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# DOWN-REGULATION OF INDUCIBLE NITRIC OXIDE SYNTHASE EXPRESSION BY BISPHENOL A

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## Introduction

Bisphenol A (BPA; 4,4'-isopropylidenediphenol) is a monomer in polycarbonate plastics and a constituent of epoxy and polystyrene resins that are used extensively in the food-packaging industry and in dentistry[1]. Environmental estrogens are a class of natural and synthetic compounds, which can mimic the function or activity of endogenous estrogen 17β-estradiol (E2). BPA has been shown to possess estrogenic properties, and is known an "endocrine disrupter" that has significant influence on sexual and reproductive development[2]. There is concern that the estrogenicity of BPA may elicit toxicity affecting mammalian developmental and reproductive processes[3,4]. However, the mechanism by which BPA causes these adverse effects is not clear.

Macrophages play a significant role in inflammation and host defense, and when activated they inhibit the growth of a wide variety of tumor cells and microorganisms. Nitric oxide (NO), a free-radical gas, is synthesized by inducible nitric oxide synthase (iNOS) and mediates diverse functions, including vasodilatation, neurotransmission, and immunoresponse[5]. The involvement of NO in nonspecific host defense, macrophage-mediated killing, and the inhibition of the proliferation of microorganisms and tumor cells have been previously demonstrated[5,6]. In macrophages, NF-κB in cooperation with other transcription factors has been found to coordinate the expression of genes encoding iNOS. Moreover, NF-κB plays a critical role in the activation of immune cells, by upregulating the expressions of many cytokines essential for immune response[7].

E2 has been shown to modulate macrophage function. E2 inhibits LPS-induced NO production and iNOS expression in macrophages[8]. However, the precise molecular mechanism of this inhibitory effect by E2 is unknown. Although BPA is widely used in the chemical industry, little is known about its effects on immune function or cytokine production. Studies on cytokine production are of considerable interest in terms of understanding of the toxicities of BPA on wildlife and human health. In terms of chemical structure, BPA is very similar to the synthetic estrogens, such as diethylstilbestrol, and BPA has been shown to mimic E2[2,3]. Recently we reported that BPA suppressed Cyp1a-1 in Hepa-1c1c7 cells[9]. In the present study, we investigated the effect of BPA on NO production and investigated its molecular mechanism in macrophages. The involvement of estrogen receptor (ER) in this process was also investigated using the ER antagonist, ICI 182.780.

## Methods and Materials

Peritoneal macrophages were isolated from BALB/C mice (female, 5-7 weeks old) and RAW 264.7 cells, a mouse macrophage cell line, cultured as described previously[10]. BPA was dissolved in dimethylsulfoxide and added directly to the culture media. Control cells were treated only with solvents, the final concentration of which never exceeded 0.1%, and this concentration did not

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show any effect on the assay systems. NO synthesis was determined by assaying the culture supernatants for nitrite using Griess reagent as described previously[10].

cDNA synthesis, semiquantitative RT-PCR for iNOS and β-actin mRNA, and the analysis of results were performed as described previously[10]. RAW 264.7 cells were transiently co-transfected with the plasmids pGL3-4κB-Luc and pCMV-β-gal, using LipofectAMINE Plus, and then the cells were treated with LPS and/or BPA. Luciferase and β-galactosidase activities were determined as described previously[10]. All experiments were repeated at least three times. Student's t-test was used to assess the statistical significance of differences. A confidence level of $< 0.01$ was considered significant.

**Results and Discussion**

Because E2 is known to inhibit LPS-induced NO release and iNOS expression in macrophages[16] and BPA has been shown to possess estrogenic properties[2,3], we decided to investigate the effects of BPA on NO production and its effects on the levels of iNOS gene expression in mouse macrophages. The potent macrophage activator LPS increased NO production compared to the control. However, BPA alone did not affect NO production, though BPA inhibited LPS-induced NO production in a dose-dependent manner in peritoneal macrophages and RAW 264.7 cells (Table 1). The BPA-mediated suppression of NO production was not due to a BPA cytotoxic effect. Cell viability was identical for cultures treated with BPA (data not shown). In addition, E2 was found to also inhibit LPS-inducible NO production, and this was consistent with earlier reports[8]. However, these effects of BPA were approximately 1,000-fold less potent than those of E2 on NO production. Regarding these results, previous studies have reported that the estrogenic activities of BPA, such as, the proliferation of MCF-7 human breast cancer cells, ER binding affinity, and yeast reporter-gene expression assays, were 1,000- or 10,000-fold lower than those of E2[11,12]. In order to assess whether the suppressive effects of BPA on LPS-inducible NO production might be influenced by the ER, ICI 182.780, a pure antiestrogen, was used, and it was found that these inhibitory effects of E2 or BPA were antagonized by ICI 182.780[13], implying that the ER mediates the suppressive effects of E2 or BPA (Fig. 1). It is known that iNOS can be transcriptionally regulated[15]. In order to determine whether BPA regulates NO production at the mRNA level, a RT-PCR assay was conducted with E2 as a positive control. Consistent with the results obtained from the NO assays, LPS-inducible iNOS mRNA levels were found to be markedly suppressed by BPA treatment (Fig. 2). Therefore, we believe that decreased LPS-inducible NO production by BPA is regulated through transcriptional activation. Several previous studies have shown that E2 treatment leads to reduced LPS-inducible NO production and iNOS expression[8], and this is confirmed by the present study (Fig. 2). The biological significance of the effect of BPA on LPS-inducible NO production needs to be determined.

