Variation in commercial rodent diets induces disparate molecular and physiological changes in the mouse uterus

Haibin Wang*,†, Susanne Tranguch*‡, Huirong Xie*, Gregory Hanley§, Sanjoy K. Das*¶, and Sudhansu K. Dey*‡**

Departments of *Pediatrics, †Pathology, ‡Cancer Biology, §Cell and Developmental Biology, and ¶Pharmacology, Vanderbilt University Medical Center, Nashville, TN 37232

Edited by George E. Seidel, Jr., Colorado State University, Fort Collins, CO, and approved May 13, 2005 (received for review March 1, 2005)

Although ovarian estrogen, estradiol-17β, is a key modulator of normal reproductive functions, natural and synthetic compounds with estrogen-like activities can further influence reproductive functions. Plant-derived phytoestrogens specifically have received much attention because of associated health benefits. However, a comprehensive understanding of the beneficial and/or detrimental impacts of phytoestrogen consumption through commercial rodent diets on uterine biology and early pregnancy at the molecular level remains largely unexplored. Using multiple approaches, we demonstrate here that exposure of adult female mice to a commercial rodent diet with higher phytoestrogen levels facilitates uterine growth in the presence or absence of ovarian estrogen, alters uterine expression of estrogen-responsive genes, and advances the timing of implantation compared with a diet with lower phytoestrogen levels. The finding that variability in phytoestrogen content in commercial rodent diets, both within and between brands, influences experimental results stresses the importance of this investigation and raises caution for investigators using rodents as animal models.

dietary phytoestrogen | embryo implantation | uterine gene expression

O
cvarian estrogen and progesterone (P4) regulate various uterine functions during the reproductive cycle and pregnancy (1). In mice, coordinated estrogen and P4 actions direct uterine cell proliferation and differentiation in a spatiotemporal manner, establishing the window of uterine receptivity for implantation. Using a delayed implantation model, we previously showed that ovarian-derived primary estrogens participate in the preparation of the P4-primed uterus to the receptive state, whereas uterine catecholestrogens formed locally from primary estrogens mediate blastocyst activation for implantation (2). These findings place both the embryo and uterus as targets for estrogen and its metabolites. Furthermore, estrogen within a very narrow range of concentration determines the duration of the window of uterine receptivity for implantation, reinforcing the idea that regulated estrogen action is critical for implantation (3). Furthermore, fetal loss frequently occurs in mice with higher estrogen levels resulting from the targeted deletion of the gene encoding estrogen sulfotransferase or 5α-reductase-1 (4, 5). These observations signal the importance of investigating the potential beneficial and/or detrimental effects of various estrogen mimics on reproductive functions.

Many natural and synthetic compounds mimic estrogen and influence uterine functions by interacting with nuclear estrogen receptors or other signaling molecules (6–8). These estrogenic compounds primarily include environmental xenoestrogens and dietary phytoestrogens. We previously showed that xenoestrogens induce an estrogen-like early phase response (6, 9, 10) and regulate expression of estrogen-responsive genes in the uterus (6, 7). More importantly, xenoestrogens induce implantation in P4-primed rats in the absence of ovarian estrogen (9, 10). These observations demonstrate that xenoestrogens can influence uterine functions. Recently, plant-derived nonsteroidal phytosteroids have received attention, because they are considered to have many health benefits (11). However, the estrogenic compounds also act as endocrine disrupters, causing developmental and reproductive disturbances (12). Thus, it is important to carefully evaluate the beneficial effects of dietary estrogens against their potential deleterious effects in estrogen-responsive target tissues.

Currently, most commercial laboratory rodent diets are formulated with soybean and alfalfa (13, 14), delivering substantial daily doses of isoflavones to animals throughout their lifespan. Recent studies show that rodents consuming these commercial diets ad libitum have high steady-state levels of isoflavones in their circulation, exceeding endogenous estrogen levels by 30,000- to 60,000-fold (15), although these levels in circulation constitute both free (bioactive) and glycosylated isoflavones. Furthermore, there is evidence that genistein, one of the isoflavones, has substantial access and effective free fractions in the sera compared with natural estradiol-17β (E2) (16). Thus, it is suspected that high dietary phytoestrogen levels will influence native estrogen actions in the uterus.

