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Biochemical and Biophysical Research Communications 305 (2003) 54–61
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cDNA microarray analysis reveals chop-10 plays a key role in Sertoli cell injury induced by bisphenol A

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Received 3 April 2003

Abstract

We examined the time course of changes in gene expression in detail using cDNA microarray analysis of mouse Sertoli TTE3 cells treated with bisphenol A (BPA). A subtoxic dose of BPA (200 μM) transiently increased intracellular Ca^{2+} concentration and time-dependently induced an increase in mRNA level of 78-kDa glucose-regulated protein, indicating that BPA induces endoplasmic reticulum stress. Of the 865 genes analyzed, 31 genes showed increased levels of expression. TaqMan analysis confirmed that the mRNA levels of chop-10, fra-2, c-myc, and ornithine decarboxylase were increased, and showed that chop-10 is the most sensitive gene. The expression level of chop-10 protein and cell injury induced by BPA were significantly reduced in stable TTE3 cells overexpressing full-length chop-10 antisense RNA. We conclude that chop-10 plays a key role in Sertoli cell injury induced by BPA.

Keywords: Bisphenol A; cDNA microarray; Sertoli cell; Cell injury; Gene expression; chop-10

Endocrine disruptors are a diverse group of chemicals found in the environment that can act as estrogens, androgens, or other hormones and that may upset normal endocrine function [1,2]. Endocrine disruptors with estrogenic activity might have adverse effects on reproductive systems in wildlife and humans. Spermatogenesis occurs within the seminiferous tubules of the testis and requires the cooperative functions of several different cell types, including Sertoli and Leydig cells. In the seminiferous tubule, 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 [3,4]. It is, therefore, postulated that endocrine disruptors may have adverse effects on the reproductive systems as a result of functional changes in Sertoli cells.

Bisphenol [2,2-bis(4-hydroxyphenyl)propane; BPA], an alkylphenol derivative, is a high production volume chemical used in the manufacture of polycarbonate plastics. BPA binds to estrogen receptors and induces estrogenic activity in a number of biological systems [5,6], 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 [7,8]. BPA has also induced male reproductive toxicities, including reductions in epididymis weight, seminal vesicle weight, sperm motility, and Sertoli cell function in in vivo models [2,9–13]. Furthermore, BPA induced cell death in testicular cells, including Sertoli cells, in in vitro models [2,14,15]. Hughes et al. [14] showed that alkylphenols, including BPA, induced cell death in mouse Sertoli cells (TM4) and inhibited endoplasmic reticulum (ER) Ca^{2+}-ATPase activity and Ca^{2+}-uptake in rat testis microsomes and mobilized intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the intact TM4 cells. Quite recently, we showed that a significant decrease in mRNA of estrogen receptor α and an increase in mRNAs of ornithine decarboxylase (ODC), chop-10, 78-kDa glucose-regulated protein (GRP78), oxidative stress-induced protein, and inducible nitric oxide synthase were found in mouse Sertoli cells (TTE3) treated with BPA [15]. However, the molecular mechanisms by which BPA

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doi:10.1016/S0006-291X(03)00708-3

induces Sertoli cell toxicity are still unclear. In the present study, to identify genes that are differentially expressed in response to BPA-induced cell damage, we investigated the detailed time course of changes in gene expression using cDNA microarray technique.

Materials and methods

Cell characteristics and cell culture. TTE3, a Sertoli cell line, established from transgenic mice harboring temperature-sensitive simian virus 40 large T-antigen [16] was used as a Sertoli cell model. TTE3 cells showed a temperature-sensitive growth phenotype reflected by the oncogene. The cells did not show any colony forming activity in soft agar and did not form any tumors in nude mice, indicating that TTE3 cells are only immortalized and not transformed. The cells expressed Sertoli cell-specific mRNAs encoding steel factor, inhibin α, transferrin (TF), follicle-stimulating hormone receptor, and sulfated glycoprotein-2. The expressions of both vimentin and zonula occludens-1 were observed in the cytoplasm and on the boundaries of the cells, respectively [16]. TTE3 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS) on a collagen type I-precoated culture vessel (Asahi Techno Glass, Tokyo, Japan) at a permissive temperature (33 °C).

Incubation of the cells with compounds. BPA (purity: 99.7%, Wako Pure Chemical Industries, Osaka, Japan) at final concentrations of 0–400 μM was dissolved in dimethyl sulfoxide (DMSO) (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 the compound for 0–24 h at 33 °C. The cells treated with 0.1% DMSO served as control.

