Effect of prenatal exposure to the endocrine disruptor bisphenol A on mammary gland morphology and gene expression signature

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Abstract

Bisphenol A (BPA), known as an environmental endocrine disruptor, is widely used as a plasticizer. This study aims to investigate whether exposure in utero to BPA alters the architecture, proliferative index, and genomic signature of the rat mammary gland during critical stages of development. Pregnant rats were gavaged with 25 μg BPA/kg body weight (BW; low-dose group) or 250 μg BPA/kg BW (high-dose group) from day 10 post-conception to delivery. Female litters were euthanized at 21, 35, 50, and 100 days, and mammary glands were collected. Analysis of gland morphology was performed from whole-mounted mammary tissue, while proliferative index was determined by detection of bromodeoxyuridine incorporation in the epithelial cells. Genomic profiles were obtained by microarray analysis, and some genes were validated by real-time RT-PCR. BPA exposure induced changes in the mammary gland that were time and dose specific. High-dose exposure resulted in architectural modifications, mainly in the number of undifferentiated epithelial structures of the breast tissue. Proliferative index did not show remarkable differences by the effect of BPA. Low and high doses of BPA changed the gene expression signature of the mammary gland following a different fashion: low dose had the highest effect by 50 days, while high dose had a highest influence on gene expression by 100 days. Both doses presented a significant cluster of up-modulated genes related to the immune system at the age of maximal changes. Moreover, high-dose exposure induced changes in genes related to differentiation suggesting alterations in the normal development of the gland. The increase of undifferentiated structures and the changes in the gene expression profile at different ages suggest that prenatal exposure to BPA can affect the susceptibility of the mammary gland to transformation.


Introduction

Breast cancer is an estrogen-dependent malignancy whose incidence is steadily increasing in most western societies and in recently industrialized countries (Althuis et al. 2005). The mammary gland is influenced by many hormones and among them, estrogens are playing a key role in promoting the proliferation of both the normal and the neoplastic epithelium (Russo & Russo 1997, 1998). Hormones induce developmental changes in the mammary gland that permanently modify the architecture and the biological characteristics of the gland, and changes in the estrogenic environment during critical stages of development can play a role in the future susceptibility to develop breast cancer (Russo & Russo 1997, 1998). Several lines of evidence in both humans and animal models suggest that in utero exposure to an estrogenic environment increases breast cancer risk later in life (Hilakivi-Clarke et al. 2001, Birnbaum & Fenton 2003). Moreover, the effect that estrogenic compounds can have on cancer susceptibility depends on the timing of environmental exposure. Prenatal exposure to the non-steroidal estrogen diethylstilbestrol has been associated with increased breast cancer risk (Boylan & Calhoon 1979), whereas neonatal exposure reduced the incidence of spontaneous (Lamartiniere & Holland 1992) and induced mammary tumors (Shellabarger & Sool 1973, Nagasawa et al. 1974). Thus, exposure to estrogenic compounds, especially in utero, acting as endocrine-disrupting chemicals might have potential adverse effects on hormone-sensitive organs such as the breast.

Bisphenol A (BPA) is used in the manufacture of polycarbonate plastics and epoxy resins that are found in a wide variety of common products including reusable food and drink containers, baby bottles, protective liners in metal canned foods and beverages, dental composites and sealants, and many other products (Welshons et al. 2006). There is controversial data about the safety of these products. Extensive safety research on BPA has shown that consumer products made with BPA are safe for their intended uses and pose no known risks to human health (Haughton et al. 2002, Gray et al. 2004). On the contrary, a large number of in vivo studies have reported adverse effects concerning low-dose exposures to BPA, even below the current reference dose (Welshons et al. 2006). The differences in the published data can be related to
the doses used or the developmental period when the compounds were administered.

