RESEARCH ARTICLE

In utero and postnatal exposure to isoflavones results in a reduced responsivity of the mammary gland towards estradiol

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Scope: Exposure scenarios during different stages of development of an organism are discussed to trigger adverse and beneficial effects of isoflavones (ISO). The aim of this study was to investigate how in utero and postnatal ISO exposure modulates the estrogen sensitivity of the mammary gland and to identify the underlying molecular mechanisms.
Methods and results: Therefore, rats were exposed to either ISO-free (IDD), ISO-rich (IRD) or genistein-rich diet (GRD), up to young adulthood. Proliferative activity (PCNA expression) in the mammary gland at different ages and the estrogen sensitivity of the mammary gland to estradiol (E_2) or genistein (GEN) in adult ovariectomized animals was determined and compared with different treatments. Treatment with E_2 resulted in a significant lower proliferative and estrogenic response of the mammary gland in IRD and GRD compared with IDD. This correlates to a change in the gene expression pattern and a decrease in the ratio of estrogen receptor alpha (ERα) beta (ERβ).
Conclusions: Our results provide evidence that in utero and postnatal exposure to a diet rich in ISO but also to GEN reduces the sensitivity of the mammary gland toward estrogens and support the hypothesis that in utero and postnatal ISO exposure reduces the risk to develop breast cancer.

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1 Introduction

The incidence of breast cancer in eastern Asia is approximately three times lower than in Western countries [1]. It has been hypothesized that nutrition plays an important role in the prevention of hormone-dependent breast cancer [2]. The traditional East Asian diet includes soy, which is the main source of isoflavones (ISO), belonging to the class of phytoestrogens. Soy ISO show a polyphenolic non-steroidal structure and their hydroxyl groups at C-7 of the A-ring and C-4′ of the B-Ring of the ISO skeleton exhibit a distance similar to those of the endogenous estrogens. Because of this similarity, ISO exert the ability to bind to both estrogen receptor subtypes (ER), with a higher affinity to the ERβ, and subsequently initiate estrogen-dependent transcription [3].

The estrogenic potency of ISO is much lower compared with estrogens, but the circulating concentration of ISO can reach much higher levels after ingestion of soy food or soy-derived dietary supplements. The daily uptake of soy

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Abbreviations: DAI, daidzein; **ER,** estrogen receptor; **E_2,** estradiol; **GRD,** genistein-rich diet; **GEN,** genistein; **IDD,** isoflavone-depleted diet; **IRD,** isoflavone-rich diet; **ISO,** soy isoflavone; **OVX,** ovariectomized control group; **PCNA,** proliferating cell nuclear antigen; **PND,** postnatal day; **PR,** progesterone receptor; **s.c.,** subcutaneously

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ISO in the various Asian countries range around 20–50 mg, resulting in ISO plasma concentrations of 870 nM while the Western population ingests 1 mg ISO per day [4], which leads to plasma concentrations of ISO as low as 10 nM [5]. Intake of ISO via supplements normally range between 20 and 80 mg/day, resulting in ISO plasma concentrations in the range of 1–3 μM.

Whether effects of ISO are beneficial or not is controversially discussed. A protective effect of ISO toward bone health is described in animal studies [6–8], although questionable for the human situation as neither a randomized double-blind placebo controlled study nor a recent meta-analysis were able to provide evidence [9, 10]. A benefit of ISO on hot flushes is also controversially discussed [11, 12].

The same controversy exists regarding the outcome of ISO intake in respect to breast cancer. Negative impact for example could be shown in an animal model which mimics the key aspects of postmenopausal conditions by inoculating MCF-7 breast cancer cells into immunodeficient nude mice. Here, an increase in breast tumor growth was detected after treatment with genistein (GEN) [13]. In vitro for MCF-7 breast cancer cells a biphasic effect on cell growth dependent on concentration could be observed in response to GEN [14]. The National Toxicology Program (NTP) conducted a two-year multigenerational GEN study in Sprague–Dawley rats, which was recently reviewed by Doerge [15]. The key results relevant to this study were that depending on the study arm a trend for increased incidences of mammary adenoma and adenocarcinoma could be observed in the 5 and 100 ppm GEN feeding group, which although the absolute numbers were relatively low, reached statistical significance in the 500 ppm feeding group [15–17]. However, there is also evidence that in utero and postnatal or neonatal ISO or GEN exposure may protect against breast cancer [18, 19]. In addition, soy ISO intake was found to be inversely correlated to mortality and recurrence from breast cancer.

To understand these controversial results it is important to have a closer look toward the risk factors involved in the development of breast cancer. Risk factors for breast cancer are early onset of menarche, late onset of menopause and high amounts of free circulating estradiol (E_2) in the serum of postmenopausal women [20–22]. ISO exert the ability to increase the amount of sex hormone-binding globuline, which results in a reduction of free, bioavailable circulating estrogens [23]. This conforms to the fact that compared with Western women the free serum-estradiol levels of Asian women are 40% lower [24]. Beside the concentration of circulating estrogens also the estrogen sensitivity of the non-malignant breast tissue may be altered by ISO. There is evidence that ISO may influence the development of the mammary gland, starting already in utero. A rudimentary gland is present at birth, and during puberty, hormone-dependent development of the mammary gland occurs [25]. Various studies suggest a protective effect of ISO if the ingestion starts before onset of puberty [26, 27]. Furthermore, it has been shown that prepubertal exposure to GEN alters the development of the mammary gland [28]. Hence, it seems reasonable that the exposure to ISO has to begin before pubertal breast development starts in order to exert protective effects as recently proposed [29].

