Altered Mammary Gland Development in Male Rats Exposed to Genistein and Methoxychlor

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Genistein (GE) is a prevalent phytoestrogen whose presence in human and animal foods may affect biological actions of synthetic endocrine active compounds. We have previously reported that in utero and lactational exposure to high doses of GE or the endocrine active pesticide methoxychlor (MXC) caused mammary epithelial proliferation in 21-day-old male rats. Combined exposure to GE and MXC resulted in significant feminization of the male mammary glands. The goals of the current study were to evaluate mammary responses to GE and MXC at the adult stage and investigate relevant mechanisms. Following in utero, lactational exposure (through maternal diet), and direct dietary exposure, the inguinal mammary gland of male rats (90 days of age) was found to exhibit significant morphological alterations in the groups treated with GE and/or MXC compared to the control. GE exposure (at 300 and 800 ppm concentrations) caused lobular enlargement and epithelial proliferation, whereas MXC exposure (800 ppm) led to ductal elongation and lobular enlargement. Combining the two treatments caused prominent proliferation of both ducts and alveoli; secretory material was seen in readily recognizable alveolar lumens, which are absent in untreated male mammary. We also surveyed gene expression in the mammary tissue using a cDNA microarray and evaluated relevant protein factors. The results indicated that the treatment effects are likely due to interactions between steroid hormone receptor–mediated signals and growth factor–driven cellular pathways. The distinctive responses associated with the GE+MXC combination were likely linked to enhanced actions of insulin-like growth factor 1 and related downstream pathways.

Key Words: endocrine disruptor; genistein; methoxychlor; mammary; microarray; insulin-like growth factor 1; β-catenin; casein.

Encompassing a large number of environmental chemicals, endocrine active compounds (EAC) interact with the endocrine system through a multitude of mechanisms. EACs can originate from various sources. In addition to synthetic compounds, dietary components, phytoestrogens in particular, have been extensively characterized for their ability to interact with the endocrine system.

Dietary exposure to phytoestrogens is common for both animals and humans; exposures occur through regular dietary intake or through nutritional supplements of phytoestrogens. One of the most widely available phytoestrogens is the isoflavone genistein (GE), which is found in soybeans and other plant products. The estrogenic action of GE is based on its ability to activate estrogen receptor (ER) α and β (Casanova et al., 1999; Kuiper et al., 1998). GE at concentrations normally found in animal food can be biologically active and capable of causing developmental alterations in experimental animals (Casanova et al., 1999).

The biological actions of one chemical may be influenced by the presence of another. The frequency of human and animal exposure to GE makes it important to understand the potential of GE to interact with other chemicals in causing biological responses (Anderson et al., 1999; Brown and Setchell, 2001). Because of the ubiquity of phytoestrogens in animal and human foods, any exposure to environmental EACs is essentially an exposure to an EAC mixture. To explore the influence of phytoestrogen on the actions of synthetic EACs, we have studied the effects of exposures to combinations of GE and the synthetic EAC methoxychlor (MXC). MXC is a pesticide widely used in agricultural and household applications. Exposure to MXC at high doses is known to alter sexual and reproductive development in laboratory animals (Chapin et al., 1997; Gray et al., 1989).

We previously reported that exposure to either GE or MXC resulted in extensive developmental alterations in both male and female reproductive tracts (You et al., 2002a). Both GE and MXC advance the onset of puberty in the female rat as indicated by the early advent of vaginal opening. The combined estrogenic effects of the two compounds on reproductive tract of female rats were largely additive of their individual effects. In the male, MXC exposure delayed the onset of preputial separation, a landmark for male pubertal onset. GE by itself was without detectable effect on male pubertal onset; when combined with MXC, however, GE potentiated (increased the magnitude of) the effect of MXC (You et al., 2002a). While the
underlying mechanisms for the potentiation are unclear, these findings highlighted the influence of phytoestrogens on biological responses to synthetic EACs.