Several studies have reported high potential of BPA for human exposure, due to an increase in products based on epoxy resins and polycarbonate plastics (Kang et al. 2006). Actually, this xenoestrogen has been found in 95% of urinary samples in a human reference population in the United States (Calafat et al. 2005), in maternal and fetal plasma, in placental tissue at birth (Ikezuki et al. 2002) and in human colostrum (Kuruto-Niwa et al. 2007). These data suggest accumulation of BPA in early fetuses and significant exposure during the prenatal period. Furthermore, in normal women and women with ovarian dysfunction, there is a positive relationship between the serum levels of BPA and androgens, which may be due to an effect of androgen on the metabolism of BPA (Takeuchi et al. 2006). Although it is difficult to determine doses for experimental studies, the doses chosen for this investigation (25 and 250 μg BPA/kg body weight (BW) to pregnant rats) were based on reported human exposures, experimental studies in rats, and the US Environmental Protection Agency (US EPA) maximum acceptable dose. Blood sera from human pregnant women have been reported to be 0.46–19 μg/l (Schonfelder et al. 2002, Kuroda et al. 2003, Welshons et al. 2006). Exposure assessments have ranged from 0.2 μg/l (ng/g tissue) in human fetal cord serum up to 105 μg/l in human placenta (Kuroda et al. 2003). Within the United States, an exposure of up to 50 μg/kg per day (50 ppb) is considered safe by the US-EPA.

On the other hand, experimental animal models have shown an estrogenic effect of BPA, and thus an endocrine-disrupting action that may have long-term effects on the endocrine system, influencing tumor development later in life (Birnbaum & Fenton 2003). Prenatal exposure to BPA has been associated with hormonal, morphological, functional, and behavioral anomalies related to reproduction (Adriani et al. 2003), including alterations in the testosterone levels at birth in rats (Tanaka et al. 2006), in brain development in female mice, but not in males (Tando et al. 2007), in rat prostate development (Ramos et al. 2001), in the male and female genital tract (Maffini et al. 2006), and in the tissue organization of the rat and mouse mammary gland (Markey et al. 2001, Munoz-de-Toro et al. 2005). Of special interest, recent data have suggested an effect of BPA on mammary transformation (Durando et al. 2007, Murray et al. 2007). Thus, perinatal exposure to BPA increased the susceptibility to N-nitroso N-methylurea (NMU)-induced preneoplastic lesions during puberty (Durando et al. 2007). Moreover, in utero exposure to BPA induced preneoplastic and neoplastic lesions in the adult mammary gland, even in the absence of any other carcinogenic insult (Murray et al. 2007).

We have previously demonstrated in rodent models that modifications in the susceptibility of the mammary gland to transformation are accompanied by structural and molecular changes of the gland. Pregnancy and pregnancy-mimicking hormonal treatments prevent the development of chemically induced mammary carcinomas (Russo & Russo 1997, 1998) by the differentiation of the mammary gland that results in elimination of undifferentiated terminal end buds (TEBs) and formation of lobules, resulting in inhibition of cell proliferation and, more importantly, the induction of a specific genomic signature characterized by the up- or down-regulation of specific clusters of genes (Russo et al. 2006). Using these parameters as biomarkers for assessing the potential risk of the mammary gland for developing cancer, we have conducted this work with the aim to understand whether in utero exposure to environmentally relevant levels of BPA may affect the developmental pattern and the proliferative activity of the rat mammary gland and, moreover, to identify genomic changes during different stages of the development of this tissue.

Materials and Methods

Experimental design

All animal studies were conducted in accordance with the University of Alabama at Birmingham Guidelines for Animal Use and Care. From Charles River (Raleigh, NC, USA), 8-week-old female Sprague–Dawley CD rats (Rattus norvegicus) were bred and maintained on phytoestrogen-free AIN-93G diet (Harlan Texlad, Madison, WS, USA). Pregnant female rats (ten per treatment group) were gavaged with 25 μg BPA/kg BW (low-dose group), 250 μg BPA/kg BW (high-dose group), or an equivalent volume of sesame oil (control group) on days 10–21 post-conception. The offspring was transferred to surrogate dams immediately after birth. The female offspring were weaned at day 21 and continued on AIN-93G diet until day 70 where they were switched to AIN-93M diet. Females were processed at 21, 35±1, 50±1, and 100±2 days. For the latter three ages, all females were killed in the estrous phase. The fourth abdominal mammary glands were rapidly dissected from live ketamine/xylazine anesthetized animals. The ‘mammary tree’ was trimmed via free access.

Mammary gland morphological analysis

The excised mammary glands were processed for morphological analysis as whole mounts. Tissues were fixed in 10% neutral-buffered formalin, defatted in acetone, re-hydrated, stained in alum carmine, dehydrated in a series of graded alcohols, cleared in xylene, and coverslipped with mounting media. The total number of epithelial structures (TEBs, terminal ducts (TDs), alveolar buds (ABs), and lobules type 1 (Lob 1)) was determined under an Olympus microscope using a 40X magnification objective. These structures were...
recognized according to criteria previously established and counted in the zone C, opposite to the nipple, which is the most actively growing area of the mammary gland (Russo & Russo 1996). Data collected were analyzed using ANOVA and two-tailed unpaired t-tests.

