Activation of Southern White Rhinoceros (*Ceratotherium simum simum*) Estrogen Receptors by Phytoestrogens: Potential Role in the Reproductive Failure of Captive-Born Females?

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The captive southern white rhinoceros (SWR; *Ceratotherium simum simum*) population serves as an important genetic reservoir critical to the conservation of this vulnerable species. Unfortunately, captive populations are declining due to the poor reproductive success of captive-born females. Captive female SWR exhibit reproductive problems suggested to result from continual ovarian follicular activity and prolonged exposure to endogenous estrogen. However, we investigated the potential role of exogenous dietary phytoestrogens in the reproductive failure of SWR by cloning and characterizing *in vitro* phytoestrogen binding and activation of recombinant SWR estrogen receptors (ESR). We compared those characteristics with recombinant greater one-horned rhinoceros (GOHR; *Rhinoceros unicornis*) ESR, a species that receives similar captive diets yet reproduces relatively well. Our results indicate that phytoestrogens bind rhino ESR in a manner similar to other vertebrate species, but there are no differences found in phytoestrogen binding affinity of SWR ESR compared with GOHR ESR. However, species-specific differences in ESR activation by phytoestrogens were detected. The phytoestrogen coumestrol stimulated greater maximal activation of SWR ESR1 than GOHR ESR1. SWR ESR2 were also more sensitive to phytoestrogens and were activated to a greater extent by both coumestrol and daidzein. The concentrations in which significant differences in ESR activation occurred (10^{-7} to 10^{-5} M) are consistent with circulating concentrations measured in other vertebrate species. Taken together, these findings suggest that phytoestrogens potentially pose a risk to the reproductive health of captive SWR. However, additional studies are needed to further clarify the physiological role of dietary phytoestrogens in the reduced fertility of this species. (*Endocrinology* 153: 1444–1452, 2012)

Within the last century, the global southern white rhinoceros (SWR; *Ceratotherium simum simum*) population has grown from 20–200 individuals (1, 2) to a current estimated population exceeding 20,000 (3). Nevertheless, the International Union for Conservation of Nature lists SWR as near threatened due to persistent poaching to meet the high international demand for illegal rhino horn for use in traditional medicine (4). Recovery of SWR has primarily relied on *in situ* efforts, such as protection of wild stocks from poaching and sport hunting and the creation of game reserves. *Ex situ* programs in the form of captive breeding colonies have also proven valuable to the SWR recovery and continue to serve as genetic reservoirs that protect this species against further pressure (5, 6).

In captive environments that socially and spatially resemble natural conditions, founding members (F_0) of

SWR captive breeding colonies reproduced well throughout much of the 1980s and 1990s. More recently, however, the captive SWR population has been declining because most F_0 females are deceased or beyond their reproductive life span, and the captive-born (F_1) females have exhibited poor reproductive success (5, 6). The cause and extent of low fecundity among female F_1 SWRs is both unclear and controversial, but it is generally accepted that less than one third of all F_1 females have reproduced (5–8). Behavioral studies conducted on captive SWR suggest that both F_0 and F_1 females engage in similar sociosexual behaviors toward males and that male SWR are equally likely to breed F_1 females or F_0 females (6). A survey of 21 institutions noted that there is no difference in copulatory behavior of F_0 and F_1 female SWR, yet a large disparity in the percentage of successful breedings between F_1 females (39%) and F_0 females (79%) exists (6). Examinations into luteal and adrenal activity have also shown no differences between F_0 and F_1 females (7, 9, 10).

A multiinstitutional examination of captive female SWR revealed that many individuals exhibit reproductive pathologies that likely contribute to reduced fertility (11). The most prevalent reproductive problems include varying degrees of cystic endometrial hyperplasia, cervical and uterine leiomyomas, uterine adenomas, mesovarial tumors, and paraovarian cysts (11). In addition, luteal activity of approximately 75% of all female SWR was characterized as either erratic or absent (9–11). Hermes *et al.* (11) suggested that nonreproductive periods, resulting from some unidentified characteristic of the captive environment, lead to continual ovarian follicular activity and prolonged exposure to lifetime levels of estrogen that would be higher than would occur if the individual underwent at least one successful pregnancy. This exposure is proposed to lead to the development of the observed estrogen-responsive reproductive pathologies, which further confound successful reproduction. Although similar phenomena are described or suggested in other vertebrate species (12, 13), the authors do not consider a potential role of exogenous estrogenic compounds in the poor reproductive success of SWR, which can similarly affect the female reproductive tract (14, 15).

Dietary phytoestrogens are one group of exogenous estrogenic compounds to which SWR are potentially exposed. Defined as compounds produced by plants that exhibit estrogenic actions, phytoestrogens consist of two major classes: isoflavonoids and lignans. Lignans found in a variety of plants and plant products, including seeds, fruits, and grains are converted to the weak phytoestrogens enterodiol and enterolactone in the mammalian gut (16). The isoflavonoids coumestrol, genistein, daidzein, and their metabolites exhibit moderate to strong estro-genic activity and are found in legumes, such as alfalfa and soy (17). The Rhinoceros Species Survival Plan husbandry manual (18) recommends that SWR receive 1.5% body mass/d (~34.5 kg) of primarily mixed grasses to reflect wild SWR diets. To compensate for potentially low protein and nutrient levels in grasses, the Species Survival Plan and others suggest supplementing captive SWR diets with as much as 20% (of total grass given) alfalfa and up to 33% (of total calories) from herbivore pelleted food concentrates derived from alfalfa meal (18, 19). In addition to high levels of alfalfa products, many commercially produced pellets also contain soy meal or other soy derivatives as major ingredients (Tubbs, C., personal observation). Indeed, a survey of captive SWR diets suggests many institutions follow these feeding guidelines as 39 North American zoos report SWR diets consisting, on average, of 58% mixed grasses, 24% commercial pellets, and 15% alfalfa (20). Therefore, many captive SWR are likely exposed to significant isoflavonoid concentrations throughout their lifetime.