Information whether the ISO exposure during puberty and in further developmental stages alters the sensitivity of breast tissue toward estrogens or ISO in postmenopausal woman is still limited. However, as recently reviewed the degree of maturation of terminal end buds appears to play a key role [30]. While studies investigating environmental endocrine disrupters, e.g. bisphenol A, suggest that early embryonic exposure significantly impacts on tissue (patho)physiology and hormone responsiveness later in life by delaying maturation [31], the tumor-preventive effect of neonatally administered GEN appears to occur through advanced differentiation of these glandular structures which is paralleled by alterations in cell proliferation and apoptosis as well as upregulation of tumor-suppressor genes (reviewed in [18, 30]). Taking into consideration that manufacturers advertise dietary soy supplements as an effective alternative to the conventional HRT without adverse side effects, understanding consequences of an early ISO exposure seems of fundamental importance.

The aim of this study was to investigate the effect of in utero and postnatal ISO exposure on estrogen sensitivity of the mammary gland in adult intact and ovariectomized (OVX) female rats. The animals were fed diets containing different amounts of ISO. The ISO exposure was initiated in utero, maintained during neonatal, and prepubertal stages of development and adhered until adulthood. It is well known that an increased cell proliferation rate is associated with a high risk of developing breast cancer. For that reason the proliferation in the mammary gland was determined as a biological endpoint for estrogen sensitivity. In addition, the expression of the progesterone receptor (PR), the estrogen receptor α (ERα) and estrogen receptor β (ERβ) and PS2 was investigated.

2 Materials and methods

2.1 Experimental animals and study design

All animal handling and experimental conditions were carried out according to the ''Institutional Animal Care and Use Committee guidelines'' regulated by the German federal law for animal welfare (Permission Number 50.203.2-K15).

Wistar rats were obtained from Janvier (Le Genest St Isle, France) and kept under controlled conditions of temperature (20°C ± 1), relative humidity (50–80%) and illumination (12 h dark, 12 h light). Female rats were mated and the dams (7 in each dietary group) were fed one of the three diets during pregnancy and nursing. After weaning the female offspring of each nutrition group (30 each group) was randomly assigned to five treatment groups (6 each group),

to make sure that pups from different mothers generate a group. These rats had ad libitum access to the appropriate diet and water. The first subgroup was sacrificed on postnatal day (PND) 50 (puberty), the second on PND 80 (adult). The other three subgroups of each nutrition group were ovariectomized at day 80. After 14 days of hormonal decline an uterotrophic assay was performed at day 94. These rats then received 17β-estradiol (E_2; 7.8 μg/kg b.wt./day), genistein (GEN; 19.6 mg/kg b.wt./day), or the vehicle solely (OVX) subcutaneously for three days before they were sacrificed (PND 97). The estrogenic compounds were solved in 20% DMSO/peanutoil.

2.2 Diets

The animals had free access to one of three diets containing different amount of ISO: an ISO-depleted diet (IDD; Ssniff R/M-H Ssniff GmbH, Soest, Germany), an ISO-rich diet (IRD; Harlan Teklad 8604 rodent diet, Harlan Winkelmann, Borchen, Germany), or an IDD supplemented with 700 μg GEN (GRD; 4′,5,7-trihydroxyisoflavone, LC Laboratories, Woburn, USA). The compositions of the diets are depicted in Table 1 of Supporting Information. Indicated by the manufacturers the protein source in the Ssniff R/M-H diet is cereals and potatoes, in the Harlan Teklad 8604 rodent diet soy, fish meal and yeast. The choice of these diets and the respective ISO content was based on results of former studies. In previous experiments of our laboratory, an oral dose up to 50 mg GEN per kg did not affect the uterine wet weights [32], while an oral GEN dose of 100 mg/kg results in effects on several tissues in the animals [33]. This very high dose is representative for an exposure scenario depicting supplementation with soy extracts or pure GEN. On the contrary, the source of the high content of ISO in the used IRD is dehulled soybean meal. Based on the data from previous studies, where ISO-content of rodent diets from different vendors was analyzed and compared [34], the ISO-rich diet (IRD) from Harlan–Winkelmann was chosen because of its high ISO content. This diet depicts more an exposure scenario of an ISO-rich nutrition like consumed in eastern Asia.

Given the ISO content and daily food consumption (18–20 g/d/animal), the average oral intake resulted in 13.5 mg/kg b.wt. for daidzein (DAI) and 15.7 mg/kg b.wt. for GEN per adult animal in the IRD group, 42 mg/kg b.wt. per adult animal and day for GEN in the GRD group, and <0.1 mg DAI or GEN/kg b.wt./day in the IDD group.

2.3 ISO standards

The ISO aglycones daidzein, genistein, glycitein, their corresponding 7-*O*-ß-glucosides daidzin, genistin, glycitin, as well as 6″-*O*-acetyl-daidzin, -genistin and -glycitin and 6″-*O*-malonyl-daidzin, -genistin and -glycitin were obtained from Wako Chemicals GmbH (Neuss, Germany). Purity of the standard compounds was not $<97\%$ (determined by HPLC/DAD analysis at 250 nm).

2.4 Tissue preparation

After weighing, animals were decapitated and blood was collected. The mammary gland and the uterus were removed and the uterus wet weights were determined. Specimens of each tissue were either snap frozen in liquid nitrogen for mRNA and protein preparation or fixed and embedded in paraffin for histological analysis.