Proliferative index analysis

Animals were injected with bromodeoxyuridine (BrdU) 2 h before euthanasia. Cycling animals received the BrdU injection while in estrous. The abdominal mammary glands were fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned at 4 μm thickness. Tissue sections were mounted on positively charged slides and immunocytochemically reacted with anti-BrdU monoclonal antibody (BioGenex, San Ramon, CA, USA) using an automatic slide stainer (BioGenex). Incorporated BrdU was visualized using the streptavidin–biotin-labeling system with 3,3′-diaminobenzidine (DAB) as a color reaction substrate (BioGenex). The proliferative index was determined quantitatively under Olympus BX40 microscope (60× magnification objective) as the percentage of DAB-positive cells within specific epithelial structures, i.e., TEBs, TD and ducts, Abs, and Lob 1. Data from different groups were analyzed by ANOVA and unpaired t-tests.

Determination of gene expression profile by microarrays

Total RNA from frozen abdominal mammary glands was extracted by TRIzol reagent (Invitrogen) according to manufacturer’s instructions. The quality and the quantity of each sample were individually verified by spectrophotometry using NanoDrop 2.5.4. (NanoDrop Technologies, Wilmington, DE, USA) and by capillary electrophoresis using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The eight samples in each group were then pooled to reduce them to four samples per group.

Fluorescent cRNA probes were prepared using Agilent Low Input RNA Fluorescent Linear Amplification Kit (Agilent Technologies) in the presence of Cy3–dCTP or Cy5–dCTP (Perkin–Elmer, Wellesley, MA, USA), and then purified using RNeasy Mini Kit (Qiagen Inc). Eight picograms of Cy3 and Cy5 from labeled control probe and labeled BPA group probe respectively, were used to hybridize an Agilent 60-mer oligo–microarrays slide containing 22,000 features. Slides were hybridized at 60 °C for 18 h in SureHyb chambers (Agilent Technologies). Slides were then washed and scanned.

The images obtained were analyzed with Feature extraction software (Agilent Technologies) to verify the quality of the hybridization by the report of the outlier data. The intensity of the spots was measured using ImaGene 5.6 software (BioDiscovery Inc., El Segundo, CA, USA). Spots with pixels with very low or high intensity, or with saturated pixels, were then removed from the defined features and background. Log ratios of the red and green channels were calculated, as well as a log ratio error and a P value for each feature. These metrics assessed the level of confidence in order to determine whether one gene was or was not differentially expressed in this experiment. For statistical analysis, we used GeneSight 4.1.6 software (BioDiscovery). The lowest method that calculates the normalization curve which measures the potential log ratio bias across the entire range of spot intensities was selected for normalization. We determined the genes with $P \leq 0.05$. The methodology used to adjust the $P$ values does not allow the determination of the false discovery rates. The confidence analyzer established confidence levels, beyond which genes were selected as differentially regulated, by pooling the replicate differences from a population together into an empirical distribution of residuals.

We performed functional analysis of the genes obtained as differentially expressed using Protein ANalysis THrough Evolutionary Relationships (PANTHER). The genes were annotated to known function and mapped to PANTHER biological process, molecular function, and biological pathways. The lists of the clusters for differentially expressed genes at different ages and by the different doses of BPA were statistically compared with the R. norvegicus genes reference list (NCBI) to look for under- and over-represented functional categories. Each list was compared using the binomial test (Cho & Campbell 2000) and the Bonferroni correction for multiple testing.

Gene expression analysis by real-time RT-PCR

Several genes found differentially expressed in the mammary glands of rats exposed prenatally to BPA were chosen to validate gene expression results by quantitative real-time RT-PCR. All RT-PCRs were performed on the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the fluorescent Taqman methodology (TaqMan One Step RT-PCR Master Mix Reagents, Applied Biosystems). Total RNA (100 ng) was used for each reaction in a total volume of 50 μl according to the manufacturer’s protocol. The thermal cycling conditions comprised 30 min at 48 °C, 10 min at 95 °C, and 40 cycles of 15 s denaturation at 95 °C and 60 s annealing at 60 °C. The end point used in the real-time RT-PCR quantification, C$_t$, was defined as the PCR cycle number at which each assay target passes the threshold. Each gene was normalized using β-actin as a control gene, and data were analyzed using two-tailed unpaired t-test.

