for endometrial carcinoma while taking Tam for their breast cancer remains unknown. Genetic polymorphisms and metabolism of antiestrogens may lead to increased uterine sensitivity to Tam in women taking the antiestrogen for breast cancer as well as change their mortality rates.[5–9] Collectively, these observations suggest that genetic factors can influence sensitivity to hormones and antihormones.

Estrogens, Tam and other selective estrogen receptor modulators (SERMs) exert their actions through ERs (ERα and ERβ), which belong to the nuclear receptor superfamily of transcriptional regulatory proteins related by structure and function.[10,11] Estrogen binding allows ER to dissociate from heat shock proteins and form dimers that bind to specific DNA sequences, named estrogen response elements, located in the promoters of target genes.[12] Altered transcription of specific estrogen responsive genes can cause increased proliferation and growth of target tissues. Receptor levels and ER activity are thought to play a key role in target tissue responsiveness to estrogen and Tam.[13]

Recently, a large number of ER co-activators and co-repressors have been identified.[14,15] Furthermore, several investigators have shown that estradiol and SERMs influence the way co-regulator proteins bind

to agonist-activated ER and enhance or repress gene transactivation in a number of ways.[16–18] Although co-regulators are fairly ubiquitously expressed, expression levels vary widely among tissues and organs, and co-regulators are thought to influence the functional activity of 17β-estradiol (E_2) in particular tissues.[19–21] Furthermore, ERα and co-regulator expression levels vary among uterine tissue compartments (luminal and glandular epithelium, stroma, and myometrium), which might be a determinant of the responsiveness of the different uterine cell types to estrogen and the agonist properties of mixed antagonists such as Tam.[22–24]

The response of a target tissue to estrogenic stimuli can be influenced by genetic differences in rodent strains. Among the various rodent strains that display differences in hormone sensitivity,[25–28] inbred Fischer 344 (F344) rats and outbred Sprague Dawley (SD) rats have been widely used to investigate the influence of genetic background on estrogen sensitivity. Based on pituitary tumor development[27,29–31] and the hypertrophic response of the vaginal epithelium after estrogenic stimulation,[32] the F344 rat strain is generally considered to be more sensitive to estrogen than the SD strain. To test the hypothesis that rodent strain differences can also influence Tam sensitivity, we examined the uterine response to the antiestrogen in F344 and SD rats, and determined whether differences in uterine levels of ERα and nuclear receptor co-regulators exist in the two strains.

Material and methods

Animals and treatments

The local animal care and use committee approved use and procedures performed on animals. Immature F344 and SD animals 21–23 days of age (n=84) from Harlan Sprague-Dawley (Indianapolis, IN) were ovariectomized under general anesthesia. Five days later, animals (n=6/group) were given vehicle (sesame oil; Sigma-Aldrich, St Louis, MO), 4-hydroxytamoxifen (4-OHT; Sigma-Aldrich) or E_2 (Sigma-Aldrich). Doses of 4-OHT were 1×10^{-4}, 1×10^{-3}, 1×10^{-2}, 1×10^{-1}, 1.0 and 10 mg/kg body weight (BW). E_2 doses were 1×10^{-6}, 1×10^{-5}, 1×10^{-4}, 1×10^{-3}, 1×10^{-2}, 1×10^{-1} and 1.0 μg/kg BW. Injections were given in the periscapular region for 2 days and animals were sacrificed on the third day by cervical dislocation. Reproductive tracts were collected immediately, and fat and mesentery were removed. The uterus was separated from the other reproductive tract tissues (cervix and vagina), luminal fluid was expressed and the tissue was blotted dry prior to obtaining uterine weights. To determine dry uterine weights for each animal, the right horn was isolated, weighed, placed in a 60°C oven for 2 days and reweighed. Average uterine weights were calculated per 100 g BW, which allowed us to adjust for differences in body weight for each animal. Mean values per treatment group were subjected to statistical analysis. The remaining uterine horn was placed in 4% paraformaldehyde for paraffin embedding and subsequent histomorphological and immunohistochemical analysis.