Phytoestrogens bind and activate estrogen receptors (ESR) from multiple vertebrate species (21). Furthermore, consumption of phytoestrogen-rich diets or treatment with phytoestrogens can cause deleterious reproductive effects, similar to those described in SWR. The SWR reproductive problems closely resemble those described in sheep grazed on pastures of subterranean clover (*Trifolium subterraneum*). Clover is a legume containing high concentrations of genistein and other isoflavonoids (17). Grazing on clover results in temporary infertility associated with various acute changes in female reproductive tract morphology (17, 22, 23). Permanent infertility associated with cystic endometrial hyperplasia develops in ewes after prolonged grazing on clover (24). Furthermore, lambs and ewes fed a diet primarily consisting of alfalfa, another legume containing high levels of coumestrol, also exhibit numerous reproductive pathologies identical to those described in captive SWR, including increased numbers of cervical and uterine lesions and high incidence of paraovarian cysts (25).

Similarities between the deleterious effects of phytoestrogens on sheep and other species and those described in SWR suggest that phytoestrogens may contribute to the poor reproductive success of captive SWR. To investigate this hypothesis, we cloned SWR ESR1 and ESR2 and characterized their phytoestrogen binding affinity and activation *in vitro*. We performed parallel studies using ESR from greater one-horned rhinoceros (*Rhinoceros unicornis*; GOHR), which are fed similar diets and reproduce well in captivity compared with SWR.
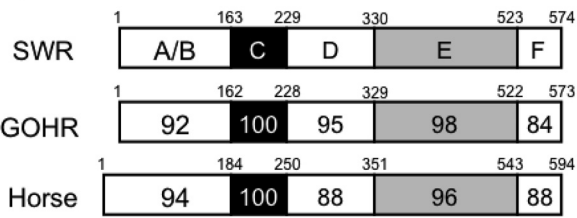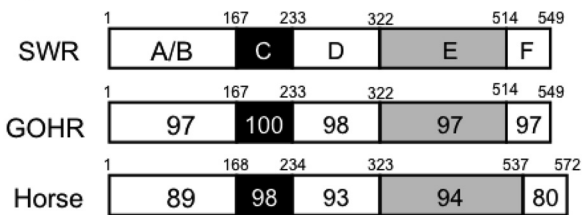
FIG. 1. Comparison of SWR, GOHR and horse ESR sequences. *Values above ESR diagrams* correspond to the number of amino acid residues in ESR functional domains A/B-F. *Values within GOHR* and *horse ESR diagrams* represent percent similarity of predicted amino acid sequences of individual functional domains compared with SWR ESR domains.

Materials and Methods

Animals

Tissue samples used for cloning estrogen receptors came from SWR and GOHR housed at the San Diego Zoo Safari Park (Escondido, CA). All tissues were collected during necropsies performed by the San Diego Zoo pathology staff on animals that died of natural causes. Tissues were immediately placed in RNAlater (Ambion, Austin, TX) upon collection and stored at −20 C until use. The San Diego Zoo's Institutional Animal Care and Use Committee approved all procedures in this study.

Cloning of rhinoceros estrogen receptors

One microgram of total RNA was extracted from SWR cervix (n = 1) and GOHR ovary (n = 1) with QIAzol (QIAGEN, Valencia, CA) and reverse transcribed using the SMART RACE cDNA amplification kit (CLONTECH, Mountain View, CA). Nested PCR was performed using primers designed against highly conserved regions of mammalian estrogen receptors (Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org) and SMART RACE adaptor oligo specific primers. Amplicons of expected size were sequenced with gene specific primers with a 3130 Genetic Analyzer and using BigDye Terminator version 3.1 sequencing chemistry (Applied Biosystems, Carlsbad, CA). Once rhinoceros ESR start and stop site identities were confirmed, PCR was performed using Platinum Taq HiFi (Invitrogen, Carlsbad, CA) with primers designed to capture the entire open reading frame (Supplemental Table 1). Full-length ESR were cloned into a TA cloning vector (Invitrogen) and transformed into TOP10 competent *Escherichia coli* (Invitrogen). Positive colonies were isolated and plasmids purified using a QIAprep spin miniprep kit (QIAGEN). Purified plasmids were sequenced as described above. Amino acid similarity between ESR and other mammalian ESR was determined using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2). Complete ESR coding sequences were subcloned using a ligase-independent method (26) into pVL1393 baculovirus transfer vector for receptor binding assays and into pcDNA3.1(+) expression vector (Invitrogen) for receptor activation assays.