2.5 Quantification of ISO derivatives in the diet by HPLC/DAD analysis

The pelleted diets IRD and IDD were crushed using mortar and pestle. Approximately, 250 mg of an accurately weighted sample of each homogenized diet powder were vortexed for 30 s in exactly 40 mL 65% v/v methanol and extracted gently for 60 min at room temperature using an overhead rotation shaker. The suspensions were centrifuged at $8600 \times g$ for 5 min and filtered using 15 mm 0.45 μm PTFE syringe filters. The filtrate was diluted by pipetting 1 mL of the solution into a 50 mL graduated flask and make up to the mark with 65% v/v methanol.

The LC-DAD analyses were performed on a Shimadzu LC system equipped with a controller (CBM-20A), a degasser (DGU-20A3), two pumps (LC-20AD), an autosampler (SIL-20AC HT), a column oven (CTO-20AC) and a diode array detector (SPD-M20A). The LC system was controlled by the software LC solution 1.24. Separation of the ISO derivatives was performed on a Phenomenex Kinetex PFP column (3.0 mm internal diameter, 100 mm length, 2.6 μm) with an oven temperature of 35°C. Solvent A was 0.1% v/v formic acid in bidest. water and solvent B was acetonitrile (VWR, LC grade). Flow rate was 0.7 mL/min, the injection volume was 10 μL. The LC gradient started with an initial period of 3 min at 10% B, increasing linearly to 45% B at 12 min, and finally to 100% at 12.5–15.5 min, re-equilibrating the system in a 7.5 min postrun (10% B). Eluent was monitored between 200 and 500 nm using diode array detection. Peaks were integrated at 250 nm. The identity of each compound was confirmed by the retention time and the UV–Vis spectra. The limit of quantification (LOQ) and lowest calibration point was 0.8 nmol for all target analytes.

2.6 Measurement of the GEN and DAI serum concentrations

GEN and DAI were quantified in the serum samples (100 μL) by capillary gas chromatography–mass spectrometry (1200 Varian Triple Quadruole system) as previously

described in detail [35] with the exception that the derivatization of the ISO was performed using *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide with 1% *tert*-butyldimethylchlorosilane (Sigma-Aldrich, Taufkirchen, Germany). The two stable isotopically labeled ISO, [3,4,8-$^{13}C_3$]daidzein, and [3,4,1′-$^{13}C_3$]genistein (Nigel Botting, University of St. Andrews, UK) were used as internal standard compounds.

2.7 Western Blot analysis

Frozen mammary gland tissue specimen was powdered and homogenized in buffer (623.5 nM Tris pH 8 EDTA) containing enzyme inhibitors (5 mg/mL aprotonin, 5 mg/mL leupeptin, 1 mg/mL pepstatin-A, 5 mg/mL antipain, 100 mM pefac in 0.5 M EDTA pH 8). Protein concentrations were measured with the method of Lowry [36] (D_c Protein Assay, Bio-Rad). Equal amounts of samples (40 μg protein) were loaded on 4–12% Bis-Tris NUPAGE® Novex Gels (Invitrogen Life Technologies, Karlsruhe, Germany). For the electrophoresis the MES Buffer was used (Invitrogen Life Technologies). After electrophoresis, the proteins were transferred onto nitrocellulose membranes and blocked with 5% BSA in phosphate-buffered saline solution at room temperature for 1 h. The protein expressions of actin and Proliferating Cell Nuclear Antigen (PCNA) were quantitative detected using specific antibodies (Anti Actin A 5060, Sigma-Aldrich, Taufkirchen, Germany; Anti PCNA M0879, Dako, Glostrup, Denmark). As species-specific antibodies the Horseradish Peroxidase-conjugated Polyclonal Rabbit Anti Mouse (Dako, Glostrup, Denmark) and Polyclonal Swine Anti Rabbit (Dako) were used. The visualization of the blot signals was performed with the chemiluminescent POD-substrate and a Fluorchem Luminescent Imager. The densitometrical analysis was performed with the Image J program (ImageJ 1.33u, National Institute of Health, USA, http://rsb.info.nih.gov/ij/). Actin was used as the reference protein and served as the loading control.

2.8 Immunhistochemical analysis

The paraffin-embedded mammary glands were cut into 7 μm sections and were mounted on slides coated with polylysine (Menzel Gläser, Hilden, Germany). The mammary gland tissue was cleared, hydrated and antigen retrieval was performed using Tris-EDTA. After overnight incubation in Tris-EDTA at 60°C, the mammary gland tissues were washed four times with phosphate-buffered saline (PBS). Then the tissue was incubated with a solution consisting of 0.5 M ammoniumchloride in 0.25% Triton-X/PBS for 10 min. After four wash steps the unspecific binding sites were blocked with 5% BSA for 1 h. Then the mammary gland tissue sections were incubated with the first antibody (Anti PCNA M0879, Dako; Anti PR 1408, Beckman Coulter, Marseille, France; Anti ERβ (H-150) sc-8974, Santa Cruz Biotechnology, Santa Cruz, USA) at 4°C overnight. After four wash steps the second antibody (Polyclonal Rabbit Anti Mouse biotinylated, Dako; Polyclonal Goat Anti Rabbit biotinylated, Dako) was incubated for 1 h at room temperature. To visualize the binding of the PCNA antibody, the tissue sections were incubated with FluoroLink™Cy™3 labelled streptavidin (PA 43001, Amersham Biosciences). To depict the percentage of proliferating nuclei, all nuclei were stained with DAPI (4′,6-Diamidino-2-phenylindole, D 9542, Sigma-Aldrich, Taufkirchen, Germany). Per slide 300 nuclei were analyzed for PCNA expression and the percentage of PCNA-positive nuclei was calculated. In order to visualize the binding of the PR and the ERβ antibody, the tissue sections were incubated with Streptavidin-Biotinylated Horseradish Peroxidase Complex (RPN1051, GE Healthcare, Buckinghamshire, UK) and stained with Diaminobenzidin (DAB). Totally, 300 nuclei were counted per slide, and the percentage of PR-positive nuclei was calculated. Staining of ERβ was utilized by densitometrical analysis using the Image J program (ImageJ 1.33u, National Institute of Health, http://rsb.info.nih.gov/ij/).