Histomorphometrics

To examine uterine histomorphology, sections were cut at 6 μm, mounted on Super Frost Plus slides (Fisher Scientific, Houston TX) and stained with H & E. The Spot Imaging System (Diagnostic Instruments, Sterling Heights, MI) was used to photograph and analyze the sections. Cell heights, measured from the top of cell to the basement membrane, were calculated for 40 luminal epithelial cells per animal (10 cells per tissue section, four sections per animal), using Scion Imaging software (Scion, Frederick, MD). ANOVA analysis was performed using the SPSS statistical software (Claritas, Troy, NY). The *post hoc* LSD test was used to compare mean cell heights in each group. All error bars represent SD from the mean.

RNA Isolation, cDNA probes and Northern blot analysis

To examine ER and co-regulator expression in reproductive tract tissues prior to treatment, uteri and vaginas were collected form a second group of control F344 and SD animals (n=12). Total cellular RNA was isolated from whole uterine or vaginal tissues using TRI reagent, according to the protocol provided by the manufacturer (Molecular Research Center, Cincinnati, OH). Total uterine or vaginal RNA was pooled by strain for Northern blot analysis as described previously.[24] The constructs used in this study have been described previously,[24,33] unless otherwise noted. Briefly, the following cDNAs were labeled with [^{32}P]CTP using Rediprime II kit (Amersham Life Science, Arlington Heights, IL) and used for Northern analysis: full-length rat ERα, rat GRIP1,

rat SRC-1, rat p300, rat SPA, mouse CARM1,[34] human REA[35] and rat Uba3.[33] The 36B4 cDNA, a constitutively expressed gene in the uterus,[33,36] was used to correct for differences in loading between blots. All Northern blot experiments were performed twice.

Immunohistochemistry

Uterine sections (6 μm) from paraffin-embedded blocks were mounted on Superfrost Plus slides and dried to room temperature. To assure that tissues from each strain were treated similarly, five sections from F344 and SD rats were placed on the same slide. Sections were hydrated in a series of washes from xylene to dH_2O. Antigen retrieval was accomplished by heating the sections and a $1\times$ Dako Target Retrieval solution (Dako, Carpinteria, CA) to boiling and allowing to cool to room temperature, followed by 3 washes in 3% H_2O_2 in $1\times$ PBS with 0.1% Tween (PBST) for 10 min each. Sections were washed in PBST 3 times for 10 min each. Sections were blocked with undiluted normal goat serum (Sigma-Aldrich) for 20 min, washed in PBST 3 times for 5 min each and then incubated overnight at 4°C with the anti-ERα rabbit polyclonal antibody (PA1-309; Affinity Bio Reagents, Golden, CO). The following day, sections were washed in PBST (3×10 min each) and secondary antibody was added in a 1:400 dilution of goat anti-rabbit antibody (BA1000; Vector, Burlingame, CA.). One hour later, sections were washed in PBST (3×5 min each). Sections were then incubated in ABC Elite reagent (Vector) for 1 h at room temperatur and then washed in PBST (3×5 min each). Sections were reacted with nickel-intensified diaminobenzidine reagent (DAB kit; Vector) for 3 min. Sections were washed in dH_2O (3×5 min each), followed by dehydration in graded alcohols and xylene, and then coverslipped. Immunohistochemistry experiments were repeated 3 times on individual animals. Intensity of ERα staining (low=1+; high=3+) was determined for 100 cells/animal.

Western analysis and protein isolation

Protein was isolated from F344 and SD control uterine tissue using a general lysis buffer containing 25 mM HEPES, pH 7.4, 1% Triton X-100 (Tween 20), 0.5% SDS, 0.5% deoxycholate, as well as the protease inhibitors 1% apropotin, 0.1% leopeptin, 10 μm pepstain, and 1 mM phenylmethylsulfonyl fluoride. Western analysis was carried out using a 12% polyacrylamide gel. Equal amounts of protein, 40 μg, were loaded per lane for both animals. Proteins were transferred to a PVDF-Plus membrane (Osmonics, Westborough, MA). Membranes were blocked with 5% powdered milk in $1\times$ Tris buffered saline with 0.1% Tween (TBST), washed in TBST (3×10 min each) and then incubated in primary antibody 1:1000 (anti-ERα, PA1-309; Affinity Bioreagents) overnight at 4°C. The next day, membranes were washed in TBST (5×5 min each) and incubated for 1 h with secondary peroxidase-coupled goat anti-rabbit antibody, 1:5000 dilution (Bio-Rad, Hercules, CA). The membrane was then washed in TBST (5×5 min each) and placed in a 1:1 mix of SuperSignal chemiluminescent substrate A and B (Pierce, Rockford, IL) for 5 min. Protein expression was assessed using autoradiography. Optical densities of the autoradiograms for ER were compared to GAPDH (Chemicon, Temecula, CA). Image analysis was performed using Molecular Analyst Software (G5670; Bio-Rad). Western blot experiments were performed twice.

