INTRODUCTION

Bisphenol A (BPA) is a synthetic monomer of plastic used in consumer products and, in terms of volume, is one of the most abundantly produced chemicals worldwide. BPA has been shown to exhibit weak estrogenic activity by binding to estrogen receptors, and BPA ingested during pregnancy has been detected in the fetal serum and full-term amniotic fluid passing through the placenta (Schönfelder et al., 2002). Therefore, embryotoxicity caused by BPA is the subject of intense research.

DNA methylation is recognized as a contributor to epigenetic regulation of transcription and is essential for embryogenesis and development. DNA methyltransferases (DNMTs) are known to establish and/or maintain proper DNA methylation patterns. Among the DNMTs, DNMT3a and 3b act as “de novo methyltransferases,” which establish methylation patterns during early development (Okano et al., 1999), and DNMT1 acts mainly as a “maintenance methyltransferase,” which preferentially methylates hemimethylated DNA after DNA replication (Bestor, 2000). On the other hand, methyl-CpG binding protein 2 (MECP2), which has 2 splice variants, MECP2_e1 and MECP2_e2, is the founding member of a family of proteins that contain a closely related methyl-CpG binding domain. Once bound to methylated CpG sites, MECP2 recruits corepressors and chromatin remodeling proteins; this process is widely believed to assist in the transcriptional silencing process and act as a long-range regulator of methylated genes (Jones et al., 1998). Recent reports suggest that BPA can act to alter DNA methylation in several tissues (Kundakovic and...
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more, Chahrour et al. (2008) demonstrated that lack of or overexpression of MECP2 altered the expression levels of thousands of genes in the hypothalamus that serve many functions, such as in the autonomic nervous system and hormonal secretion from the pituitary gland. So far, many experiments have been performed to clarify the long-lasting effects of BPA. For example, prenatal or neonatal exposure of rodents to BPA induces aberrations in serum gonadotropin/steroid hormone levels (Cardoso et al., 2011), significant earlier onset of puberty, and alteration in estrous cyclicity (Howdeshell et al., 1999; Maffini et al., 2006). In addition, anatomical evidence of alterations in the sexual dimorphism of the hypothalamic region has been reported in rats exposed perinatally to BPA (Funabashi et al., 2004). In terms of the molecular mechanism underlying the long-lasting effects of BPA, we hypothesized that this agent can exert adverse effects on proper functional development in prenatal or neonatal hypothalamic cells through alteration of the epigenetic regulation of transcription.

The purpose of the present study was to examine whether BPA directly induces adverse effects on the DNA methylation system and CpG-DNA-mediated gene transcription in developing hypothalamic cells, particularly with respect to the gene expression of Dnmts (Dnmt1, Dnmt3a, and Dnmt3b) and Mecp2 isoforms e1 and e2. We used the embryonic mouse hypothalamic cell line N-44 (mHypoE-N44) as the fetal hypothalamic cell model, and assessed dose-dependent effects of BPA on gene expression with real-time reverse transcription polymerase chain reaction (real-time RT-PCR).

**MATERIALS AND METHODS**

**Cell culture of mHypoE-N44 cells and incubation with BPA**

The mHypoE-N44 cells, an embryonic mouse hypothalamic cell line (CELLutions Biosystems, Inc., Toronto, ON, Canada) were cultured as previously described (Warita et al., 2013). BPA (Junsei Chemical Co., Tokyo, Japan) at final concentrations of 0.02 μM, 0.2 μM, 2 μM, 20 μM, and 200 μM was dissolved in dimethyl sulfoxide (DMSO; final concentration, 0.1%). According to a previous report by Kim et al. (2009) on cytotoxicity of BPA in neural differentiation using an in vitro lactate dehydrogenase release assay, the cells treated with < 200 μM BPA for 12 hr did not show notable adverse effects. Therefore, treatment of cells with < 200 μM BPA for 3 hr is considered to have no effect on cell viability. In addition, the 0.1% vehicle DMSO was not toxic to the mHypoE-N44 cells and had no effect on cell viability or cell division. Prior to treatment with BPA, the mHypoE-N44 cells were cultured in phenol red-free minimum essential medium alpha (MEMα; Invitrogen-Gibco, Carlsbad, CA, USA), supplemented with 0.5% charcoal-stripped fetal bovine serum (FBS) for 24 hr at 37°C. They were subsequently incubated at 37°C in phenol red-free MEMα supplemented with 0.5% charcoal-stripped FBS and BPA for 3 hr. The cells treated with 0.1% DMSO served as controls, and we performed statistical analyses on the results of 3 independent replicate experiments.

