DNA Microarray Analysis of Differentially Expressed Genes Responsive to Bisphenol A, an Alkylphenol Derivative, in an In Vitro Mouse Sertoli Cell Model

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ABSTRACT—To identify genes elicited by bisphenol A (BPA) in Sertoli cells, we carried out a microarray analysis of TTE3 cells (a mouse Sertoli cell line) treated with BPA. BPA (100, 200 and 400 μM) induced cell death concentration-dependently, with levels being 25%, 33% and 96%, respectively. Of the 1,081 genes analyzed, 3 genes showed decreased levels of expression while the remaining 10 genes showed increased levels in the cells treated with a subtoxic dose of BPA (200 μM). The expressions of six genes were confirmed by the TaqMan assay. These findings suggest that DNA microarray analysis is a useful tool for investigating the molecular mechanisms of the toxic effects of BPA in testicular cells.

Keywords: Bisphenol A, Microarray, Sertoli cell

2,2-Bis(4-hydroxyphenyl)propane (bisphenol A, BPA), an alkylphenol derivative, is a high-production volume chemical used in the manufacture of polycarbonate plastics. BPA binds to estrogen receptors (ERs) and induces estrogenic activity in a number of biological systems, which shows that BPA may act as an environmental estrogen, although its estrogenic activity was about four orders less potent than that of 17β-estradiol (1). In the seminiferous tubule in testis, the blood-testis barrier is created by adjacent Sertoli cells at the basal compartment, and spermatogenic cells are in continuous contact with Sertoli cells, which exert a variety of functions crucial for germ cell differentiation (2). BPA is reported to induce male reproductive toxicities such as reductions in epididymis weight, seminal vesicle weight, sperm motility and Sertoli cell function in in vivo models (3, 4). Furthermore, BPA induced cell death in many kinds of cells including Sertoli cells in in vitro models (5). Although many such toxicological and pharmacological findings concerning the male reproductive toxicity induced by BPA exist, the molecular mechanisms by which BPA induces this toxicity are still unclear. Recently, we have succeeded in establishing a Sertoli cell line, TTE3, using temperature-sensitive simian virus 40 (tsSV40) large T-antigen transgenic mice (6). With the help of DNA microarray technology, the expression of hundreds to thousands of genes can be assayed simultaneously, allowing scanning differential expression of a large number of genes (7). The purpose of the present study was to identify genes responsive to BPA in an in vitro mouse Sertoli cell model using DNA microarrays.

TTE3 cells showed a temperature-sensitive growth phenotype reflected by the tsSV40 large T-antigen and a non-tumorigenic activity. The cells expressed Sertoli cell-specific mRNAs encoding steel factor, inhibin α, transferrin, follicle-stimulating hormone receptor and sulfated glycoprotein-2 (6). The cells were cultivated in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) on a collagen type I-precoated culture vessel at a permissive temperature (33°C). BPA (purity: 99.7%; Wako Pure Chemical Industries Ltd., Osaka) was dissolved in dimethyl sulfoxide (final concentration: 0.1%). The cells were cultured with DMEM supplemented with 0.5% FBS for 24 h at 33°C, followed by incubation in DMEM supplemented with 0.5% FBS and BPA for 24 h at 33°C. The cell viability was determined using a tetrazolium compound, 4-[3-(4-idophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3- benzene disulphonate (1a) sodium salt (WST-1), according to our previous report (8).