Results

Mammary gland architecture

The mammary gland architecture was evaluated by quantification of epithelial TDs structures, i.e., TEBs, TDs, Abs, and Lob 1. The total number of these epithelial structures in the zone C of the mammary gland (Russo & Russo 1996) at different ages of development in the control and BPA-exposed rats is shown in Fig. 1. At 21 days, the main epithelial structure of the mammary gland was the TEB, and its number decreased over time in the three groups, whereas the number...
of TD increased proportionally with age in all groups. The amount of Lob 1 increased from 21 to 50 days.

The number of TEB was significantly increased in the high-dose group related to the low-dose group at 21 days of age. We also observed an increase in the number of TD proportional to BPA dose, being significantly higher in the high-dose group compared with control group at 21 and 100 days. The number of AB showed little differences over time or in relation to the exposure to BPA. Regarding the lobular structures, the number of Lob 1 was significantly higher in the high-dose group in comparison with low-dose and control groups by 35 days of age (Fig. 1).

**Proliferative index**

The proliferative index in the different epithelial structures was evaluated by incorporation of BrdU into the DNA of proliferating cells and immunological detection in paraffin sections of the mammary gland. Comparison of BPA exposed groups with controls did not show major influence of BPA on cell proliferation index (data not shown).

**Gene expression profile**

The scatter plots of the microarrays performed from the mammary glands of BPA-exposed rats compared with the controls showed high correlation coefficients (>0.95) between experimental and control conditions at all ages, indicating the high reproducibility of the methodology and that most of the 20 000 expression sequences examined were similarly expressed between the treatment groups and the controls (data not shown). We determined the genes with \( \geq 0.5 \log \text{base 2 differences (1.4-fold)} \) at \( P<0.05 \) (Supplemental Tables 1 and 2 see supplementary data in the online version of the Journal of Endocrinology at http://joe.endocrinology-journals.org/content/vol196/issue1). We are aware of the limitations of this screening methodology in general, and the conditions used in this study in particular, including the statistical analysis chosen and the low cutoff used. Nevertheless, we considered these data of interest as it allowed us the finding of genes that we validated by the more reliable real-time RT-PCR, thus providing an insight into the effects that BPA can have on the mammary gland.

For both treated groups (low and high doses), the number of genes with changes in the gene expression was low at 21 days and lower at 35 days. By 50 days, the number of up-modulated genes increased in both groups, and at 100 days the high-dose group shows the maximum number of modulated genes. Thus, at 21 days, in the low-dose group (25 µg), we found 31 genes up-modulated (16 of them known genes) and two known down-modulated genes, one of them being Gad1. In the high-dose group (250 µg), we obtained 65 up-regulated genes, 22 of them known and included genes related to cell differentiation. By 35 days of...
age, the number of genes in the low-dose group decreased to two known up-modulated and three down-modulated (one known). At that age, the high-dose group presented 29 up-modulated genes (11 of them known) and 19 down-modulated (with five known genes, including Gad1). At 50 days in the low-dose group, we obtained 189 up-modulated genes, 68 of them known including genes with a role in cell death (Eis1, Ripk3, and Stk17b) or immune function and response to stress (Cd3d, cathepsin E, Cts5, Cd5, and Sod2), and three down-modulated genes (one known, the growth arrest specific 6). The high-dose group had 88 up-modulated sequences (40 known genes, some related to differentiation as fatty acid-binding protein 3, Fabp3, insulin-like growth factor, Igf2, whey acidic protein, Wap, Tnni2, Povlh, and Myl2) and five down-modulated genes (two of them known, Gad1 and growth arrest-specific gene 6, Gas6). At 100 days, the number of genes found in the low-dose group was reduced to five unknown up-regulated genes and six down-regulated sequences (five of them known genes). In the high-dose group, we obtained 330 up-modulated genes (114 known) and 91 down-modulated expression sequences (42 known genes). Among the up-regulated genes, we found an important cluster of them related to immune response (Cd3d, Cts5, Cd5, Lth, Cxcl10, Cd5, Mef2, Cd2, A2m, and Il1b). Interestingly, at that age, we obtained as down-regulated genes, some that were related to differentiation (casein κ (Csn10) and Fhl1) including some that were found as up-modulated by 50 days (Fabp3, Wap, and crystallin α-B, Cryab; Supplemental Tables 1 and 2).

