Estrogen Receptor α and β Expressions in Hypothalamus-pituitary-ovary Axis in Rats Exposed Lactationally to Soy Isoflavones and Bisphenol A

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Objectives This paper aims to investigate the uterotrophic activities of lactational exposure to combination of soy isoflavones (SIF) and bisphenol A (BPA) and to examine estrogen receptor α (ERα) and estrogen receptor β (ERβ) expressions in hypothalamus-pituitary-ovary axis and uterus. Methods Maternal rats that were breeding about 8 litters were randomly divided into four groups with seven dams in each group. Dams in different treatment groups received corn oil (control), 150 mg/kg BW of SIF, 150 mg/kg BW of BPA or combination of 150 mg/kg BW of SIF and 150 mg/kg BW of BPA, respectively, from postnatal day 5 to 11 (PND5-11) by gavage. On PND12 and PND70, 10 female litters were killed and hypothalamus, pituitary, ovary and uterus were collected. ERα and ERβ expressions in these organs were detected with Western blotting assay. And vaginal opening time and estrus cycle were examined in animals fed for PND70. Results On PND12, the relative uterine weight of rats treated with ISF or BPA or their combination was significantly higher than that of untreated rats (P<0.05). But the relative uterine weight of rats in the co-exposure group was slightly lower than that in the group only exposed to SIF or BPA. On PND 70, however, the relative uterine weight in each treatment group was not statistically different from that in the control group (P>0.05). Vaginal opening time and estrus cycle in groups treated with SIF or BPA or their combination were similar to those in the control group (P>0.05). Exposure to SIF or BPA or their combination could up-regulate or down-regulate ERα and ERβ expressions in hypothalamus, pituitary, ovary and uterus on PND12 and PND70. These regulation patterns for ERα and ERβ were different in different organs at different time points. Conclusion Lactational exposure to ISF or BPA or their combination could induce uterotrophic responses in neonate rats, which disappeared in later life. But these data fail to suggest a possibility for synergic actions between SIF and BPA. It was also demonstrated that the uterotrophic effects of SIF and BPA exposure might, at least, involve modification of ERα or ERβ expressions in the hypothalamus-pituitary-ovary axis. Key words: Soy isoflavones; Bisphenol A; Combinatory actions; Estrogen receptors

INTRODUCTION

Soy isoflavones (SIF) has been identified as a naturally-occurring and estrogen-like phytoestrogen, which is widely contained in various diets including infant soy formula. Franke et al. found that soybean intake resulted in a rapid and dose-dependent increase in genistin and daidzein derivatives in milk[1]. Previous studies conducted in our laboratory demonstrated that lactational exposure to SIF could cause estrogen-like effects on the reproductive system in neonate female rats, and its mechanisms might be, at least, involved with modifications of steroid receptor transcription in the reproductive system[2]. Bisphenol A (BPA) is a chemical used in the manufacture of polycarbonate and epoxy resins and the largest source for human exposure to it is food containers (e.g. milk, water, and infant bottles) and medical devices[3]. It is reported that estimated intakes of BPA in breast-fed infants and those fed with infant formula were 0.028 and 0.055 mg/kg BW, respectively[4]. BPA, as an endocrine disruptor, is demonstrated to have estrogen-like actions and thus result in reproductive and developmental toxicity in numerous studies[5]. It is clearly suggested that infants may be simultaneously exposed to SIF and BPA. It is, however, unknown whether this simultaneous exposure can induce an additive or synergic action or results with an increase of toxicity. It is found recently that a mixture of

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xenoestrogens could cause estrogenic effects despite each individual component of the mixture at a concentration below their respective no observable effect level (NOEL) for estrogenicity in vitro\textsuperscript{[6]}. Schmidt et al. found that genistein (a potent isoflavone), in contrast to BPA, did not exhibit any antiestrogenic properties when administered orally to ovariectomized rats with ethinylestradiol\textsuperscript{[7]}. In another study using uterotrophic assay in immature rats, BPA was shown to act as an antagonist of genistein\textsuperscript{[8]} but data available now are insufficient to identify the interaction between BPA and isoflavones or even their joint effects on the reproductive system when tested in a binary mixture.