Production of recombinant estrogen receptors

Recombinant SWR and GOHR ESR were produced as described previously (27). Briefly, full-length ESR subcloned into the pVL1393 baculovirus transfer vector were cotransfected with linearized AcV EPA viral DNA into Sf21 insect cells to yield recombinant baculovirus. Positive ESR baculovirus clones were selected and used to infect 50 ml of Sf21 cell suspension (1×10^6 cells/ml). After 72 h of incubation at 27 C, cell suspensions were centrifuged for 10 min at $700 \times g$. Cell pellets were resuspended in 50 ml of a high-salt buffer containing 400 mM KCl, 10 mM Tris, 1 mM Na_2MoO_4, 1.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1.5 mM dithiothreitol, 10% glycerol, 10 mg/ml BSA (TEDG) and subjected to three freeze-thaw cycles with each followed by a $12,000 \times g$ centrifugation at 4 C for 30 min. Clarified lysates were stored at −80 C until use in receptor binding assays described below.
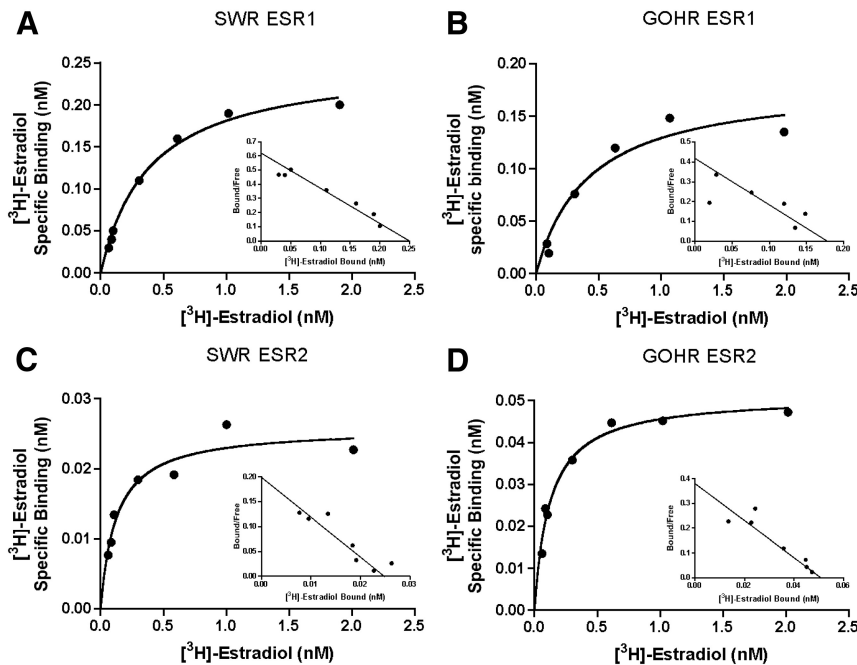
FIG. 2. Characteristics of [³H]E₂ binding to recombinant rhinoceros ESR. Representative saturation and Scatchard plots of specific [³H]E₂ binding to SWR ESR1 (A), GOHR ESR1 (B), SWR ESR2 (C), and GOHR ESR2 (D). Mean K_d for the receptors were the following: SWR ESR1, K_d = 0.41 ± 0.08 nM; GOHR ESR1, K_d = 0.49 ± 0.13 nM; SWR ESR2, K_d = 0.16 ± 0.02 nM; GOHR ESR2, K_d = 0.14 ± 0.02 nM. Results of assays performed in triplicate are typical of three to four independent experiments.

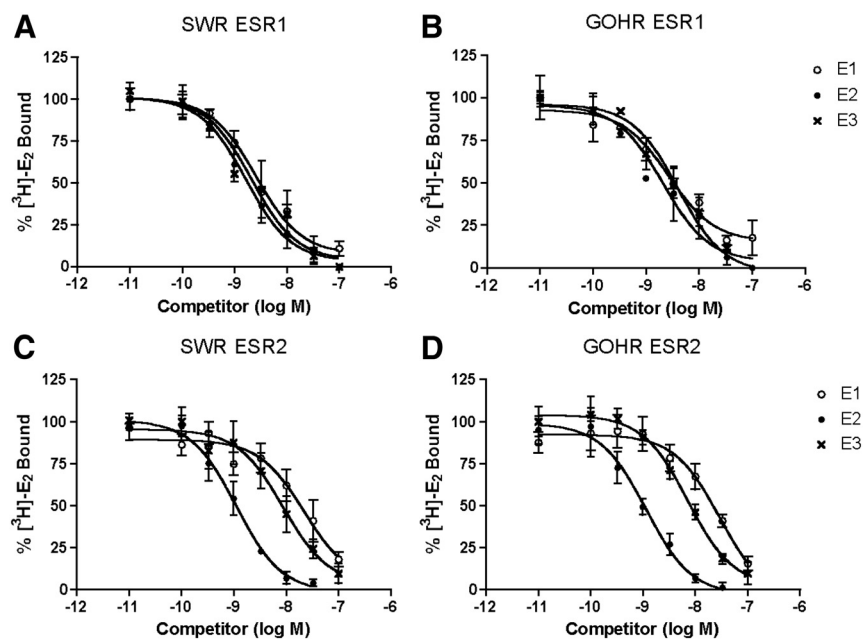**FIG. 3.** Binding of endogenous estrogens to recombinant rhinoceros ESR. SWR (A and C) and GOHR (B and D) ESR were incubated in triplicate with 1 nM (ESR1) or 0.5 nM (ESR2) in the absence (total binding) or presence (nonspecific binding) of 100-fold excess nonradiolabeled E_2. Additional samples were incubated in triplicate with the same concentrations of $[^3H]E_2$ with increasing concentrations (10^{-11} to 10^{-7} M) of E_1, E_2, or E_3. Data are presented as the mean $\pm$ SEM of the percentage of specific $[^3H]E_2$ binding (total binding minus nonspecific binding) displaced by each concentration of the competitors (ESR1 n = 3; ESR2 n = 4).