Microarray analysis was performed by Atlas glass mouse 1.0 microarrays (Clontech Laboratories, Inc., Palo Alto,
CA, USA) that include 1,081 mouse DNA fragments and a list of these genes is available at Clontech Web site (http://www.clontech.com/atlas/genelists/index.shtml). cDNA probes were prepared by reverse transcription with Cy3-dUTP or Cy5-dUTP from mRNAs from cells exposed to 0 (control) or 200 μM BPA, respectively, by using an RNA Fluorescence Labeling Core Kit (Takara Shuzo Co., Kyoto). In some experiments, the control sample was labeled with Cy5; and in others, it was labeled with Cy3, with essentially identical results. cDNA probe solutions containing both Cy3- and Cy5-labeled cDNA probes were applied to the microarrays, and the microarrays were placed in a humidified chamber at 65°C for 16 h. Then, the microarrays were sequentially washed in 2× SSC (150 mM NaCl and 15 mM sodium citrate) containing 0.2% SDS (sodium dodecyl sulfate) for 5 min twice at 55°C and in 0.05× SSC for 1 min once at room temperature. The microarrays were scanned with a ScanArray Lite (Packard BioChip Technologies) was used for image analysis. Genes were considered to be positive-expressed if the signal/background ratio was >3.0. The average of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) Cy3 and Cy5 signal gives a ratio that was used to balance or normalize the signals.

TaqMan 5′ nuclease fluorogenic quantitative polymerase chain reaction (PCR) assay was performed according to the manufacturer’s instructions (Applied Biosystems Japan K.K., Tokyo). Based on the data base (Genbank accession no. D78645, M10624, M87039, U40930, U85259 and X67083), specific primers and probes were synthesized.

Data are shown as means ± S.D. Statistical analysis was carried out using Dunnett’s multiple conversion test or Student’s t-test, and P values less than 0.05 were regarded as significant.

The effects of BPA on the cell viability of TTE3 cells were examined. Although BPA at concentrations of 25 and 50 μM did not affect cell viability, higher concentrations of the compound (100 – 400 μM) significantly decreased cell viability in a concentration-dependent manner, as follows: BPA decreased cell viability by 25, 33 and 96% at concentrations of 100, 200 and 400 μM, respectively (Fig. 1). Considering the effects of a subtoxic dose of BPA on the basis of these data, 200 μM BPA was chosen for the further studies. We monitored the differentially expressed genes responsive to BPA in TTE3 cells by using commercial glass microarrays hybridized with fluorescence dye-labeled cDNA probes synthesized from mRNAs of the cells that had been treated with and without 200 μM BPA. Genes were considered up- or down-regulated if the averaged fold change was 2.0 or greater in two different experiments. Of the 1,081 genes examined, changes in mRNA levels were detected in 13 genes (3 decreased and 10 increased). In these genes, estrogen receptor α (ERα) or ornithine decarboxylase (ODC), chop-10, 78-kDa glucose-regulated protein (GRP78), oxidative stress-induced protein (OSIP) and inducible nitric oxide synthase (iNOS) were down- or up-regulated in the cells exposed to this compound, with their levels being 0.37-fold lower or 3.4, 3.2, 3.0, 2.8 and 2.4-fold higher, respectively, than that of the control group (BPA 0 μM) (Table 1). To verify the results of the microarrays, we performed the TaqMan assay, a quantitative PCR. Six genes were selected from the genes that were differentially decreased or increased by microarray analysis. As in the case of microarray analysis, mRNA expression levels of ERα or ODC, chop-10, GRP78, OSIP and iNOS were significantly down- or up-regulated in the cells exposed to BPA (200 μM), with the levels being 0.24-fold lower or 10.9, 19.7, 9.9, 4.2 and 23.5-fold higher than that of the control group (BPA 0 μM) (Fig. 2).

Cell death (33%) was induced in the cells treated with a subtoxic dose of BPA (200 μM) for 24 h. Of the 1,081 genes examined under the experimental condition, changes in mRNA levels were detected in 13 genes, with these genes containing 4 stress response proteins such as ODC, chop-10, GRP78 and OSIP. Elevation of the mRNA levels of these 4 genes was confirmed by a quantitative PCR technique. ODC (9), chop-10 (10), GRP78 (11) and OSIP (12) are the genes overexpressed by mammalian cells exposed to cytotoxic agents or to a variety of stresses such as endoplasmic reticulum and oxidative stress. Hughes et al. (5) showed that alkylphenols including BPA induced cell death in mouse Sertoli cells (TM4) and inhibited endoplasmic reticulum Ca2+-ATPase activity and Ca2+-uptake in rat testis microsomes and mobilized intracellular Ca2+ in the
Table 1. Down- and up-regulated genes of TTE3 cells treated with BPA