Measurement of $[Ca^{2+}]_i$. The cells were cultured in a glass-bottomed dish coated with collagen type I and were loaded with HR buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.13 mM $MgCl_2$, 1 mM Na_2HPO_4, 5.5 mM glucose, and 10 mM Hepes–NaOH, pH 7.4) containing 5 mM Fura-2/AM (Dojindo Laboratory, Hamamatsu, Japan) for 30 min at 25 °C. After washing once with the HR buffer, digital imaging of Fura-2 fluorescence was carried out using an inverted microscope and digital image processor (Argus 50/Ca, Hamamatsu Photonics, Shizuoka, Japan) as reported previously [17,18]. The fluorescence ratio (340/380 nm) at 510 nm of emission wavelength was converted to $[Ca^{2+}]_i$ using the equation of Grynkiewicz et al. [19].

Analyses of cell viability. The cell viability was determined using a tetrazolium compound, 4-[3-(4-idophenyl)-2-(4-nitrophenyl)-2H-5-tetra-zolio]-1,3-benzene disulfonate (1a) sodium salt (WST-1, Wako), which is reduced by lactate dehydrogenase to a highly water-soluble formazan dye [20]. The cells were cultured in Daigo's T medium (without phenol red, Wako) containing 91 μM WST-1 and 19 μM 1-methoxy-5-methylphenazinium methylsulfate (Wako) at 37 °C. After 3-h culture, the formazan dye concentration was determined from the absorbance at 450 nm.

Separation of mRNA. Total RNA was extracted from the cells using an RNeasy Total RNA Extraction Kit (Qiagen K.K., Tokyo, Japan). Then, RNA samples were treated with RNase-free DNase (Qiagen) for 30 min at room temperature. mRNAs were extracted from the RNase-treated samples using an Oligotex-dT30 mRNA Purification Kit (Takara Shuzo, Kyoto, Japan).

cDNA microarray analysis. cDNA microarray analysis was performed by IntelliGene mouse expression glass microarrays (Version 1.0, Takara Shuzo), which were spotted with 564 cDNA fragments of mouse known genes and approximately 301 expressed sequence tags (ESTs). A list of these genes is available at Takara's website (http://www.takara.co.jp/english/index.htm). cDNA probes were prepared by reverse transcription with Cy3-dUTP (Amersham Pharmacia Biotech, Tokyo, Japan) or Cy5-dUTP (Amersham Pharmacia) from mRNAs from cells exposed to 0 (control) or 200 μM BPA for 3, 6, 12, and 24 h, respectively, by using an RNA Fluorescence Labeling Core Kit (Takara Shuzo). In some experiments, control sample was labeled with Cy5; and in others, it was labeled with Cy3, with essentially identical results. After treatment with RNase H, cDNA probes were purified by gel filtration. Hybridization and washing of the microarray were carried out according to the manufacturer's instructions. In brief, cDNA probe solutions containing both Cy3- and Cy5-labeled cDNA probes were applied to the microarrays, and the microarrays were covered with a spaced glass coverslip (Takara Shuzo) and 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 for 5 min twice at 55 °C, in 2× SSC containing 0.2% SDS for 5 min once at 65 °C and in 0.05× SSC for 1 min once at room temperature. The microarrays were scanned in both Cy3 and Cy5 channels with a ScanArray Lite (Packard BioChip Technologies, Billerica, MA). QuantArray software (Packard BioChip) 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 (8 spots each) gives a ratio that was used to balance or normalize the signals [21].

TaqMan assay. TaqMan 5′ nuclease fluorogenic quantitative polymerase chain reaction (PCR) assay was performed according to the manufacturer's instructions. Reverse transcriptase (RT) reaction was carried out with mRNA by using an $oligo(dT)_6$ primer. Based on the databases, the specific primers and probes were designed (Table 1). Temperature cycling conditions for each primer set consisted of 2 min at 50 °C and 10 min at 95 °C followed by 40 cycles for 15 s at 95 °C and 1 min at 60 °C. All reactions were performed in an ABI Prism 7700 Sequence Detector (Applied Biosystems Japan K.K., Tokyo, Japan). TaqMan rodent G3PDH control reagent was purchased from Applied Biosystems. Fold induction was normalized by G3PDH.

Establishment of stable cell lines. A full-length mouse chop-10 cDNA fragment was produced by PCR from a murine testis cDNA library (Takara Shuzo) using the following primer set; forward, 5′-ATGGCAGCTGAGTCCCTGCCTTT-3′ (75–97) and reverse, 5′-TCATGCTTGGTGCAGGCTGA-3′ (581–562). These oligonucleotides were synthesized referring to the database (GenBank Accession No.: X67083). The PCR product was cloned into a pCRII vector by the TA Cloning Kit (Invitrogen) and was confirmed by direct sequencing. A pcDNA3.1/ASchop-10 vector was constructed as follows; murine chop-10 cDNA (both ends are *Eco*RI sites) was introduced in the antisense orientation, downstream (*Eco*RI site) of the CMV promoter of pcDNA3.1 (Invitrogen). This pcDNA3.1/ASchop-10 vector was able to express complementary antisense RNA vs. murine chop-10 cDNA. When a stable cell line expressing antisense RNA vs. the murine chop-10 gene and the corresponding empty vector were developed, TTE3 cells were transfected with either pcDNA3.1/ASchop-10 or pcDNA3.1 using Effectene Transfection Reagent (Qiagen) according to the manufacturer's instructions. Stable transfectants were selected in DMEM containing 10% FBS and 1 mg/ml neomycin (Invitrogen), and were cloned by colony formation. This procedure was performed a total of two times. The cloned cells were maintained in DMEM containing 10% FBS and 0.5 mg/ml neomycin. The expression of antisense RNA derived from chop-10 cDNA was confirmed using specific primers to RT-PCR.