EACs affect many physiological processes, especially in the reproductive system where hormones drive sexual and reproductive processes. Among the highly hormone-sensitive reproductive organs is the mammary gland, whose growth and function require tight control by the endocrine milieu. In our previous study, we examined mammary development in 21-day-old rats exposed to GE and MXC during gestational and lactational stages through maternal dietary administration of the chemicals (You et al., 2002b). We found that, compared to the female, the male rat mammary glands were particularly sensitive to the treatments. Both GE and MXC, when given alone, altered the growth pattern of mammary gland in male rats; however, the most dramatic effects were seen in the group exposed to combined GE and MXC. Male mammary glands in the combined treatment group exhibited prominent lobuloalveolar growth that is typical of female in early pregnancy and normally unseen in male mammary gland (You et al., 2002b).

This study is a follow-up to our early report on the effects of GE and MXC on prepubertal mammary development. We examined the development of mammary gland and gene expression profile in GE- and MXC-treated male rats following their exposure to the two compounds in utero, through lactation, and by diet after weaning until reaching adult age (90 days). Results from this study indicated that such exposures, especially the one involving MXC, have a profound impact on the proliferation and differentiation of the male mammary glandular tissue.

MATERIALS AND METHODS

Animals and treatment. The animals and treatments of this experiment were previously described (You et al., 2002a,b). Pregnant female Sprague-Dawley rats (Charles River Lab, Raleigh, NC) were delivered to the CIT animal facility on gestation day (GD) 0, which was defined as the day that they were found to be sperm-positive. The animals were randomized, based on body weight, into treatment groups. The treatment diets were produced and stored in -2°C. The pregnant dams were housed with their respective dams until postnatal day (PND) 22, when the offspring were weaned. Following weaning, the offspring rats were fed the prescribed amounts of GE and MXC with a base diet, a custom-prepared soy-concentrate diet, a diet with 800 ppm MXC, or with 800 ppm GE and 800 ppm MXC. The pregnant dams were provided with deionized water ad libitum.

Histology. The inguinal mammary glands from three male rats in each treatment group were dissected and fixed in 10% neutral buffered formalin for 24 h. Fixed tissue samples were trimmed and embedded into paraffin blocks. To properly embed larger pieces of the gland tissue, it was necessary to cut the tissue into halves. The samples were placed flatly in the paraffin bed to enable frontal longitudinal cut. The samples were cut into 5-μm sections and processed for either hematoxylin and eosin staining or immunohistochemical staining for the proliferating cell nuclear antigen (PCNA). The immunohistochemistry procedure was previously described (You et al., 2002b).

Radioimmunoassay for serum hormones. Concentrations of serum hormones were determined using commercially available radioimmunoassay kits. The kits for testosterone and estradiol were from DSL (Webster, TX); kits for rat specific luteinizing hormone (LH), follicular stimulating hormone (FSH), growth hormone (GH), IGF-1, and prolactin were from Amersham Life Sciences (Buckinghamshire, UK). All RIA procedures were carried out according to the manufacturer’s instructions; duplicate samples were used in all assays. Hormone concentrations were expressed as mean ± standard deviations. The data were analyzed using two-way analysis of variance with GE and MXC each as a treatment factor.

Immunoblotting. Immunoblotting followed a previously described procedure (You et al., 1999). Total protein extracts (5–20 μg/sample, depending on the antigen) from mammary gland tissue were denatured and fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% polyacrylamide. Proteins were transferred to nitrocellulose membranes, blocked for nonspecific binding, and incubated with primary antibodies for specific proteins. The primary antibodies used were the following: rabbit anti-IGF-1 receptor (IGF-1R), rabbit anti-insulin receptor, rabbit anti-epidermal growth factor receptor, rabbit anti-progesterone receptor (Santa Cruz Bio-technology, Santa Cruz, CA), mouse anti-IGF-2 Receptor (BD Transduction, San Jose, CA), rabbit anti-androgen receptor, mouse anti-prolactin receptor (Affinity BioReagent, Golden, CO), rabbit anti-β-catenin (UBI, Lake Placid, NY), and rabbit anti-β-casein (a generous gift from Dr. Margot Ip, Roswell Park Cancer Institute, Buffalo, NY). Following incubation with primary antibody, membranes were incubated with horseradish peroxidase-linked anti-rabbit or anti-mouse (where appropriate) IgG secondary antibodies and visualized on film exposed to enhanced chemiluminescence (Hyperfilm-ECL, Amersham Life Sciences).