Receptor binding assays

For all binding experiments, cell lysates were incubated with $[^3H]$estradiol-17β (E_2; PerkinElmer, Waltham, MA) in TEDG
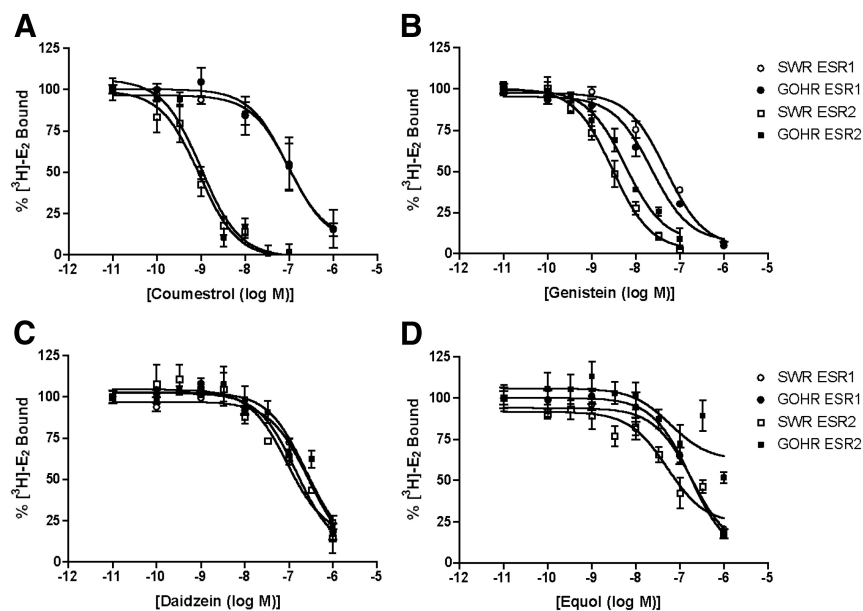

FIG. 4. Binding of phytoestrogens to recombinant rhinoceros ESR. SWR and GOHR ESR were incubated with 1 nM (ESR1) or 0.5 nM (ESR2) in the absence (total binding) or presence (nonspecific binding) of 100-fold excess nonradiolabeled E_2. Additional samples were incubated in triplicate with the same concentrations of $[^3H]E_2$ with increasing concentrations (10^{-11} to 10^{-6} M) of coumestrol (A), genistein (B), daidzein (C), or equol (D). Data are presented as the mean $\pm$ SEM of the percentage of specific $[^3H]E_2$ binding (total binding minus nonspecific binding) displaced by each concentration of the competitors (n = 3).

buffer in the absence (total binding) or presence (nonspecific binding) of 100-fold excess nonradiolabeled E_2 (Steraloids, Newport, RI) in a 96-well plate for 20 h at 4 C. Receptor-bound $[^3H]E_2$ was separated from free by incubation with charcoal-dextran (5:0.5%) in TEDG for 5 min and centrifugation at $1000 \times g$. Specific binding was determined by subtracting the mean radioactivity collected from the supernatant of nonspecific binding wells from total binding wells. Optimal receptor concentrations (those that specifically bound 5–10% of total radioactivity added) were determined as described previously (28) and were 1:32 and 1:64 (lysate-TEDG) for SWR and GOHR ESR1, respectively (data not shown). The same procedure was used for ESR2; however, BSA was excluded from the TEDG buffer to reduce nonspecific $[^3H]E_2$ binding. The optimal ESR2 concentrations were determined to be 1:8 for both SWR and GOHR lysates (data not shown). Saturation and Scatchard analyses were performed by incubating diluted lysates with increasing concentrations of $[^3H]E_2$ (0.03–2 nM) with or without 100-fold excess nonradiolabeled E_2. Specific E_2 binding for each concentration tested was calculated as described above.

In competitive binding experiments, SWR and GOHR ESR were incubated in triplicate with $[^3H]E_2$ (1 nM for ESR1, 0.5 nM for ESR2) and 0.01 nM to 1 μM of estrone (E_1), estriol (E_3), testosterone, progesterone, or cortisol (Steraloids), the synthetic estrogens/antiestrogens ethinylestradiol (EE_2), diethylstilbestrol (DES), or ICI182780 (Sigma, St. Louis, MO) or phytoestrogen standards coumestrol, genistein, enterodiol, enterolactone (Sigma), daidzein, or equol (Indofine Chemical, Hillsborough, NJ). Displacement of $[^3H]E_2$ from ESR by each test compound concentration relative to that of a 100 nM nonradiolabeled E_2 treatment was then fit to a one-site competition curve. The IC_{50} for each ligand-receptor combination was calculated as the concentration at which 50% of $[^3H]E_2$ binding was inhibited.

Cell culture and receptor activation assays

Human embryonic kidney 293 (HEK 293) cells were maintained in MEM supplemented with 10% fetal bovine serum at 37 C and 5% CO_2. Cells (100 μl of 6×10^5 cells/ml) were added to each well of a 96 well plate. After 24 h cells were cotransfected with 5 μg pCMX-β-galactosidase (β-gal), 5 μg pGL2–3xERE luciferase reporter plasmid [Addgene plasmid 11354; (29)], and 0.5 μg of ESR-pcDNA3.1(+) expres-

sion plasmid (Invitrogen) per plate using TransIT 2020 transfection reagent (Mirus Bio L.L.C., Madison, WI) and incubated for an additional 24 h. Transfected cells were then treated in triplicate with 10^{-14} to 10^{-6} M of test compound or appropriate vehicle control [0.01% EtOH or dimethylsulfoxide (DMSO)] in MEM supplemented with 10% charcoal-resin stripped fetal bovine serum. After 24 h cells were lysed and assayed for luciferase and β-gal activity as described previously (30). Luciferase activity was normalized to β-gal activity and fold activation for each ligand concentration was calculated relative to the vehicle control. The fold activation was then normalized to 1 nM E_2, which was run in triplicate on every plate. The relative activation by each test compound was then fit to a sigmoidal dose-response curve to calculate EC_{50} and maximum activation.

