The intake, as well as serum and urinary concentrations, of phytoestrogens is high in countries where incidence of prostate cancer is low, suggesting a chemopreventive role for phytoestrogens. Their significance could be explained by the ability to antagonize the action of more potent endogenous estrogens in initiation or promotion of tumor formation. We have studied estrogenicity and antiestrogenicity of dietary soy and two phytoestrogens, coumestrol and daidzein, in our neoDES mouse model for the study of prostatic neoplasia. Soy was chosen because it is rich in phytoestrogens, is widely used in Oriental diets, and has antiestrogenic and anticarcinogenic properties in the neoDES mouse when given from fertilization onward. In short-term tests with adult animals, no evidence for estrogenicity or antiestrogenicity (capability to antagonize the action of 17β-estradiol) of soy was found when development of epithelial metaplasia and expression of c-fos protooncogene in prostate were used as end points of estrogen action. Estrogenic activity of coumestrol and daidzein on c-fos expression was subtle. Coumestrol, either given alone or in combination with 17β-estradiol, had no effect on development of epithelial metaplasia. These marginal or missing effects in adult males could be interpreted by assuming that the neonatal period is more critical for estrogenic or antiestrogenic action of soy and phytoestrogens. Once initiated, estrogen-related lesions would develop spontaneously. Alternatively, the chemopreventive action of soy is not due to antiestrogenicity of soy-derived phytoestrogens. — Environ Health Perspect 103(Suppl 7):123–127 (1995)

Key words: accessory sex glands, c-fos, coumestrol, daidzein, estrogen, male, mouse, phytoestrogens, prostate, soy

Introduction

Dietary soy has chemopreventive properties in several animal models for cancers (1–3). There is also suggestive evidence that dietary soy (tofu) is chemopreventive in human prostate cancer (4). Using the developmentally estrogenized mouse model (5,6), we have shown that the estrogen-related inhibition of prostatic growth is reduced and the development of dysplastic changes is delayed in the prostate when the animals are kept on soy-containing feed from fertilization onward (7). Both the number of animals showing dysplasia and also the severity of the alterations in dysplastic epithelium were lower in animals given soy. Morphologically the dysplastic lesions in the prostate of neoDES animals are similar to prostatic intraepithelial neoplasia (PIN) in human prostate (8). Although no progression to carcinomas with invasion of surrounding tissues or metastasis could be demonstrated, the morphological changes and increased expression of protooncogenes in developmentally estrogenized mice suggest an increased potential for benign and malignant growth (Table 1).

Soy is rich in phytoestrogens, weakly estrogenic nonsteroidal compounds. Their urinary excretion (particularly that of isoflavones) correlates with the ingestion of soy in our animal experiments (7). In humans, urinary excretion and serum concentrations of phytoestrogens are higher in countries where the incidence of prostate cancer is low (9,10). This suggests that phytoestrogens may account for the chemopreventive action of soy in prostate carcinogenesis.

The mechanisms of the possible chemopreventive action of phytoestrogens are not known. Their significance could be explained by their ability to antagonize the action of more potent endogenous estrogens in initiation or promotion of carcinogenesis, although currently no direct evidence for this action is available. The mouse model based on neonatal diethylstilbestrol treatment (neoDES model) is particularly suitable for testing this hypothesis. In neoDES mice the histological response to 17β-estradiol in terms of metaestrogenic transformation and the 17β-estradiol-induced expression of c-fos protooncogene are greatly enhanced. Based on these end points of estrogen action, we have tested the estrogenicity and antiestrogenicity of dietary soy and two structurally different phytoestrogens, coumestrol and daidzein, both known to be present in soy.

Materials and Methods

Animals and Diets

Outbred Han-NMRI mice (produced by the Animal Quarters, Institute of Biomedicine, University of Turku, Turku, Finland) were used throughout the study. The test protocols were approved by the Turku University Committee on the Laboratory Animal Center. The animals were given free access to feed and tap water. A soyfree diet, where soy is substituted with casein, was purchased from Finnewos (Helsinki, Finland) and Special Diet Services, (Witham, Essex, United Kingdom). A standard soy-containing laboratory feed for mice (Ewos R3) containing cereals, wheat germ, wheat middlings, roasted soy meal (7%), fish protein concentrate, fodder yeast, minerals, animal and vegetable fat, vitamin concentrate,
and trace element concentrate was prepared by Finnewos, Helsinki, Finland. The animals were kept on soyfree diet, and the soy-containing diet was used only in the experiments.

