Abstract. The aim of this study was to explore the effect of bisphenol A (BPA) on the EGFR-STAT3 pathway in breast cancer. We applied 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) cytotoxicity assay to the analysis of the responsiveness of MCF-7 cells to BPA. Gene expression was assayed at the transcriptional and translational levels by reverse transcription-PCR and Western blotting. We explored the effects of BPA on MCF-7 cell proliferation through inhibition of the related genes, STAT3, using RNA interference, and EGFR, using its inhibitor AG1478. The optimal concentration and time point of BPA-induced proliferation in MCF-7 cells are 1 µM and 24 h, respectively. BPA significantly increased the expression of STAT3 at a concentration of 1 µM following treatment for 48 h and the expression of STAT3 was down-regulated after blocking EGFR. When STAT3 was blocked in MCF-7 cells, BPA did not appear to induce cell proliferation. Treatment with BPA (1 µM) in the presence of AG1478 for 48 h resulted in the stimulation of cell growth in MCF-7 cells, similar to that of the BPA alone treatment. BPA increases STAT3 expression, which is a major factor in the pathway of BPA-induced proliferation, and STAT3 activation contributes to BPA-induced breast cancer cell proliferation. However, EGFR mediates negative signaling for BPA-induced breast cancer cell proliferation.

Introduction

Breast cancer has the highest incidence of female neoplastic diseases and sporadic breast cancer is the most common cancer diagnosed in American and Northern European women (1). The occurrence and development of breast cancer is a hormone-dependent and multi-step process, which may be induced by various factors such as hormones, chemicals, radiation, viruses and genetic factors, and according to certain reports, by environmental factors as well (2). Certain environmental chemicals may simulate estrogens completely or partly by binding to their receptors. Savis et al hypothesized that environmental estrogens (EEs) are likely to be related to breast and ovarian cancer (3). Bisphenol A (BPA) was shown to be an EE and is widely used in plastic products, including food and water containers, baby bottles, linings of metal food and beverage cans, medical tubing, epoxy resins and dental fillings (4). Neonatal exposure to BPA is associated with higher body weight, increased breast and prostate cancer incidence and altered reproductive function (5). Experimental evidence indicates that exposure to BPA during childhood increases the risk of breast cancer in the adult female (6). As a consequence of this widespread opportunity for exposure, 95% of Americans carry detectable levels of BPA (7), which is currently one of the highest volume chemicals produced in the world (8). BPA may be digested and absorbed in the human body and increases the actual loading doses of estrogen (4), thereby increasing the incidence of breast, ovarian and endometrial cancer.

The mechanism by which BPA exerts its biological actions remains to be determined. The majority of studies have examined the manner in which BPA stimulates breast cancer cell proliferation and the related research on BPA is focused on estrogen receptors (ERs) (9), chemoresistance (10) and the apoptosis proteins Bcl-2 and Bax (11). However, the potential

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Key words: breast cancer, bisphenol A, EGFR, STAT3, RNA interference
effect of BPA on signal pathways have received little attention. Few studies investigating BPA signal pathways exist. Park et al (12) have reported that EE-activated ERK1/2 or p38 is unlikely to be involved in cell growth in estrogen-responsive ovarian cancer cells. Certain studies have confirmed the effectiveness of the activated EGFR-STAT3 pathway in breast cancer (13) and the cooperated efforts of STAT3 and nuclear EGFR of controlling cell transcription (14). EGFR is involved in a variety of downstream signaling pathways including MEK>ERK, PI3K>AKT, STAT, p38 and JNK (15,16). It has been suggested that the JAK/STAT pathway is one of the significant downstream pathways for EGFR signaling (13). In addition, the existence of EGFR-independent signaling for STAT3 activation has been reported, enhancing the importance of STAT3 activation in tumor growth (17). Therefore, in this study, we inhibited the expression of STAT3 with RNA interference, and of EGFR with the highly selective Tyrophostin AG1478 in breast cancer MCF-7 cells, to explore the effect of BPA on the EGFR-STAT3 signaling pathway and its mechanism of carcinogenesis.