The present study is therefore designed to explore possible effects of exposure to SIF or BPA or their combination on reproductive development in neonate female rats. Expressions of ER\textalpha{} and ER\textbeta{} in the hypothalamus-pituitary-ovary axis and uterus would also be examined to explore their potential mechanisms.

**MATERIALS AND METHODS**

**Reagents**

Soy isoflavones (SIF, 80%, genistin: daidzin: glycitin=13:5:2, extracted from soy beans) was purchased from Sichuan Guanhua Biochem Ltd. (Sichuan, China). Bisphenol A (BPA, >99%) was bought from Sigma Chemical Co. (Missouri, USA) and dissolved in corn oil. Soy-/alfalfa-free diet (SAFD)\textsuperscript{[9]} was formulated in Laboratory Animal Centers, Chinese Academy of Medical Science (Beijing, China). Monoclonal antibodies for ER\textalpha{}, ER\textbeta{} and \beta{}-actin were obtained from Chemicon International Inc. (California, USA). T-PER tissue protein extract reagent was acquired from Pierce Biotechnology Inc. (Illinois, USA). PVDF membrane was procured from Amersham Biosciences (New Jersey, USA) and Lumiglo reserve chemiluminescent substrate was got from KPL Inc. (Maryland, USA).

**Animals and Experimental Design**

According to our previous study\textsuperscript{[3]} with some modifications, 9-week old virgin female and stud male Sprague-Dawley rats were obtained from Vital River Laboratory Animal Institute, Beijing. After their acclimation to the local animal house conditions (23±1 °C; 12-h light/12-h dark cycle; SAFD feed) for 5 days, female rats were placed with stud male rats in a one-for-one manner in stainless cages overnight. Females with a vaginal plug observed the next morning were recorded as their gestation day 0 (GD0) and were housed one per cage. The day of delivery was defined as Postnatal Day (PND) 1. On PND4, seven dams were randomly assigned to each of the four treatment groups. The mean litter size and sex ratio in each group did not vary significantly. The dams in each of three treatment groups were treated by gavage with 150 mg/kg BW of SIF (Group SIF), 150 mg/kg BW of BPA (Group BPA), combination of 150 mg/kg BW of SIF and 150 mg/kg BW of BPA (Group B+S) daily from PND5 to PND11, respectively. Corn oil served as the vehicle in the control group (Group CTL). It was demonstrated that 150 mg/kg BW of SIF, the dose used, could create significant effects in this exposure model\textsuperscript{[3]}. During the study, SAFD feeds and tap water were provided for maternal animals ad libitum. On PND12, 10 female pups in each group were obtained randomly and sacrificed after anaesthesia with Pentobarbital Sodium injection. Each dam provided at least one female pup for sacrifice. The rest in each groups were continuously fed with SAFD feed and sacrificed on PND70. The care and treatment of experimental animals were in accordance with the “Guiding Principles in the Use of Animals in Toxicology” adopted in 1989.

**Vaginal Opening Time and Estrous Cycle**

The rest female pups were examined daily for vaginal opening time after weaning on PND21. The appearance of a small “pin hole” or a vaginal thread, as well as complete vaginal opening was recorded on the days they were observed. However, the day for complete vaginal opening was the endpoint used in the analysis for the age of vaginal opening. From PND56 to PND69, vaginal cytology was assessed at 9:00 AM daily by vaginal lavage to monitor cytological cyclicity and thus determine estrous cycle of female pups.

**Body Weights and Uterus Weights**

Female pups were weighed on PND1, PND12, PND20, PND30, PND40, PND50, PND60, and PND70. The uteri from sacrificed pups on PND12 and PND70, carefully dissected free of adhering fat and mesentery, were weighed. One uterine horn from each animal was dissected and kept under -80 °C for Western blotting analysis. Similarly, hypothalamus, pituitary and ovary were also sampled and kept under -80 °C for Western blotting analysis.