**Neonatal Estrogenization with Diethylstilbestrol (neoDES Treatment)**

Male pups were injected sc with 2 μg of diethylstilbestrol (DES) in 20 μl of corn oil per day for the first 3 days after birth.

**Induction of Metaplastic Reaction**

Adult neoDES mice (3–5 months of age) were castrated under barbiturate anesthesia. In soy experiments the animals were divided into four groups: group 1 continued on the soyfree diet; group 2 was transferred to the soy-containing diet; group 3 received an sc implant with 50 μg estradiol and continued on the soyfree diet; and group 4 received an sc implant with 50 μg estradiol and was transferred to the soy-containing diet. In experiments with coumestrol, the animals were divided into four groups: group 1 received the vehicle, 20 μl of dimethyl sulfoxide (DMSO) per day sc; group 2 received 25 μg of estradiol in DMSO sc per day; group 3 received 50 μg of coumestrol in DMSO sc per day; and group 4 received both estradiol and coumestrol. After 10 days (soy experiment) and 7 days (coumestrol experiment) the animals were sacrificed; the urethroprostatic blocks were removed and used for histologic preparations. Tissue blocks were fixed whole in Bouin’s fixative, dehydrated, and embedded in paraffin. Serial horizontal 6-μm sections with 200-μm intervals were cut through the tissue block, from lower urethra up to the upper half of the urinary bladder. The sections were stained with routine hematoxylin and eosin, dehydrated, and mounted with Permount. The

sections were carefully studied for the presence of squamous epithelial metaplasia in the periurethral collecting ducts and proximal parts of coagulating gland, dorsolateral prostate, and seminal vesicles.

**Induction of c-fos Expression**

Adult (3–5 months of age) neoDES mice were castrated under barbiturate anesthesia and, after 7 days, they were injected sc with 17β-estradiol (100 μg/animal), coumestrol (200 μg/animal), or daidzein (200 μg/animal) diluted in corn oil (100 μl/animal). The controls received the vehicle only. In the soy experiment, the animals were kept on the soy diet from castration onward. Animals were sacrificed 3, 6, or 12 hr after injection and urethroprostatic blocks were removed. Tissues were transferred to a petri dish containing phosphate-buffered saline (PBS) and prostate lobes, seminal vesicles, and prostatic urethra were dissected under a microscope. Tissue samples were immediately frozen in liquid nitrogen and stored at −70°C. Total RNAs were extracted with the single step method (11). Fifteen-microgram aliquots of total RNA were size-fractionated in 1% agarose/formaldehyde gels and blotted onto nylon membrane (GeneScreen, DuPont, NEN, Boston, MA). The filters were hybridized and washed as suggested by the manufacturer. The 32P-labeled c-fos antisense RNA probe was synthesized from the insert in the pGEM vector (Promega, Madison, WI) according to the manufacturer’s instructions, using Sp6 RNA polymerase; the radiolabeled probe was added directly to the prehybridization mixture. The c-fos probe was kindly provided by George Stancel (University of Texas, Houston TX). This mouse c-fos probe was originally obtained by digestion and subcloning of mouse pcfos-3 (12). For quantitation, the signal intensities of autoradiographic films were scanned with the Microcomputer Imaging Device (MCID) by using M4, version 2.1 software program (Imaging Research Inc., Ontario, Canada). The intensity values of c-fos were corrected by the corresponding intensity values obtained after hybridization with mouse 28S ribosomal RNA probe. The 4.8 kb SalI–EcoRI fragment of mouse 28S ribosomal RNA cDNA was 32P-dCTP labeled by random priming.

**Results**

**Effects of Dietary Soy and Coumestrol on Development of Squamous Epithelial Metaplasia**

An extensive squamous metaplasia was observed in the periurethral collecting ducts, as well as in the periurethral parts of coagulating glands, when castrated neoDES animals fed a soy-free diet were treated with estrogen pellets (50 μg of 17β-estradiol per pellet) for 10 days or with daily injections (25 μg of 17β-estradiol per day) for 7 days (Figure 1A,B).

When adult neoDES animals were kept on a soy-containing diet for 10 or 21 days after castration, no signs of squamous epithelial metaplasia could be observed. Soy feeding did not prevent the metaplastic reaction induced by 17β-estradiol implants (Figure 1C,D).

Coumestrol (50 μg per day for 7 days given sc) did not induce squamous metaplasia in neoDES animals. Neither did it inhibit the metaplastic reaction induced by 17β-estradiol injections (Figure 1E,F). Thus, based on squamous epithelial metaplasia, there is no evidence that either coumestrol or dietary soy are estrogenic or antiestrogenic.