Statistical analyses

Data represent mean ± SEM of at least three independent experiments. All curve fitting and statistical analyses were performed using GraphPad Prism Software (version 5.03; San Diego, CA).

Results

Identification of SWR and GOHR ESR

Nucleotide sequences with a high degree of sequence homology to other vertebrate ESR were identified as SWR and GOHR ESR1 and ESR2 (SWR ESR1 GenBank accession no. JN997452, SWR ESR2 GenBank accession no. JN997453, GOHR ESR1 GenBank accession no.

JN997454, and GOHR ESR2 GenBank accession no. JN997455). Rhinoceros ESR are most similar to horse ESR (Fig. 1), which are the only members of the order *Perrisodactyla* for which ESR have been identified. SWR and GOHR ESR1 are encoded by 1725- and 1722-bp open reading frames, respectively. Predicted amino acid sequences of SWR and GOHR are 96% identical overall, with the DNA binding (Fig. 1C), hinge (Fig. 1D), and ligand binging domains all sharing 95% or greater sequence identity. The majority of ESR1 sequence variability between the two species is in the transactivation domain (A/B; 92%) and the F domain (84%; Fig. 1). Both SWR and GOHR ESR2 open reading frames are 1650 bp and encode predicted amino acid sequences that are 97% identical. The predicted amino acid sequences of corresponding ESR2 functional domains A/B-F from SWR and GOHR were each at least 97% identical with one another (Fig. 1).

Binding characteristics of SWR and GOHR ESR

The binding characteristics of $[^3H]E_2$ to recombinant SWR and GOHR ESR were consistent with those of other vertebrate ESR (Fig. 2, A–D). The mean dissociation constants (K_d) for each receptor were 0.41 ± 0.08 nM for SWR ESR1, 0.49 ± 0.13 nM for GOHR ESR1, 0.16 ± 0.02 nM for SWR ESR2, and 0.14 ± 0.02 nM for GOHR ESR2. Mean maximal binding capacity values for the dilutions of lysate that specifically bound 5–10% of the total radioactivity added were 0.25 ± 0.05 nM for SWR ESR1, 0.21 ± 0.06 nM for GOHR ESR1, 0.03 ± 0.01 nM for SWR ESR2, and 0.05 ± 0.01 nM for GOHR ESR2.

Binding of endogenous steroids, synthetic estrogens, and antiestrogens and phytoestrogens are summarized in Supplemental Table 1. There were no species-specific differences in receptor binding affinity detected for any individual compound using IC_{50} 95% confidence intervals. Relative binding affinities were calculated by dividing the IC_{50} for individual compounds by the IC_{50} for E_2. Of the endogenous steroids tested, SWR ESR1 bound E_2 with the highest affinity followed by E_3 and E_1 (Fig. 3A). For GOHR ESR1, E_2 bound with the highest affinity, followed by E_1 and then E_3 (Fig 3B). Both ESR2 bound E_2 with the highest affinity, whereas E_3 and E_1 showed moderate affinity for the receptors (Fig. 3, C and D). Of the
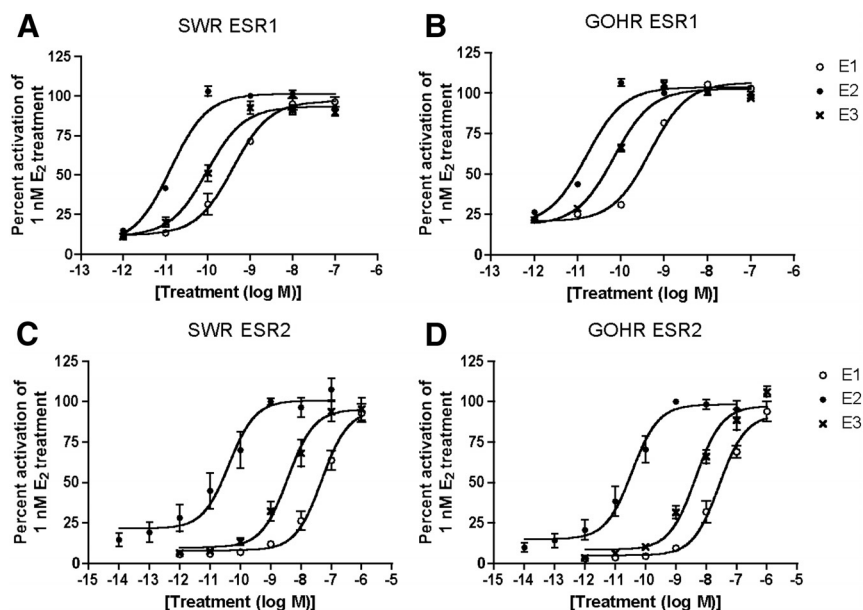

FIG. 5. Activation of recombinant rhinoceros ESR by endogenous estrogens. HEK 293 cells were transiently cotransfected with SWR (A and C) or GOHR (B and D) ESR and estrogen response element-luciferase and β-gal reporter plasmids. Cells were then treated in triplicate with increasing concentrations (10^{-14} to 10^{-6} M) of E_1, E_2, E_3, or vehicle (0.01% EtOH). Luciferase activity of treatments relative to vehicle-only treatment and normalized to β-gal activity was determined and used to calculate fold receptor activation. Data are presented as mean ± SEM of the fold activation of each treatment divided by the fold activation of a 10^{-9} M E_2 treatment (n = 3).

synthetic estrogens and antiestrogens tested, EE_2 bound rhinoceros ESR1 with the highest affinity ($EE_2 >$ DES $>$ ICI182780), whereas DES bound ESR2 with much higher affinity than EE_2 and ICI182780. The relative binding affinity of DES to rhinoceros ESR2 was approximately 2–4 times higher than E_2.