Microarray procedures. Clontech Atlas Rat Toxicology 1.2 arrays (Clontech, Palo Alto, CA) were used to determine gene expression in the mammary gland tissue samples obtained from adult male rat offspring. Total RNA was isolated from frozen mammary tissue samples that were snap-frozen in liquid nitrogen at necropsy; three individual rats were used in each of the four treatment groups (control, MXC 800 ppm, GE 800 ppm, and a combination of MXC 800 ppm and GE 800 ppm). RNAzol B reagent (Tel-Test, Friendswood, TX) was used to obtain the RNA samples through phenol-chloroform extraction and isopropanol precipitation. Reverse transcription was performed by mixing 2 μg total RNA, [32P]-dCTP (ICN, Casta Mesa, CA), CDS primer mix (Clontech),
RESULTS

Mammary Gland Growth Pattern

The growth pattern of mammary gland in the male rats at the adult stage was markedly changed by treatments of GE, MXC, or their combinations GE+MXC. The epithelial development in whole-mount samples within each treatment group was largely consistent. Compared to the controls, rats in all treated groups displayed greater total glandular areas (Fig. 1). GE at both 300 and 800 ppm moderately increased the glandular size and enhanced the glandular tissue density (Figs. 1A, 1C, and 1E); MXC greatly facilitated the longitudinal growth of the ducts in the fat pads (Figs. 1B, 1D, and 1F). Markedly different changes were seen in the group GE+MXC (Fig. 1E). In this combinational group, the mammary gland increased in density and size, occupying most of the fat pad with prominently increased ductal branches and alveolar mass.

In tissue sections of control rats, sexual dimorphism of the mammary morphology was evident (Fig. 2). The male mammary glands contained larger and contiguous lobular epithelial clusters that were not seen in the mammary structure of the female. In the male mammary glands, lobules often lacked tubular or ductal orientation. The cells forming alveoli were characterized by abundant, foamy, and eosinophilic cytoplasm. Acinar lumens were mostly indistinct. In contrast, female mammary glands were characterized by tubuloalveolar structures scattered within the hypodermis. Tubular ducts and alveolar structures in female mammary contained well-defined lumens, lined by a single layer of cuboidal epithelium.

Consistent with whole-mount appearance, both MXC and GE treatments caused in the male mammary glands changes in microscopic structures (Fig. 3). In MXC-treated male rats, the mammary tubuloalveolar acini were increased in number and enlarged in volume. The ducts became more elongated; the ductal walls were lined with increased layers of epithelial cells. The alveoli were enlarged and increased, with lumens visible (Figs. 3B, 3D, and 3F). The GE treatment, in contrast to MXC, had limited effects on ductal elongation and epithelial hyperplasia. GE at 800-ppm caused more prominent alveolar proliferation than at 300-ppm (Figs. 3A, 3C, and 3E). Immunohistochemical staining for PCNA revealed a greater number of proliferating cells in the group receiving the GE 800 ppm and MXC 800 ppm combination compared to the controls (data not shown).

The effects of MXC and GE on female mammary glands were mainly proliferative in nature. The MXC treatment caused mild ductal and alveolar proliferation; when exposed to both compounds, the glandular tissue contained varying degree of fibrosis (data not shown).

