During Development, 17α-Estradiol Is a Potent Estrogen and Carcinogen

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Neonatal administration of estradiol-17β (E2-17β) increases the nuclear DNA content in the mouse reproductive tract. Similar responses have been demonstrated for synthetic estrogens such as diethylstilbestrol. One of the questions raised regarding environmental estrogens such as organochlorines is whether they are potent enough to result in abnormal changes such as those demonstrated by both natural and synthetic estrogens. To test this hypothesis, female BALB/c mice were treated neonatally (days 1–5) with either E2-17β or estradiol-17α (E2-17α), an inactive stereoisomer in adult reproductive tissues. We also proposed whether neonatal administration of (E2-17α) was tumorigenic and whether the effects were age dependent. To answer these questions, one set each of 10-day-old treated and control mice received short-term secondary administration of E2-17β, E2-17α, or cholesterol. Carcinogenic tumors from intact BALB/c mice were examined histologically and by flow cytometry at 70 days of age and by histology alone at 18 to 22 months of age. The results include several important findings: a) like E2-17β, neonatal E2-17α treatment induced persistent vaginal cornification, hypspadias, vaginal concretions, and hyperproliferation in nearly 100% of the animals regardless of the secondary treatment for most groups; b) neonatal E2-17α treatment increased the nuclear DNA content of cervicovaginal epithelium at one-half the level (mean DNA index of 1.02 vs 1.04) and incidence (22 vs 46% of the animals) of E2-17β; c) short-term secondary treatment with E2-17α, unlike E2-17β, did not significantly increase the increase in DNA content (13% for E2-17α vs 37 and 56% for control and E2-17β, respectively); and d) neonatal administration with E2-17α induced adenocarcinomas in the reproductive tract in 25% of the animals. Therefore, the biological effects (estrogenic potency) of E2-17α may be age dependent. — Environ Health Perspect 105(Suppl 3):577–581 (1997)

Key words: carcinogen, cervicovaginal, endocrine disruptor, estradiol, estrogen, development, neonatal, mouse

Introduction

Natural and synthetic estrogens are carcinogenic in humans and rodents (1–3). In particular, the perinatal mouse treated with sex steroid hormones has been used extensively to study both the etiology and potential danger of in utero steroid exposure to induce morphological, developmental, biochemical, and carcinogenic changes in the human reproductive tract (1,3–5). Neonatal treatment of female mice with 17β-estradiol (E2-17β) results in permanent, irreversible morphological and biochemical changes in the cervicovaginal (CV) epithelium including hyperplastic proliferation of the epithelium with extensive cornification and downgrowths followed by subsequent neoplastic development (3). We have previously demonstrated that increased nuclear DNA content in the CV epithelium precedes neoplastic development in neonatally E2-17β-treated mice (6). The latency period for the appearance of this change in DNA ploidy can be shortened from 8 months to 40 days with secondary E2-17β administration (6).

Several studies concentrating on estrogens, both in vitro and in vivo, have hypothesized that genotoxic effects (7–9), altered gene expression (10), and catechol metabolites of estrogen (5,11–13) are examples of the potential mechanisms of estrogen-induced carcinogenesis. The role of estrogen and its receptor is better understood in promotion; it is unclear what role, if any, the receptor plays in estrogen-induced carcinogenesis (3,5,14,15). Several studies have demonstrated that the estrogen potency of a compound alone is a poor predictor of its carcinogenic potential (14,16,17). In contrast, weak estrogen compounds as well as antiestrogenic compounds, as determined by assays using postnatal tissues, have induced changes similar to those induced by E2-17β in the neonatal mouse (1,3–5,13,14).

The present studies were undertaken to determine if a weak estrogen administered during a critical period of development results in an increase in the nuclear DNA content in the CV epithelium. Specifically, the early effects of neonatal treatment with the weakly estrogenic stereoisomer of E2-17β, estradiol-17α (E2-17α), were compared to the early E2-17β–induced effects by both histological and flow cytometric analysis. To determine whether the effects of E2-17α were age dependent, animals were treated secondarily with E2-17α, E2-17β, or cholesterol and compared. The ultimate tumorigenicity of E2-17α was also investigated.