RT-PCR. Based on the database, PCR primers were designed; G3PDH (GenBank Accession No.: M32599) forward and reverse, 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ (51–76) and 5′-CATGTAGGCCATGAGGTCCACCAC-3′ (1033–1010), respectively; antisense chop-10 forward and reverse, 5′-TCATGCTTGGTGCAGGCTGA-3′ (581–562; mouse chop-10 gene) and 5′-TAGAAGGCACAGTCGAGG-3′ (in pcDNA3.1), respectively. The PCR products for G3PDH and antisense chop-10 were predicted to be 983 and 599 bp in length, respectively. RT-PCR was performed by using an OneStep RT-PCR Kit (Qiagen). Temperature cycling conditions for each primers

Table 1
Nucleotide sequences of TaqMan primers and probes for target genes

Gene	Orientation	Nucleotide sequence (position)	GenBank Accession Nos.
chop-10	Sense	5′-CGTCCCTAGCTTGGCTGACA-3′ (247–266)	X67083
	Antisense	5′-GGCCATAGAACTCTGACTGGAATC-3′ (344–321)	
	Probe	5′-Fam-AGAGGTCACACGCACATCCCAAAGC-Tamra-3′ (284–308)	
fra-2	Sense	5′-CTGCAGCCAAGTGTCGGAA-3′ (410–428)	X83971
	Antisense	5′-GCAGCTCAGCAATCTCTTTCTG-3′ (520–499)	
	Probe	5′-Fam-TCTCCGCCTGCAGCTTCTCTGTCAG-Tamra-3′ (466–490)	
GRP78	Sense	5′-TCATCGGACGCACTTGGAA-3′ (307–325)	D78645
	Antisense	5′-AACCACCTTGAATGGCAAGAA-3′ (374–354)	
	Probe	5′-Fam-ACCCTTCGGTGCAGCAGGACATCA-Tamra-3′ (328–351)	
c-myc	Sense	5′-CCTAGTGCTGCATGAGGAGACA-3′ (1317–1338)	X01023
	Antisense	5′-TCCACAGACACCACATCAATTTC-3′ (1409–1387)	
	Probe	5′-Fam-CACCACCAGCAGCGACTCTGAAGAAG-Tamra-3′ (1344–1369)	
ODC	Sense	5′-GGTTCCAGAGGCCAAACATCT-3′ (1933–1953)	M10624
	Antisense	5′-GCTCTCCTGGGCACAAGACA-3′ (2072–2053)	
	Probe	5′-Fam-CACGGCCAATGTGGCAACTCATG-Tamra-3′ (1966–1988)	
TF	Sense	5′-CCCATTGGCTTGCTCTTCTG-3′ (448–467)	AF440692
	Antisense	5′-CCCGAGAAGAAACTGGACACA-3′ (524–504)	
	Probe	5′-Fam-TGTCGGAGCCCCGCAGTCCT-Tamra-3′ (473–492)	

consisted of 30 min at 50 °C and 15 min at 95 °C followed by 35 cycles for 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, with a final extension for 10 min at 72 °C. Reaction was performed in a thermocycler (GeneAmp PCR System 9700; Applied Biosystems). PCR samples were electrophoresed through 2% agarose gels, and after staining with ethidium bromide, product bands were visualized under ultraviolet light.

SDS–polyacrylamide gel electrophoresis and Western blot. The cells were washed once with phosphate-buffered saline and placed into 50 mM Tris–HCl buffer (pH 8.0) containing 50 mM NaCl and 1% NP-40, and homogenized by an ultrasonic disruptor. SDS–PAGE and Western blotting were carried out as described elsewhere [22,23]. In short, the cell lysate was separated by SDS–PAGE using a 10% gel and electrotransferred to nitrocellulose membranes. The membranes were blocked with TBS (0.9% NaCl and 10 mM Tris–HCl, pH 7.4) containing 4% Block Ace (Dainippon Pharmaceutical, Osaka, Japan) at room temperature for 30 min. The membranes were then incubated with rabbit polyclonal anti-chop-10 (Santa Cruz, CA) or mouse monoclonal anti-G3PDH (Organon Teknika, Durham, NC) in TBS containing 0.8% Block Ace at 4 °C for 18 h, washed five times in TBS containing 0.1% Tween 20, and exposed to peroxidase-conjugated goat anti-rabbit IgG (Organon Teknika) or peroxidase-conjugated goat anti-mouse IgG (Organon Teknika) in TBS containing 0.8% Block Ace at room temperature for 1 h. Immunoreactive proteins were visualized by a luminescent image analyzer (LAS-1000 plus, Fujifilm, Tokyo, Japan) using an enhanced chemiluminescence detection system (ECL plus, Amersham Pharmacia). Bands of target proteins were quantified by densitometry using Multi-Analyst software (Japan Bio-Rad Laboratories, Tokyo, Japan). Fold induction was normalized by G3PDH.