Our research program was relocated to Vanderbilt University (Nashville, TN) 3 years ago after 30 years at the University of Kansas Medical Center (Kansas City, KS). Certain well established results on uterine gene expression in response to ovarian steroid hormones and reproductive functions that we persistently observed in Kansas became variable after we moved to Vanderbilt. The major changes between the animal facilities include differences in mouse diets, caging and bedding systems, environmental conditions, SPF (specific pathogen-free) status, and, presumably, water quality. Considering the finding that maternal dietary factors influence the pregnancy outcome with respect to the sex ratio of the offspring (17), we thought that a change in the source of the diet was a potential cause of the discrepancy. Previous reports using immature rodents have shown that dietary estrogenic compounds advance female sexual development (18) and interfere with uterine growth in response to estrogen (19). However, the potential impacts of phytoestrogen consumption through commercial diets on uterine biology and early pregnancy at the molecular level remains to be comprehensively elucidated. To address these questions, we initiated a study using two different rodent diets with different phytoestrogen levels from Purina Mills LabDiet. Using molecular, cellular, and physiological approaches, we show here that exposure of adult mice to higher phytoestrogen levels via dietary intake facilitates uterine growth in the absence or presence of ovarian estrogen,}

This paper was submitted directly (Track II) to the PNAS office.
Abbreviations: E2, estradiol-17β; P4, progesterone.
†H.W. and S.T. contributed equally to this work.
**To whom correspondence should be addressed at: Department of Pediatrics, Division of Reproductive and Developmental Biology, Vanderbilt University Medical Center, MCN-D4100, Nashville, TN 37232-2678. E-mail: sk.dey@vanderbilt.edu.
© 2005 by The National Academy of Sciences of the USA
alters uterine expression of estrogen-responsive genes, and advances the window of implantation in adult mice.

Materials and Methods

Animals and Diets. Mice were housed according to Institutional Animal Care and Use Committee and National Institutes of Health guidelines. Six-week-old female CD1 mice purchased from Charles River Breeding Laboratories were housed with a light-dark cycle of 12 h:12 h. Mice were randomly distributed into two groups and fed the commercial rodent diets Purina 5V00 or 5001, which contain similar dietary ingredients but varying levels of isoflavones (www.labdiet.com/indexlabdiethome.htm). The Purina 5V00 diet contains <50 ppm total isoflavones, whereas the Purina 5001 diet contains >400 ppm total isoflavones (13, 15). Food and water were provided ad libitum to all mice. Assuming a daily consumption of 4 g of pelleted ration per adult mouse, 200 and 1,600 μg of total isoflavones are daily consumed from the 5V00 and 5001 diets, respectively. Normally, mice at Vanderbilt are maintained on the Purina 5001 diet.

Uterotrophic and Cell-Proliferation Assays. Mice were on Purina 5V00 or 5001 diets for 6 weeks. They were then ovariectomized and divided randomly into several groups. After 2 more weeks of consumption of the respective diets, mice were injected s.c. with sesame oil (0.1 ml per mouse), P4 (2 mg per mouse), estradiol-17β (E2; 100 ng per mouse), or a combination of the same doses of P4 and E2. The control group of mice received no treatment. Mice were killed at 6 and 24 h after steroid treatments, and body weight was recorded. BrdUrd solution (40 mg per kg of body weight; Zymed) was i.p. injected 2 h before sacrifice. Uterine horns were dissected free of adhering fat and mesentery. After weighing, one uterine horn was fixed in 10% buffered formalin for immunostaining, and the contralateral horn was flash-frozen for gene analysis. The uterine growth response with respect to uterine wet weight and ratio of uterine weight to body weight was analyzed by t test followed by analysis of variance and analysis of covariance with SPSS Software (SPSS, Chicago).

Implantation “Window” and Delayed Implantation. Mice were fed Purina 5V00 or 5001 diets for 8 weeks. To induce pregnancy, females were placed overnight with fertile males and checked for vaginal plugs the next morning (vaginal plug = day 1). To study the effects of diet on the timing of implantation, pregnant females were killed at 2000, 2200, or 2400 hours on day 4 evening, and the number of implantation sites as demarcated by blue bands was recorded after i.v. injections of a Chicago blue B dye solution (20). To confirm pregnancy, uteri devoid of any sign of implantation were flash-frozen for gene analysis. The uterine growth response with respect to uterine wet weight and ratio of uterine weight to body weight was analyzed by t test followed by analysis of variance and analysis of covariance with SPSS Software (SPSS, Chicago).