Materials and methods

Cells, drugs and antibody. The human breast cancer MCF-7 and MDA-MB-231 cells were purchased from ATCC (Manassas, VA, USA). Phenol red-free 1640 medium, RPMI-1640, Opti-MEM medium, fetal bovine serum (FBS) and pancreatic enzyme were purchased from Gibco (Invitrogen, Carlsbad, CA, USA): BPA was kindly provided by Dr Huang (Jilin University): Tyrophostin AG1478 was purchased from Shanghai Baijin Biological Co. (China), and Lipofectamine 2000 transfection reagent was purchased from Invitrogen: β-actin, STAT3 (sc-482) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Design and synthesis of STAT3 siRNA. The siRNA design and synthesis service was provided by GenePharma, Shanghai Co. (China). According to a series of Genbank accession numbers, the sequence information of the eight sites selected in designing siRNA includes: siRNA1, STAT3-homo-683 (GCAGGAGUCUAGAACSAAATT); siRNA2, STAT3-homo-1148 (GGAGCUUUAGAAACUAAATT); siRNA3, STAT3-homo-376 (CCACUUUGGGUGUUCAUAATT); siRNA4, STAT3-homo-1244 (CGUCCAUUACUAAATT); siRNA5, STAT3-homo-1358 (CCCCGAAUUUUAACAUCUATT); siRNA6, STAT3-homo-1815 (GGAGCCUGGGUGAAUAAATT); siRNA7, STAT3-homo-1964 (GGUACUACUGGCUUUAATT); siRNA8, STAT3-homo-2422 (GAAACGACUGAGGGCGUCAATT).

Cell culture. MCF-7 and MDA-MB-231 (control group) cells were routinely maintained and grown in RPMI-1640 medium supplemented with 10% FBS at 37°C in a humidified atmosphere of 5% CO₂ in an incubator and in phenol red-free 1640 medium for 4 days to exhaust the phenol red prior to initiating the experiment. Cells were seeded in 96-well plates at a density of 3x10⁴ cells/well and were continued to be cultured by 24 h for 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) assay.

Cell proliferation assay. BPA was dissolved in dehydrated alcohol at stock concentrations of 10 mM and was diluted to a working concentration of 1,000 nmol/l with phenol red-free 1640 medium prior to treatment. BPA was then 10-fold diluted into 10⁻¹-10⁻⁶ M. MCF-7 and MDA-MB-231 cells were plated at a density of 6,000 and 8,000 cells/well, respectively, in 96-well plates in plating medium, with the setting of 5 repetitive samples for each dose and the dehydrated alcohol group as the control group. Cells were treated with BPA for 24, 48 and 72 h, respectively, and changes in proliferation relative to vehicle treatment were examined by MTT assay. MTT was added at a final concentration of 0.5 mg/ml for 2 h. Following medium aspiration, the formazan dye was extracted with DMSO and absorbance at 490 nm was determined for each well with a microplate reader. Measured counts were regarded as relative indices of proliferation by normalizing to untreated cell controls.

Quantitative reverse transcription-PCR assay. Total RNA was isolated using TRIzol and cDNA was synthesized (SuperScript III reverse transcriptase) with the use of oligo (dT) primers from Invitrogen. Samples were run in triplicate on Stratagene M x 2005P (Agilent, Santa Clara, CA, USA), using the following variables: denaturation at 95°C for 3 min followed by 40 cycles of amplification (95°C for 30 sec and 62°C for 40 sec). We applied Primer Premier 5 software to design the STAT3 primer. The relative expression levels of STAT3 were normalized with a GAPDH housekeeping gene as the internal control. Primers for PCR amplification were: STAT3, forward: 5’-TCCATCACCTCTACATGCTGACG-3’ and reverse: 5’-TCCAGGAAGATTACCAAACC-3’; GAPDH, forward: 5’-CATGAGAATGATGACACGACCT-3’ and reverse 5’-AGTCTTTCCAGATACCAAAGT-3’.

Immunoblotting. MCF-7 cells were harvested, washed twice with cold phosphate-buffered saline (PBS), and then lysed with lysis buffer [PBS containing 1% Triton X-100, complete protease inhibitor cocktail (Roche, Germany) and RNase inhibitor (New England Biolabs, Ipswich, MA, USA)] at 4°C for 30 min. The protein concentration was determined using the Pierce (Rockford, IL, USA) bichinonic acid (BCA) protein assay. We added 5 µl 2X SDS-PAGE loading buffer to 80 µg protein/well, and the samples were then electrophoresed in 10% SDS-PAGE minigels. We used a PVDF membrane to transfer the proteins in transfer buffer [25 mM Tris base, 0.2 M glycine and 20% methanol (pH 8.5)]. After being transferred to the PVDF membrane, samples were sealed in blocking buffer (1X TBS, 0.1% Tween-20 and 5% w/v non-fat dry milk) for 1 h and incubated overnight with mouse monoclonal antibody to STAT3 (1:2000). Following incubation with goat anti-rabbit secondary antibody (1:1000), detection was performed using SuperSignal chemiluminescence reagents (Pierce).