Statistical analysis. Data are shown as means ± SD. Statistical analysis was carried out using Student's *t* test and *P* values less than 0.05 were regarded as significant.

Results

BPA-induced ER stress and cell injury

We examined the effects of BPA on the cell viability of TTE3 cells and found that BPA at concentrations of 200 and 400 μM significantly decreased cell viability in a time-dependent manner, with the cell viability of 90.6% and 58.6%, 72.8% and 16.1%, 69.7% and 20.6%, or 59.3% and 5.3% after 3, 6, 12, or 24 h of the compound treatment, respectively (Fig. 1A). This finding suggests that BPA induces cell injury of Sertoli cells [14,15]. BPA has been reported to inhibit ER Ca^{2+}-ATPase activity and Ca^{2+}-uptake in rat testis microsomes and mobilized $[Ca^{2+}]_i$ in the intact TM4 cells [14]. Basal $[Ca^{2+}]_i$ of the TTE3 cells was about 100 nM. BPA at concentrations of 100, 200, and 400 μM induced a significant increase in $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 1B) as in the case of TM4 cells [14]. Considering the effects of a subtoxic concentration of BPA on the basis of these data, we chose 200 μM BPA for the remainder of our studies. Alteration in Ca^{2+} homeostasis in ER caused ER stress and a high level of GRP78 expression is indicative of ER stress [24,25]. To study whether BPA induces ER stress, we tested the effects of this compound on mRNA level of GRP78. BPA at a concentration of 200 μM induced a marked increase in the mRNA level of GRP78 in a time-dependent manner (Fig. 1C) [15], indicating that BPA induces ER stress. It has been reported that TF is expressed in normal Sertoli [26] and TTE3 cells [16]. BPA (200 μM) time-dependently decreased the mRNA level of TF (Fig. 1D), showing that BPA decreases the function of Sertoli cell.

A time course cDNA microarray analysis

To identify genes that are differentially expressed in response to ER stress and cell injury induced by BPA in TTE3 cells, we carried out the cDNA microarray analysis of cells either exposed to 200 μM BPA or not for 3,