All phytoestrogens displayed relatively low binding affinity ($<10\%$ of E_2) for ESR1 (Supplemental Table 1). However, nearly complete displacement of $[^3H]E_2$ from SWR and GOHR ESR1 occurred after treatment with high concentrations (10^{-7} to 10^{-6} M) of coumestrol, genistein, or daidzein (Fig. 4, A–C). In contrast, the binding affinity of coumestrol to ESR2 was greater than that of E_2. Genistein showed moderate affinity for ESR2, whereas daidzein bound with low affinity. Similar to ESR1, near complete displacement $[^3H]E_2$ from ESR2 was achieved by 10^{-8} to 10^{-6} M of each phytoestrogen tested. Equol, a phytoestrogen metabolite, has low binding affinity for all four ESR. Finally, there was no detectable binding to any rhinoceros ESR by the lignans enterodiol and enterolactone.

Activation of SWR and GOHR ESR by endogenous estrogens and phytoestrogens

Activation of rhinoceros ESR was determined by measuring the activity of ESR-mediated transcription of the pGL2–3xERE luciferase reporter plasmid. Maximal in-duction of luciferase activity for ESR1s was 5- to 7-fold over vehicle-only controls and 20- to 40-fold over controls for ESR2 (data not shown). For all four ESR, E_2 is the most potent agonist, followed by E_3 and then E_1 (Fig. 5, A–D). The concentration of the three endogenous estrogens required to reach EC_{50} were $E_1 = 3.89 \times 10^{-10}$, $E_2 = 1.32 \times 10^{-11}$, and $E_3 = 9.49 \times 10^{-11}$ for SWR ESR1; $E_1 = 4.70 \times 10^{-10}$, $E_2 = 1.58 \times 10^{-11}$, and $E_3 = 6.95 \times 10^{-11}$ for GOHR ESR1; $E_1 = 4.88 \times 10^{-8}$, $E_2 = 4.23 \times 10^{-11}$, and $E_3 = 3.67 \times 10^{-9}$ for SWR ESR2; and $E_1 = 2.68 \times 10^{-8}$, $E_2 = 3.54 \times 10^{-11}$, and $E_3 = 4.46 \times 10^{-9}$ for GOHR ESR2.

All four rhinoceros ESR were activated by coumestrol, genistein, daidzein, and equol. At high concentrations (10^{-6} to 10^{-5} M), all four compounds were capable of activating ESR at levels near or slightly above that of a 10^{-9} M E_2 treatment (Figs. 6 and 7 and Supplemental Table 2). Coumestrol was the most potent phytoestrogen agonist despite having a relatively low binding affinity for SWR and GOHR ESR1 (Fig. 6A). Furthermore, coumestrol treatment at 10^{-7} to 10^{-5} M resulted in greater activation of SWR ESR1 compared with GOHR ESR1 (Fig. 6A). In contrast, activation of GOHR ESR1 by equol was greater than that of SWR ESR1 at a concentration of 10^{-5} M (Fig. 6D). There were no significant differences between SWR and GOHR ESR1 activation by genistein or daidzein (Fig. 6, B and C).

Similar to ESR1, all phytoestrogens tested were agonists for SWR and GOHR ESR2 (Fig. 7, A–D and Supplemental Table 2). The most potent ESR2 agonist was also coumestrol, which induced a significantly higher activation response for SWR ESR2 at 10^{-6} to 10^{-7} M than for GOHR ESR2 (Fig. 7A). The activation of SWR ESR2 was also significantly higher than the activation of GOHR ESR2 at 10^{-5} M for daidzein and equol (Fig. 7, C and D). There was no difference between SWR and GHOR ESR2 activation in response to genistein (Fig. 7B).