Small interfering RNA knockdown. Knockdown of STAT3 was performed using specific small interfering RNA (siRNA) pools targeting STAT3 purchased from GenePharma, Shanghai Co. (China). Cells were divided into mock, blank (normal cells) and negative control groups. Introduction of siRNA was performed with Lipofectamine 2000 according
Concentration of BPA (nM)

Figure 1. MCF-7 cells were treated with BPA for 24, 48 and 72 h, followed by increasing BPA concentrations from $10^1$ to $10^5$ nM. Cytotoxicity was determined by the MTT assay. (A–C) Effect of BPA-induced proliferation following treatment for 24, 48 and 72 h, respectively. The relative proliferation rates of the BPA treatment group are shown as the percentage of the dehydrated alcohol group.

Results

Promoting cell proliferation by BPA in a dose-dependent manner. To determine the effect of BPA on cell proliferation, MCF-7 and MDA-MB-231 (control group) cells were treated with BPA to determine the optimal concentration and application time. Cytotoxicity was determined by the MTT assay following treatment with BPA for 24, 48 and 72 h. Cells were treated with either vehicle or 1 µM BPA and incubated for 24 h resulting in a significant increase of proliferation (Fig. 1A), which continued to the time point at 48 h (Fig. 1B). As demonstrated in Fig. 1C there was no effect on proliferation when treated with 1 µM BPA for the 72-h treatment. Treatment with 100 µM BPA showed a strong cytotoxic effect, although there was no significant effect of BPA-induced proliferation on MDA-MB-231 cells (Fig. 1).

Effect of BPA on the activation of STAT3. To investigate the effect of BPA on STAT3 activation, MCF-7 cells were treated with 1 µM BPA for 24, 48 and 72 h, respectively. According to the results of Western blotting, as demonstrated in Fig. 2,
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the expression of STAT3 was significantly up-regulated with 1 µM BPA treatment for 48 h (Fig. 2).

**Overview of the transfection efficiency.** As shown in Fig. 3 MCF-7 cells were transfected with FAM-labeled NC-siRNA and monitored with a fluorescent microscope (magnification, x200). The transfection efficiency was 80%, which was determined by counting the percentage of fluorescence-bearing cells (Fig. 3).

**Effective fragment of the screening results.** To achieve effective RNA interference, we transfected MCF-7 cells with 8 STAT3 interference segments. As shown in Fig. 4, after combining the results of RT-PCR and Western blotting, we selected the 5th oligo (siRNA5) to carry out the subsequent experiments (Fig. 4).

**STAT3 expression following BPA and STAT3 siRNA5 treatment.** To probe the interference effect and BPA-promoted STAT3 expression in MCF-7 cells, we performed a combined treatment of BPA and STAT3 siRNA5, and the expression of STAT3 was detected following a 48-h treatment. As shown in Fig. 5, given the results of the RT-PCR and Western blotting, the efficiency of RNAi5 for the targeted knockdown of STAT3 is approximately that of the STAT3 siRNA5 alone treatment group (group B). As compared to the normal breast cancer cells (group A) and BPA with the negative control treatment group (group D), BPA promoted the expression of STAT3. In addition, treatment with 1 µM BPA in the presence of STAT3 siRNA5 and vehicle (group C) suggests that BPA is not capable of activating STAT3 after siRNA5 activity (Fig. 5).

**MTT assay following STAT3 siRNA5 and BPA treatment.** To establish the relevance of STAT3 in the signaling pathway of BPA-induced cell growth, the cytotoxicity of MCF-7 cells was determined by the MTT assay following the combined treat-
Figure 6. MCF-7 cells were treated with BPA and STAT3 siRNAs for 48 h to determine the cytotoxicity by the MTT assay. A, blank group: MCF-7 cells; B, STAT3 siRNAs; C, BPA (at the working concentration of 1 µM) + dehydrated alcohol + STAT3 siRNAs; D, BPA (at the working concentration of 1 µM) + dehydrated alcohol + negative control; E, dehydrated alcohol (the volume is equal to BPA, which was at working concentration) + STAT3 siRNAs; F, negative control (the volume is equal to BPA that was at working concentration) group.