Serum Hormones

Blood samples from the adult rats were collected at necropsy to determine concentrations of hormones known to be involved in mammary gland growth and differentiation. There was no statistically significant difference in estradiol, testosterone, GH, FSH, LH, or prolactin concentrations following either GE or MXC treatment (two-way ANOVA), although in some cases the average hormone values (such as in testosterone and prolactin) were rather different (Table 1). GE at 800 ppm, however, was shown to significantly reduce serum IGF-1 concentration (two-way ANOVA).

Gene Expression Profiles

Clontech’s Atlas Rat Toxicology 1.2 cDNA expression array was used to profile the gene expression patterns in the male...
mammary glands exposed to GE and MXC. This array set contains specific probes for 1176 unique genes that are generally known to be implicated in cellular responses to stress and toxicity (BD Biosciences, 2001). The samples represented the mammary glandular tissues embedded into the surrounding fat pads; thus the data is associated with both the mammary epithelial structures and fat pad. The tissue samples were individually processed; no samples were pooled.

FIG. 1. Whole-mount samples of inguinal mammary gland from 90-day-old male rats. The rats were exposed to GE, MXC, and their combinations in utero and lactationally through maternal dietary exposure and, after weaning, directly through diet. The control group (A) consumed a phytoestrogen-free base diet; treatments for other groups are as follows: (B) MXC 800 ppm; (C) GE 300 ppm; (D) GE 300 ppm + MXC 800 ppm; (E) GE 800 ppm; (F) GE 800 ppm + MXC 800 ppm. Magnification: 2×.
Three rats were included from each of the following groups: control, MXC 800 ppm, GE 800 ppm, and GE+MXC. A total of 12 microarray hybridizations were performed.

All hybridizations were successful, with consistently low background and high signal-to-background ratio. The signal-to-background ratio (unnormalized, untransformed intensity values) ranged from 1 to 620 (arbitrary unit), with a median value of 3.6 and 90-percentile value of 95. All housekeeping genes were well represented. The unprocessed data from the cDNA array experiment has been deposit at the web address: http://ftp.ciit.org/public/rattox1.2excel.txt. (Group 1, 2, 3, and 4 denote to the control, MXC, GE 800 ppm, and combination group, respectively.)

In the control group (consisting of three individual rats, array 1–3), the amounts of mRNA for the 1176 interrogated genes were distributed in an approximately normal fashion following log2 transformation. The pattern of normal distribution was less consistent in the MXC 800 ppm (array 4–6) and GE+MXC (Array 10–12). Array 4 (of MXC 800 ppm) and array 7 (of the GE group) contained a large number of genes with very low levels of expression and thus deviated from the apparent normality of data distribution (Fig. 4). This lack of normality in the log2-transformed gene distribution was greatly enhanced in the GE+MXC group, suggesting a treatment-related disturbance of normal gene expression pattern.

Volcano plot analyses identified gene expression changes associated with GE and MXC by two criteria: fold change in gene expression over the control levels and the statistical significance of the differences between the treated groups and the control. Figure 5 presented this data using the thresholds of two fold changes (up or down) and p value of 0.05.

Using Venn diagrams tool in GeneSpring, we generated gene lists that were affected by the three treatments. There were 10, 13, and 83 down-regulated genes unique for the groups of GE, MXC, and GE+MXC, respectively; there were 23, 5, and 47 genes that were up-regulated in the three respective groups. Among the genes responding to the treatments, several genes of the heat shock protein (HSP) family were significantly up-regulated by all the treatments; no HSP was down-regulated in any of the treatment groups. Similarly, several oncogenes and proto-oncogenes were up-regulated in the treatment groups. Also affected were the expression of many growth factors and growth factor receptors. For instance, the IGF-1R expression was up-regulated in all three treatment groups, with undetectable change in the IGF-1 gene. Among steroid hormone receptors, the androgen receptor was down-regulated in the GE-treated animals, ERα was increased in the GE group, and both ERα and ERβ were increased in the GE+MXC group. Another group of genes that were affected are the mitogen activated protein kinases; the ERK1, 2, and 3 expression decreased, and the JNK and ERK4 expression increased among the treatment groups. Also responding to the treatments were some of the genes for enzymes involved in steroid and xenobiotic metabolism.

