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-17 β , 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

Ovarian 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 5 α -reductase-1 (4, 5). These observations signal the importance to investigate 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 phytoestro-

gens 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-17 β (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) $\approx$ 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-17 β ; 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

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 E_2 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 $ER\alpha$ expression.

Estrogen normally down-regulates $ER\alpha$ because of enhanced receptor processing and degradation (25). Using immunohistochemistry, we indeed observed that dietary phytoestrogens have pronounced inhibitory effects on $ER\alpha$ expression (Fig. 4*C* and *D*). The results show that signal intensity of $ER\alpha$ is much higher in epithelial and stromal cells of oil-treated ovariectomized mice on the Purina 5V00 diet and a single injection of E_2 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 $ER\alpha$ 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 $ER\alpha$ 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*, $\approx 45\%$ 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 adminis-

Fig. 5. Commercial rodent diets alter the window of uterine receptivity for implantation. (*A*) Higher dietary phytoestrogen levels advance the timing of the attachment reaction. Numbers within the bar indicate the number of mice with blue bands per mice examined. (*B*) Representative photographs of uteri with or without implantation (blue bands) on the respective diets. (*C*) Recovered unimplanted blastocysts from mice consuming the low phytoestrogen diet showing normal morphology. (Scale bar, 50 μ m.) (*D*) Dietary phytoestrogens alters the uterine responsiveness to native E_2 in initiating implantation in delayed implanting mice. Numbers within the bar indicate the number of mice with blue bands per mice examined. (*E*) *In situ* hybridization of *HB-EGF* in uteri devoid of blue bands at 2200 hours on day 4 of pregnancy on the respective diets. Representative dark-field photomicrographs of longitudinal uterine sections are shown at $\times 40$. Arrow indicates the site of the blastocyst. myo, myometrium; le, luminal epithelium; ge, glandular epithelium; s, stroma.

tration 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 E_2 treatment at a dose as low as 1.5 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 E_2 , whereas only 3 ng of E_2 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 uterine responsiveness to ovarian estrogen.

Because an extremely low level of estrogen initiates implantation in coordination with P_4 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.