Figure 7. BPA and AG1478 were treated together to explore the effect of AG148 and BPA on EGFR. Cells were treated with BPA and AG148 for 48 h. Cytotoxicity was determined by the MTT assay. The mock group was DMSO + dehydrated alcohol group; AG1478 group was at the working concentration of 10 µM; AG148 and BPA groups were at their working concentration of 10 and 1 µM, respectively; BPA group was at the working concentration of 1 µM.

Figure 8. Cells were treated with AG148 for 48 h. MCF-7 cells were collected to assay the expression of STAT3 after blocking EGFR. AG group, STAT3 expression, which was treated with AG1478 at the working concentration of 10 µM; Mock group, MCF-7 cells and DMSO.

Effect of AG1478 on BPA-induced proliferation in MCF-7 cells. To elucidate the relevance of EGFR in the signaling pathway of BPA-induced proliferation, the specific inhibitor AG1478 was pre-treated for 20 min with prior to BPA treatment. As shown in Fig. 7, treatment with AG1478 alone at a dose of 10 µM did not alter the cell growth of MCF-7 cells. However, treatment with 1 µM BPA in the presence of AG1478 for 48 h resulted in the stimulation of cell growth in MCF-7 cells similar to that of the BPA alone treatment (Fig. 7).

Effect of AG1478 and BPA on the expression of STAT3 in MCF-7 cells. To investigate whether STAT3 expression correlated to EGFR, cells were treated with AG1478 for 48 h. The real-time PCR and Western blotting results showed an approximately 34 and 72% decrease, respectively, compared to the normal samples, which indicated that the expression of STAT3 was down-regulated after being treated with AG1478. P<0.05 was considered to be statistically significant (Fig. 8).

Discussion

A number of investigators have indicated that BPA promotes breast cancer MCF-7 cell proliferation in the concentration range of 1x10^{-7} to 1x10^{-5} M (18). When BPA is at nanomolar doses it often displays stronger estrogen-like activities than E_{2} (estradiol) itself (19). Samuelsen et al (20) confirmed that BPA often exhibits an inverted U-shaped curve, observed when treating cells with increasing doses of BPA. Cell proliferation was unchanged in the presence of 10 nM BPA, increased >40% with 100 nM BPA, peaked with 1 µM BPA and declined at higher doses. These studies are in agreement with our data, suggesting that MCF-7 cells proliferate significantly with 1 µM BPA treatment for 24 h. In addition to the optimal concentration of BPA for promoting cell proliferation, we further investigated the optimal treatment period, which is 24 h, and the proliferation effect which may continue to 48 h.

We also demonstrated that BPA only promotes the proliferation of ER-positive MCF-7 cells, but not of ER-negative MDA-MB-231 cells, suggesting specificity of this effect for the ER-mediated pathway. BPA induced the transcriptional activity of ERE and mimicked the effect of E_{2} activity on cell proliferation (12). The specific ERs of BPA-induced proliferation remain to be clarified; however, hypotheses include: i) BPA binds differently within the ligand-binding domain of ERs or ERβ and recruits dissimilar coregulators (21); ii) BPA elicits rapid responses by binding to membrane-anchored ERs, or G-protein-coupled receptor 30 (GPR30) (22), and its chemoresistance is probably through the non-classical GPR30 and members of the ERR family (10); iii) BPA binds to estrogen-related receptor γ (ERRγ), an orphan nuclear receptor belonging to the ERR family of receptors that do not directly bind E_{2} (23). Moreover, Okada et al (24) have shown that BPA was found to bind at high affinity to ERRγ. Matsushima et al (25) have demonstrated that ERRγ behaves as a constitutive activator of transcription, and that BPA binds markedly to ERRγ (but not to ER), preserving the ERRγ's basal constitutive activity.