Results

To investigate Tam sensitivity in F344 and SD rats, uterine weight was assessed three days after treatment with 4-OHT, the active metabolite of Tam, at various doses (Figure 1). In SD but not F344 rats, 1×10^{-3} mg/kg BW 4-OHT increased ($p<0.05$) uterine dry weight. The next log dose of 4-OHT (1×10^{-2}) increased ($p<0.05$) uterine weight in both strains (Figure 1A). Similar results were obtained using whole uterine and right horn uterine wet weights (data not shown). At 1×10^{-3} mg/kg BW 4-OHT increased ($p<0.05$) uterine luminal epithelial cell heights in SD rats compared to F344 animals and vehicle-treated animals (Figure 2; upper panel), further demonstrating that SD and F344 rat strains exhibit differences in sensitivity to 4-OHT at the level of the uterine growth response.

In both SD and F344 strains, administration of the two lowest doses of E_2 (1×10^{-6} and 1×10^{-5} μg/kg BW) had no effect on uterine weight (Figure 1B). Treatment with 1×10^{-4} μg/kg BW of E_2 increased ($p<0.05$) uterine weight in both rat strains, suggesting no difference in estrogen sensitivity between strains (Figure 1B). However, when uterine histomorphology was assessed, uterine epithelial cell height was increased ($p<0.05$) by 1×10^{-5} μg/kg BW of E_2, in F344 rats but not SD animals (Figure 2; lower panel), corroborating reports by us and