Functional analyses

Functional analysis of the modulated genes was performed using PANTHER (Fig. 2). The gene ontology of the sequences found modulated by the effect of prenatal exposure to BPA revealed that the low-dose group presented significant over-representation of genes with a function in cell proliferation and differentiation, cell communication, signal transduction, immunity, protein metabolism and modification, and apoptosis by 50 days. The high-dose group showed several over-represented functional categories, at 21 days (cell proliferation and differentiation, and cell communication), 50 days (developmental processes), and mainly at 100 days (cell proliferation and differentiation, developmental processes, cell communication, signal transduction, immunity, protein metabolism and modification, apoptosis, and cell adhesion). At this age, muscle development and contraction categories were also over-represented among the down-modulated genes.

Validation of gene expression by real-time RT-PCR

For the validation of the microarray results, we assessed the gene expression by real-time RT-PCR of genes related to different functions, such as cell communication (Gad1), cell proliferation and growth (Fhl1, Gas6, Ets1, and Igfbp2), response to stress (Cryab, Sod2, and Dnaja1), components of the cytoskeleton (Kbtbd10 and Coro1a, related to cell motility), immunity (Cts5, Cd3d, Cd53, and Slpi), apoptosis (Birc3), and genes related to mammary gland differentiation (Fabp3, Wap, Gasn10, and Ldb1).

As shown in Fig. 3, real-time RT-PCR confirmed most of the genes found in the microarrays and chosen for further analysis. We observed down-regulation of Gad1 at different times, including some end points where we did not obtain changes by microarrays analysis (35 and 50 days of animals exposed to low dose). This gene was significantly down-modulated by effect of low dose at 21, 35, and 50 days, and by effect of high dose at 100 days. We also strongly confirmed the up-regulation of immune system genes (significant up-modulation of Cts5, Cd3d, and Cd53, and a trend in the case of Slpi), mainly at 50 days in the group exposed to low dose and at 100 days in the animals exposed to high dose. Genes related to cell differentiation were found significantly up-regulated in high-dose exposed rats by 50 days (Fabp3), but down-regulated by 100 days (Fabp3, Kbtbd10, Csn10, and Wap). Wap was also found down-modulated in low-dose group by 100 days. Other genes of interest were also significantly modulated, as Gas6 in low- and high-dose groups by 50 days; Coro1a by 50 days in low-dose group; Fhl1, Cryab, and Coro1a in high-dose group by 100 days (Fig. 3). Sod2 had a discrete and close to significance up-modulation in low-dose group by 50 days. Finally, we did not find significant modulation of Igfbp2, Dnaja1 or Ets1 in low-dose group and Bin3 in high-dose group, by real-time RT-PCR.

Discussion

In this study, we have investigated the effects of prenatal exposure to BPA on the rat mammary gland at different ages of development. Our data indicate that exposure to this xenobiotic induced subtle but important modifications on the mammary gland that were dose- and time dependant. BPA exposure, mainly to high dose (250 μg), induced changes in the number of the most undifferentiated epithelial structures (TEB and TD), together with modifications in the expression of several genes at different ages, long after the end of the exposure. Low dose also modified the gene expression profile of the gland as a function of age.

The effects of BPA on maturation and mammary gland development have previously been investigated with contradictory results, probably due to methodological questions. In concordance with previous reports in mice, with specific detailing regarding the identification of the different morphological components, i.e., TEB, TD, and lobules (Markey et al. 2001, Munoz-de-Toro et al. 2005), we have found changes in the number of these epithelial structures, mainly when BPA is administered in utero at high dose. The main structures affected by BPA exposure are the undifferentiated ones. We have previously demonstrated in rat models that the induction of mammary carcinomas requires that the
carcinogen acts on the most undifferentiated structures, the TEB. The higher susceptibility of TEB to neoplastic transformation is attributed to the fact that this is composed of active proliferating epithelium (Russo et al. 1983). Thus, our results suggest subtle but important modifications in the morphology of the mammary gland, as an increase in the number of the structures that are target for carcinogens can have an effect on breast cancer susceptibility later in life.