Studies have shown that BPA induced significant changes in the phosphorylation state of STAT members (26), and the effects of EEs on the phosphorylation state of transcription...
factor STATs suggest that these compounds lead to changes in gene expression secondary to the modulation of kinase/phosphatases (27). Our data have confirmed that BPA up-regulated STAT3, therefore we postulated that STAT3 is a key gene in BPA-induced cell proliferation. It has been confirmed that the effect of BPA (10 μM) on stimulation of cell growth was not blocked by pretreatment with inhibitors of ERK1/2 or p38 in a dose-dependent manner, and that MAPK is unlikely to be involved in EE-induced cell growth in estrogen-responsive ovarian cancer cells (12). EGFR is the common upstream regulator of ERK1/2, p38 and STAT3; therefore, we speculated the BPA-induced cell proliferation acts through the EGFR-STAT3 pathway.

We postulated that STAT3 plays a role in BPA-induced proliferation, based on the fact that STAT3 is an oncogenic gene constitutively activated in approximately 30-60% of primary breast cancers (28). We inhibited the STAT3 gene with an RNAi technique, as it has been confirmed that STAT3 inhibition using RNA interference leads to reduced cell proliferation (29). We used STAT3 siRNA5, which markedly knocks down the expression of STAT3 with an efficiency of almost up to 100%. Pretreatment by knocking down STAT3 eradicated the stimulation of cell growth by BPA in MCF-7 cells. Although there are complicated signal pathways in BPA-induced proliferation in breast cancer cells, our results indicated that the activation and transmission of STAT3 are vital elements.

In their study, Berclaz et al (13) have shown that there is a marked correlation between nuclear STAT3 and EGFR expression in breast cancer. STAT3 is the convergence of multiple oncogenic tyrosine kinase signaling pathways, such as EGFR, IL-6/JAK and Src (30). However, given that EGFR could signal to a variety of downstream effectors, the role of EGFR in BPA-promoted cell proliferation has not been fully elucidated. We explored the effect of BPA on EGFR with its inhibitor Tyrophostin AG1478 to ensure the possible signaling pathway of BPA. Tyrophostin AG1478 is an artificial micro-molecular protein tyrosine kinase inhibitor, which is highly selective for EGFR (31). The in vitro experiment indicated that AG1478 was capable of blocking the EGFR signaling pathway, inhibiting proliferation of numerous tumor cells and inducing cell apoptosis (32). In addition, Tyrophostin AG1478 is a potential EGFR-targeted therapeutic agent for breast cancer (33). In our study, AG1478 did not neutralize the stimulatory effect of MCF-7 cell proliferation by BPA, indicating that EGFR inhibition was not able to affect the proliferation of MCF-7 cells. Therefore, EGFR mediates negative signaling for BPA-induced breast cancer cell proliferation. In this study, we explored the relationship of ER and BPA-induced proliferation in MCF-7 cells following inhibition of EGFR, and whether ER and EGFR are non-redundant pathways remains to be further confirmed.

Our study also demonstrated that the expression of STAT3 is down-regulated after blocking EGFR, and the inhibition indicates that EGFR-STAT3 is highly correlated. It has been confirmed that the activation of EGF receptor tyrosine kinases is associated with the activation of STAT proteins (13). In addition, the recruitment sites of STAT3 within the EGFR have been identified and characterized, indicating the direct association of the two molecules (17). Notably, the combined treatment of AG1478 and siRNA inhibited STAT3 expression resulted in opposite effects on cell proliferation. Our data have demonstrated that AG1478 treatment results in an approximately 70% decrease in the expression of STAT3, and that the 30% gene expression of STAT3 is sufficient for cell proliferation, underlining the crucial role of STAT3 in cell proliferation. However, the inactivation of the STAT3 gene by siRNA acts potently and inhibits BPA-induced MCF-7 cell proliferation. Thus, STAT3 activation contributes to BPA-induced breast cancer cell proliferation.

Our study concentrated on the effect of BPA on the EGFR-STAT3 pathway in breast cancer MCF-7 cells. We confirmed that BPA increases STAT3 expression, which is a significant factor in the pathway of BPA-induced proliferation, and that STAT3 activation contributes to BPA-induced breast cancer cell proliferation. However, EGFR mediates negative signaling for BPA-induced breast cancer cell proliferation. These data emphasize a previously unrecognized signaling pathway of BPA in its carcinogenesis, thereby adding powerful support to the increasing realization of the adverse effects of BPA on human health. Given the hazard of BPA and the importance of STAT3 as a signal transducer and activator, the mechanism of STAT3-mediated BPA-induced proliferation should be further investigated.

Acknowledgements

This study was supported by the Department of Science and Technology of the Jilin Province, Project Number: 201105094.

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