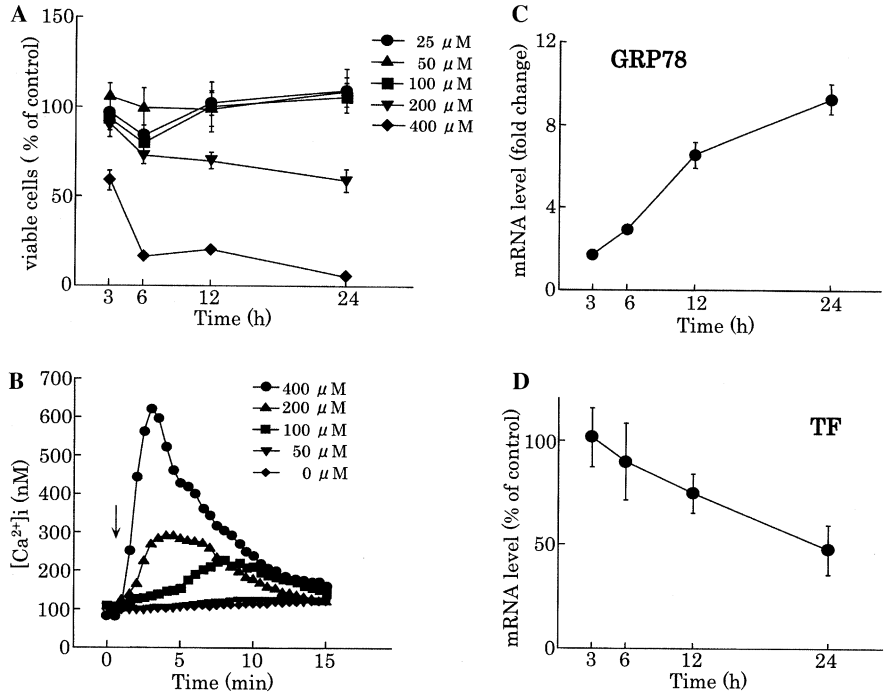

Fig. 1. Effects of BPA on cell viability (A), $[Ca^{2+}]_i$ (B) and mRNA levels of GRP78 (C), and TF (D). (A) TTE3 cells were incubated with BPA (0–400 μM) for 3–24 h at 33 °C. The cell viability was determined by WST-1 assay. The cells treated with 0 μM BPA served as control (100%). The data represent means ± SD for 4 wells. (B) The cells were loaded with Fura-2/AM. Fura-2 fluorescence was carried out using a digital image processor. The fluorescence ratio (340/380 nm) at 510 nm of emission wavelength was converted to $[Ca^{2+}]_i$. BPA (0–400 μM) was added to the cells at time 0.5 min (↓). The data represent means of 56 cells. (C,D) The cells were incubated with BPA (200 μM) for 0–24 h at 33 °C. RT reaction was carried out with mRNA. TaqMan assay was performed according to the manufacturer's instructions. mRNA level was normalized by G3PDH. Data indicate means ± SD for four different experiments.

6, 12, and 24 h. Genes were considered up- or down-regulated if the average of fold change was 2.0 or greater in duplicate experiments. Of the 865 genes analyzed, 31 genes showed increased levels of expression, whereas no down-regulated genes were detected (Table 2). As shown in Fig. 2, we classified the genes we identified as follows: (1) early genes 6 h (peak expression 6 h); (2) early genes 12 h (peak expression 12 h), and (3) late genes (peak expression 24 h). Several transcription factors (Pbx1, BRF1, fra-2, and c-myc) and stress response genes (MyD116 and ODC or HSC70, HSP90, and chop-10) were included in the groups of early genes 6 h and early genes 12 h or late genes, respectively. During experiment periods, the transcript chop-10 changed the most, with expression levels of 6.3, 9.6, 9.2, and 10.4 at 3, 6, 12, and 24 h, respectively. To verify the results of the microarrays, we performed a TaqMan assay, which is a quantitative PCR. Four genes such as c-myc, fra-2, ODC, and chop-10 were selected from the genes that were differentially induced by BPA in the microarray experiments from the viewpoint of variation ratio. Although the expression profile of these genes was not completely comparable to that found by microarray analysis, the mRNA expression levels of these four genes were significantly up-regulated in the cells exposed to BPA (200 μM) over experiment periods, which was similar to the results of microarray analysis (Fig. 3). Among these four genes, chop-10 was the most sensitive and variable gene in terms of being responsive to BPA.

Roles of chop-10 on BPA-induced cell injury

To elucidate whether the expression of chop-10 is required for the progression of ER stress and cell injury induced by BPA in TTE3 cells, we developed a cell line expressing full-length chop-10 antisense RNA. RT-PCR analysis revealed that mRNA for antisense chop-10 gene was expressed in chopR14 cells but not in TTE3 or mock cells (Fig. 4A). Moreover, Western blot analysis showed that BPA (200 μM) induced a significant increase in chop-10 protein level in a time-dependent manner in mock cells. On the other hand, the expression level of chop-10 protein induced by the same concentration of this compound in chopR14 cells was markedly reduced compared to the level in mock cells, with the inhibition percentage being 46.8% and 67.5% at 12 and 24 h, respectively (Figs. 4B and C). In mock cells treated with BPA (200 μM), cell viability and the mRNA level of TF were significantly decreased at 12 and 24 h. On the other hand, in chopR14 cells treated with the same concentration of this compound, both decreased parameters were significantly restored at either 12 or 24 h