Immunohistochemistry. Cell proliferation in formalin-fixed, paraffin-embedded sections (5 μm) was detected by using a Zymed BrdUrd staining kit. Immunolocalization of progesterone receptor (PR) and estrogen receptor-α (ERα) was performed by using a monoclonal mouse anti-PR antibody (Zymed) and a polyclonal rabbit anti-ERα antibody (Santa Cruz Biotechnology), respectively. A Histostain-Plus (DAB) kit (Zymed) was used to visualize the antigen. Reddish brown deposits indicate sites of positive immunostaining.

Fig. 1. Commercial rodent diets alter uterine growth responses. (A) Adult ovariectomized mice show comparable body weights after 8 weeks of feeding the Purina diet 5V00 or 5001 (P > 0.05). (B and C) Mice consuming the Purina 5001 diet have markedly higher uterine wet weights than those on the Purina 5V00 diet (*, P < 0.005). (D–G) Uterotrophic assay with adult ovariectomized mice on the respective diets treated with oil, E2, P4, or E2 plus P4. Numbers within the bar indicate the number of mice examined (*, P < 0.05; **, P < 0.005).

In Situ Hybridization. In situ hybridization was performed in frozen sections (10 μm) as previously described by us (21).

RT-PCR. RNA extraction, RT and comparative PCR reactions (25 cycles) have previously been described by us (22). The abundance of LF (lactoferrin), LIF (leukemia inhibitory factor), PR (nuclear progesterone receptor), ERα (nuclear estrogen receptor α), and sFRP2 (secreted frizzled related protein 2) mRNAs was corrected against rPL7.

Results and Discussion

Commercial Diets Differentially Influence Uterine Growth Responses. To examine whether variable phytoestrogen levels in commercial rodent diets influence uterine growth, mice were fed ad libitum for 8 weeks with either of the two brands of Purina mouse chows, 5V00 or 5001 with low or high phytoestrogen levels, respectively. They were ovariectomized 2 weeks before sacrifice. Although mice consuming either diet had comparable body weights (Fig. 1A, P > 0.05), uterine wet weights of mice on the Purina 5001 diet were markedly higher than those on the Purina 5V00 diet (Fig. 1B and C, P < 0.05), indicating higher estrogenicity of the
commercial rodent diets alters uterine responsiveness to native estrogen.

The uterus is comprised of heterogeneous cell types that respond differentially to estrogen and progesterone. Whereas estrogen primarily stimulates uterine epithelial cell proliferation in adult mice, progesterone dampens this effect of estrogen. In contrast, progesterone in combination with estrogen and even estrogen alone to some extent can stimulate stromal cell proliferation. To further explore the impact of dietary phytoestrogens on uterine responses, we examined uterine growth responses to progesterone alone or to a combined treatment of progesterone and estrogen in ovariectomized mice. Again, uterine weights in females on the Purina 5001 diet were significantly higher in response to progesterone when compared with those on the 5V00 diet (Fig. 1 F and G, P < 0.005). However, mice consuming either diet had similar uterine weight increases at 24 h of combined progesterone and estrogen treatment (Fig. 1 F and G, P > 0.05).

Analysis of BrdUrd staining showed stromal cell proliferation in response to progesterone in the presence of higher phytoestrogen levels (Fig. 2C). These results suggest that dietary phytoestrogens mimic native estrogen in stimulating progesterone-induced stromal cell proliferation. Minimal luminal epithelial proliferation noted at 24 h after combined progesterone and estrogen treatment suggests that dietary phytoestrogens have little effect on progesterone’s inhibitory effect on estrogen-induced epithelial proliferation. However, BrdUrd staining was rarely observed in stroma cells in mice on either diet at 24 h of combined treatment with progesterone and estrogen (Fig. 2C). One cause of this poor stromal cell proliferation could be due to the lack of progesterone priming. Thus, we examined the impact of dietary phytoestrogens on stromal cell proliferation in progesterone-primed ovariectomized mice in response to steroid hormones. As expected, BrdUrd labeling was noted in stromal cells when mice on the Purina 5V00 diet were primed with progesterone for 48 h and then challenged with progesterone or with both estrogen and progesterone for 24 h (Fig. 2D). In contrast, this effect was not observed in mice on the Purina 5001 diet with higher phytoestrogen levels (Fig. 2D). These observations demonstrate that exposure to a commercial rodent diet with higher phytoestrogen levels alters uterine stromal cell responsiveness to progesterone. To elucidate the underlying molecular mechanism of estrogenic actions of these diets, we next analyzed differential expression of estrogen-responsive genes in the uterus.

