Regulation of HOXA10 expression by phytoestrogens

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First published September 19, 2006; doi:10.1152/ajpendo.00167.2006.—HOXA10 is necessary for normal development of the Müllerian duct, and continued adult expression in the uterus is necessary for female fertility. HOXA10 expression is altered by diethylstilbestrol, leading to uterine anomalies. Other endocrine disruptors may potentially lead to reproductive anomalies or dysfunction by altering HOXA10 expression. Here we investigated the effect of isoflavones on HOXA10 expression after in utero or adult exposure in the mouse. Genistein, but not daidzein, regulated HOXA10 mRNA and protein expression in the adult mouse uterus. In contrast, in utero genistein or diadzein exposure had no lasting effect on HOXA10 expression in the exposed offspring. Reporter gene expression driven by the HOXA10 estrogen response element was increased in a dose-responsive manner by genistein, but not daidzein. Neither estrogen receptor-α nor estrogen receptor-β binding to the HOXA10 estrogen response element was affected by genistein or daidzein. In utero exposure to isoflavones is unlikely to result in HOXA10-mediated developmental anomalies. Adult genistein exposure alters uterine HOXA10 expression, a potential mechanism by which this agent affects fertility. Phytoestrogen; endocrine disruption; development

Hox genes impart developmental identity to multiple vertebrate embryonic axes, including the central nervous system, vertebral, limb, and the reproductive tract (35, 47). Hox genes encode transcription factors and regulate a battery of downstream genes that lead to appropriate position-specific tissue identity. The patterning of the developing Müllerian duct depends on the ordered expression of Hox genes (67). We have previously shown that genes of the Hox cluster are differentially expressed in the developing Müllerian duct and lead to its differentiation into discrete adult structures (67). HOXA9 is expressed in the anlage of the oviduct, HOXA10 in the presumptive uterus, and HOXA11 in the developing lower uterine segment and cervix. Diethylstilbestrol (DES) exposure in utero results in altered Hox gene expression, a molecular mechanism by which uterine anomalies occur in exposed female offspring (8). A number of agents with estrogen-like activity induce organ-specific developmental changes in the female reproductive tract. DES, bisphenol A, and methoxychlor all alter uterine Hox gene expression, potentially making this a common mechanism by which these agents lead to reproductive tract anomalies (8, 20, 61).

Compounds derived from plants can also act as estrogens. Included are the isoflavones genistein and daidzein (4, 32, 39, 40, 50). These compounds are found in soy-based products, including infant formula and dietary supplements, resulting in frequent human exposure (22). Asian diets often include high levels of soy, leading to higher exposure than Western diets. Developmental exposure to genistein and daidzein has been shown to result in alterations in the female reproductive tract of rodents (4, 9, 29, 32, 39, 40, 50). Sprague-Dawley rats treated in utero with genistein have lower uterine weights and increased progesterone receptor (PR) expression (12, 28, 71). Rats similarly treated in utero were also noted to have smaller anogenital distances than controls (39). These effects occurred at concentrations well below that attained in the serum of humans consuming a high-soy diet. Adverse effects of human isoflavone exposure have not been documented.

In the present study, we investigated the effects of the isoflavones genistein and daidzein on HOXA10 gene expression in the murine uterus after exposure in utero, after adult exposure. We and others have previously shown that exposure to several xenoestrogens in utero and in vitro results in altered uterine HOXA10 expression mediated through the estrogen receptor (ER) (2, 8, 13, 20, 45, 61). We hypothesized that isoflavones may also disrupt this pathway, leading to altered HOXA10 expression with potential adverse reproductive consequences. Here we determined the ability of genistein or daidzein to alter HOXA10 expression.

METHODS

Chemicals. 17β-Estradiol (1,3,5[10]-estratriene-3,17β-diol), genistein, 4',7-dihydroxyisoflavone (daidzein), and 4',5,7-trihydroxyisoflavone (genistein) were purchased from Sigma-Aldrich (St. Louis, MO).