Immunoblots

We evaluated through immunoblotting a set of proteins that are known to be involved in mammary growth regulation. No significant change was found in the protein levels of IGF-2 receptor, insulin receptor, EGF receptor, prolactin receptor, androgen receptor, and progesterone receptor (data not shown). However, we detected treatment-associated changes in the levels of IGF-1 receptor, β-catenin, and β-casein (Fig. 6). Treatment with GE and MXC increased the level of IGF-1 receptor protein, most significantly in the GE+MXC group. β-catenin, a key signaling protein in the Wnt pathway (and a downstream effector of IGF-1) that regulates cell proliferation and differentiation, was markedly and exclusively increased in the GE+MXC group. In addition, out of three animals

FIG. 2. Sexual dimorphism of the mammary glands in untreated adult rats. Inguinal mammary gland from 90-day old male rats (A) displays contiguous epithelial cell clusters forming lobular structures that lack lumens. The inguinal mammary gland in virgin female of same age (B) showed well developed lobuloalveolar structures with single-layer epithelial cells surrounding well-defined lumen spaces. Magnification: 100×.
FIG. 3. Treatment effects of GE and MXC on male mammary gland. The rats were exposed to GE, MXC, and their combinations (GE+MXC) in utero and lactationally through maternal dietary exposure and, after weaning, directly through diet. The control group (A) consumed a phytoestrogen-free base diet (SAFD, as described in Materials and Methods); treatments for other groups are as follows: (B) MXC 800 ppm; (C) GE 300 ppm; (D) GE 300 ppm + MXC 800 ppm; (E) GE 800 ppm; (F) GE 800 ppm + MXC 800 ppm. (A) The control rats contained alveoli that were composed of cells with abundant, foamy, and eosinophilic cytoplasm (arrow); alveolar lumens are mostly absent. (B) MXC caused elongated ducts and enlarged alveoli with lumens clearly visible (arrow). (C and E) Enlargement and hyperplasia of alveoli, some of which were dilated with secretory material within lumens (arrows), were the main features in the GE treated groups. (D and F) Ductile elongation and hyperplasia of both ducts and alveoli were prominent in the MXC+GE groups, with increased secretory activity seen in F (arrows). Magnification: 400×.
examine each treatment group. One rat in the MJC group and two in the GE + MJC group had significantly elevated β-
-casein, a milk protein and mammary differentiation marker that was not detected in control male rat mammary gland. One
needs to note that, similar to the cDNA array assay, these
immunoblots were based on samples from total mammary
tissue without separation of the fat pad and the epithelium;
these results are thus not cell type-specific.

**DISCUSSION**

We have previously observed that in utero and lactational
exposure to maternally dosed GE and MJC affect mammary
gland development in male rats at the prepubertal stage (You
et al., 2002b). We found that postnatal mammary glands of the
male, but not female, rat were highly sensitive to the treatment.
While both GE and MJC enhanced proliferation of the
gland.