Commercial Diets Alter Estrogen-Responsive Gene Expression in the Uterus. We previously showed that an acute exposure to xenoestrogens alters uterine expression of estrogen-responsive genes in mice (7). Thus, it is conceivable that chronic exposure to dietary phytoestrogens would also influence uterine expression of estrogen-responsive genes. We used comparative RT-PCR to analyze uterine mRNA levels of estrogen-related genes in mice (7). We used a well-known estrogen-target gene, sFRP2, all well-known estrogen-target genes (7, 25–30), in adult ovariectomized mice after they were fed diets containing low (5V00 diet) or high (5001 diet) phytoestrogens. There was increased uterine accumulation of estrogen-related genes in mice consuming the Purina 5001 diet with concurrent decreased levels of sFRP2 (Fig. 3 A and B). These results indicate that a higher background level of phytoestrogens in commercial rodent diets alters uterine expression of estrogen-responsive genes in the uterus.
superimposed on native E2 activity. This estrogenic activity of phytoestrogens influence the functions of E2 and P4 on uterine observation further suggests that background levels of dietary Purina 5001 diet (Fig. 3). Indeed, LF mRNA levels returned to basal levels in oil-treated mice on the 5V00 diet. The levels in mice fed the 5001 diet, however, remained high in the epithelium (Fig. 3). This observation further suggests that background levels of dietary phytoestrogens alter estrogen-responsive genes in response to E2 and/or P4. The next question was to address the mechanism by which dietary phytoestrogens influence these genes. These changes are similar in nature to those previously observed for native estrogen (7, 25–30).

Given our finding of differential effects of these two diets on estrogen-mediated uterotrophic activity, we anticipated that higher phytoestrogen levels would also affect uterine expression of estrogen-responsive genes in response to E2 and/or P4. Indeed, in situ hybridization results show differential temporal expression profiles for LF mRNA in mice on Purina 5V00 and 5001 diets (Fig. 3 C and D). E2 induces a biphasic LF expression in the mouse uterus (7, 31). After E2 treatment, an early increase in LF mRNA levels between 1 and 2 h is followed by a decline at 4–6 h. A more intense expression reappears between 12 and 24 h (7, 31). Consistent with these results, a very low basal level of LF mRNA was detected in the luminal epithelium of mice on the Purina 5V00 diet at 6 h after exposure to steroid hormones. Representative dark-field photomicrographs of longitudinal uterine sections are shown at ×40. In mice on the 5V00 diet, a strong inducer of uterine LF (Fig. 3 C and D). P4 counteracts E2 induced up-regulation of uterine LF (31, 32). Indeed, LF mRNA levels returned to basal levels in oil-treated mice on the 5V00 diet. The levels in mice fed the 5001 diet, however, remained high in the epithelium (Fig. 3 D). This observation further suggests that background levels of dietary phytoestrogens alter estrogen-responsive genes in response to native estrogen and/or P4. The next question was to address the mechanism by which dietary phytochemicals affect uterine gene expression.

The effects of estrogen in the uterus are primarily mediated by nuclear ERα (33); levels of uterine ERβ in mice are extremely low (6, 34). There is evidence that phytoestrogens bind ERs and modulate a variety of estrogen-dependent processes (35, 36). It is also possible that diets with higher phytoestrogen levels exert their estrogenic activity by regulating ERα and PR expression, which then affects uterine responsiveness to estrogen and P4. Indeed, up-regulation of PR and down-regulation of ERα mRNA levels were noted in ovariectomized mouse uteri after chronic exposure to the Purina 5001 diet with higher phytoestrogen levels (Fig. 3 A and B).