Animals. Nulliparous reproductive age female CD1 mice were obtained from Charles River (Wilmington, MA). The mice were housed in a temperature-controlled room (22°C) with a 14:10-h light-dark cycle. Food (Purina Chow; Purina Mills, Richmond, IN) and water were provided ad libitum. All mice weighed 20–25 g. The Yale University Animal Use and Care Committee approved all animal experiments.

For the uterotrophic assay and measurement of adult HOXA10 expression, on day 0 (1 wk after arrival), bilateral ovariectomy (Ovx) was performed via a single middorsal incision. On day 14 (2 wk after bilateral Ovx), the mice were randomly divided into four groups of three mice each. Three groups received either E2 (0.5 mg/kg), genistein (2 mg/kg), or daidzein (2 mg/kg) dissolved in dimethylsulfoxide. Two groups received a combination of each isoflavone and E2. The control group of Ovx animals was treated with vehicle only. All injections were intraperitoneal.

For in utero exposure, CD-1 mice were treated with genistein (2 or 0.2 mg·kg⁻¹·day⁻¹), daidzein (2 mg·kg⁻¹·day⁻¹), the combination of E2 and genistein (2 mg·kg⁻¹·day⁻¹), or vehicle control by intra-peritoneal injection on day 1 until parturition (day 21 of gestation). Detection of vaginal plug was considered day 0 of pregnancy. Analysis was performed on 2-wk-old-exposed female offspring.

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Animals were killed by cervical dislocation under anesthesia using CO₂ inhalation. Uteri were removed, and wet weight was determined. The uteri were then fixed in 4% formalin and embedded in paraffin for histological and immunohistochemical analysis.

For histological analysis, 5-μm sections were stained with hematoxylin and eosin. For immunohistochemical analysis of HOXA10 expression, slides were deparaffinized and dehydrated through a series of xylene and ethanol washes, followed by permeabilization in 95% cold ethanol. After a 5-min rinse in distilled water, slides were steamed in 0.01 M sodium citrate buffer for 20 min, cooled for 20 min, and rinsed in phosphate-buffered saline with 0.1% Tween-20 (PBST), and sections were circumscribed with a hydrophobic pen. Endogenous peroxidase was quenched with 3% hydrogen peroxide for 5 min followed by PBST. Nonspecific binding was blocked with 1.5% normal horse serum in PBST for 1 h at room temperature. HOXA10 antibody (sc-17159) was purchased from Santa Cruz Biotech (Santa Cruz, CA). Slides were incubated with the primary antibody overnight at 4°C. Normal goat IgG (Santa Cruz) was used as a negative control. Horse α-goat biotinylated secondary antibody was purchased from Vector Laboratories and applied for 1 h at 4°C. Slides were washed in 1× PBST, incubated in ABC Elite (Vector) for 15 min at room temperature, washed in 1× PBST, and incubated for 5 min in diaminobenzidine (Vector). A 20-s exposure to hematoxylin was used as a counterstain. All slides were processed simultaneously. Slides were rehydrated through 3-min ethanol and xylene washes and mounted with Permount.

Real-time RT-PCR. Quantitative real-time RT-PCR was performed using the Lightcycler SYBR green RT-PCR kit from Roche. One microgram of total RNA was reverse transcribed in 20 μl of reaction mixture containing 10 mM each of dATP, dCTP, dGTP, and dTTP; 20 pmol each of forward and reverse primers; 0.8 U of avian myeloblastosis virus-reverse transcriptase; and 10× avian myeloblastosis-reverse transcriptase buffer for 30 min at 61°C. The HOXA10 intron-spanning primers were selected using the primer selection program Primer3 developed by the Whitehead Institute for Biomedical Research. The primers selected yield a 211-bp reaction product located the 5' end of the transcription site of the HOXA10 gene (2).