| Gene                        | Genbank accession no. | exp. 1 | exp. 2 | mean |
|-----------------------------|-----------------------|--------|--------|------|
| Down-regulated genes        |                       |        |        |      |
| Hormone receptors           |                       |        |        |      |
| estrogen receptor α (ERα)   | U85259                | 0.32   | 0.41   | 0.37 |
| Protease inhibitors         |                       |        |        |      |
| tissue inhibitor of metalloproteinase 2 | X62622          | 0.49   | 0.34   | 0.42 |
| Cytoskeleton and motility proteins |                 |        |        |      |
| decorin                     | X53929                | 0.47   | 0.38   | 0.43 |
| Up-regulated genes          |                       |        |        |      |
| Cell cycle-regulating kinases |                     |        |        |      |
| cyclin-dependent kinase 7   | U11822                | 2.2    | 2.0    | 2.1  |
| Phospholipases and phosphoinositol kinases |       |        |        |      |
| phospholipase C γ 1         | X95346                | 2.1    | 2.2    | 2.2  |
| G proteins                  |                       |        |        |      |
| G-α-13 guanine nucleotide regulatory protein | M63660          | 2.3    | 2.2    | 2.3  |
| Transcription activators & repressors |                 |        |        |      |
| activating transcription factor 3 | U191118          | 2.1    | 2.4    | 2.3  |
| activating transcription factor 4 | M94087            | 3.4    | 2.9    | 3.2  |
| Cell signaling and extracellular communication proteins |             |        |        |      |
| inducible nitric oxide synthase (iNOS) | M87039          | 2.3    | 2.4    | 2.4  |
| Stress response proteins    |                       |        |        |      |
| oxidative stress-induced protein (OSIP) | U40930          | 3.3    | 2.2    | 2.8  |
| 78-kDa glucose-regulated protein (BipGRP78) | D78645        | 3.5    | 2.5    | 3.0  |
| Chop-10                     | X67083                | 2.5    | 3.8    | 3.2  |
| ornithine decarboxylase (ODC) | M10624            | 3.1    | 3.7    | 3.4  |

The cells were exposed to 200 μM of BPA for 24 h. Microarray analysis was performed. Details of experimental conditions described in the text.

Fig. 2. Verification of the microarray results with TaqMan assay. Six genes were selected from the genes that were differentially increased or decreased by the microarray analysis. TTE3 cells were incubated with BPA (0 or 200 μM) for 24 h at 33°C. The TaqMan assay was performed. Each expression level was normalized by G3PDH. The data represent the mean ± S.D. for 3 – 4 different experiments. *P<0.05 vs BPA (0 μM) (Student’s t-test).
intact TM4 cells. We, therefore, postulate that increase in the level of transcripts of these stress-response proteins treated with BPA may relate to endoplasmic reticulum stress elicited by inhibition of endoplasmic reticulum Ca\textsuperscript{2+} ATPase by this compound.

It has been indicated that ERs are present in Sertoli cells, and that the targeted disruption of the ER gene in male mice causes alterations in spermatogenesis and infertility (13). In the present study, both microarray and TaqMan assays clearly demonstrate that the mRNA level of ER $\alpha$ was decreased in BPA-treated cells. In the same way, Aloisi et al. (14) reported that long-term exposure to BPA (40 mg/kg per day, 42 days) induced a decrease in ER-immunoreactive cells in the arcuate nucleus of lactating adult female Sprague-Dawley rats. The estrogenic activity of BPA has been demonstrated in a number of assay systems, although its activity was found to be about four orders less potent than that of 17\beta-estradiol (1). In contrast, previous observations showed that the biological effects of alkylphenols are not mediated by direct interaction with ERs (15). In the same way, the cell death and changes of mRNA expression induced by BPA were not prevented by simultaneous treatment with the specific estrogen receptor antagonist (Y. Tabuchi et al., unpublished observation).