Table 2
List of genes up-regulated by BPA

Gene	GenBank Accession No.	Fold change			
		3 h	6 h	12 h	24 h
Cell cycle-related proteins					
Growth arrest specific 5 (GAS5)	X59728	1.1	1.5	2.1	1.8
$p21^{Waf1}$	U09507	1.7	2.4	1.6	1.5
Nuclear proteins					
High mobility group protein I (HMGPI)	J04179	1.3	2.1	2.7	3.2
Histone 4 protein (H4)	M32460	1.6	3.3	4	3.4
RNA-related proteins					
Ribosomal protein S18 (RPS18)	NM_011296	1	1.4	1.7	2
Ribosomal protein L12 (RPL12)	L04280	1.1	1.4	2	2.8
dbpA murine homologue (dbpA)	D14485	1.1	1.6	2.4	2.1
DEAD box polypeptide 5 (DEAD5)	X65627	1.4	2	1.5	1.2
Stress response proteins					
HSC70	M19141	1.1	1.4	1.8	2.1
HSP90	X70887	1.3	1.7	1.7	2.2
MyD116	X51829	1.7	2	3	1.9
Ornithine decarboxylase (ODC)	M12481	2.5	6.2	9	5.5
chop-10	X67083	6.3	9.6	9.2	10.4
Transcription factors					
eIF3 p66	AB012580	1.3	1.9	2.3	2.3
Pbx1	NM_008783	1.8	2.5	1.6	1.3
Ndr1	U60593	1.8	4.1	4.6	3.6
Butyrate response factor 1 (BRF1)	M58566	2	2.6	1.4	1
fra-2	X83971	3.7	6.1	3.1	1.6
c-myc	X01023	5.1	6.7	4.9	3.8
Sui1	Z50159	1.5	1.9	2.6	2.8
Xenobiotic metabolism					
P450 (2e1)	L11650	1.2	1.5	1.5	2.3
Others					
Methenyltetrahydrofolate cyclohydrolase	NM_008638	1.4	2.5	3.1	1.3 (MHCH)
Sterol-C5-desaturase homolog (SC5DH)	AB016248	1.5	2.2	1.8	1.8
Fatty acid synthase (FAS)	X13135	2.1	3.1	2.2	1.8
Antizyme inhibitor (AZI)	AF032128	2.1	3.2	2.5	1.4
ESTs					
EST	AA530373	1.2	1.6	2	1.4
EST	AW213225	1.3	1.7	1.8	2.2
EST	AU035523	1.4	2.1	3.1	2.6
EST	AA204433	1.5	1.7	2	1.7
EST	AA794510	1.8	2	1.9	2.2
EST	AU080055	2.2	3.6	3.2	1.5

Note. TTE3 cells were incubated with BPA for 3–24 h at 33 °C. Details of experimental conditions are described under Materials and methods. Data indicate means of two different experiments.

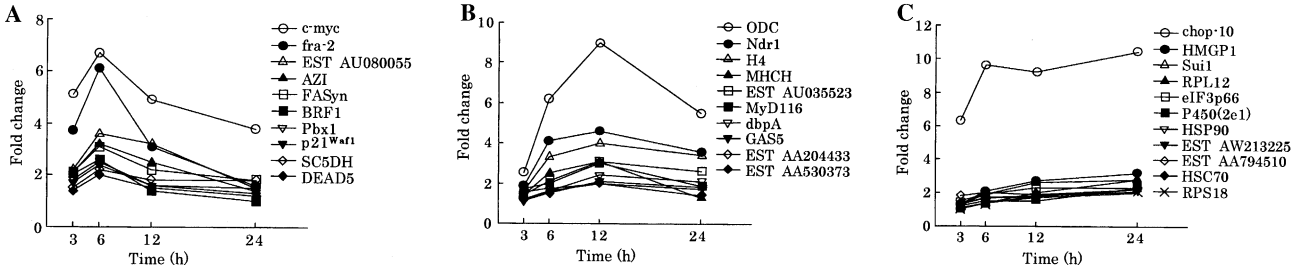

Fig. 2. A time course cDNA microarray analysis. TTE3 cells were incubated with BPA (0 or 200 μM) for 3–24 h at 33 °C. Details of experimental conditions described under Materials and methods. (A) Genes with peak expression 6 h; (B) genes with peak expression 12 h; (C) genes with peak expression 24 h. Data indicate means of two different experiments (see Table 2).

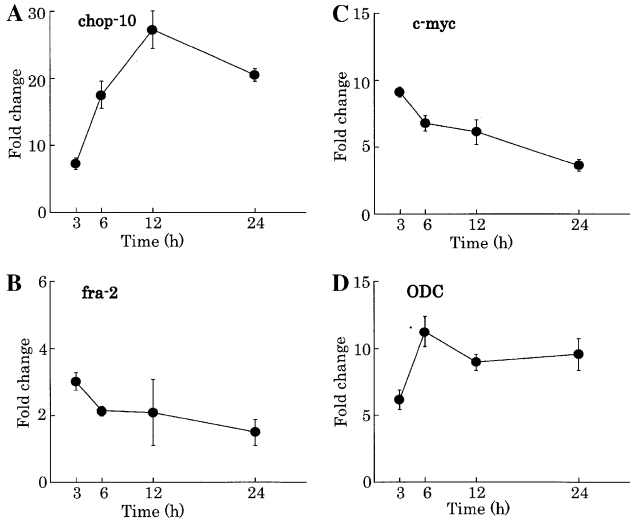

Fig. 3. Verification of the cDNA microarray results with TaqMan assay. TTE3 cells were incubated with BPA (200 μM) for 0–24 h at 33 °C. RT reaction was carried out with mRNA. TaqMan assay was performed according to the manufacturer's instructions. Each expression level was normalized by G3PDH. The data represent means ± SD for four different experiments.