Electrophoretic mobility shift assay. Electrophoretic mobility shift assays were performed as previously described (15, 69). Complementary single-stranded oligodeoxynucleotides were synthesized and annealed to incorporate the ER binding site, and flanking sequences located the 5' end of the transcription site of the HOXA10 gene (2). Nuclear extracts were prepared from Ishikawa cells growing at log-phase by the method described by Dignam et al. (18). Binding reactions were performed on ice for 30 min using 2-μg Ishikawa cell nuclear extract and 80,000 cpm of labeled DNA in a final volume of 25 μl containing the following: 25 mM HEPES (pH 7.6), 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 5 mM MgCl₂, 10 μg/ml salmon sperm DNA, and 10% glycerol. All samples were fractionated for 3 h at 200 V in a 4% nondenaturing polyacrylamide gel containing 1× Tris-borate-EDTA at 4°C. The gel was dried under a vacuum at 80°C for 45 min and exposed overnight on X-OMAT film (Kodak) and subsequently developed.

Transfection and luciferase assays. Ishikawa cells are a well-differentiated endometrial adenocarcinoma cell line that has previously been used to model human endometrial epithelial cells. Ishikawa cells express ERs, PRs, and other markers of endometrial function (26, 38, 44, 46, 63). HOX gene expression has been previously well characterized in these cells (10, 64, 66).

Ishikawa cells were maintained in 25-cm² flasks using Dulbecco’s minimal essential medium supplemented with 10% (vol/vol) fetal bovine serum. Cells were maintained at 37°C in a humidified atmosphere (5% CO₂ in air) and allowed to reach confluency. Ishikawa cells were passaged by standard methods of trypsinization and plated in 96-well culture dishes until they reached 80% confluence. The cells were then treated with serum-free, phenol red-free media for 24 h.

Preconfluent (75–80%) Ishikawa cells, in 24-well plates, were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The cells were transfected with 0.5-μg pGL3-HOXA10 estrogen response element (ERE) and treated with E₂, genistein, or daidzein, or vehicle control. All cells were cotransfected with 50 ng pcDNA3.1/LacZ to control for transfection efficiency. Experimental controls comprised Ishikawa cells transfected with either empty pGL3 control vector or no luciferase reporter construct. The controls were also assayed with or without the addition of genistein or daidzein. Transfectants were incubated for 4 h at 37°C, washed with 1× PBS, and grown for an additional 12 h. The cells were then washed with cold PBS and lysed with 1× reporter lysis buffer (Promega, Madison, WI), and lysate was collected. The lysate was snap-frozen in a dry ice/ethanol bath and microcentrifuged at maximum speed for 2 min. Luciferase activity was determined in the supernatant, using the luciferase assay kit (Promega) and luminometer. β-Galactosidase activity was determined using the B-galactosidase kit (Tropix, Bedford, MA) and luminometer. β-Galactosidase values were used to normalize luciferase values. A minimum of nine luciferase assays were performed in at least three separate experiments for each experimental condition.

RESULTS

Uterotrophic response to genistein and daidzein. The uterotrophic assay was used to confirm an estrogenic effect (or lack thereof) under the conditions used in our assay. To confirm the uterotrophic response to isoflavones, a single intraperitoneal administration of genistein (2 mg/kg), daidzein (2 mg/kg), or vehicle control was assessed after 8 h. The wet uterine weight was evaluated in three OVX animals from each group. Figure 1 shows the change in uterine weight following administration
of E2, genistein, daidzein, or control (OVX). A single injection of either E2 or genistein resulted in a rapid increase in uterine wet weight of $-1.5\text{-fold} (P < 0.05)$, consistent with previous findings. In contrast to the results in genistein-treated animals, the administration of a single dose of daidzein did not significantly increased uterine weight. Results from the treated animals were compared with control using Student’s $t$-test.