Using immunohistochemistry, we examined spatiotemporal expression of PR and ERα proteins in the uterus in response to exogenously administered E2 and/or P4 in mice on the respective diets. We found that mice on different diets exhibit differential expression of PR and ERα (Fig. 4). Whereas low levels of PR were

---

**Fig. 3.** Commercial rodent diets alter estrogen-responsive gene expression. (A and B) Comparative RT-PCR detection of LF, LIF, PR, ERα, and sFRP2 mRNAs in ovariectomized mice consuming the 5V00 or 5001 diet. Data are presented as fold induction and are relative to values from mice on the low-phytoestrogen diet 5V00. Values represent the mean ± SEM of three to four independent RNA samples. (C and D) In situ hybridization of LF in uteri of ovariectomized females on different diets at 6 and 24 h after exposure to steroid hormones. Representative dark-field photomicrographs of longitudinal uterine sections are shown at ×40. myo, myometrium; le, luminal epithelium; ge, glandular epithelium; s, stroma.

**Fig. 4.** Commercial rodent diets exert differential effects on uterine PR and ERα protein distribution in ovariectomized mice treated with E2. Representative immunohistochemical staining of longitudinal uterine sections is shown at ×400. Up-regulation of PR (A and B) and down-regulation of ERα (C and D) protein accumulation was noted in ovariectomized mouse uterus fed the 5001 diet with higher phytoestrogen levels. These experiments were repeated on three mice in each group with similar results. le, luminal epithelium; ge, glandular epithelium; s, stroma.
noted in oil-treated ovariectomized mice on the Purina 5V00 diet, much higher levels of PR were present in both epithelial and stromal cells in mice on the Purina 5001 diet. As expected, an increase in PR expression in these cell types at 6 and 24 h after E2 treatment was observed in mice on the Purina 5V00 diet. However, higher PR levels were consistently present in these cells under similar experimental conditions in mice on the Purina 5001 diet (Fig. 4 A and B). It is known that estrogen up-regulates uterine PR levels (7, 26). The present finding of increased uterine PR expression even in oil-treated mice on the Purina 5001 diet with high phytoestrogen levels clearly indicates a basal but continuous diet-derived estrogenic activity. We then asked whether dietary phytoestrogens affect uterine ERα expression.

Estrogen normally down-regulates ERα because of enhanced receptor processing and degradation (25). Using immunohistochemistry, we indeed observed that dietary phytoestrogens have pronounced inhibitory effects on ERα expression (Fig. 4 C and D). The results show that signal intensity of ERα is much higher in epithelial and stromal cells of oil-treated ovariectomized mice on the Purina 5V00 diet and a single injection of E2 dramatically down-regulated the levels at 6 h and beyond (Fig. 4 C and D). The latter finding is consistent with previous reports (25, 37). Uterine ERα levels were remarkably low in ovariectomized females on the Purina 5001 diet (Fig. 4 C and D). This finding reinforces the concept that higher background level of dietary phytoestrogens mimic functions of native estrogen in resetting its own receptor expression. Thus, differential expression of both PR and ERα in the uterus perhaps attributes to the observed differences in estrogen-mediated uterine growth and varied target gene expression resulting from dietary estrogenic factors. Because estrogen is an essential modulator of normal reproductive functions, it is important to examine physiologically relevant endpoints after exposure to potential estrogenic compounds in vivo.

Commercial Diets Alter the Window of Implantation. During normal pregnancy, implantation occurs within a limited time frame, known as the window of implantation, during which the activated stage of the blastocyst is superimposed on the receptive state of the uterus (20). In mice, the first sign of the attachment between the uterine luminal epithelium and the blastocyst trophoderm occurs at midnight on day 4 of pregnancy (21). Recent evidence shows that estrogen within a very narrow range determines the duration of the window of uterine receptivity in mice (3). Considering the estrogenicity of dietary factors in regulating uterine growth and differential gene expression, we suspected that exposure of females to higher background levels of dietary phytoestrogens would influence the window of implantation.