**Separation of total RNA and real-time RT-PCR**

Total cellular RNA was extracted from the mHypoE-N44 cells by using an RNeasy kit (Qiagen, Hilden, Germany). The extracted RNA was then subjected to real-time RT-PCR for the detection of specific gene expression as described in the Materials and Methods section.

**Table 1. Forward and reverse primer sequences for real-time RT-PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dnmt1</td>
<td>5’-CCTAGGTCCGGCTACGAGGA-3’  &lt;br&gt;  5’-TCCTCTCTCCTGAGCGGT-3’</td>
<td>137</td>
<td>Sato et al. (2006)</td>
</tr>
<tr>
<td>Dnmt3a</td>
<td>5’-CACAGGGGGTGTTACTTCTCG-3’</td>
<td>77</td>
<td>Kamei et al. (2010)</td>
</tr>
<tr>
<td>Dnmt3b</td>
<td>5’-GTCAGGATCAGGAGGAAAGT-3’</td>
<td>145</td>
<td>Sato et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>5’-TCAGGAGGGTGTTAGCCTTGCT-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mecp2_e1</td>
<td>5’-AGGAGAGACTGGAGAAAAATG-3’</td>
<td>71</td>
<td>Dragich et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>5’-CTAAACTCTAGGTGGCTGTCT-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mecp2_e2</td>
<td>5’-CTCATAAAGGTTATACAGCAGTGGAT-3’</td>
<td>184</td>
<td>Dragich et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>5’-CTCATACCTACAGGTGGCTGTCTAAGTCG-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gapdh</td>
<td>5’-AGGGCGGTTGAAACCGATTG-3’</td>
<td>123</td>
<td>Warita et al. (2013)</td>
</tr>
<tr>
<td></td>
<td>5’-TGTAGGACTCATGTAGTTGAGGTA-3’</td>
<td></td>
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</tr>
</tbody>
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Germany) according to the manufacturer’s instructions. DNase-treated RNA (1 μg) was reverse transcribed to cDNA using the Super Script III First-Strand Synthesis System (Invitrogen Corp., Carlsbad, CA, USA). Gene expression was examined using quantitative RT-PCR. The cDNA was amplified through PCR with primer sets specific for the mouse Dnmt1, Dnmt3a, Dnmt3b, Mecp2_e1, and Mecp2_e2 genes. Real-time PCR was performed using a LightCycler rapid thermal cycler system (Roche Diagnostics Ltd., Lewes, UK) with LightCycler FastStart DNA MasterPLUS SYBR Green I mix (Roche Diagnostics Ltd.). The primers used in this study are shown in Table 1. Amplification of glyceraldehyde-3-phosphate dehydrogenase (Gapdh) mRNA was used as an internal positive control, and the amounts of each mRNA were normalized to the Gapdh mRNA level in each sample.

**Statistical analysis**

Statistical analyses were performed using one-way analysis of variance (ANOVA) and Bonferroni-Dunn post hoc tests with the StatView software for Windows (version 5.0; SAS Institute Inc., Cary, NC, USA). The data for each BPA-treated group were compared with those for the controls. *P* values less than 0.05 were considered statistically significant.