2.9 RNA preparation

Frozen mammary gland tissue specimen was powdered and homogenized in TriZol. Total RNA was isolated from cells using the TriZol (Invitrogen Life Technologies) standard protocol [37] followed by cDNA synthesis with the Quantitect® Reverse Transcription Kit (Qiagen, Hilden, Germany).

2.10 Real-time RT-PCR

Quantitative real-time RT-PCR was performed in the MxPRO (Stratagene) with Platinum® Taq DNA Polymerase (Invitrogen). SybrGreen I® was used as the detection dye. The Cytochrome-c-oxidase subunit 1A (1A) was used as the housekeeping gene, and the expression of all genes was normalized to 1A. Specific primers were designed with the primer3 software (Whitehead Institute for Biomedical Research, Cambridge, UK) based on the cDNA sequences available at the EMBL database: 1A: up: 5′-CGTCACAGCCCATGCATTCG-3′, dw: 5′-CTGTTCATCCTGTTCCAGCTC-3′; PR: up: 5′-CATGTCAGTGGACAGATGCT-3′, dw. 5′-ACTTCAGACATCATTTCCGG-3′; PS2: up: 5′-GGAAAGGGTTGCTGTTTTG-3′, dw: 5′-ACAGGTGTGTATGAAGCAGGTG-3′; ERα: up: 5′-GGAAGCACAAGCGTCAGAGAGAT-3′, dw: 5′-AGACCAGACCAATCATCAGGAT-3′. The PCR program consisted of a first denaturation step at 95°C for 4 min, followed by 45 cycles of 30 s at 94°C, 30 s at 60°C and 30 s at 72°C. The fluorescence was quantified during the 72°C elongation step and the product formation was confirmed by a melting curve analysis (55–95°C). For calculation of relative rates of gene expression the $\Delta\Delta C_T$ method was used

[38]. Gene expressions were compared with those of control animals fed with the ISO-free diet.

2.11 Statistical analysis

Statistical analysis was performed using the SPSS Statistical Analysis System, SAS, Version 12.0. All data are expressed as arithmetic means with their standard deviations. First, a global Kruskal–Wallis *H*-test was performed to analyze if there are significant differences between the groups. In case of differences, a Mann–Whitney *U*-test was additionally performed to identify the groups with statistical significant variance. Statistical significance was established at $p < 0.05$.

3 Results

The experimental design of this study is depicted in Fig. 1. The animals of this experiment received one of three different diets. The ISO-depleted diet (IDD) contains no detectable amounts of ISO (<10 μg/g). The ISO-rich diet contains 248 μg GEN/g, 213 μg DAI/g and 59 μg glycitein/g (each calculated as aglykone). ISO in the diet were mainly (> 90%) present as the respective glucoside and malonylglucoside derivatives. A detailed analysis of the ISO derivatives in the diet is given in Table 2 (Supporting Information). The GEN-rich diet (GRD) based on an IDD, enriched with 700 μg GEN/g [7]. Serum concentrations of GEN and DAI were measured at PND 50 and PND 80 (Table 3, Supporting Information). As expected, the serum concentrations of GEN and DAI significantly increased with the increasing ISO content in the diet. No differences were detected between PND 50 and PND 80.

As depicted in Table 4 (Supporting Information), neither treatment nor nutrition had influenced the body weights or the heart weights. The wet weights of the uteri significantly increased after treatment with E_2 in all groups compared with their OVX groups. Additionally, IRD and GRD animals treated with E_2 showed a significant increase in the wet weights of the uteri compared with the IDD group. Treatment with GEN had no significant effect on uterine wet weights in any group.

To evaluate the influence of ISO on the proliferation of the mammary gland, the protein expression of the Proliferating Cell Nuclear Antigen (PCNA) was determined. Western blot analysis of PCNA showed a significant increase in the GRD fed animals compared with the IDD and IRD group at PND 50, but no differences were detected in PCNA expression at PND 80 in intact animals (Fig. 2).

To investigate the influence of in utero and postnatal exposure to ISO on the sensitivity of the mammary gland toward estrogen exposure mammary gland tissue obtained from rats used in an uterotrophic assay was examined. Immunfluorescence analysis of the mammary gland of OVX animals showed no differences in the PCNA expression in the OVX animals of either diet, whereas treatment with E_2 led to an increased proliferation rate (Fig. 3A). No differences could be seen in GEN-treated groups. Quantitative analysis showed a significant increase in PCNA expression in the E_2-treated groups compared with the OVX groups. After preexposure to IRD and GRD the E_2 stimulation of PCNA protein expression detectable in IDD was found to be significantly diminished (Fig. 3B). The observation was verified with Western blot analysis. As in the immunfluorescence analysis, no differences in OVX- and GEN-treated animals were detected, but a significant increase in PCNA expression in IDD-fed animals treated with E_2 (Fig. 3C). The IRD showed no increase in PCNA expression after E_2 treatment, while in the GRD group an increase in PCNA expression was detected.