Materials and Methods

Chemicals

All chemicals and reagents were of the highest grade commercially available. The
E2-17α (Steraloids, Wilton, NH) was purified by recrystallization 4 times; each recrystallization was performed using different solvent combinations than previous ones (18). The steroid was subsequently desiccated before weighing.

Animals and Treatment

Animal care was in accordance with institutional guidelines. Seventy-two newborn BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN) were separated into nine groups (Table 1). In accordance with previously described procedures for this model, the mice received daily sc injections of 25 mg of either E2-17β (Sigma Chemical Co., St. Louis, MO) or E2-17α in 0.02 ml of vehicle (sesame oil), or vehicle alone for 5 days, beginning within 15 hr of birth (3). One set each of 10-day-old E2-17β-treated, E2-17α-treated, and control mice received sc pellet implants containing either 15 mg of E2-17β and cholesterol (10:90, respectively), 15 mg of E2-17α and cholesterol (10:90, respectively), or 25 mg of cholesterol alone. A continuous release of hormone is assured for up to 12 weeks (JG Liehr, personal communication). All mice were weaned at 21 days of age. Persistent cornification was based on the vaginal smears performed from days 20 to 30. The CV tracts were analyzed at 70 days of age following 60 days of secondary treatment. This time point was chosen on the basis of previous studies that demonstrated the reduced latency period for detecting increased DNA content in the CV epithelium (6). The CV tracts were analyzed at 18 to 22 months of age in an additional three groups of animals.

Tissue Preparation and Dispersion

After removal, CV tracts were cut in half longitudinally. One-half of each tract was analyzed histologically; the other half was enzymatically and mechanically dispersed as previously described (6). This method results in a cell suspension consisting of more than 85% epithelial cells as determined by identification with antibodies against keratins (LA Jones, unpublished data).

Flow Cytometric Analysis

The nuclear DNA content of the dispersed CV epithelial cells was analyzed using a Becton-Dickinson FACScan flow cytometer (Becton-Dickinson, San Jose, CA) under conditions previously described (6). Normal BALB/c mouse lymphocytes stained under the same conditions were used as an internal diploid standard. Lymphocytes were run before and after each five unknown samples. Data were rejected if the modal channel of the lymphocytes varied by 2% or more at any time.

Flow cytometric data were obtained with Lysis II software (Becton-Dickinson). The DNA index (DI) of each sample was compared with the DI of the lymphocytes and was subsequently used to compare the samples statistically.

Histology

The other halves of the CV tracts were snap frozen in liquid N₂. The tissue samples were embedded in Tissue Tek OCT compound (Miles Diagnostic Division, Elkhart, IN). Four-micrometer sections were cut with a cryostat and placed on poly-L-lysine-coated slides. The slides were dried for 30 min at 60°C and stained with hematoxylin and eosin, and periodic acid–Schiff (PAS) without digestion.

The following parameters were used to evaluate the serial histological sections for CV abnormalities: hyperproliferation, keratinization, mild to severe dysplasia and downgrowths, and pyknosis. The presence of these parameters were scored in 10 random fields of view (×20). To control for animal variation within the groups, a mean histologic score was calculated for each parameter for each animal using the histological data from the 10 random fields of view. This mean histologic score, equal to the total number of occurrences for each parameter divided by 10, was used to statistically compare frequency rather than incidence.

Statistical Analysis

A Fisher exact test was used to compare the incidence between groups for the gross morphologic, histologic, and flow cytometric data. Using the mean histologic scores, a range test based on a Kruskal–Wallis (K-W) two-way analysis of variance (ANOVA) was used to compare the frequency of the histological parameters nonparametrically. A range test based on a K-W two-way ANOVA with multiple comparison tests was used to test nonparametrically for differences in nuclear DNA content between groups. The factors analyzed were type of initial treatment [E2-17β, E2-17α, or oil] and type of secondary treatment [(ISCN) E2-17β, E2-17α, cholesterol]. Differences between two groups were considered to be significant when the p value was ≤ 0.05. (All statistics were calculated with SPSS, Chicago, IL.)