Russo & Russo (1980), Tay & Russo (1981) and Russo et al. (1991) have postulated that the mechanism of pregnancy-induced protection is mediated by the induction of mammary gland differentiation driven by the hormonal milieu of pregnancy, which creates a specific genomic signature in the mammary gland that makes this organ permanently refractory to carcinogenesis. Hence, we have used parameters related to differentiation and proliferation activity as markers to assess modifications in the susceptibility to induced carcinogenesis by effect of BPA exposure. On the other hand, the protective effect of parity has also been proposed to be caused by persistent changes in circulating...
hormones or growth factors rather than local effects on the mammary gland (Thordarson et al. 1995, Sivaraman & Medina 2002). Other authors have proposed that the changes occurring in the mammary gland that affect its resistance to transformation, occur during the process of involution that follows pregnancy and lactation by eliminating pre-malignant cells or cells that are particularly susceptible to oncogenic transformation, thus avoiding the progression to an invasive state (Yang et al. 1999, Reddy et al. 2002, Nandi et al. 2005). This interesting hypothesis is not radically different from the one first proposed by Russo & Russo (1997) who postulated that the Lob 1 and the TEB found in the breast of nulliparous women or of young virgin rats respectively, had not completed their differentiation into Lob 2, Lob 3, and Lob 4, retaining a high concentration of stem cells called stem cells 1, which are susceptible to undergo neoplastic transformation when exposed to a carcinogenic agent. After the postmenopausal involution of the mammary gland, the architecture of the parous breast is similar to that of the nulliparous breast, containing predominantly Lob 1 composed of stem cell 2, an epithelial cell population that is refractory to transformation. It was further postulated that the degree of differentiation acquired through early pregnancy permanently changes the ‘genomic signature’ that differentiate the Lob 1 from early parous women from that of nulliparous women, shifting the stem cell 1 to the stem cell 2 that is refractory to carcinogenesis. These cells were called stem cell 2 because after post-lactational involution, the mammary epithelium remains capable of responding with proliferation and differentiation to the stimulus of a new pregnancy; however, these cells are refractory to carcinogenesis, even though they are stimulated to proliferate and to

Figure 3 Gene expression analysis by real-time RT-PCR in mammary glands from rats exposed to low and high doses of BPA. Histograms show mean fold expression (mean ± S.E.M.) related to the mean of the control group (dotted line). *Significant differences related to control group (P<0.05).
regenerate the whole mammary gland. The stem cell 2 is characterized by having a genomic signature that has been induced by the first cycle of differentiation. During the last 8 years, supporting evidence to this hypothesis has been generated by Russo et al. as well as by other researchers. Recent studies by Smith et al. (Wagner et al. 2002, Henry et al. 2004, Boulanger et al. 2005) using transgenic WAP-driven Cre and Rosa 26-fl-stop-fl-LacZ mice provided evidence of a new mammary epithelial cell population that originates from differentiated cells during pregnancy; 5–10% of this parietal-induced epithelium survives post-lactational involution after the first pregnancy. With successive pregnancies, their percentage increases, reaching 60% of the total epithelium in multiparous females. The parietal-induced mammary epithelial cells (PI-MECs) are equivalent to the stem cell 2 postulated by Russo & Russo (1997), since these cells show capacity for self-renewal and contribute to mammary outgrowth in transplantation studies. PI-MEC can function as alveolar progenitors in subsequent pregnancies, and it is thought that they would be related to differences in response to hormonal stimulation and carcinogenic agents observed between nulliparous and parous females (Wagner et al. 2002, Henry et al. 2004, Boulanger et al. 2005).

Several authors have focused on finding molecular changes as a mechanism of the pregnancy-induced protection (Russo & Russo 1994, 2004, Ginger et al. 2001, Sivaraman et al. 2001, D’Cruz et al. 2002, Ginger & Rosen 2003, Medina & Kittrell 2003, Medina 2004). These changes in the genomic signature induced by physiological process like pregnancy have significant relevance with the novel observation from our studies relating that prenatal exposure to low and high doses of BPA induced differences in the gene expression of the mammary glands, which were time- and dose specific. The prenatal exposure to the low dose (25 μg) induced the highest number of modulated genes by 50 days, which is the age of highest susceptibility of the rat mammary gland to chemically induced carcinogenesis (Russo & Russo 1997). On the other hand, the prenatal exposure to high dose (250 μg) resulted in an increasing number of modulated genes to a maximum by 100 days. Taken together, our results have indicated a higher effect with higher dose of BPA (e.g., the histopathologic changes or the modifications in gene expression by 100 days).