**Induction of c-fos Expression**

17β-Estradiol induced the expression of c-fos in neoDES mice given a soy-free diet. After the sc injection of 17β-estradiol (100 μg/animal) the increased expression was evident in the prostatic urethra and coagulating glands at 3 to 12 hr after injection (Figure 2).

Soy given to neoDES animals for 1 week before 17β-estradiol injection had no effect on the expression. Further, coumestrol and daidzein in a dose of 200 μg/animal sc showed weak estrogenic action (Figure 2), but there is no evidence that soy acts as an antiestrogen based on the induction of c-fos expression.
Discussion

Despite the greater estrogen sensitivity, no evidence for the estrogenicity of dietary soy (diet with 7% of roasted soy meal) was found in the prostate of neonatally estrogenized, adult castrated male mice when judged on the basis of development of squamous epithelial metaplasia. The lack of the estrogenicity of soy is in contrast to the findings in the immature female mouse in which the estrogenicity of diet with 7% of roasted soy meal was confirmed by the uterine growth response (7). The estrogenlike effect by dietary soy was also demonstrable on the prostatic growth in the male rat when exposed to dietary soy from fertilization onward: the size of the ventral prostate was reduced at 2 months of age (13). In the corresponding feeding experiment with the male mouse, the sizes of the sex accessory glands were reduced but the differences were not statistically significant (7).

Coumestrol, one of the most potent phytoestrogens, was also incapable of inducing metaplastic transformation in neonatally estrogenized mice; as documented earlier, it did not inhibit the prostatic growth when administered to normal adult rats (13). However, when the induction of the expression of estrogen-responsive gene, c-fos (one of the immediate early genes in mitosis) in adult neoDES mice was used as an end point, both coumestrol and daidzein showed weak estrogenlike activity. Also in rat uterus the antiestrogen tamoxifen was shown to induce a weak response in c-fos expression (14). This weak estrogenlike (or antiestrogenlike) effect is induced by phytoestrogen doses comparable to the amounts ingested by

Figure 1. Microscopic structure of prostatic collecting ducts in the posterior periurethral region of an adult castrated neoDES mouse kept on soyfree diet. (A) treated with an sc implant containing 50 μg estradiol and kept on soyfree diet for 7 days; (B) given a soy-containing diet for 10 days postcastration; (C) treated with an sc implant with 50 μg estradiol and kept on a soy-containing diet for 10 days postcastration; (D) treated with 50 μg coumestrol in DMSO sc per day for 7 days postcastration; (E) treated sc with 25 μg estradiol; and (F) 50 μg coumestrol in DMSO for 7 days postcastration.

Figure 2. Effects of 17β-estradiol, coumestrol, daidzein, and soy diet on c-fos in the prostatic urethra and coagulating gland of castrated neoDES male mice. Abbreviations: Oil, vehicle only; E2, 17β-estradiol (100 μg sc); Cou, coumestrol (200 μg sc); Dai, daidzein (200 μg sc). (A) Effect of 17β-estradiol, coumestrol, daidzein on the expression of c-fos in the prostatic urethra of castrated neoDES mice. The bars show the combined data from all experiments. Values are expressed as percentages of expression after 3-hr E2-treatment (using the corrected scanning units of c-fos relative to 28S). Each bar represents the expression in three to nine animals. (B) A representative Northern blot from one experiment. Lane 1–vehicle only (oil), 3 hr; lane 2–E2, 3 hr; lane 3–E2, 6 hr; lane 4–E2, 12 hr; lane 5–coumestrol, 3 hr; lane 6–coumestrol, 6 hr; lane 7–coumestrol, 12 hr; lane 8–daidzein, 3 hr; lane 9–daidzein, 6 hr; lane 10–daidzein, 12 hr. Each sample consists of mRNA from three animals. (C) Effect of dietary soy on the estradiol-induced expression of c-fos in the coagulating gland of castrated neoDES mice. Lane 1, soyfree diet from castration onward plus treatment with vehicle only; lane 2, soyfree diet from castration onward plus treatment with 17β-estradiol (100 μg sc); lane 3, soy diet from castration onward plus treatment with 17β-estradiol (100 μg sc). Injections were given on day 7 postcastration and all samples were taken 3 hr after injection. Corresponding ethidium bromide (EtBr) staining is shown below c-fos.
laboratory rodents in daily soy-based feed (15) and may therefore be of significance for the effects associated with the high intake of dietary soy.