Discussion

The present study characterized phytoestrogen binding affinity and activation of recombinant estrogen receptors-α (ESR1) and -β (ESR2) from SWR to investigate the potential role of dietary phytoestrogens in the previously documented low fertility of captive fe-
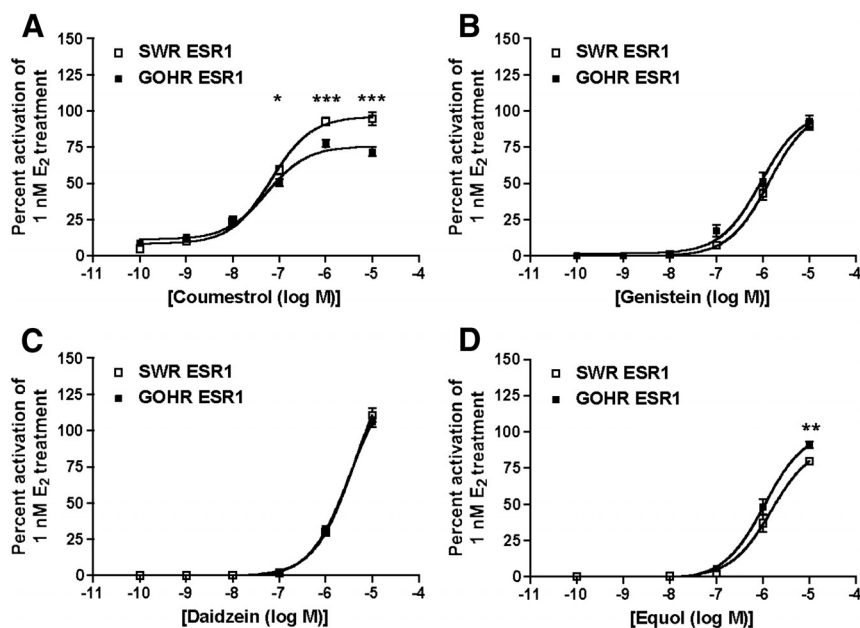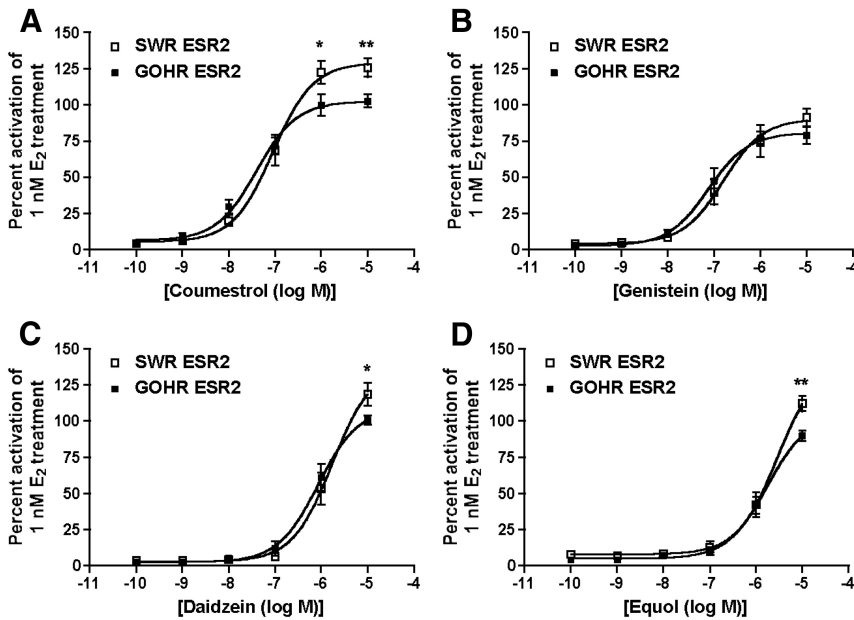
FIG. 6. Activation of recombinant rhinoceros ESR1 by phytoestrogens. HEK 293 cells were transiently cotransfected with SWR or GOHR ESR1 and estrogen response element-luciferase and β-gal reporter plasmids. Cells were then treated in triplicate with increasing concentrations (10^{-10} to 10^{-5} M) of coumestrol (A), genistein (B), daidzein (C), equol (D), or vehicle (0.01% EtOH or DMSO). Luciferase activity of treatments relative to vehicle-only treatment and normalized to β-gal activity was determined and used to calculate fold receptor activation. Data are presented as mean ± SEM of the fold activation of each treatment divided by the fold activation of a 10^{-9} M E_2 treatment. Significant differences between mean SWR and GOHR ESR1 activation was determined using a Student's t test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n = 3).

FIG. 7. Activation of recombinant rhinoceros ESR2 by phytoestrogens. HEK 293 cells were transiently cotransfected with SWR or GOHR ESR2 and estrogen response element-luciferase and β-gal reporter plasmids. Cells were then treated in triplicate with increasing concentrations (10^{-10} to 10^{-5} M) of coumestrol (A), genisein (B), daidzein (C), equol (D), or vehicle (0.01% EtOH or DMSO). Luciferase activity of treatments relative to vehicle-only treatment and normalized to β-gal activity was determined and used to calculate fold receptor activation. Data are presented as mean ± SEM of the fold activation of each treatment divided by the fold activation of a 10^{-9} M E_2 treatment. Significant differences between mean SWR and GOHR ESR2 activation was determined using a Student's *t* test (*, $P < 0.05$; **, $P < 0.01$; n = 3–4).

males. We compared SWR ESR characteristics with those of GOHR ESR, which receive similar diets yet have a higher degree of reproductive success in captivity. Binding of phytoestrogens to SWR and GOHR ESR is consistent with that of other vertebrates, with ESR2 displaying the highest affinity for the phytoestrogens tested. There were no significant differences in phytoestrogen binding affinity between SWR and GOHR ESR; however, significant differences in phytoestrogen activation of SWR and GOHR ESR were detected. Coumestrol was found to be a potent activator of both ESR1 and ESR2 and caused higher maximal activation of these receptors from SWR than GOHR. Daidzein showed greater maximal activation for SWR ESR2 compared with GOHR ESR2. Treatment with the isoflavonoid metabolite equol caused greater maximal activation of SWR ESR1 compared with a GOHR ESR1, but an opposite response was observed for ESR2. Production of equol depends on the presence of specific gut flora and can vary between species and individuals (31), and therefore, it is not currently known whether equol is physiologically relevant to SWR or GOHR. These findings suggest at the receptor level, phytoestrogens are generally more potent activators of SWR ESR than GOHR ESR. However, whether these data indicate that dietary phytoestrogens pose greater potential

for deleterious tissue-specific or organismal effects resulting in lower fertility of SWR compared with GOHR requires further investigation.