<table>
<thead>
<tr>
<th>Testosterone (ng/ml)</th>
<th>Estradiol (pg/ml)</th>
<th>GH (ng/ml)</th>
<th>FSH (ng/ml)</th>
<th>LH (ng/ml)</th>
<th>Prolactin (ng/ml)</th>
<th>IGF-1 (ng/ml)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1.79 ± 0.20</td>
<td>8.7 ± 2.5</td>
<td>12.4 ± 5.9</td>
<td>1.20 ± 0.04</td>
<td>4.19 ± 0.19</td>
<td>3.67 ± 0.50</td>
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<tr>
<td>MJC</td>
<td>3.48 ± 0.87</td>
<td>10.9 ± 2.0</td>
<td>19.0 ± 9.4</td>
<td>0.99 ± 0.03</td>
<td>3.35 ± 0.23</td>
<td>4.44 ± 1.32</td>
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<tr>
<td>GE300</td>
<td>2.49 ± 0.57</td>
<td>12.3 ± 3.5</td>
<td>10.9 ± 5.4</td>
<td>1.06 ± 0.03</td>
<td>3.25 ± 0.21</td>
<td>5.61 ± 3.34</td>
</tr>
<tr>
<td>GE300 + MJC</td>
<td>2.38 ± 0.11</td>
<td>15.7 ± 4.0</td>
<td>8.2 ± 3.4</td>
<td>1.18 ± 0.03</td>
<td>3.47 ± 0.27</td>
<td>9.08 ± 2.67</td>
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<tr>
<td>GE800</td>
<td>2.56 ± 0.55</td>
<td>8.0 ± 1.8</td>
<td>10.2 ± 12.5</td>
<td>1.07 ± 0.03</td>
<td>3.53 ± 0.18</td>
<td>7.74 ± 2.89</td>
</tr>
<tr>
<td>GE800 + MJC</td>
<td>1.66 ± 0.44</td>
<td>17.1 ± 8.0</td>
<td>11.7 ± 5.8</td>
<td>1.14 ± 0.03</td>
<td>3.75 ± 0.31</td>
<td>6.66 ± 3.48</td>
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**FIG. 4.** Histograms for the distributions of mRNA for 1176 genes in Clontech Atlas Rat Toxicology 1.2 cDNA array. Four groups were included in this survey: control, MJC 800 ppm, GE 800 ppm, and GE (800) + MJC (800). The distributions were based on background-subtracted and log2-transformed values. Each histogram plot represents an individual animal. The distributions tend to loss normality in the three treated groups as compared to the control.
mammary epithelium, exposure to their combination leads to manifestation of lobuloalveolar morphology normally seen only in differentiated female rat gland. The current study extended our initial investigation into adult male rats. Our results indicated that exposure to high-dose GE and MXC, particularly to their combination, caused marked feminization of the adult male mammary gland; such feminization is well recognizable through morphological evaluation and through detection of milk protein expression. Our gene array survey suggests that the mammary responses in the treated rats involve complex interplays between the sex hormones and local growth factors–regulated cellular pathways. Because our purpose of using the gene-array assay was to provide a broad view of gene expression profiles in relation to the treatments, we did not attempt to interpret individual gene changes.

**Endocrine Activities of Genistein and Methoxychlor**

Both GE and MXC are estrogenic in vivo (Casanova et al., 1999; Chapin et al., 1997; Gray et al., 1989; You et al., 2002a). GE is an activator for both ERα and ERβ, with higher potency toward the latter (Kuiper et al., 1998). MXC as a parent compound exhibits limited ability to activate ERs in vitro (Gaido et al., 1999; Kuiper et al., 1998). A major metabolite of

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**FIG. 5.** Volcano plots of gene expression changes in GE 800 ppm, MXC 800 ppm, and the GE (800)+MXC (800) groups. The horizontal line in each plot represents statistical significance at $p < 0.05$ regarding the changes of gene expression compared to the control. The vertical lines depict the two-fold up or down changes on a log2 scale. The letters and numbers indicate the coordinates of the particular gene on the array. The data were analyzed using a local linear regression model based on S-Plus statistical program.
MXC, 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), is an agonist for ERα and an antagonist for ERβ and androgen receptor (Gaido et al., 1999). GE and MXC can thus provide estrogenic stimulation that is normally unavailable in the male mammary tissue. This estrogenicity, perhaps in combination with the antiandrogenicity of HPTE, may serve as a primary force to feminize the male mammary gland. When GE and MXC were coadministered to rats during development, they produced expected and unexpected effects, based on their known pharmacological properties. Among the expected was the additivity in estrogenic responses in female rats (i.e., changes in vaginal opening, uterine weight, and estrous cyclicity) based on the interactions with the estrogen receptors by GE and MXC. On the other hand, the potentiation effect of GE on MXC in causing delayed male pubertal onset would not be predicated on the basis of GE- and MXC-interactions with the androgen receptor, while the MXC metabolite HPTE antagonizes AR, adding GE in the treatment does change AR response to HPTE (You et al., 2002a,b).