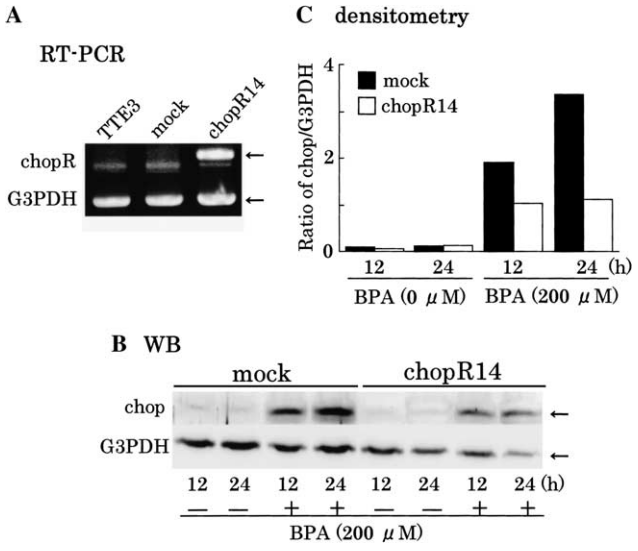

Fig. 4. Establishment of a stable cell line, chopR14, expressing antisense chop-10 RNA and effects of BPA on chop-10 protein expression in chopR14 cells. (A) mRNA from TTE3, mock (pcDNA3.1 transfection), and chopR14 cells (pcDN3.1/ASchop-10 transfection) were subjected to RT-PCR analysis using specific primers sets of antisense chop-10 (chopR) and G3PDH. (B,C) Cells were incubated with BPA (0 or 200 μM) for 12 and 24 h at 33 °C. The cell lysate was separated by SDS–PAGE, and then Western blot (WB) analysis was performed using anti-chop-10 and anti-G3PDH antibodies (B). Bands were quantified by densitometry. Fold induction was normalized by G3PDH (C).

(Figs. 5A and B). In contrast, the BPA (200 μM)-induced induction of mRNA levels of GRP78 in mock and chopR14 cells was similar (Fig. 5C).

Discussion

Recent observations showed that BPA induced cell death in many kinds of cells, including Sertoli cells in in vitro [2,14,15]. In addition, this compound inhibited ER Ca^{2+}-ATPase activity and Ca^{2+}-uptake in rat testis microsomes and mobilized $[Ca^{2+}]_i$ in the intact TM4 cells [14]. In the present study, a subtoxic dose of BPA induced a transient increase in $[Ca^{2+}]_i$ and time-dependently induced an increase of GRP78 expression. These findings indicate that BPA induces an altered Ca^{2+} homeostasis in ER, causes ER stress, and leads to injury of Sertoli cells.

Novel transcript profiling technologies allow the simultaneous measurement of changes in expression of many hundreds or many thousands of genes [27,28] and have been used for biological experiments [14,21,29,30]. In the present study, of the 865 genes analyzed, 31 genes showed increased levels of expression in TTE3 cells treated with BPA. To our surprise, no down-regulated gene was detected in the present experimental conditions. Microarray and TaqMan analyses demonstrated that chop-10 was the most sensitive and variable gene in terms of being responsive to BPA. chop-10, also known as GADD153, was originally identified as a gene induced by DNA damage and growth arrest [31] that acts as a negative modulator of CCAAT/enhancer binding protein transcriptional factors [32]. It has also been reported that chop-10 is induced in response to cellular stresses, especially by ER stress, and that it is involved in the process of cell death associated with ER stress [24,33]. Zinszner et al. [33] previously showed that the absence of chop-10 promotes increased survival of cells exposed to ER stress. To clarify whether chop-10 is required for the progression of ER stress and cell injury elicited by BPA in TTE3 cells, we established a cell line chopR14, which markedly reduced the induction of chop-10 by BPA. In chopR14 cells, BPA-induced cell injury was significantly restored, indicating that chop-10 plays a key role in Sertoli cell injury induced by BPA. On the other hand, induction of GRP78 by this compound was similar to that of mock cells. This suggests that chop-10 is not required for the progression of ER stress [33].

In this study, several transcription factors and stress response genes were elicited by BPA. Fra-2 and c-myc are members of the fos and myc family transcription factors, respectively, and these transcription factors have a very important role in varied cellular functions such as cell proliferation, transformation, and apoptosis [34–37]. Nankova et al. [36] reported the potential importance of stress-evoked increases in the expression of fra-2 gene for in vivo adaptations of the adrenal medulla in rats. Furthermore, recent papers demonstrate that c-myc promotes cell survival under stressful conditions [37]. Heat shock proteins such as HSC70 and HSP90 are