In the present study, we identified 13 differentially expressed genes in responsive to a subtoxic dose of BPA in an in vitro Sertoli cell model. To our knowledge, this is the first observation of broad-scale gene expression in the cells treated with BPA using the microarray technology. The detailed knowledge of the changes in gene expression by using microarrays will provide the basis for further understanding the molecular mechanisms of the toxic effects of BPA in testicular cells.

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REFERENCES

1 Gaido KW, Leonard LS, Lovell S, Gould JC, Babai D, Portier CJ and McDonnell DP: Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay. Toxicol Appl Pharmacol 143, 205–212 (1997)

2 Griswold MD: Interactions between germ cells and Sertoli cells in the testis. Biol Reprod 52, 211 – 216 (1995)

3 Takahashi O and Oishi S: Testicular toxicity of dietary 2,2-bis (4-hydroxyphenyl)propane (bisphenol A) in F344 rats. Arch Toxicol 75, 42 – 51 (2001)

4 Takao T, Nanamiya W, Nagano I, Asaka K, Kawabata K and Hashimoto K: Exposure with the environmental estrogen bisphenol A disrupts the male reproductive tract in young mice. Life Sci 65, 2351 – 2357 (1999)

5 Hughes PJ, McLellan H, Lowes DA, Kahn SZ, Bilmen RG, Tovey SC, Godfrey RE, Michell RH, Kirk CJ and Michelangeli F: Estrogenic alkylphenols induce cell death by inhibiting testis endoplasmic reticulum Ca\textsuperscript{2+} pumps. Biochem Biophys Res Commun 277, 568 – 574 (2000)

6 Tabuchi Y, Ohta S, Kondo T, Yanai N, Obinata M, Fuse H and Asano S: Development of the conditionally immortalized testicular Sertoli cell line TTE3 expressing Sertoli cell-specific genes from mice transgenic for temperature-sensitive simian virus 40 large T-antigen gene. J Urol 167, 1538 – 1545 (2002)

7 Tabuchi Y, Kondo T, Ogawa T and Mori H: DNA microarray analyses of genes elicited by ultrasound in human U937 cells. Biochem Biophys Res Commun 290, 498 – 503 (2002)

8 Tabuchi Y, Sugiyama N, Horiuchi T, Furuhama K and Furusawa M: Insulin stimulates production of glycoconjugate layers on the cell surface of gastric surface mucous cell line GSM06. Digestion 58, 28 – 33 (1997)

9 Pegg AE and McCann PP: Polymamine metabolism and function. Am J Physiol 243, C212 – C221 (1982)

10 Fontanier-Razzac NC, Hay SM and Rees WD: Upregulation of CHOP-10 (gadd153) expression in the mouse blastocyst as a response to stress. Mol Reprod Dev 54, 326 – 332 (1999)

11 Lee AS: The glucose-regulated proteins: stress induction and clinical applications. Trends Biochem Sci 26, 504 – 510 (2001)

12 Ishii T, Yanagawa T, Kawane T, Yuki K, Seita J, Yoshida H and Bannai S: Murine peritoneal macrophages induce a novel 60-kDa protein with structural similarity to a tyrosine kinase p56lck-associated protein in response to oxidative stress. Biochem Biophys Res Commun 226, 456 – 460 (1996)

13 Eddy EM, Washburn TF, Bunch DO, Goulding EH, Gladen BC, Lubahn DB and Korach KS: Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. Endocrinology 137, 4796 – 4805 (1996)

14 Aloisi AM, Della Seta D, Cecarelli I and Farabollini F: Bisphenol-A differently affects estrogen receptors-alphar in estrous-cycling and lactating female rats. Neurosci Lett 310, 49 – 52 (2001)

15 Harris RM, Waring RH, Kirk CJ and Hughes PJ: Sulfation of "estrogenic" alkylphenols and 17beta-estradiol by human platelet phenol sulfotransferases. J Biol Chem 275, 159 – 166 (2000)