A well-established marker for estrogen action in the mammary gland is the progesterone receptor (PR) bearing an estrogen response element (ERE) in its promoter [39].

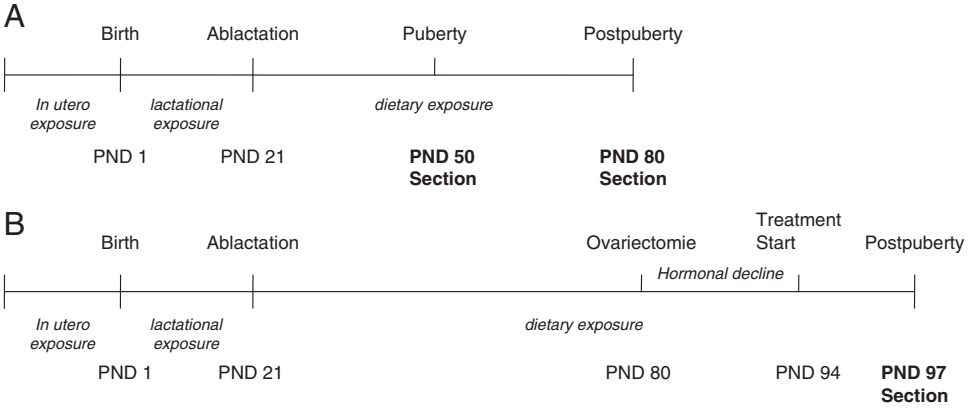

Figure 1. Timeline and experimental setting of the study depicting modes of isoflavone exposure (in utero, lactational, and dietary) and point of time for analysis. (A) In intact PND 50 and PND 80 the ISO content were measured in the serum and the proliferation were determined in the mammary gland. (B) After avariectomie on PND 80, animals were treated with E_2, GEN, or the vehicle from PND 94 up to PND 97 and were sacrificed. In these animals the proliferation and the estrogenic response of the mammary gland were determined.

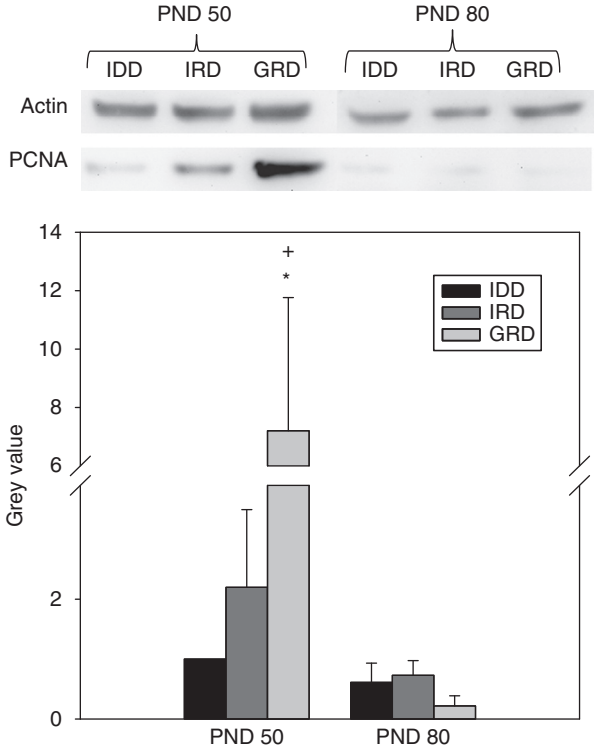

Figure 2. Protein expression of PCNA in the mammary gland of intact 50 and 80 day-old rats. Depicted is a representative Western Blot of PCNA in intact animals and the quantitative analysis of the Western blots. IDD PND 50 served as the control and were set to one. The bars shown are mean ± SD. Six rats were included to each group. * = sign. versus IDD PND 50 ($p<0.05$); + = sign. versus IRD PND 50 ($p<0.05$); (Kruskal–Wallis *H*-test followed by a Mann–Whitney *U*-test).