According to the classical hypothesis, the cellular effects of estrogens are mediated by the intracellular ER, which serve as transcription factors. ER belongs to the superfamily of ligand-activated transcription factors, the nuclear receptors. E2-ER complexes bind to the genomic estrogen response elements. The estrogen-occupied receptor interacts with additional transcription factors and components of the transcription initiation complex to modulate gene transcription. No regions homologous to the consensus sequence of the estrogen response elements have been identified in the 5'-flanking regions of the iNOS genes. However, estrogens may inhibit the transcription of the iNOS genes by interacting with other sequences. Previous studies have shown

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that nuclear hormone receptors, including glucocorticoid receptor and progesterone receptor, might repress NF-κB activity[14]. NF-κB is a member of the Rel family, and is a common regulatory element in the promoter region of iNOS and cytokines such as, TNF-α and IL-1[7]. To further investigate the role of BPA on iNOS gene expression, the effect of BPA on NF-κB-dependent gene expression was assessed using the luciferase reporter gene assay. RAW 264.7 cells were transiently transfected with a plasmid containing 4 copies of the NF-κB binding sites and the luciferase activities were measured. Consistent with NO production and iNOS mRNA measurement, BPA also significantly decreased NF-κB-dependent luciferase activities in a dose dependent manner and co-treatment with ICI 182.780 recovered the decreased activity by BPA (Fig. 3). These results indicate that the down-regulation of the iNOS genes by BPA is mediated by the inhibition of NF-κB activation.

Our results indicated that BPA inhibition of LPS-inducible iNOS expression in macrophages is mediated through the NF-κB sites of the iNOS gene. However, the precise mechanisms by which BPA suppress iNOS expression in macrophages remains unknown. The current study suggests the possibility that BPA might act as an immunomodulator. Although information concerning the level of exposure to BPA in humans is limited, further in vivo study is necessary to understand whether BPA affect NO production and ultimately immune function.

**Table 1.** Effects of BPA on NO Production

| Treatment | Nitrite (μM) | |
|---|---|---|
| | Macrophages | RAW 264.7 |
| Control | 2.12 ± 0.22* | 2.33 ± 0.27* |
| BPA 50 μM | 2.34 ± 0.25* | 2.28 ± 0.28* |
| LPS 0.5 μg/ml | 15.27 ± 1.53 | 42.28 ± 4.37 |
| LPS + BPA 1 μM | 12.39 ± 1.43 | 37.14 ± 3.87 |
| LPS + BPA 10 μM | 8.23 ± 0.91* | 20.16 ± 2.16* |
| LPS + BPA 50 μM | 4.81 ± 0.52* | 7.91 ± 0.87* |
| LPS + E2 1 nM | 9.87 ± 1.13* | 29.22 ± 3.22* |
| LPS + E2 10 nM | 6.61 ± 0.83* | 18.45 ± 2.06* |

Values are expressed as means ± S.D. of three individual experiments, performed in triplicate. *$P < 0.01$, significantly different from the LPS.

**Fig. 1**

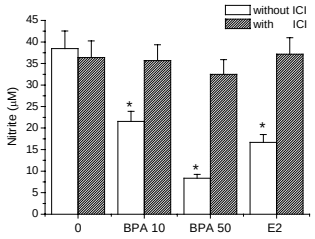


**Fig. 2**

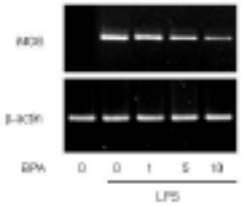


**Fig. 3**

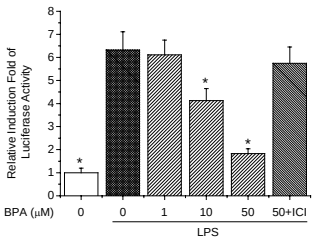

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**Fig. 1.** The effects of ICI 182,780 on NO production. RAW 264.7 cells were treated with BPA (10, 50 μM), E2 (10 nM), or ICI 182,780 (ICI; 100 nM) in the presence of LPS (0.5 μg/ml). Supernatants were harvested 24 h later and assayed for NO production. Values are expressed as mean ± S.D. of three individual experiments, performed in triplicate. *$P$ < 0.01, significantly different from the LPS.

**Fig. 2.** The effects of BPA on iNOS mRNA expression. RAW 264.7 cells were treated with LPS (0.5 μg/ml) and/or BPA (1, 10, 50 μM) for 6 h. One of three representative experiments is shown.

**Fig. 3.** Effects of BPA on NF-κB-dependent luciferase gene expression. RAW 264.7 cells were transiently co-transfected with pGL3-4κB-Luc and pCMV-β-gal. After 18 h, the cells were treated with LPS (0.5 μg/ml) and/or BPA (1, 10, 50 μM), ICI 182,780 (ICI; 100 nM) for 18 h, harvested and their luciferase and β-galactosidase activities determined. Luciferase activities were expressed relative to the control. Each bar shows the mean ± S.D. of three independent experiments, performed in triplicate. *$P$ < 0.01, significantly different from the LPS.

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