However, the effect on gene expression profile at 50 days was more evident with the exposure to low dose. In the literature, BPA has shown either a dose–response effect (Yoshino et al. 2004) or an inverted U-dose response (Takai et al. 2000), depending on the experimental model studied. Our results by 50 days can be related to the profound changes that the mammary gland is facing at that age, resulting from an intense hormonal activity (Russo & Russo 1997). Low dose may be inducing changes that, although more subtle than high dose, can be magnified in the period of high hormonal activity. These modifications could be related to epigenetic changes in the mammary gland stem cells, which can be manifested during differentiation.

Interestingly, in both experimental conditions (low and high doses), the age with the maximal number of modulated genes presented an important cluster related to immune response, including Ctse (Sealy et al. 1996), Cd53 (Tohami et al. 2004), Cd3d (Fischer et al. 2005), or secretory leukocyte peptidase inhibitor (Slpi; King et al. 2003). In addition, the cytoskeletal protein Coronin-1A (Coro1a), also found up-modulated by effect of low and high doses at 50 and 100 days respectively, has been reported to play a role in T cell differentiation/activation events (Nal et al. 2004). We have verified the increased expression levels of some of those genes, such as Ctse, Cd3d, Cd53, and Coro1a and we also observed moderate up-modulation of Slpi by real-time RT-PCR. It has long been known that the immune system is regulated by the gonadal steroids, such as estrogen, androgen, and progesterone (Grossman 1984), and modulation by estradiol of Ctse (Gladson et al. 1998) and Slpi (Chen et al. 2004) has been reported. These results are in accordance with others reported in the literature, as there is evidence that administration of BPA in mice has a modulating activity on immune response (Youn et al. 2002, Lee et al. 2003, Yamashita et al. 2003). Prenatal exposure in mice also increased immune responses in adulthood (Yoshino et al. 2004). Our results also indicate that exposure to BPA has an effect on immune surveillance long after the end of the treatment. Considering that the expression of some of those genes has been found modified in different tumors or tumor cell lines (Sealy et al. 1996, King et al. 2003, Yunta & Lazo 2003, Kluger et al. 2005, Wild et al. 2005), we cannot rule out the possibility that changes in their expression can affect the susceptibility to transformation.

We also found differences in genes related to differentiation depending of the age tested, mainly in animals exposed to high dose of BPA. Interestingly, the high dose induced up-regulation of breast differentiation markers at 50 days but down-regulation by 100 days. That is the case of Fabp3, which has been related to mammary differentiation (Yang et al. 1994, Hu et al. 1997). This gene is an identical homolog to mammary-derived growth inhibitor (MDGI), a breast tumor growth suppressor gene capable of inhibiting tumor cell proliferation (Huynh et al. 1995). In previous studies, in human breast tissue, we observed a highly MDGI expression in the most differentiated lobular structures (Lob 4), and that its expression was silenced in breast cancer progression (Hu et al. 1997). We also observed this kind of change in the expression of the mammary differentiation marker Wap (Dandekar et al. 1982). Other differentiation markers were found down-modulated by 100 days, as cas10 and other genes like kdelh repeat and BTB (POZ) domain containing 10 (Kbad10), Cryab that has been reported as down-modulated in breast tumors and metastasis (Seitz et al. 2006); and four and a half LIM domains 1 (Fhl1), suggested as a tumor suppressor gene (Shen et al. 2006). Thus, the observed changes in the expression of these genes by effect of high dose of BPA suggest a molecular context in the mammary glands compatible with a lower degree of differentiation. The lower degree of differentiation markers by 100 days in the high-dose group is
in accordance with the increase in the undifferentiated structures by this age, as TDs.