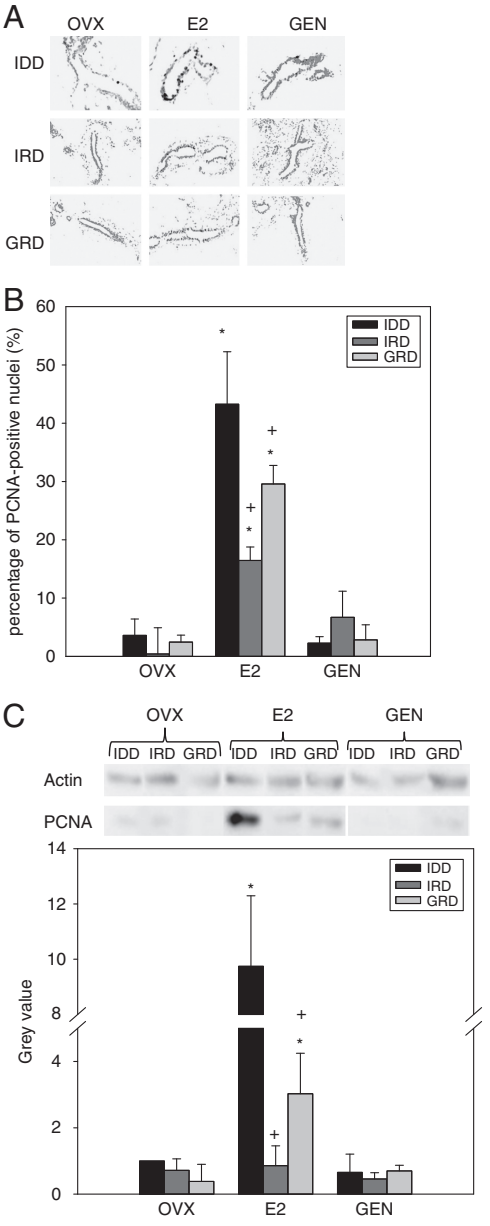

Figure 3. Protein expression of PCNA in the mammary gland of ovariectomized animals. (A) Representative pictures of immunhistochemical stained sections of the mammary gland. Proliferating nuclei were stained with PCNA labelled with Cy3 (black). Stained grey with DAPI are all nuclei. (B) Quantitative analysis of PCNA staining. (C) Depicted is a representative Western Blot of PCNA in the mammary gland of OVX rats and the quantitative analysis of the Western blots. IDD OVX served as the control and were set to one. The bars shown are mean ± SD. Six rats were included to each group. * = sign. versus IDD OVX ($p<0.05$); + = sign. versus IDD E_2 ($p<0.05$); (Kruskal–Wallis *H*-test followed by a Mann–Whitney *U*-test).

Immunhistochemical analysis of PR expression in the mammary gland revealed only a slight expression in OVX- and GEN-treated groups. E_2 treatment results in a significant increase of PR staining in all groups (Fig. 4A). Quantitative analysis of the immunhistochemical staining indicated that the increase was significant higher in the animals which received in utero and postnatal an IDD (Fig. 4B). This observation was confirmed by semi-quantitative analysis of PR mRNA expression (Fig. 4C). None of the GEN-treated groups showed differences in the mRNA expression of PR compared with the OVX groups.

Beside PR, the expression of the estrogen receptor α (ERα) and the PS2 gene was measured in the mammary gland using real-time RT-PCR. For ERα it has been demonstrated that mRNA and protein expression show a good regulation in this tissue [40]. As shown in Fig. 5A, in utero and postnatal exposure to an IRD showed only a slight increase in ERα mRNA expression in OVX animals, while treatment with E_2 resulted in a significant increase in ERα expression in the animals fed in utero and postnatal an IDD compared with those fed an IRD or GRD. PS2 expression (Fig. 5B) was increased in OVX IRD and GRD animals compared with OVX IDD. E_2 treatment resulted in a significant decrease in PS2 expression in the IDD group but

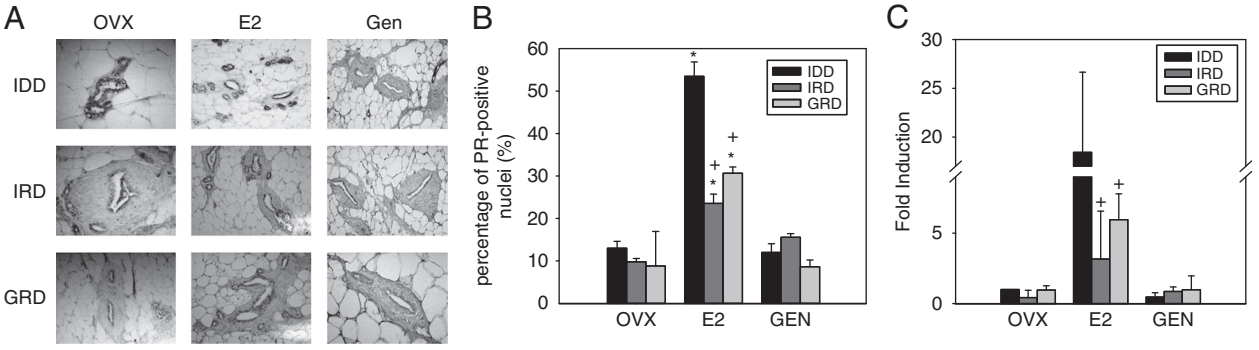

Figure 4. Expression of the progesterone receptor (PR) in the mammary gland of 97 days-old ovariectomized rats. (A) Protein expression of the PR. Depicted are representative pictures of immunhistochemical stained sections of the mammary gland. (B) Quantitative analysis of PR staining. (C) mRNA expression of the PR in the mammary gland. IDD OVX served as the control and were set to one. The bars shown are mean ± SD. Six rats were included to each group. + = sign. versus tDD E2, * = sign. versus IDD OVX ($p < 0.05$); (Kruskal–Wallis *H*-test followed by a Mann–Whitney *U*-test).

in a strong stimulation of PS2 expression in the IRD and GRD group. In response to GEN treatment, no significant differences could be measured.

To reveal differences in the expression patterns of the estrogen receptors, the protein expression of estrogen receptor β (ERβ) were analyzed by immunhistochemistry (Fig. 6). ERβ staining of the mammary gland showed only marginal differences in its expression in the OVX groups (Fig. 6A). After treatment with E_2, an increase in expression could be detected in IRD and GRD animals but not in animals receiving an IDD. This observation was confirmed by densitometrical analysis (Fig. 6B). The highest expression of ERβ was measured after treatment of GRD animals with GEN.