To address this question, we first examined the timing of the initiation of the embryo-uterine attachment reaction in normal pregnant mice after an 8-week exposure to each respective diet. We observed that females on the Purina 5001 diet with higher phytoestrogen levels exhibit an ~4-h advancement in the attachment reaction (blue reaction) when examined on day 4 late evening. As shown in Fig. 5 A and B, ~45% of mice on the Purina 5001 diet showed distinct blue bands (implantation sites) as early as 2000 hours on day 4, whereas none of the mice on the Purina 5V00 diet showed any signs of implantation even at 2200 hours. We recovered unimplanted morphologically normal blastocysts from mice on the Purina 5V00 diet with lower phytoestrogen levels (Fig. 5 C), indicating normal preimplantation embryo development. This observation demonstrates that a certain level of phytoestrogens in commercial diets resets the window of implantation by affecting uterine receptivity. Similar to the additive estrogenic effects of dietary phytoestrogens in triggering uterine early responses, the present in vivo results provide evidence that the potency of estrogenic compounds predicts their action on implantation (23). Although an acute administration of Kepone, a xenoestrogen, at a high dose initiates implantation in rats (10), we do not know whether an acute exposure of mice to dietary phytoestrogens at background levels will alter implantation status. Nonetheless, our present study suggests that beneficial or adverse effects of maternal exposure to dietary phytoestrogens may occur very early in pregnancy.

The uterus is extremely sensitive to estrogen levels in ensuring on-time implantation (3). Our present observation of an altered window of implantation by dietary phytoestrogens may reflect a change in estrogen responsiveness of the uterus to implantation. Therefore, using a physiologically relevant delayed implantation model, we further explored the influence of dietary phytoestrogens on the minimum level of estrogen required for the initiation of the attachment reaction. Using suboptimal estrogen doses, we observed that 43% of mice on the Purina 5001 diet showed implantation (blue reaction) after E2 treatment at a dose as low as 0.5 ng per mouse (Fig. 5 D), whereas none of the mice on the Purina 5V00 diet showed any blue reaction at the same dose. In fact, only 67% of the females on the low-phytoestrogen diet showed implantation even when treated with 10 ng of E2, whereas only 3 ng of E2 induced implantation in 100% of the mice on the high-phytoestrogen diet (Fig. 5 D). These differences in doses of estrogen required to induce implantation under different diets further confirm that background levels of dietary phytoestrogens alter uterine functions by altering estrogen responsiveness to ovarian estrogen.

Because an extremely low level of estrogen initiates implantation in coordination with P4 in mice, it is suggested that the estrogen-mediated effects in the uterus are locally amplified by various growth factors. This idea is consistent with the expression of several...
growth factors and their receptors in the uterus and/or embryo around the time of implantation (1). We have shown that delayed implanting mouse uteri exhibit differential expression of implantation-related genes after exposure to different physiological levels of native estrogen (3). Considering the impact of dietary phytoestrogens on the window of implantation, we speculated that females on diets with variable phytoestrogen levels would show differential expression of implantation-related genes. HB-EGF (heparin-binding EGF-like growth factor) is a well-characterized implantation marker gene and responsive to estrogen in the mouse uterus (21, 31). Thus, we examined the effects of diet on uterine HB-EGF expression during the attachment reaction. As shown in Fig. 5E, whereas a lower level of HB-EGF mRNA was detected in the luminal epithelium at the site of the blastocyst apposition at 2200 hours on day 4 in mice on the Purina 5V00 diet, a more intense expression was noted in those consuming the Purina 5001 diet containing higher phytoestrogen levels. These results further show that dietary phytoestrogens influence uterine gene expression during implantation.

In summary, the present study demonstrates that commercial rodent diets with variable phytoestrogen levels alter uterine growth response, estrogen-responsive gene expression, and the window of implantation in mice. This investigation raises an issue as to whether commercial rodent diets should be standardized to generate more uniform experimental results among various laboratories. Our present work specifically raises a cautionary note to investigators whose research is sensitive to exogenous sources of estrogens, such as research in steroid hormonal regulation of target cell gene expression, regulation of the cardiovascular system, bone metabolism, aging, and gynecological cancers.

We thank Purina Mills LabDiet for providing us with Purina 5V00 rodent diet and Fuhua Xu for statistical analysis. This work was supported in parts by the National Institutes of Health Grants to S. K. Dey (HD12304, HD33994, DA06668, and CA77839) and S. K. Das (ES07814 and HD37830). S. K. Dey is the recipient of Method to Extend Research in Time Awards from the National Institute of Child Health and Human Development and the National Institute on Drug Abuse.