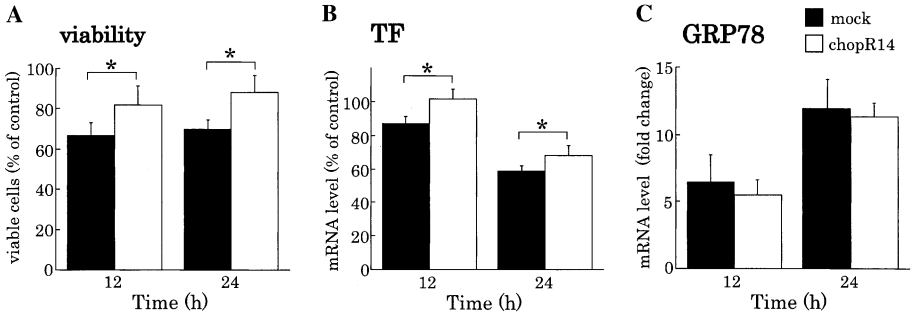

Fig. 5. Effects of chop-10 reduction using chopR14 cells in BPA-induced cell injury. Cells were incubated with BPA (0 or 200 μM) for 12 and 24 h at 33 °C. (A) The cell injury was determined by WST-1 assay. The cells were treated with 0 μM BPA served as control (100%). The data represent means ± SD for 4 wells. (B) (TF) and (C) (GRP78), RT reaction was carried out with mRNA. TaqMan assay was performed according to the manufacturer's instructions. Each expression level was normalized by G3PDH. The data represent means ± SD for four different experiments.

molecular chaperones that participate in important cellular processes and appear to play a critical role in the protection from cellular damage associated with various stresses [38–40]. For example, in neuroblastoma and fibroblast cells, overexpression of HSC70 protects cells from damages induced by oxidative stress [39]. Neuroblastoma cells were protected from 3-hydroxykynurenine-induced cytotoxicity by prior elevation of HSPs expression, and the protective effect was abolished by HSP90 antisense oligonucleotides [40]. ODC is a key enzyme in polyamine biosynthesis and is induced by toxic stimuli [41], and polyamines are known to have a protective role against various types of stress-induced cell deaths [42,43]. In this study, we show that fra-2, c-myc, HSC70, HSP90, and ODC expressions are significantly induced by BPA in TTE3 cells. It is, therefore, highly likely that induction of these transcripts protected cells from cell injury induced by BPA.

In this study, genes identified by using microarray were classified as one of the three types (Fig. 2), and a transcription factor c-myc and stress response genes, including ODC or HSC70, were included in the groups of early genes 6 h and early genes 12 h or late genes, respectively. In addition, use of the TaqMan technique confirmed that the induction of c-myc expression occurred earlier than did the induction of the stress response genes. It is well known that ODC is one of the transcriptional targets of c-myc [44]. More recently, Park et al. [37] reported that enhanced expression of c-myc protects against cell death caused by cellular insults through ODC induction. Moreover, Shoji et al. [45] showed that HSC70 expression was positively regulated by the c-myc gene in murine erythroleukemia cells. Therefore, it seems likely that either ODC or HSC70 may be induced by BPA through c-myc induction.

More recently, we identified 13 differentially expressed genes that are responsive to BPA (24-h exposure) in TTE3 cells using Clontech's Atlas glass mouse 1.0 microarrays that include 1.081 mouse DNA fragments [15]. In these 13 genes, only two genes, including chop-10 and ODC, could be detected by the present microarray analysis. This discrepancy may be due to different experimental conditions, including the type of microarray, DNA labeling reagent, and exposure period of BPA.

Although the actions of BPA are assumed to be mediated through estrogen receptors, its estrogenic activity was found to be about four orders less potent than that of 17β-estradiol [5–8]. In contrast, previous observations showed that the biological effects of alkylphenols are not mediated by direct interaction with estrogen receptors [46]. In the same way, the cell death and changes of mRNA expression induced by BPA were not prevented by simultaneous treatment with ICI 182, 780, a specific estrogen receptor antagonist (Tabuchi et al., unpublished observation). Here, the results indicate that BPA induces alteration in Ca^{2+} homeostasis in ER, causes ER stress, and leads to injury of Sertoli cells, and that chop-10 plays a key role in Sertoli cell injury that BPA induces. Moreover, we newly identified many up-regulated genes whose relationship to the biological effects induced by this compound have not yet been reported. These findings cause us to postulate that BPA may disrupt the male reproductive system by altering Ca^{2+} homeostasis in the ER of Sertoli cells without direct interaction with estrogen receptors [14], and the differentially expressed genes identified here, especially chop-10, are likely to be involved in the cell damage that BPA induces in Sertoli cells. Further studies will be necessary to clarify these mechanisms of BPA in in vivo animal models.

In conclusion, detailed knowledge of the changes in gene expression using cDNA microarrays will provide a basis for further understanding of the molecular mechanisms of BPA's toxic effects in testicular cells.

Acknowledgments

This study was supported in part by a Grant-in-Aid from the Japanese Ministry of Education, Culture, Sports, Science and Tech-

nology and by grants from Takeda Science Foundation and Toyama Daiichi Bank Foundation.

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