Results

Early Histological Effects of Neonatal Treatment

Neonatal treatment with E2-17α induced premature vaginal opening, persistent cornification, clitoridoid hypospadias, and concretions in the vaginal lumen equivalent to the incidence observed in the E2-17β-treated animals (Table 1). Gross morphological changes in the entire genital
tract, characteristic of E2-17β treatment, were also seen in all the E2-17α-treated animals (Figure 1). Neonatal treatment with E2-17α induced hyperproliferation and mild dysplasia and downgrowths in the CV tract indistinguishable from those changes described by Takasugi and Bern (3) and seen in the E2-17β-treated animals. No significant histological difference was observed between the two treated groups in either the incidence/animal and the prevalence/animal for hyperplasia, dysplasia, and pyknosis. A significant relationship existed between the presence of pyknosis and the presence of mild to severe dysplasia (p < 0.01). With the exception of secondary administration of E2-17β inducing hyperproliferation in the oil-treated animals, secondary administration of either E2-17β or E2-17α did not significantly alter the histological changes.

Flow Cytometric Analysis

As shown in Table 2, the incidence of significantly increased DI (DI > 1.05) was significantly higher in the neonatally estrogen-treated animals when compared to controls. Several E2-17α-treated animals also possessed an increased DI (Figure 2) but at a lower level and incidence. As shown in Table 3, a higher mean DI and a significantly increased incidence was observed with subsequent short-term secondary E2-17β administration (p < 0.01). In contrast, secondary E2-17α administration did not significantly alter the DI of any animals. The main effects in the two-way ANOVA (INIT, SCND) were both significant at p < 0.001. The second-order interaction (INIT X SCND) was also significant at p = 0.007.

Tumorigenicity

Two of the eight E2-17α-treated animals sacrificed between 18 and 24 months of age developed tumors. Both tumors were classified as adenosquamous (Figure 3). In addition, areas having the potential to eventually become tumors were found in two additional animals. The incidence of tumor formation in these studies was significantly increased (p < 0.001) over that expected for controls (1:5000 (LA Jones, unpublished data)) and approximately one-half of that expected for an equal dose of E2-17β (Table 4).

Discussion

This study demonstrated that neonatal treatment with E2-17α can induce teratogenic and morphological changes equivalent to those induced by neonatal treatment with E2-17β in the CV tract. Even though neonatal administration with E2-17α is tumorigenic, the incidence may be lower than with E2-17β. Furthermore, the increased nuclear DNA content suggests that neonatal treatment with E2-17α induces potential neoplastic conditions in the CV epithelium (6) and that these changes play a role in the subsequent tumor formation. Our findings are in agreement with those of Forsberg and Kalland (19), who described the presence of heterotropic columnar epithelium in an outbred strain following neonatal treatment with E2-17α. The additional abnormalities described in the present study, particularly the increased DNA ploidy and tumorigenesis, indicate that the estrogenic potency of a compound may be increased in neonatal tissues, as evidenced by the inability of secondary E2-17α administration to augment the increased DNA ploidy. Many studies in a variety of systems have demonstrated that E2-17α is a weak or inactive estrogen. For example, a significantly lower relative binding affinity for the estrogen receptor in both human endometrium and rat uterus and a corresponding potency of 6% were observed when compared to E2-17β (20). The potency of E2-17α in stimulating cell growth and prolactin production was approximately 100-fold lower and the binding affinity was 10-fold lower in rat

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Table 2. Incidence of DNA ploidy changes based on neonatal treatment.

| Treatment | Number of animals | Mean DI (per group) |
|-----------|-------------------|---------------------|
| Oil       | 22                | 1.00                |
| 17β-E2    | 28                | 1.04                |
| 17α-E2    | 22                | 1.02                |

*Initial neonatal treatment on days 1 to 5 with daily sc injections. *Values with different superscript numbers are significantly different using the Fisher exact test (p < 0.05).