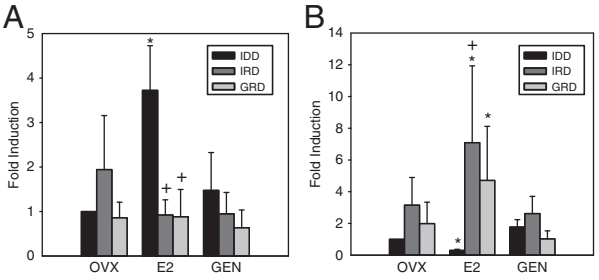

Figure 5. mRNA expression of the estrogen receptor α (ERα) (A) and PS2 (B) in the mammary gland of 97 day-old ovariectomized rats. IDD OVX served as the control and were set to one. The bars shown are mean ± SD. Six rats were included to each group. * = sign. versus IDD OVX ($p < 0.05$); + = sign. versus IDD E_2 ($p < 0.05$); (Kruskal–Wallis *H*-test followed by a Mann–Whitney *U*-test).

4 Discussion

Epidemiological studies suggest that soy consumption is associated with a lower risk of developing breast cancer in the Asian population. It is hypothesized that the exposure to ISO has to start before adolescence sets in to exert protective effects. Studies investigating the effects of in utero and postnatal exposure to ISO are still inconsistent [28]. The present study was performed to analyze the impact of in utero and postnatal exposure to ISO on the mammary gland. In order to make sure that the offspring was exposed to ISO already in utero the dams received one of three different diets, respectively. Although the diets differ in protein and fat content, both IRD and GRD produce similar effects. Thus, we can assume that these differences do not influence the estrogenic response of the mammary gland (Table 1, Supporting Information). The long-term exposure to IRD and GRD consequently resulted in increased serum concentrations of GEN (Table 3, Supporting Information). In case of the IRD increased concentrations of DAI and its colonic microbiota-derived metabolite equol have additionally to be taken into account. Unfortunately, we have not determined the serum equol concentration in our study, but its remarkable formation in rodents is well described. For instance, Poulsen et al. found almost equally high DAI and equol plasma concentrations in ovariectomized rats fed a diet supplemented with DAI [41].

As we observed a strong influence of ISO and GEN exposure on mammary gland biology the question arises about a potential developmental origin of these effects. Previous studies suggested that the placental transfer of ISO is high, whereas the lactational transfer is low to negligible [15, 42]. On the other hand, even if the lactational transfer seems to be low, quite significant alterations of the proteome can be achieved in mammary glands of prepubertal animals which were fed by lactating dams which were exposed to a diet containing 250 ppm of GEN [43]. Applying these findings to our study, it has to be assumed that in utero exposure most likely contributes to the effects observed; however, a contribution to the effects through lactational exposure cannot be excluded.

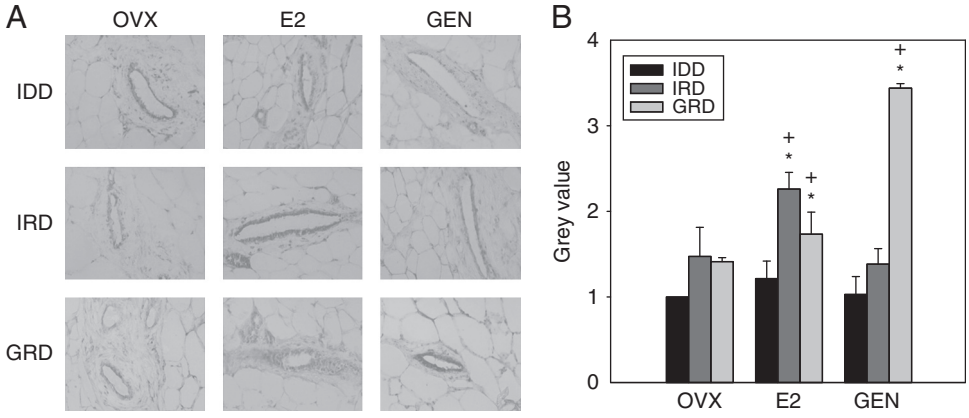

Figure 6. Expression of the estrogen receptor β (ERβ) in the mammary gland of 97 day-old ovariecotmized rats. (A) Protein expression of the ERβ. Depicted are representative pictures of immunhistochemical stained sections of the mammary gland. (B) Densitometric analysis of ERβ staining. IDD OVX served as the control and were set to one. The bars shown are mean ± SD. Six rats were included to each group. * = sign. versus IDD OVX ($p<0.05$); + = sign. versus IDD E_2 ($p<0.05$); (Kruskal–Wallis *H*-test followed by a Mann–Whitney *U*-test).

As shown in Table 4 (Supporting Information) and as reported before [7], no differences in the uterine wet weights could be detected in 50 and 80-day-old intact animals, indicating a low estrogenic potential of the different diets.

In the first part of the present study the mammary gland was analyzed at postnatal day (PND) 50 and at PND 80. As shown in Fig. 2 no differences could be observed between the different dietary groups of 80-day-old adult intact animals, whereas significant differences were observed at day 50, where PCNA expression was strongly stimulated in the GRD group. A recent review on xenobiotic exposure and breast cancer risk in animal models came to the conclusion that acceleration or delay of glandular maturation appears to be a crucial point regarding whether exposure to a xenobiotic leads to prevention or increases the risk to develop breast cancer [30]. A possible explanation for our effect may indeed be an acceleration of the differentiation program of the mammary gland in response to GEN treatment which has already been shown by other groups [28]. In fact, in a recent neonatal exposure study a transient, statistically not significant increase in Ki-67 immunoreactivity, occurring earlier than the increase in PCNA immunoreactivity in our study, was reported in response to GEN which decreased statistically significant later in life [43].