Effects of genistein and daidzein on adult uterine gene expression. To evaluate the acute effect of adult isoflavone exposure on HOXA10 expression, OVX mice were treated with E2, genistein, daidzein, or vehicle control as described. Eight mice were treated in each group. Adult exposure consisted of a single intraperitoneal administration with extraction of uterine RNA after 8 h. Figure 2 demonstrates HOXA10 mRNA expression measured using real-time RT-PCR, normalized to actin expression, and presented as fold induction compared with that obtained from the uteri of vehicle control animals. Differential expression was compared by ANOVA on ranks and post hoc Dunn’s test. Eight hours after exposure, E2 treatment decreased and genistein increased HOXA10 mRNA ($P < 0.05$), whereas no effect was detected after treatment with daidzein or vehicle control. The combination of E2 and genistein resulted in HOXA10 mRNA expression at a level intermediate between that induced by either agent alone.

To explore further the effect of genistein on other markers of uterine proliferation and differentiation, we determine its effect on known estrogen responsive genes. E2 is known to increase uterine C3 expression (16) and, as shown in Fig. 3, significantly increased C3 expression here. Genistein increased C3 mRNA expression also, but by only a minimal amount compared with E2, E2 and genistein each decreased both ER-α and PR expression by a similar amount. E2, but not genistein, induced proliferating cell nuclear antigen expression significantly more than control.

Efig. 3. Effect of adult isoflavone exposure on uterine gene expression. Complement C3 (C3), proliferating cell nuclear antigen (PCNA), progesterone receptor (PR), and estrogen receptor (ER)-α mRNA levels are shown, as determined by quantitative RT-PCR after treatment with vehicle CTL, E2 (0.5 mg·kg$^{-1}$·day$^{-1}$), or GEN (G; 2.0 mg·kg$^{-1}$·day$^{-1}$). GEN had a similar effect to GEN on ER and PR expression but induced significantly less C3 and PCNA expression. Significant difference from control ($^{*}P < 0.05$). Significant difference between the effect of E2 and GEN ($^{#}P < 0.05$).
Ishikawa cells treated with daidzein demonstrated increased luciferase activity only at concentrations above the biologically relevant range. Genistein’s effect on HOXA10 expression likely involves activation of transcription through the HOXA10 ERE.

Effects of genistein and daidzein on ER binding to the HOXA10 ERE. We have previously shown that ER-α and ER-β bind to the HOXA10 ERE and that the affinity of binding is not altered by DES (2). Here we investigated the effect of genistein and daidzein on ER binding to the HOXA10 ERE. Electrophoretic mobility shift assay was used to assess ER-α and ER-β binding to the HOXA10 ERE in the absence and presence of genistein and daidzein. ER-α and ER-β from Ishikawa cell nuclear extract have previously been shown to bind and retard electrophoretic mobility of this element in the presence of \(10^{-8}\) M \(E_2\) (2). As demonstrated in Fig. 7, migration of the HOXA10 ERE through polyacrylamide was retarded by Ishikawa nuclear extract. The location of the shifted complex corresponds to the previously defined ER/ERE complex (2). Treatment with \(10^{-6}\) M \(E_2\) results in a shifted complex. The addition of neither \(10^{-6}\) M daidzein or genistein altered migration of ER or ER binding to the HOXA10 ERE. Neither isoflavone alters HOX gene expression by altering ER binding.

DISCUSSION

Genistein and daidzein are naturally occurring isoflavones found in soy and soy-based products. Soy use is increasing in the Western hemisphere, and soy consumption has remained high in many Eastern countries. Diets containing high levels of these isoflavones have been associated with improved cognitive function and lipid profiles (33, 34). Reduction in breast and prostate cancer as well as cardiovascular disease have also been attributed to isoflavone consumption (7, 36, 62, 70). Despite the promotion of their widespread use, the effects of isoflavones on the reproductive tract have not been well defined. In particular, the effect of these agents on the expression

RT-PCR was performed using uterine RNA obtained from the 2-wk-old animals. Figure 5 demonstrates a lack of effect of either genistein or daidzein on HOXA10 mRNA levels. Each group consisted of 10 female offspring from at least six separate litters.