Table 3. Incidence of DNA ploidy changes based on secondary treatment without neonatal oil-treated animals.

| Treatment | Number of animals | Mean DI (per group) |
|-----------|-------------------|---------------------|
| Cholesterol | 19                | 1.04                |
| 17β-E2    | 16                | 1.05                |
| 17α-E2    | 15                | 1.01                |

*Secondary treatment from days 10 to 70, with sc pellet implants. *Values with different superscript numbers are significantly different using the Fisher exact test (p < 0.05).
pituitary cells than the potency of E$_2$-17$\beta$ (21). Even though the binding for type I estrogen receptor sites by E$_2$-17$\alpha$ is diminished, it is unable to compete for type II sites (22). The induction of gene expression such as c-fos in rat uterus and guinea pig endometrium is specific for E$_2$-17$\beta$ (23, 24). On the other hand, several studies have demonstrated similar effects between the two stereoisomers such as growth inhibition of MCF-7 cells (25). Furthermore, although strain differences have been documented, it is unclear whether the epithelial cells in the CV tract of BALB/c mice possess estrogen receptors on days 1 to 3 after birth (26). Likewise, it is not clear whether these effects are receptor mediated. The role of primitive nondiscriminatory receptors, orphan receptors, or the mesenchyme cannot be discounted (5,13-15,19). Other possible nongenomic mechanisms of action for E$_2$-17$\alpha$ include conversion to E$_2$-17$\beta$ (27), common reactive metabolites with E$_2$-17$\beta$ (5,11-13), aneuploidy (5,7-9), and the rapid release of intracellular calcium (28). Conversion of E$_2$-17$\alpha$ to E$_2$-17$\beta$ and/or prolonged administration of E$_2$-17$\alpha$ was proposed by Clark and associates (27), who observed a uterotrophic effect in mature rats. Unlike E$_2$-17$\beta$, chronic treatment with E$_2$-17$\alpha$ did not induce Leydig cell hyperplasia in BALB/c mice (29). It is unclear which mechanism(s) is playing a role in the changes observed in this study.

More important, these results strengthen the concerns put forth by McLachlan and colleagues that weak environmental estrogentic compounds, as judged by assays using postnatal tissues, may have dramatic potant estrogenic effects in developing perinatal tissues (1,4,5,13,30). These perinatal estrogenic effects include functional, teratogenic, and carcinogenic changes in hormone-target tissues. In the present study, the potency of E$_2$-17$\alpha$ was age dependent in that the effects observed were significant and similar to those observed for E$_2$-17$\beta$ when E$_2$-17$\alpha$ was administered neonatally, including tumorigenesis. In contrast, the potency and effects resulting from secondary treatment of E$_2$-17$\alpha$ were not like those of E$_2$-17$\beta$. Findings resulting from the neonatal mouse model may apply to other hormone-sensitive tissues, particularly during development, and may provide predictors for hormonal carcinogenesis (4). For example, only one out of three breast cancer patients has an identifiable risk factor. While some investigators have implicated the exposure to high concentrations of endogenous prenatal estrogen in the development of breast cancer (31), it may also be suggested that exposure to weak environmental estrogens during a critical period of development may also play a role (1,4,5,13,32,33). Further studies are both under way and needed to further define the mechanism of action of estrogen and steroid-induced carcinogenesis in this model.

Table 4. Relative tumorigenicity comparison.

| Treatment* | Number of animals | With tumors (%) |
|-----------|------------------|-----------------|
| Oil       | -5000            | 1 (0.0002)*     |
| 17$\beta$-E$_2$ | -5000          | -2500 (50)*     |
| 17$\alpha$-E$_2$ | 8               | 2 (8)*          |

*Initial neonatal treatment on days 1 to 5 with daily sc injections of either 25 $\mu$g of steroid in vehicle (sesame oil) or vehicle alone. Approximation of combined results over the past 20 years. Actual values obtained during this current investigation.

Figure 3. Photomicrographs of hematoxylin and eosin staining illustrating an adenosquamous tumor induced by neonatal E$_2$-17$\alpha$ administration in the CV tract from a 24-month-old animal that received no further treatment. (A) Note the squamous components (arrow [x63]). (B) Also note the glandular components (arrow [x500]).
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