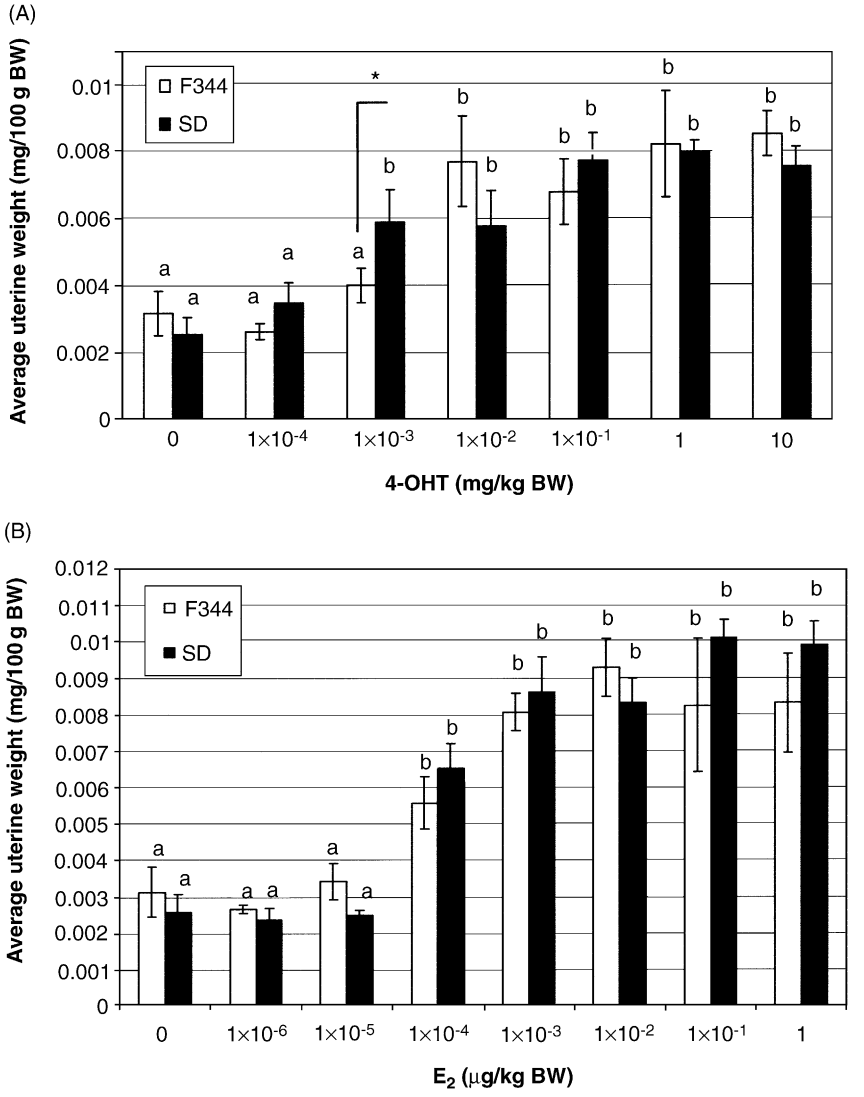

Figure 1. Effects of 4-OHT and E_2 on uterine weights of SD and F344 rats. Rats were treated with vehicle or (A) Tam (1×10^{-4}–10 mg/kg BW) or (B) E_2 (1×10^{-6} to 1 μg/kg BW). Uterine weight was increased ($p < 0.05$) by 1×10^{-3} mg/kg BW Tam in SD but not F344 rats (indicated by the asterisk). No difference between strains in uterine weight after E_2 treatment was seen; however, an increase in the uterine weights of both strains was seen at the 1×10^{-4} μg/kg BW dose. Values not different than controls are indicated by an 'a'; values greater ($p < 0.05$) than control are denoted by a 'b'.

others[29,32] that differences in estrogen sensitivity exist between the two strains.

Receptor levels have a profound influence on target tissue responsiveness and the difference in uterine sensitivity might simply be explained by a difference in the tissue concentration or distribution of ER in the two rat strains. To examine this possibility, levels of ERα, the dominant form of the ER in the rodent uterus, were analyzed in reproductive tract tissues from vehicle-treated animals. Northern blot analysis of total RNA and densitometric tracing of the resulting autoradiograms revealed no difference in uterine ER mRNA levels in F344 and SD animals (Figure 3A). Likewise, Western analysis of ER also showed that receptor protein levels in the uterus were not different between strains (Figure 3B). Receptor immunostaining was localized to the nucleus in uterine cells of both strains, and the number of glandular and luminal epithelial cells given a 3+, 2+ and 1+ staining intensity was similar in F344 and SD rats (Figure 4).

The levels of nuclear receptor co-regulator expression in the reproductive tract and other tissues are thought to be associated with hormonal