On the other hand, some genes related to growth were modulated by the effect of BPA exposure. Gas6, which exerts an anti-apoptotic and proliferative action (Hafizi & Dahlback 2006), was down-regulated in both low- and high-dose groups by 50 days of age. Gas6 has been reported to be induced by estrogen in normal and tumor mammary epithelial cells (Mo et al. 2007). Fhl1, also related to cell growth, was down-modulated in the mammary glands from 100-day-old rats prenatally exposed to high dose. As mentioned above, this gene is suggested to be a tumor suppressor gene (Shen et al. 2006). Other genes of interest were related to important cellular functions, like stress response. That is the case for Cryab, down-modulated by 100 days. Cryab is a small heat shock protein that seems to modulate filament organization under conditions of physiological stress (Head & Goldman 2000). Moreover, Cryab has been found down-modulated in breast tumors and metastasis (Seitz et al. 2006) and its deregulation in our experimental tissues can be related to changes in the differentiation status of the mammary gland by the effect of high dose of BPA. Finally, we found significant down-regulation of Gad1 at different ages. The enzyme encoded by the gene Gad1 is responsible for catalyzing the production of γ-aminobutyric acid (GABA). Estrogen modulates mRNA and protein expression of GAD1 and GAD2 in adult rat hippocampus concomitantly with negative and positive controls of luteinizing hormone release (Wagner et al. 2001, Nakamura et al. 2004), and GAD1 gene expression changes in discrete regions of the rostral preoptic area during estrous cycle and with age (Cashion et al. 2004). Studies in nonhuman primates also reported the role of Gad1 in control of puberty (Kasuya et al. 1999). GABAergic system is also involved in hormonal regulation and pathogenesis of breast cancer (Opolski et al. 2000) and other types of neoplasia (Matuszek et al. 2001, Azuma et al. 2003, Moon et al. 2004). These lines of evidence suggest that, although the function that Gad1 plays in the mammary gland is unknown, a long-term down-regulation in such tissue by effect of BPA exposure can have an effect in the physiopathology of that gland.

To our knowledge, this is the first microarray analysis of BPA effect in breast cells in vivo, and few studies have addressed the effects of this xenoestrogen in mammary cells in vitro. Estradiol and BPA induced transcriptional changes in estrogen responsive human breast cancer cells derived from the MCF-7 cell line. In accordance with our results, several genes with a role in growth and development were modulated exclusively by BPA, but not by estradiol (Singleton et al. 2006). Human MCF-7 and T47D mammary carcinoma cells have been described to change their transcriptional profile in response to several compounds, including estradiol, BPA, and genistein. These natural and synthetic estrogenic compounds showed a high degree of similarity inducing changes in the expression profile, including up-regulation of genes related to cell division, growth, inhibition of apoptosis, transcription, signal transduction, or adhesion (Buterin et al. 2006). According to the estrogenic activity reported for BPA, the effects observed in our study can be mediated through the estrogen receptors. Even the differences described regarding the genes activated by estradiol, and BPA can be related to the differential ability of each ER ligand to recruit specific coactivator sets (Singleton et al. 2006). However, ER-independent mechanisms also have to be considered. Actually, BPA can modulate gene expression in estrogen-insensitive ER-null C4-12 cell line (Singleton et al. 2004). BPA has been described to strongly bind the human estrogen-related receptor γ, which has an exceptionally broad specificity of DNA sequence recognition and is strongly expressed in the brain during development (Takayanagi et al. 2006). Other studies have also indicated the potential of BPA to disrupt thyroid hormone action (Zoeller et al. 2005)

In conclusion, BPA exposure induced changes in the mammary gland that were time- and dose specific. Both doses tested modified the gene expression signature of this tissue, with the higher number of up-modulated genes at 50 days in the low-dose group, while the high-dose group presented the maximum gene expression changes by 100 days. Functional analyses of the differentially expressed genes revealed that the genes modified by both doses had similar roles. An important cluster of up-regulated genes had a function in immunity and defense. The morphological study also indicated a higher number of undifferentiated structures by effect of high-dose exposure. Moreover, in these groups, the gene expression of several differentiation markers and cytoskeleton-related genes was found up- or down-modulated when compared with control depending on the age tested. This data suggest a “shift” in the normal development of the mammary gland, pointing to the importance of the status of differentiation of the gland if affected by a carcinogen. Recent evidence has shown an increase the susceptibility of the mammary gland to transformation by effect of BPA (Durando et al. 2007, Murray et al. 2007). The changes that we have observed in the expression of genes related to immune system, proliferation and differentiation, growth or response to stress, among others, can be one of the mechanisms by which BPA may change such susceptibility.

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