**Western Blotting Analyses for ER\textalpha{} and ER\textbeta{}**

Frozen hypothalamus, pituitary, uterus and ovary were homogenized in 5× volumes (per g wet weight tissue) of ice-cold T-PER tissue protein extract...
reagent. The protein concentrations of the lysates were measured with Coomassie blue assay and were adjusted to 4.0 mg/mL. Proteins were separated by SDS/PAGE in 10% gels when loading at 40 µg per lane. After electrophoresis, proteins were transblotted onto PVDF membranes in the transfer buffer. The blotted membranes were probed with primary ERα or ERβ antibodies (with dilution of 1:500 in PBS) overnight at room temperature, followed by addition of β-actin antibody (with dilution of 1:2000 in PBS) and incubation with secondary antibodies (with dilution of 1:5000 in PBS) for 1 h. Chemiluminescent detection was conducted after addition of LumiGLO chemiluminescent substrate. For control blots, membranes were incubated in the dilution buffer with the blocking peptides.

**Statistical Evaluation**

ANOVA in SPSS (SPSS, Inc, Chicago, IL, USA) was used to analyze body weights, uterus weights, vaginal opening time and estrous cycle followed by testing for variance homogeneity. A two-tailed probability value of 5.0% (P<0.05) was considered statistically significant between the control and treated groups.

**RESULTS**

**Body Weights and Uterus Weights**

Changes in body weights and uterus weights of female pups were shown in Table 1 and Table 2. When compared to Group CTL, Group B+S on PND12 had lower body weights but without statistical differences. There were no statistically significant differences in body weights from PND1 to PND70 at about 10-day intervals between the control and SIF-BPA-and B+S-treated groups (P>0.05). As shown in Table 2, on PND12, females in Groups SIF, BPA and B+S had significantly higher relative uterus weights than animals in Group CTL (P<0.05) with an increase of 13.9%, 24.8%, and 11.8%, respectively. On PND70, however, administration of SIF, BPA or combination of SIF and BPA did not significantly increase relative uterus weights although Group SIF and Group B+S had higher relative uterus weights than Group CTL (P>0.05).

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<td><strong>Body Weights of Female Rats Lactationally exposed to Combination of Soy Isoflavones and Bisphenol A (g)</strong></td>
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*Note. Values are mean±SD.*

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<td><strong>Uterus Weights, Vaginal Opening, and Estrous Cycle in Rats Lactationally exposed to the Combination of Soy Isoflavones and Bisphenol A</strong></td>
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*Note. Values are mean±SD. Relative weights were calculated by dividing uterus weight by body weight and multiplying by a factor of 100. Compared with Group CTL, †P<0.05, ‡P<0.01; compared with Group B+S, *P<0.05, ††P<0.01.*

**Vaginal Opening Time and Estrous Cycle**

As shown in Table 2, compared with Group CTL, Group BPA and Group BPA had shortened their vaginal opening time while Group B+S had prolonged it without statistical differences (P>0.05). In addition, female rats treated with SIF or BPA or combination of SIF and BPA did not witness significant changes in their duration of estrous cycle, compared with that of untreated animals (P>0.05).

**Western Blot Analyses of ERα**

Immunoreactive bands of ERα in the hypothalamus-pituitary-ovary axis and uterus detected with Western blot assays were shown in Fig 1. On PND12, compared with Group CTL, Group
SIF showed a weaker ERα expression in the hypothalamus while a stronger expression in the ovary, and similar expressions were observed in the pituitary and the uterus. In Group BPA, however, there was a down-regulated level of ERα expression in the hypothalamus while an up-regulated level in the pituitary and similar levels in the ovary and the uterus. Female rats treated with the combination of SIF and BPA showed an increase of ERα expressions in the hypothalamus and ovary and similar ERα expressions in the pituitary and uterus, compared with animals untreated.