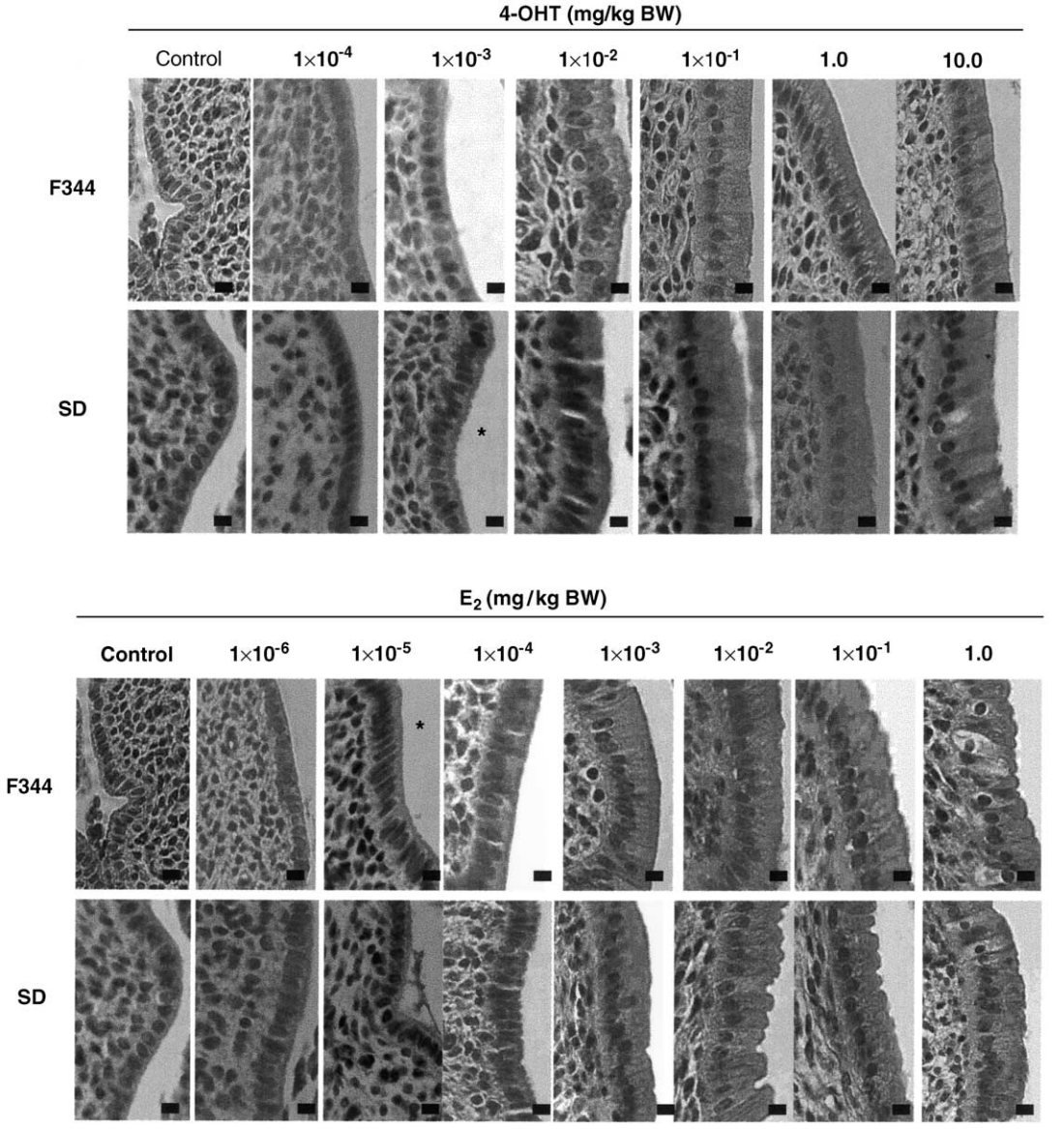

Figure 2. Uterine histomorphology in F344 and SD rats. Representative uterine sections stained with H & E for all dose groups are shown. Control sections were from vehicle-treated immature F344 and SD rats 5 days post ovariectomy. In SD but not F344 rats, luminal epithelial cell height was increased ($p < 0.05$) by 1×10^{-3} mg/kg BW 4-OHT (asterisk). In F344, but not SD rats, epithelial cell height was increased ($p < 0.05$) in the E_2 1×10^{-5} μg/kg BW dose group (asterisk). Calibration bars = 1 μm.

responsiveness.[24,37,38] The difference in sensitivity to Tam might be explained by differences in uterine co-regulator expression, and we examined this possibility by examining the expression of several known ER co-activators and co-repressors. Northern blot analysis using specific probes SRC-1, GRIP1, SPA, CARM-1, P300, REA and Uba3 detected bands of predicted sizes in both the uterus and vagina of either rat strain (Figure 5). Co-regulator levels were consistently higher in the uterus compared to the vagina, regardless of strain (Figure 5). Between the two rat strains, some differences in uterine co-activator levels were observed. For example, higher uterine expression of CARM1 was observed in SD rats and expression of GRIP1 was higher in F344 animals. Expression of SPA, a protein thought to be a mediator of the mixed agonist–antagonist activity of tamoxifen, was higher in the F344 uterus compared to the SD uterus. No difference in SRC-1 or P300 expression was observed between strains.