The weak estrogenicity of coumestrol seen in the adult male is again contradictory with the findings in female rodents. It is very well documented that coumestrol is a potent estrogen in the developing female reproductive tract. It had DES-like effects in the neonatal female mouse (16) and promoted uterine growth in immature rats when given in doses (per body weight) similar to those we used (15, 17–19). In adult ovariectomized rats, coumestrol showed partial estrogen agonism; it was uterotrophic but did not induce ovum implantation in mated, ovariectomized gestagen-maintained animals (20). The conflicting findings on the hormonal potency of soy or coumestrol cannot yet be explained, but they could be due to sex- or age-related differences in the uptake or metabolism of phytoestrogens.

It is also intriguing that in adult male mice soy did not block the estrogen-induced metaplastic transformation or expression of c-fos protooncoprotein, and coumestrol did not inhibit the development of metaplasia in 17β-estradiol-treated animals. This is in conflict with the idea of the antiestrogenicity of soy seen as reduction of the growth inhibition of the prostate and prevention of dysplastic development after neonatal estrogenization and the reduction of the estrogen-induced growth of immature uterus (7). One could interpret these findings by assuming that in males the neonatal or prepubertal period would be more critical for estrogen- and antiestrogen actions. Once initiated, the estrogen-related lesions would develop spontaneously.

It is not easily conceivable how soy or phytoestrogens could antagonize the estrogen action at the target cells. Phytoestrogens (coumestrol, daidzein, genistin) are clearly estrogen agonists in breast cancer cells in vitro and act through the estrogen receptor (ER)-mediated mechanisms (21, 22). Phytoestrogens with high binding affinities for estrogen receptor are also most active biologically (e.g., they enhance cell proliferation) (23). Phytoestrogens are supposed to act as antiestrogens by competing with more potent endogenous estrogens for the binding to ER. Dietary estrogens representing three structurally different groups (coumestans, isoflavonoids, and resorcylic acid lactones) had additive effects with 17β-estradiol in the presence of the concentration giving submaximal stimulation, and none of the phytoestrogens we studied (at concentrations below 1 μM) reduced the proliferation rate of breast cancer cells, i.e., had antiestrogenic effects in the presence of 17β-estradiol (22).

In addition to the interaction with ER, dietary estrogens or structurally related compounds might compete with endogenous estrogens for the active site of the estrogen-biosynthesizing and estrogen-metabolizing enzymes and thus reduce the concentration of biologically active endogenous estrogens. Coumestrol and genistein have been shown to inhibit the reduction of estrone to 17β-estradiol by estrogen-specific 17β-hydroxysteroid oxidoreductase type 1 (EC 1.1.1.62, also known as 17β-hydroxysteroid dehydrogenase type 1, 17β-HSD type 1) (24). This enzyme is expressed in steroidogenic cells such as ovarian granulosa cells and placental trophoblasts, as well as in some target tissues of estrogen action, such as normal and malignant breast and endometrium. The antibody against estrogen-specific 17β-hydroxysteroid oxidoreductase stains the uterine epithelium in the mouse (25) as well as in man (26). The immunostaining of epithelium extends to the periurethral parts of the dorsolateral lobes, coagulating glands, and seminal vesicles in the mouse. These are also the sites where metaplastic epithelium and most dysplastic lesions are found. Changes in the 17β-oxidoreduction status of endogenous estrogens (estradiol and estrone) may considerably modify the biological activities of these hormones. This would have biological significance if continuous estrogen stimulation were needed for the development of dysplasia. At present, there is no direct evidence to support this hypothesis.

Moreover, the possibility remains that the chemopreventive action of soy is not due to the antiestrogenicity of soy-derived phytoestrogens. At very high concentrations, phytoestrogens are reported to have effects not related to estrogen action. Genistein, an isoflavonoid phytoestrogen, is a potent inhibitor of both estrogen receptor negative and positive breast cancer cells (27) and of tyrosine protein kinase activity of several growth factor receptors and oncogenes that may be associated with tumor cell growth (28). Further, the inhibition of DNA topoisomerase II has been suggested as an alternative mechanism for the action of isoflavones (29). Genistein is also an inhibitor of angiogenesis, which may partly explain the possible antitumor activity of phytoestrogens (30). The relevance of these mechanisms for understanding the possible antiestrogenic action of soy or soy-derived estrogens in the male mouse is not known. It is noteworthy that soy had no general inhibitory effect on prostatic growth in neonatally untreated animals (7). In addition to phytoestrogens, soybeans contain several other potential anticarcinogenic agents such as protease inhibitors, phytosterols, saponins, and inositol hexaphosphate (1, 31); their possible roles as chemopreventive agents cannot be excluded in the present model.

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