There were different changes of ERα expressions in the hypothalamus-pituitary-ovary axis and uterus on PND70. As shown in Fig. 1, female rats in the SIF treatment group had weaker immunoreactive bands of ERα in the ovary and uterus than animals in the control group. But the SIF treatment group and the control group had similar band intensity for ERα expressions in the hypothalamus and the pituitary. Group BPA had similar changes of ERα expressions to Group B+S when compared with the control group. For instance, immunosignals of ERα proteins in the pituitary in Group BPA and Group B+S were stronger than that in Group CTL. ERα immunosignals in the ovary and uterus in these two treatment groups were weaker than that in the control group. However, there were no significant changes of ERα protein immunosignals in the hypothalamus in these two treatment groups, compared with the control group.

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**DISCUSSIONS**

Since information about combinatory actions of
EFFECTS OF SIF AND BPA ON ESTROGEN RECEPTORS

Endocrine disruptors is limited and results obtained in a few studies are inconsistent, we have studied the combinatory effects of the natural phytoestrogens SIF and the industrial chemical BPA on estrogen sensitive parameters in neonate female rats exposed lactationally; the models were shown to be sensitive to SIF in our previous study [10]. In this study, lactational exposure to SIF or BPA could significantly increase relative uterine weights, but the uterotrophic activity was lower when SIF and BPA were co-administered lactationally (Table 2). Therefore, it could be concluded that no synergic effect or additive effect existed while there might be antagonistic effect between SIF and BPA. Our data were largely in agreement with Tinwell and Ashby’s results obtained from immature rats, demonstrating that BPA had the ability to antagonize the uterotrophic effects of genistein [8]. These results had identified that BPA acts, at least in the uterus, as a functional antagonist to isoflavones. But in another study conducted by Wade et al. SIF and BPA showed a synergic effect [11]. These inconsistent results obtained from different studies resulted from many factors, such as the age of animals used, the administration routes of test materials, the endpoints observed, the doses of each material and their ratios, and so on [8,11].

Results in the present study have demonstrated that uterotrophic effects of SIF and BPA as well as their combinatory actions after lactational exposure might be mediated by regulation of ERα or ERβ expressions in the hypothalamus-pituitary-ovary axis and uterus. And an interesting tissue specific difference in the regulation of ERα or ERβ was clearly presented, which had also been found out in other studies [1,12]. As shown in Fig. 1 and Fig. 2, ERα or ERβ expressions in hypothalamus, pituitary, ovary and uterus were different even in the same treatment group. We could not present combinatory actions to ER expressions between SIF and BPA although co-administration of SIF and BPA could significantly enhance ERα expressions in the hypothalamus and ovary and reduce ERβ expression in the pituitary. Due to the fact that many studies have got different results in this respect [13-14], further research is required to elucidate conclusively the regulation of endocrine disruptors on estrogen receptors in different tissues.

![Western blot analyses of ERβ protein, with β-actin as internal reference, in hypothalamus, pituitary, ovary and uterus. Bands (left to right) in each immunoblotting represent group CTL, SIF, BPA and B+S. Although SIF or BPA and their combination had uterotrophic activities on PND12 when female rats were lactationally exposed on PND5-11, these treatments did not affect significantly vaginal opening time and estrous cycle and relative uterine weight on PND70, which might demonstrate that effects resulted from lactational exposure to SIF or BPA disappeared in later life. The increase of endogenous estrogen was considered as the main explanation for this change. However, lactational exposure to SIF or BPA still up-regulated ERα expression in the pituitary and ERβ expression in the...](image-url)
hypothalamus and down-regulated ERα expression in the ovary and ERβ expression in the uterus on PND70, which suggested that these exposures might affect protein expressions or even molecule levels in the later life.

In conclusion, lactational exposure to SIF or BPA and their combination could produce uterotoxic actions in early life, which might be involved with the regulation of ERα or ERβ expressions in the hypothalamus-pituitary-ovary axis and uterus. Nevertheless, it is worth mentioning that these results should be extrapolated prudently to humans since human exposure to these endocrine disruptors is considerably lower and different\textsuperscript{115-16}. Potential hazard identified at high experimental doses in rodents is not necessarily predictive of a relevant risk for humans at real exposure levels.

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