Significant differences in SWR and GOHR ESR activation by daidzein, coumestrol, and equol occurred at concentrations at least 100-fold higher (100 nM to 10 μM) than maximally activating E_2 concentrations (≥1 nM). Nevertheless, these concentrations fall within the defined ranges of circulating concentrations of phytoestrogens for other species (21). Consumption of soy-based commercial diets results in peak serum total isoflavone concentrations of 1–10 μM in mice, rats (32), and humans (33). In rats fed diets supplemented with daidzein, mean circulating total daidzein concentrations after 21 d were 300–400 nM (34). Furthermore, the majority of consumed daidzein is converted to equol, which was present at concentrations of 0.5–4.4 μM (34). Circulating levels of coumestrol after consumption of alfalfa vary, depending on species, and are typically low. Circulating coumestrol concentrations of 4–20 nM have been reported in sheep, goats, cows, and mice (35–39). Interestingly, sheep fed medic hay (*Medicago littoralis*), a legume in the same genus as alfalfa, exhibit elevated coumestrol levels of 100–150 nM (40), which corresponds to the lowest concentration of coumestrol that results in higher activation of SWR ESR1 compared with GOHR ESR1. These studies demonstrate that concentrations of phytoestrogens at which rhinoceros ESR are differentially activated *in vitro* reflect the physiologically relevant circulating concentrations in other vertebrates, but additional studies are necessary to determine their relevance in SWR and GOHR.

Many factors influence bioavailability of phytoestrogens and warrant consideration when evaluating their effects *in vivo*. First, given the multitude of naturally occurring phytoestrogens and the diversity of captive diets, SWR are certainly exposed to a greater variety of phytoestrogens than those tested here. Furthermore, production of phytoestrogens by plants can be modulated in response to disease, grazing pressure, and season (17), thereby affecting phytoestrogen concentrations in captive feeds. Examining the activation of SWR ESR by crude and fractionated captive food extracts will assist in identifying phytoestrogens and food items of greatest concern (41, 42). Metabolism of phytoestrogens or phytoestrogen pre-

cursors in the gut or by the liver can also significantly alter their estrogenicity and subsequent effects on reproductive health. As mentioned above, production of the phytoestrogen metabolite equol depends on the presence of certain gut flora and is not ubiquitous among vertebrates (43) or individuals of the same species (31). Furthermore, the weak phytoestrogen 4-methoxycoumestrol is converted to the more potent coumestrol, resulting in estrogenic responses in sheep (40). The establishment of feeding trials and subsequent measurement of circulating phytoestrogen concentrations in SWR is therefore necessary to gain a better understanding of the metabolism of phytoestrogen in this species. Finally, binding of endogenous estrogens and estrogenic chemicals to blood binding proteins can affect their bioavailability at specific tissues. SHBG and α-fetoprotein display low binding affinity for major phytoestrogens compared with endogenous estrogens, which could result in relatively higher bioavailability (21). Differences in the sequestering capacity of SWR and GOHR blood binding proteins represent yet another mechanism that may impact the ability of phytoestrogens to cause reproductive harm in these two species. Each of these pathways must be investigated to more clearly establish a physiological role for phytoestrogens in the poor reproductive success of SWR.

Reproductive effects of phytoestrogens can be characterized as activational or organizational (22). Activational effects occur after short-term exposure and can include sub- or infertility, hypertrophy of estrogen-responsive tissues, and alteration of hormonal cycles (22). They are often temporary and fertility can be restored after the removal of phytoestrogen sources from the diet. Prolonged exposure to phytoestrogens at all life stages, but especially during fetal development, can result in organizational effects. These are far more detrimental to the reproductive health of an individual and can include altered neuroendocrine function, morphological changes in the reproductive tract, increased incidence of reproductive tumors, and permanent infertility. Similar effects occur after developmental exposure to E_2 or other estrogenic substances, such as DES (14, 22). The high incidence of severe reproductive pathologies like advanced cystic endometrial hyperplasia, leiomyoma of the cervix and uterus, uterine adenomas, tumors of the mesovarium, and ovarian cysts implies organizational estrogenic effects exist in the captive SWR population. However, these pathologies are found in both F_0 and F_1 females. Whether prolonged exposure of SWR to estrogenic compounds as adults can result in organizational effects, as is the case in sheep (19), or whether these pathologies are attributable in some degree to activational effects remains unclear. Nevertheless, the striking reduction in fertility of F_1 females whose fetal development occurred within a captive environment compared with F_0 females that developed in the wild makes a strong argument that a currently unknown organizational effect occurs in captive SWR (6). If these effects result from exposure to phytoestrogens, the degree to which they are organizational *vs.* activational will resolve whether reduction or complete removal of phytoestrogenic sources from feeds can increase in fecundity within the current captive SWR population.

Although the population of wild SWR has been steadily increasing, this species still faces many challenges. For example, SWR poaching reached its highest levels in more than a decade in 2010 and continues to rise (3). Of perhaps greater concern is the likely low genetic diversity of the SWR population as a result the severe reduction in their numbers nearly a century ago. Indeed, genetic variability of SWR is lower than that of black, Sumatran, and GOHR rhinoceros based on microsatellite analyses (44). Whether this represents the effects of a population bottleneck and subsequent genetic drift is unclear but suggests that the SWR population is potentially vulnerable to stochastic events such as disease or climatological change. Therefore, further investigation into the cause of the poor reproductive success of SWR will assist in maintaining self-sustaining *ex situ* captive breeding programs that remain critical to the conservation of this species.

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