**RESULTS AND DISCUSSION**

The developmental period is an important time when elaborate DNA methylation patterns are programmed. To examine the effects of BPA on the DNA methylation system and CpG-DNA-mediated gene transcription in embryonic hypothalamic cells, we performed dose-dependent exposure of these cells to BPA and analyzed the changes in gene expression of Dnmts and Mecp2 isoforms e1 and e2. In this study, Dnmt1 and Dnmt3a mRNA expression in the 200 μM BPA-treated cells was significantly lower (*P* < 0.0001) than that in the controls (Fig. 1). In contrast, Dnmt3b mRNA levels were markedly and significantly increased in cells treated with 200 μM BPA (*P* < 0.0001) compared with that of the controls. Previous in vitro investigations on fetal rat hypothalamic cells demonstrated that BPA induces changes in both dendritic and synaptic development at 0.1 μM BPA (Yokosuka et al., 2008). Synaptic function in neurons is reported to be regulated by DNMTs (Feng et al., 2010; Smrt and Zhao, 2010). Based on the results given by Yokosuka et al. (2008), we originally predicted that BPA has some effect on Dnmts at a concentration close to 0.1 μM BPA. Contrary to our expectations, BPA affected the gene expression of Dnmts at relatively high doses.

Our results suggest that BPA has the potential to directly alter the gene expression levels of Dnmts in embryonic hypothalamic cells; however, these effects are not seen at the doses at which dendritic and synaptic development are affected. The previously reported effects of low doses of BPA on dendritic and synaptic development in embryonic hypothalamic cells are considered to be induced by a mechanism other than alteration of gene expression of Dnmts. Interestingly, gene expression of Dnmt3a and Dnmt3b showed opposite responses to BPA. Although
both enzymes act as de novo methyltransferases during the developmental period, responsiveness of these genes to BPA may not correlate to one another. Further research is needed to determine how BPA affects the epigenome, but it is suggested that alteration of gene expression of Dnmts, which are key players in the establishment of genomic methylation, may lead to the disruption of normal maturation of hypothalamic functions.

Mecp2_e1 mRNA levels in the 20 μM and 200 μM BPA-treated cells increased significantly (P < 0.001 and P < 0.0001, respectively) relative to the controls (Fig. 2). On the other hand, a significant increase in Mecp2_e2 mRNA was detected only in the 200 μM BPA-treated cells compared with the control cells (P < 0.0001). MECP2 regulates thousands of genes in the hypothalamus (Chahrour et al., 2008), and therefore the increase in Mecp2 gene expression elicited by BPA is expected to have enormous consequences in the hypothalamus. Martinowich et al. (2003) reported that MECP2 binds selectively to the promoter region of the brain-derived neurotrophic factor (Bdnf) gene and functions to repress the expression of the BDNF protein, which is highly expressed in hypothalamic tissue (Katoh-Semba et al., 1997). In our previous work (Warita et al., 2013), Bdnf mRNA expression in mHypoE-N44 cells significantly decreased at the same BPA dose that the increase of Mecp2 gene expression was observed in this study. Taking into consideration the current results, increased MEC2P2 elicited by BPA offers a clue as to the mode of reduction of Bdnf mRNA expression in embryonic hypothalamic cells. Dragich et al. (2007) demonstrated that MEC2P2_e1 and MEC2P2_e2 have distinct expression patterns within different brain areas and developmental stages and that MEC2P2_e1 is much more abundant than MEC2P2_e2. However, thus far, functional differences between the 2 isoforms have not been adequately investigated. In our results, the gene expression of Mecp2_e1 is more sensitive than that of Mecp2_e2 to BPA. Further investigation will be needed to determine the biological significance of these results.

Collectively, our results suggest that gene expression of Dnmts and Mecp2 are less susceptible to lower doses of BPA in developing hypothalamic cells. However, as BPA concentration increases, this agent has the potential to alter gene expression of key players that provide stability and flexibility of epigenetic gene regulation. From the results of in vivo experiments using rodents, such as in utero and/or neonatal exposure to BPA, it is unclear what concentration of BPA the embryonic hypothalamic cells are directly exposed to because of various compounding factors, such as drug metabolism in the dam, fetus, or neonate itself. However, alteration of gene expression of Dnmts and Mecp2 in developing hypothalamic cells is suspected as one of the causative factors of the previously reported long-lasting adverse effects observed in the rodents treated with high doses of BPA prenatally or neonatally.

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REFERENCES

Effects of bisphenol A on embryonic mouse hypothalamic cells in vitro