1. Dey, S. K., Lim, H., Das, S. K., Reese, J., Paria, B. C., Daikoku, T. & Wang, H. (2004) *Endocr. Rev.* **25**, 341–373.
2. Paria, B. C., Lim, H., Wang, X. N., Liehr, J., Das, S. K. & Dey, S. K. (1998) *Endocrinology* **139**, 5235–5246.
3. Ma, W. G., Song, H., Das, S. K., Paria, B. C. & Dey, S. K. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 2963–2968.
4. Tong, M. H., Jiang, H., Liu, P., Lawson, J. A., Brass, L. F. & Song, W. C. (2005) *Nat. Med.* **11**, 153–159.
5. Mahendroo, M. S., Cala, K. M., Landrum, D. P. & Russell, D. W. (1997) *Mol. Endocrinol.* **11**, 917–927.
6. Das, S. K., Taylor, J. A., Korach, K. S., Paria, B. C., Dey, S. K. & Lubahn, D. B. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 12786–12791.
7. Das, S. K., Tan, J., Johnson, D. C. & Dey, S. K. (1998) *Endocrinology* **139**, 2905–2915.
8. Collins-Burow, B. M., Burow, M. E., Duong, B. N. & McLachlan, J. A. (2000) *Nutr. Cancer* **38**, 229–244.
9. Johnson, D. C., Kogo, H., Sen, M. & Dey, S. K. (1988) *Toxicology* **53**, 79–87.
10. Johnson, D. C., Sen, M., Kogo, H. & Dey, S. K. (1990) *Proc. Soc. Exp. Biol. Med.* **195**, 44–50.
11. Setchell, K. D. & Cassidy, A. (1999) *J. Nutr.* **129**, 758S–767S.
12. Whitten, P. L., Lewis, C., Russell, E. & Naftolin, F. (1995) *J. Nutr.* **125**, 771S–776S.
13. Thigpen, J. E., Setchell, K. D., Ahlmark, K. B., Locklear, J., Spahr, T., Caviness, G. F., Goetz, M. F., Haseman, J. K., Newbold, R. R. & Forsythe, D. B. (1999) *Lab. Anim. Sci.* **49**, 530–536.
14. Murphy, P. A., Farmakalidis, E. & Johnson, L. D. (1982) *Food Chem. Toxicol.* **20**, 315–317.
15. Brown, N. M. & Setchell, K. D. (2001) *Lab. Invest.* **81**, 735–747.
16. Nagel, S. C., vom Saal, F. S. & Welshons, W. V. (1998) *Proc. Soc. Exp. Biol. Med.* **217**, 300–309.
17. Rosenfeld, C. S., Grimm, K. M., Livingston, K. A., Brokman, A. M., Lamberson, W. E. & Roberts, R. M. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 4628–4632.
18. Thigpen, J. E., Haseman, J. K., Saunders, H. E., Setchell, K. D., Grant, M. G. & Forsythe, D. B. (2003) *Comp. Med.* **53**, 607–615.
19. Boettger-Tong, H., Murthy, L., Chiappetta, C., Kirkland, J. L., Goodwin, B., Adlercreutz, H., Stancel, G. M. & Makela, S. (1998) *Environ. Health Perspect.* **106**, 369–373.
20. Paria, B. C., Huet-Hudson, Y. M. & Dey, S. K. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 10159–10162.
21. Das, S. K., Wang, X. N., Paria, B. C., Damm, D., Abraham, J. A., Klagsbrun, M., Andrews, G. K. & Dey, S. K. (1994) *Development (Cambridge, U.K.)* **120**, 1071–1083.
22. Daikoku, T., Tranguch, S., Friedman, D. B., Das, S. K., Smith, D. F. & Dey, S. K. (2005) *Mol. Endocrinol.* **19**, 683–697.
23. Huet, Y. M. & Dey, S. K. (1987) *J. Reprod. Fertil.* **81**, 453–458.
24. Kirkland, J. L., Gardner, R. M., Ireland, J. S. & Stancel, G. M. (1977) *Endocrinology* **101**, 403–410.
25. Rosser, M., Chorich, L., Howard, E., Zamorano, P. & Mahesh, V. B. (1993) *Biol. Reprod.* **48**, 89–98.
26. Couse, J. F., Curtis, S. W., Washburn, T. F., Lindzey, J., Golding, T. S., Lubahn, D. B., Smithies, O. & Korach, K. S. (1995) *Mol. Endocrinol.* **9**, 1441–1454.
27. Das, S. K., Tan, J., Raja, S., Halder, J., Paria, B. C. & Dey, S. K. (2000) *J. Biol. Chem.* **275**, 28834–28842.
28. Pentecost, B. T. & Teng, C. T. (1987) *J. Biol. Chem.* **262**, 10134–10139.
29. Shen, M. M. & Leder, P. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 8240–8244.
30. Bhatt, H., Brunet, L. J. & Stewart, C. L. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 11408–11412.
31. Wang, X. N., Das, S. K., Damm, D., Klagsbrun, M., Abraham, J. A. & Dey, S. K. (1994) *Endocrinology* **135**, 1264–1271.
32. McMaster, M. T., Teng, C. T., Dey, S. K. & Andrews, G. K. (1992) *Mol. Endocrinol.* **6**, 101–111.
33. Hewitt, S. C. & Korach, K. S. (2003) *Reproduction* **125**, 143–149.
34. Tan, J., Paria, B. C., Dey, S. K. & Das, S. K. (1999) *Endocrinology* **140**, 5310–5321.
35. Branham, W. S., Dial, S. L., Moland, C. L., Hass, B. S., Blair, R. M., Fang, H., Shi, L., Tong, W., Perkins, R. G. & Sheehan, D. M. (2002) *J. Nutr.* **132**, 658–664.
36. Safford, B., Dickens, A., Halleron, N., Briggs, D., Carthew, P. & Baker, V. (2003) *Regul. Toxicol. Pharmacol.* **38**, 196–209.
37. Shupnik, M. A., Gordon, M. S. & Chin, W. W. (1989) *Mol. Endocrinol.* **3**, 660–665.