Effects of genistein and daidzein on reporter expression driven by the HOXA10 ERE. We have recently identified an ERE in the HOXA10 gene that regulates expression in response to \(E_2\) (2). To determine whether the effect of genistein and daidzein on HOXA10 expression was mediated by this ERE, we investigated the effect of these isoflavones on reporter gene expression driven by the HOXA10 ERE. Ishikawa cells were transfected with the pGL3 promotor/HOXA10 ERE construct. Genistein or daidzein were used to treat cells at varying concentrations, and, after 12 h, luciferase activity was measured in the cellular lysate. Figure 6 demonstrates the luciferase activity induced by each compound. Cells treated with genistein demonstrated a dose-responsive increase in luciferase activity. Maximal activity was attained at genistein concentrations of \(10^{-11}\) M and greater. The \(K_d\) 50 was \(10^{-14}\) M.

Fig. 4. Effect of in utero isoflavone exposure on HOXA10 expression. Pregnant mice were treated from day 1 until parturition with \(E_2\) (0.05 mg·kg\(^{-1}\)·day\(^{-1}\)), GEN (2 mg·kg\(^{-1}\)·day\(^{-1}\)), or vehicle control (DMSO). HOXA10 expression was determined in the uteri of the female offspring. Immunohistochemistry was used to semiquantitatively assess level of expression as well as spatial localization. Representative photomicrographs are shown; \(n=6\) animals per treatment. A: representative photomicrograph obtained from \(E_2\)-treated animals. B: section from GEN-treated animal. C: section from vehicle control-treated animal. D: negative control omitting primary antibody. No changes in HOXA10 expression were noted in the GEN or \(E_2\) treatment groups compared with control. Bar = 10 \(\mu\)m.

Fig. 5. Quantitative RT-PCR analysis of uterine HOXA10 expression after in utero treatment. Pregnant mice were treated as described from day 1 until parturition with vehicle control (DMSO; CTL), GEN (0.2 mg·kg\(^{-1}\)·day\(^{-1}\); G0.2), GEN (2 mg·kg\(^{-1}\)·day\(^{-1}\); G2), DA1 (D), \(E_2\), or the combination of \(E_2\) and GEN (G+2). HOXA10 expression was measured in the uteri of the offspring. None of the treatments significantly altered HOXA10 expression.
of genes essential to uterine development and fertility is not well characterized.

Exposure to estrogens during embryogenesis can permanently alter reproductive tract development, leading to abnor-

Fig. 6. HOXA10 estrogen response element (ERE) reporter expression in response to GEN or DAI. We investigated the effect of GEN and DAI on reporter gene expression driven by the HOXA10 ERE. Ishikawa cells were transfected with the pGL3 promoter/HOXA10 ERE construct. GEN or DAI were added at varying concentrations, and, after 12 h, luciferase activity was measured in the cellular lysate and normalized. Cells treated with GEN demonstrated a dose-responsive increase in luciferase activity. Maximal activity was attained using $10^{-11}$ M and higher GEN with a $K_d$ 50 of $10^{-14}$ M. Ishikawa cells treated with DAI demonstrated increased luciferase activity only at concentrations above the biologically relevant range.