Estrogen promotes mammary gland proliferation and differentiation by stimulating ductal morphogenesis and alveolar development. Estrogen plays a key role in regulating reproductive status-specific mammary development in the female. While the male mammary gland takes a drastically different developmental path, it does, nonetheless, possess the capacity to respond to estrogen. Male mice transgenically expressing high level of aromatase, the enzyme producing estrogens, develop mammary glands similar to differentiated female glands (Li et al., 2002). Those male rats, with their high estrogen production, develop mammary glands characterized by alveoli filled with secretory material and expressing casein. Such mammary phenotype of the aromatase-transgenic mice is similar to what we observed with the male rats in the GE+MXC group. Thus, it is the estrogenic action of GE and MXC that facilitated the ductal growth and branching, which are not seen in normal male glands.

What needs to be emphasized here is that, while the GE concentrations used in this study are relevant to animal and human exposures under various dietary scenarios, the MXC dose was chosen to elicit significant toxicological responses; the 800 ppm MXC dose is not expected to represent environmental exposures.

Sexual Dimorphism and Male Mammary Gland Responses to Genistein and Methoxychlor

The male mammary gland is regarded as a rudimentary organ without ascribed functions; as a result, research on the development and endocrine responses of male mammary glands has been limited. The male mammary gland nonetheless undergoes significant growth and development from embryonic to adult stage. While the mammary gland is well recognized as a sexually dimorphic organ, the characteristics of such dimorphism has been poorly explored (Cardy, 1991).

Mediators of the Genistein and Methoxychlor Effects on Mammary Gland

We now know that relatively little morphological difference can be identified between the male and female mammary glands in rats during the embryonic and early postnatal stages (Cardy, 1991; You et al., 2002b). Thereafter, growth of the male and female mammary glands embarks on different pathways. Sexual differences in mammary morphology start to appear at the peripubertal stage, when allometric growth of the mammary gland manifests as a part of the overall pubertal developmental process (Hovey et al., 2002). Mammary glands in immature male rats are similar to those seen in female immature rats, characterized by tubular epithelium and bud formation. The lumen structures in prepubertal males disappear during maturation as the production of gonadal steroid rises. The structures of mammary glands in mature male rats are distinctively different from both mature female glands and immature male glands. Mature female rat mammary glands are characterized by scattered tubular ducts and distinct alveolar lumens that are lined by cuboidal epithelial cells; in contrast, the mature male mammary gland lacks such alveolar structures. Mammary glands in mature male rats are characterized by restricted glandular area and lobules of cellular clusters devoid of lumen. Restricted glandular regions results in smaller size of the mammary gland compared to mature females. Within glandular structures, the male mammary gland contains contiguous epithelial cells in irregularly shaped lobular groups of epithelial cells (Astwood et al., 1937; Cardy, 1991).

Our previous (You et al., 2002b) and current studies demonstrated that the male mammary gland is sensitive to EACs at various developmental stages. In addition to being susceptible to GE and MXC through gestational and lactational exposures (You et al., 2002b), shorter-term perinatal exposure also caused responses (unpublished data). These responses are distinct for GE, MXC, and their combination, suggesting additional pharmacological effects other than the estrogenicity of those compounds underlying these effects. GE and MXC, when combined, produced a response pattern that differs from the effects caused by either compound alone. Such results argue for recognition that phytoestrogen has the potential to modulate biological responses to other endocrine-active compounds. Thus, dietary GE should be considered as an important experimental parameter in pharmacological and toxicological studies.
of the sex hormone receptors. In the current model of male mammary response, ER activation by the test compounds is probably the major factor in the manifestation of steroid hormone-like effects of both GE and MXC. On the other hand, neither alterations in the serum levels of steroids and other mammotrophic hormones nor changes in the levels of receptor protein in the mammary tissue were found to be associated with the observed responses.