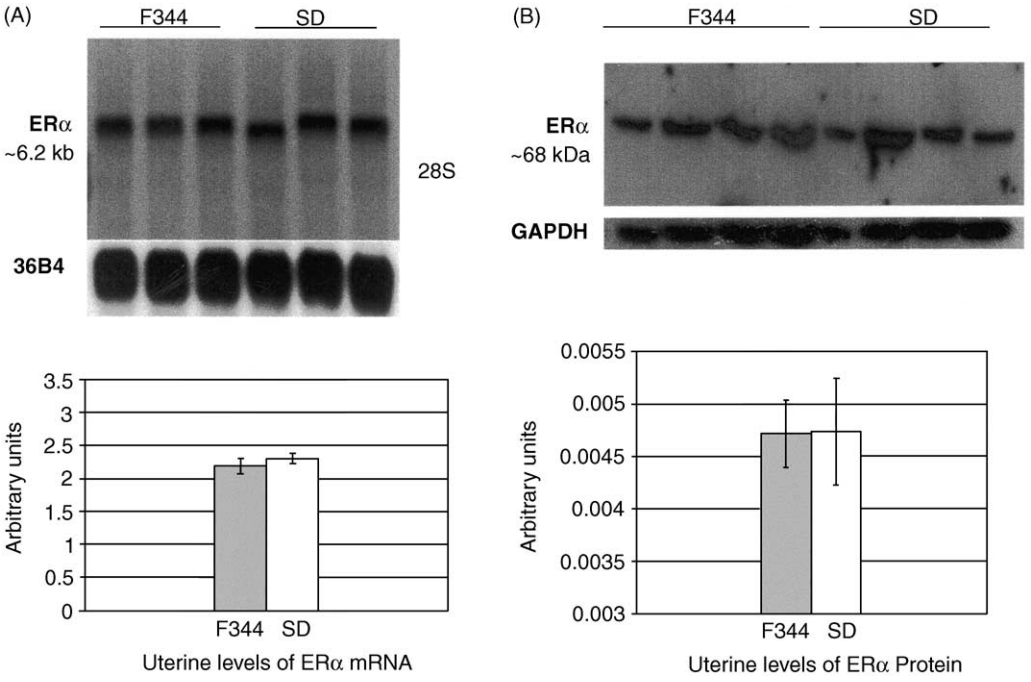

Figure 3. Levels of ERα mRNA and protein in uterine tissue of the F344 and SD rats. (A) Representative Northern analysis using 20 μg of total RNA per lane (three different pools) and probed with and an ERα-specific cDNA. The corresponding loading control, 36B4, is shown below. The ERα:36B4 ratio was used for the densitometric analysis. (B) Representative Western blot analysis of uteri from individual rats ($n=4$/strain) with loading control GAPDH. The densitometric analysis of the western analyses is shown below the blot.

Discussion

Our finding that the uterus of F344 rats responded to a lower dose of E_2 than the SD rat uterus is consistent with those of others on increased estrogen sensitivity of inbred F344 rats compared to the outbred SD rat strains.[27,32,39,40] Thus, because F344 rats are more sensitive to E_2 and other exogenous compounds, we began this study with the assumption that the F344 strain would be more sensitive than the SD stain to the estrogen-like activity of Tam. However, based on the observations that uterine weight and epithelial cell heights were increased by a lower dose of 4-OHT in SD rats, the outbred rat strain appears to be more sensitive to the antiestrogen than the inbred F344 animals. While this is the first report of strain differences in uterine sensitivity to the antiestrogen, administration of high doses of the parent compound Tam to SD rats caused uterine metaplasia in the SD and not the F344 rats,[46] supporting the notion that differences in 4-OHT sensitivity exist between these two rat strains. SD rats have also been shown to be more sensitive than F344 rats to Tam-induced DNA adduct formation in the liver,[41] perhaps due to differences in metabolism of the drug by the cytochrome 450 enzyme pathway.[7,41–43] Yamasaki *et al.* showed that SD rats were more sensitive to flutamide, an antiandrogen, than F344 rats, based on increased weights of accessory sex organs.[44] Based on these observations, we suggest that the inbred F344 rats may be more sensitive to estrogenic compounds, but the outbred SD strain displays increased sensitivity to antihormones and compounds with mixed agonist and antagonist activity, including Tam. However, because SD rats given estrogen plus DMBA showed increased mammary gland tumor development compared to F344 animals,[45] tissue differences in hormone responsiveness between strains may exist under different experimental settings, and there is a need for caution in interpreting the data and drawing finite conclusions.

Higher levels of ERα would provide a mechanism(s) to explain why the SD rat uterus is more sensitive to tamoxifen than the F344 uterus. However, we observed no difference in uterine ERα levels between the two strains, suggesting that other factors must account for the increased sensitivity of the SD rat uterus to the antiestrogen. For example, proteins that affect receptor activity, such as a higher ratio of co-activator to co-repressor expression, increased co-activator expression levels or decreased expression