To study if in utero and postnatal exposure to IRD or GRD alters the estrogen responsiveness of the mammary gland, in OVX animals serving as a model system of the situation in postmenopausal woman, an uterotrophic assay was performed. As shown in Table 4 (Supporting Information), E_2 exposure always resulted in an increase in uterine wet weights, but the uterine weights of IRD and GRD were significantly higher than in IDD animals. These data indicate that the estrogen responsiveness of the uterus was highly modulated by long-term ISO exposure. Interestingly, no influence on uterine proliferation could be observed between the dietary groups after E_2 treatment, whereas the water homeostasis was highly affected. Möller et al. speculated that the methylation pattern of genes associated with transcellular water transport in the uterus were changed after in utero and postnatal ISO exposure [44].

In the mammary gland of OVX IRD, IDD and GRD, similar to intact 80-day-old animals, there were no significant differences in PCNA expression. In order to stimulate cell-proliferation in mammary gland tissue, animals were treated with E_2 for 3 days subcutaneously (s.c). In line with published data [45–47] the protein expression of PCNA in the mammary gland increased significantly in response to the E_2 treatment in all dietary groups. In contrast, exposure to GEN had no effect in treatment groups. This confirms a previous study from our laboratory [45], where neither s.c. nor oral treatment with GEN affected the PCNA expression and only slight effects on the PR expression in the mammary gland of adult OVX Wistar rats could be observed.

A key result of the study presented here is the observation that the E_2-induced stimulation of mammary gland PCNA expression was significantly lower following dietary exposure of animals in IRD and GRD groups if compared with IDD-fed animals. This strengthens the hypothesis that pretreatment with ISO accounts for a lower sensitivity of mammary gland tissue toward estrogen treatment.

A possible explanation for this altered sensitivity may be the anatomy of the mammary gland. Several studies have investigated the effects of in utero, perinatal and prepubertal exposure toward ISO on the morphology of the mammary gland [28]. Whereas results obtained for in utero, perinatal and prenatal exposure scenarios are not consistent, it has been shown that prepubertal exposure to GEN increases mammary tissue differentiation by leading to a reduction in the number of terminal end buds (TEB) and an increase in the number of differentiated lobules [48–51]. It is possible

that the lack of increased proliferation after treatment with E_2 in the IRD-fed group is a result of a reduced number of TEB in the mammary gland.

Taken further, the present results show that not only proliferation, but also the expression of a variety of estrogen-sensitive genes is altered. The expression patterns of the PR (Fig. 4) and ERα (Fig. 5A) are very similar to those of the PCNA expression. Both receptors have been implicated in the etiology and the pathogenesis of breast cancer. In the normal mammary gland, progesterone is needed for lobulo-alveolar development and ductal branching, while estradiol regulates ductal elongation. Russo et al. [52] reported that the content of ERα and PR in the lobular structures in the breast is directly proportional to the rate of cell proliferation. In normal resting mammary glands, the percentage of ERα-positive cells is generally low, and increases in proliferative benign disease, particularly when associated with atypia [53]. Additionally, the dual expression of the ER and the proliferating marker Ki-67 seemed to be the manifestation of an important early molecular change in the development of malignant breast neoplasia [54]. Furthermore, an important function of the ERα in the epithelium is the induction of PR [55], and it is known that co-expression of both is inversely associated with breast cancer risk among postmenopausal women [56].

Remarkable is the observation regarding the regulation of ERβ by E_2 in the different nutritional groups. It is hypothesized that one function of ERβ is to counter that of ERα [57]. In vitro studies showed a reduction in estrogen stimulated proliferation after introduction of the ERβ expression vector into representative ERα-positive breast cancer cell lines MCF-7 and T47D [58, 59]. In line with these observations is our finding that the reduced sensitivity of the mammary gland to respond to E_2 treatment by proliferation in GRD- and IRD-fed animals was associated with an elevated expression of ERβ (Fig. 6) and a decreased expression of ERα (Fig. 5A). Also notable is the fact that the expression pattern of the PS2 gene is similar to that of ERβ (Fig. 5B). There are speculations that high expression levels of PS2 may be protective against the development of breast cancer. Transgenic mice, which overexpress PS2 in their mammary gland, do not develop tumors [60]. Our observation that PS2 expression can be induced by ISO is in line with results of Hargreaves et al. [61] showing that treatment of premenopausal women with dietary soy supplements for two weeks results in an increased expression of PS2 in the breast. Interestingly, the control of the PS2 gene also depends upon epigenetic factors [62, 63] as the tissue-specific methylation of its proximal promoter/enhancer region correlates directly with its expression [64, 65]. The increased mRNA expression of PS2 in the IRD fed group could lead to the suggestion, that ISO could exert their protective effects via epigenetic modulation. Indeed, recently a variety of studies has shown epigenetic changes induced by ISO [66–68] having positive effects on breast or prostate cancer.

In summary, our results provide evidence that in utero and postnatal exposure to a diet rich in soy ISO in their natural chemical form as β-glucoside conjugates as well as to a diet solely enriched with GEN in the aglycone form may alter the gene expression of the mammary gland, which consequently results in a changed sensitivity of this tissue towards estrogens. This observation leads to the hypothesis that in utero and postnatal ISO exposure may reduce the risk to develop breast cancer in a protective manner. To clarify the underlying mechanisms, i.e. if the altered susceptibility of the mammary gland is a result of epigenetic mechanisms during the development further studies are needed.

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