Many of the gene expression changes detected by the microarray assay may represent nonspecific stress responses. Nonetheless, several observed changes in the gene and protein expression have shed important light on potential mechanisms. A key change is the increase expression of IGF-1 receptor in the mammary tissue of treated male rats, especially in the GE+MXC group. The IGF-1R mediates the actions the growth hormone-IGF-1. IGF-1 plays a fundamental role in mammary gland development, as IGF-1 signaling is required at all stages of mammary gland development (Laban et al., 2003). In the absence of IGF-1 (e.g., in IGF-1-null mice), the mammary tissue does not respond to estrogen (Ruan and Kleinberg, 1999). IGF-1R is mostly found in the ductal epithelium, with higher levels of expression often found in mammary tumors compared to normal tissue (Laban et al., 2003). Estrogen enhances the responses of mammary epithelial cells to IGF-1 by up-regulating IGF-1R expression (Lee et al., 1999). GE was shown to up-regulate the expression of IGF-1R in human breast cancer cells (Chen and Wong, 2004). Thus, the estrogenic actions of GE and MXC may account for the enhanced mammary gland when both compounds were applied together. Our data indicate that the effect of MXC on IGF-1R expression is not as strong as that of GE, perhaps reflecting a weaker MXC activation of the ERs than GE.

The consequences of enhanced IGF-1 signaling in GE- and MXC-treated mammary gland may rest at least partially on the dramatically increased β-catenin protein. β-Catenin is a key component of the Wnt-canonical signaling pathway that regulates genes involved in transcription, cell cycle, tissue modeling, and morphogenesis (Smalley and Dale, 2001). IGF-1 is shown to enhance the stability of β-catenin by a mechanism involving tyrosine phosphorylation (Playford et al., 2000); IGF-1 also stimulates the β-catenin pathways through two independent mechanisms: inhibition of the GSK-3β-associated multiprotein complex and enhancing MAPK (ERK) activities (Desbois-Mouthon et al., 2001). β-Catenin binds to Fc/LeF1 transcriptional factors and regulates cyclin D1 and other β-catenin-dependent genes that are linked to tumorigenesis (Conacci-Sorrell et al., 2002; Moon, 2004). Transgenic expression of β-catenin causes precocious lobuloalveolar development in both male and female mice (Imbert et al., 2001). In the male where ovarian hormones are absent, β-catenin induces lobuloalveolar growth without ductal branching and elongation (Imbert et al., 2001). We found prominent increase of β-catenin protein in the GE+MXC combination group; this increase was accompanied by decreased mRNA of β-catenin in the microarray assay. Our observation is consistent with the known pattern of β-catenin regulation: the function of β-catenin is based on its protein stability; activation of the Wnt or IGF-1 pathways inhibits GSK phosphorylation of β-catenin, reducing its ubiquitination-driven degradation. One point to note is that β-catenin is a proto-oncogene. Various gene defects would result in extended half-life of β-catenin; cellular transformation may ensue as a consequence (Smalley and Dale 2001).

Taken together, the male mammary phenotype following combined exposure to GE and MXC is a result of interactions between growth factor-mediated signals and steroid hormone-mediated signals. Enhanced expression of IGF-1R likely increased the sensitivity of the male mammary gland to the estrogenic actions of the GE and MXC; in addition, β-catenin also likely played an important role in the development of female-like alveolar structures.

It appears that the mammary gland of male rat is a rather sensitive organ to external endocrine stimuli. The sensitivity suggests a potential utility of this organ system in detecting the effects and investigating the relative mechanisms of environmental EACs. Whether male human beings may be susceptible to such effects remains unknown.

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