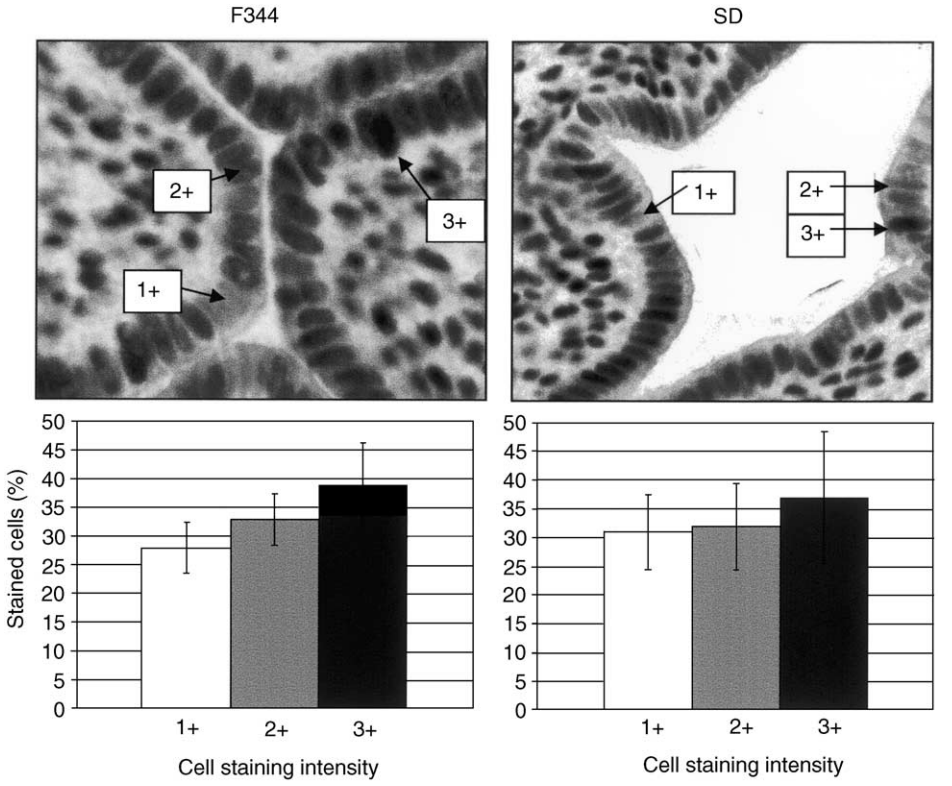

Figure 4. Cellular patterns of immunohistochemistry for ERα in uterine tissue of F344 and SD rats. Representative sections showing the pattern of uterine ER immunohistochemical staining in F344 (left) and SD (right). Arrows point to examples of cells assigned 1 + to 3 + staining intensity. Histograms show average percent cells stained 1 +, 2 + or 3 + per F344 and SD uterus (n=6 animals/group). Magnification is × 40.

levels of co-repressors could all contribute to differences in hormonal responsiveness. Our observation that co-regulator expression levels were consistently higher in the uterus compared to vagina regardless of strain support the concept that differential expression of co-regulators can contribute to hormone sensitivity in various tissues, including the reproductive tract.[24] Although an overall strain difference in uterine co-regulator expression was not observed, some interesting differences in expression of particular co-regulators were seen. CARM1 expression, which was more highly expressed in the uterus of SD rats, has been shown to enhance the ability of the p160 family member GRIP1 to co-activate ER.[46,47] Preferential recruitment of CARM1/GRIP1 complexes when ER is liganded by Tam could enhance receptor transactivation function and play a role in the observed strain difference in uterine growth following 4-OHT treatment. The agonist activity of Tam in normal tissues has been shown to be enhanced by SPA, a co-activator presumed to play a key role in tissue-specific sensitivity to mixed antiestrogens.[48,49] Thus, SPA levels might correlate with sensitivity to antiestrogens, but SPA expression was higher in F344 rat uterus compared to the SD uterus. However, we did not examine whether SPA expression is regulated at the protein level.

In summary, our findings have shown for the first time that differences in uterine sensitivity to Tam exist in rats of different genetic backgrounds. This information may be important in helping us gain a better understanding of why some women develop endometrial cancer while taking Tam for breast cancer treatment or prevention. In addition, the results re-emphasize the importance of considering strain differences when designing *in vivo* drug studies.