Fig. 7. ER binding to the HOXA10 ERE is not altered by GEN or DAI. Nuclear extracts were prepared from Ishikawa cells. We have previously demonstrated an electrophoretic mobility shift generated by both ER-α and -β using Ishikawa cell nuclear extract and the HOXA10 ERE. Binding reactions were performed on ice for 30 min using 2 μg Ishikawa cell nuclear extract and 80,000 cpm of labeled DNA. Lane labeled 0 contains labeled probe only. Lane labeled E2 contains labeled probe, nuclear extract, and E2. Lane labeled E2+G contains E2 and GEN. Lane labeled E2+D contains E2 and DAI. No change in the shifted complex is detected with the addition of GEN or DAI.
nal function in the adult (20, 23). The developing female fetus is uniquely sensitive to the effects of estrogenic compounds. DES exposure is an established model for the lasting effects of developmental estrogen exposure on uterine development (52). The carcinogenic and teratogenic effects of prenatal exposure to DES are well characterized in humans (25, 31, 41, 54). Similarly, mice treated with DES in utero or neonatally have reproductive tract defects (24, 51, 53, 68). In mice, alterations in HOX gene expression caused by DES exposure have been shown to be associated with uterine developmental defects. Altered HOX gene expression may serve as a marker of exposure to some endocrine disruptors.

Although genistein is known to affect the rodent reproductive tract, the mechanism is not completely understood. The functional effect of genistein exposure in rodent models varies by route, dose, and timing of administration (17, 27, 37). Higher doses of genistein induce uterine hypertrophy, as demonstrated by increased uterine weight, and also disrupt female fertility and uterine histology (28, 55). Furthermore, as with DES exposure, uterine carcinoma can be induced in rodents treated with high doses of genistein (3, 17, 51). ER expression is not modulated by genistein, except at high concentrations (12). The response generated by genistein is distinct from that in response to E2; global gene expression profiling in the rat has demonstrated unique uterine gene expression profiles in animals exposed to E2 or genistein (1, 11, 42, 43, 49). The occurrence of endocrine disruption by genistein is documented; however, the involvement of Hox genes in the process has not previously been investigated.

Here we investigated the effect of isoflavones on HOXA10 expression. This gene is known to be essential for normal uterine development (8, 64). Targeted disruption of HOXA10 has a profound effect on reproductive function; however, it results in only subtle anatomic or histological uterine defects (5, 60). Additionally, HOXA10 expression is essential in the adult for normal endometrial receptivity and embryo implantation (10). Treatment of adult mice with uterine HOXA10 antisense diminishes pregnancy rates and litter size (6). Simi-
larly, women with conditions that decrease embryo implantation often have diminished HOXA10 expression (10, 14, 58, 65). Alteration of developmental or adult HOXA10 expression may have health implications. Here we demonstrate the ability of genistein to alter HOXA10 expression after treatment of the adult mouse but not after in utero exposure. Whereas E2 decreased the expression of HOXA10 in the adult, genistein increased it and blocked the repressive effect of E2. Genistein had a similar effect to E2 on ER and PR expression, indicating that modulation of these receptors does not underlie this regulation. Nor does genistein treatment lead to altered binding of ER to the Hox ERE, yet the ERE is regulated by genistein. It is likely that conformational changes in the receptor liganded to either E2 or genistein regulate differential transcriptional activity.

In humans, no clinical uterine effect (e.g., endometrial thickness or bleeding) has been noted in menopausal women ingesting genistein (56, 59). The effect of genistein ingestion in pregnancy on the developing human female reproductive tract has not been examined. Genistein is detectable in second-trimester human amniotic fluid (21). Here we demonstrate that, unlike DES, genistein exposure in utero did not result in a lasting alteration of HOXA10 expression. The concentrations used were above those reported in women (21, 57). Based on this result, it is unlikely that human females exposed to genistein in utero are at risk for HOX gene-mediated developmental anomalies. Genistein has long been known to affect female fertility in rodents (19, 30, 48). Altered uterine Hox gene expression is a likely molecular mechanism contributing to the decreased fertility in the adult. In contrast, developmental exposure more likely results in fertility deficits through its effect on the central nervous system or ovarian function. The effect of genistein use on fertility is less well characterized in reproductive aged women. Adult HOXA10 expression is also necessary for endometrial receptivity and fertility in the human (6, 64). The alteration of adult endometrial HOXA10 expression by genistein may have implications for human fertility.

GRANTS

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REFERENCES

GENESTEIN REGULATES HOXA10 EXPRESSION