Conclusion

We have shown that SD and F344 rat strains display different uterine sensitivities to Tam compared to E_2. Overall, this model may be useful to shed light on the complex issue of why some women are at increased risk for the development of endometrial carcinoma while taking Tam for breast cancer treatment and

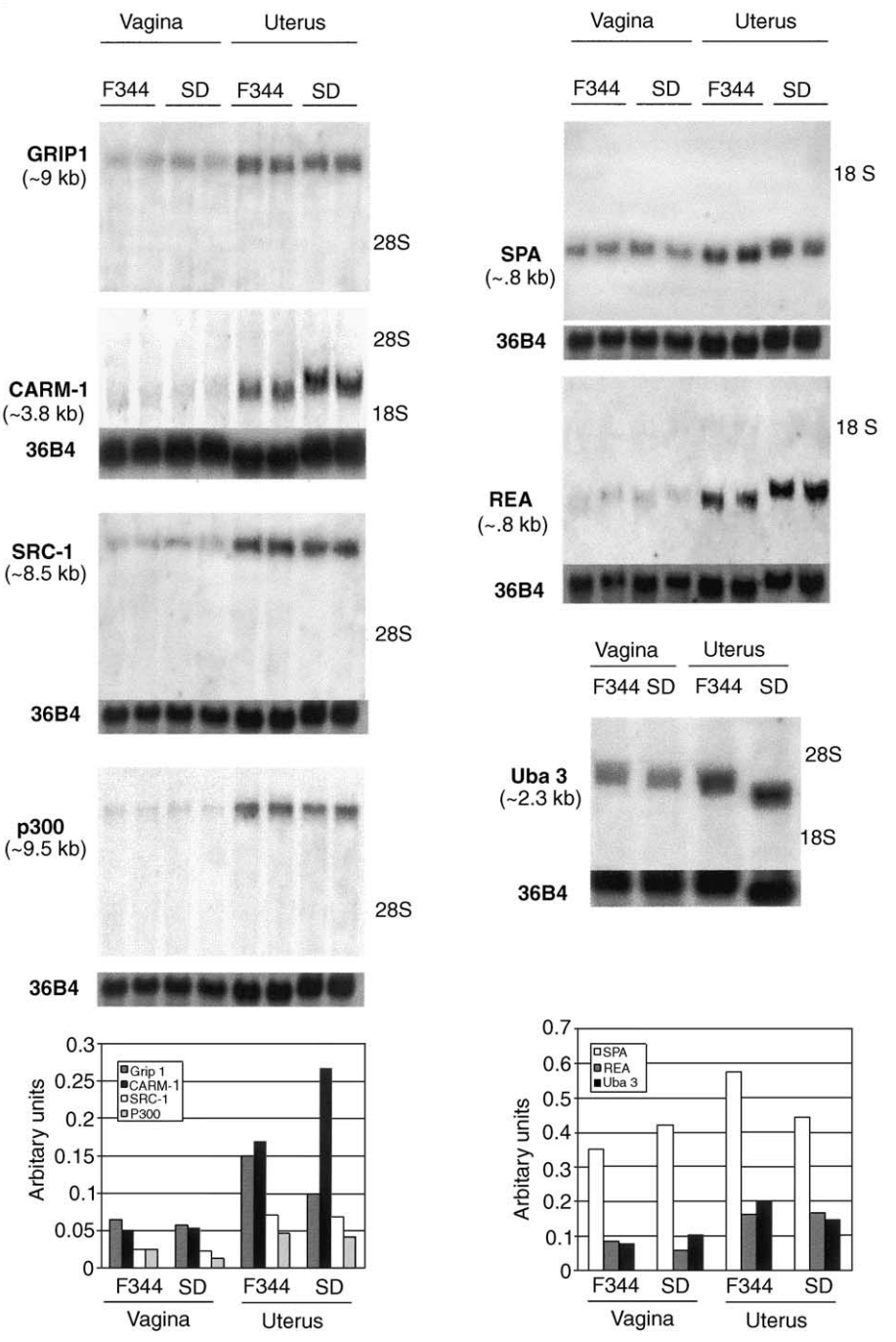

Figure 5. Co-regulator mRNA expression in the uterus and vagina of F344 and SD rats. Northern analysis was performed with 20 μg of total RNA loaded per lane and representative autoradiographs are shown. 36B4 expression was used to correct for differences in sample loading. The graph represents relative expression of the mRNA levels as a ratio of co-regulator to 36B4 signal intensity.

prevention. Although only a limited set of ER-associated genes was examined in the present study, we are currently taking a large-scale genomics approach to examine gene expression in the reproductive tracts of the different rodent strains. In the long term, we believe this kind of information may contribute to improved estimates for risk:benefit ratios for Tam therapy and perhaps allow for more individualized approaches for breast cancer treatment and prevention.

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