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Toxic Effects of Bisphenol S Showing Immunomodulation in Fish Macrophages

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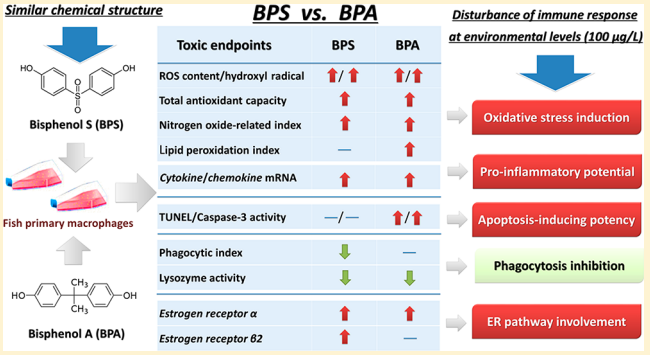
Supporting Information

ABSTRACT: Bisphenol S (BPS), a structural analogue of bisphenol A (BPA), has been increasingly used as a common replacement of BPA due to health concerns regarding the former. However, mounting evidence suggests that BPS has similar endocrine-disrupting effects as BPA, and likewise, its presence in the environment may pose considerable risks to ecosystems and human health. Using fish primary macrophages (fpMQs), we here evaluated the immunomodulatory effects of BPS and its mechanisms of action associated with estrogen receptors (ERs). Following BPS exposure at environmentally relevant concentrations from 0.1 to 1000 μg/L, we observed approximate concentration-dependent increases in nitric oxide and reactive oxygen species generation and total antioxidant capacity as well as the gene expression of inflammatory cytokines in fpMQs. BPS impaired phagocytic capability but enhanced fpMQ activation levels in response to lipopolysaccharide stimulation and promoted apoptosis, indicating an impact on cell functions. At a concentration of 100 μg/L, BPS and BPA showed comparable pro-inflammatory potential with both up-regulating the production of free radicals and cytokine expression; however, BPS had no significant potency with regards to inducing lipid peroxidation and apoptosis, different from BPA's effects. Moreover, BPS induced both *erα* and *erβ2* expression in fpMQs, whereas BPA induced only *erα* expression. This study demonstrates that, similarly to BPA, exposure to low doses of BPS significantly disturbs the immune response of fpMQs in vitro and first reveals overlapping but different roles of ERs in response to BPS and BPA.



INTRODUCTION

Bisphenol S (BPS), a common bisphenol A (BPA) replacement, has been used in a range of products due to health concerns regarding the former,[1] e.g., in baby feeding bottles, in thermal receipt paper, and in food packing and personal care products.[2−4] BPS is a structural analogue of BPA but more-stable at high temperatures and less bio- and photodegradable.[5−8] As an emerging environmental contaminant, the distribution of BPS has been reported worldwide.[4,9−11] For example, levels of BPS detected in surface waters of the Adyar River and Buckingham Canal in India have been found to range from ND to 7200 ng/L and from 58 to 2100 ng/L, respectively,[12] whereas levels of 4.1 to 160 ng/L have been recorded in Taihu Lake, China.[13] Further, a recent examination of human urine samples from the United States and Asian countries indicated that, whereas 93% had detectable levels of BPA, 81% had detectable levels of BPS.[9] It is thus expected that in the near future the presence of BPS will be as ubiquitous as that of BPA in the environment, and like BPA, it may pose considerable risks to ecosystem and human health.[12,14,15]

In a systematic review of 32 previous in vitro and in vivo studies, Rochester and Bolden[16] have indicated that BPS has similar endocrine-disrupting effects (estrogenic, anti-estrogenic, androgenic, and anti-androgenic) in the same order of magnitude as BPA. BPS, similarly to BPA, appears to act both as a weak estrogen via classical nuclear receptor models and as a strong estrogen via extranuclear-located estrogen receptors (ERs) or a new membrane estrogen receptor, GPER.[15,17−20] Additional receptors, such as the thyroid hormone receptor[21,22] and PPARγ,[23] are also involved in the

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action BPS. In terms of ER binding, BPS has a basic structural feature in common with BPA,[24] namely, a para-hydroxyl group on each of the phenol rings,[25] which may explain the common signaling pathways activated by BPS and BPA, as well as their similar low-dose effects and invariably nonmonotonic dose−response relationships. For instance, low-dose BPS exposure can interfere with early brain development in embryonic zebrafish,[21] induce precocious hypothalamic neurogenesis and concomitant hyperactive behavior in fish larvae,[26] and also exert female-specific pro-arrhythmic effects[27] and obesogenic effects in rodents,[28] which are remarkably similar to the effects reported for BPA.

According to the potential of BPS to induce oxidative stress both in vivo[29,30] and in vitro,[31,32] along with recent evidence on its pro-inflammatory effects on a mouse macrophage cell line,[33] the immunotoxicity of BPS has been implicated in its broad adverse effects. However, reliable results to substantiate the immunotoxic effect of BPS and the signaling pathways via which BPS exerts its mode of action (MOA) are still limited. In teleost, head kidney macrophages are important components in both nonspecific and specific defense and can serve as the first line of defense against invading pathogens, engulfing and destroying them by producing reactive oxygen species (ROS), and also work via the release of several pro-inflammatory cytokines important in promoting the initiation of host immune responses.[34] Also, the changes observed in head kidney macrophages have provided a useful early biomarker of low-level xenobiotic exposure.[34] Using fish head kidney macrophages as a model, we herein thoroughly explored the dose−response modulatory effects of BPS on macrophage response and functions as well as the associated ER signaling pathways and also compared the effects and MOA of BPS with those of BPA at the same mass concentrations. Our aim was to reveal whether BPS acts similarly to BPA in terms of its cell immunotoxicity potency and to identify the pathways via which its effects are mediated.

MATERIALS AND METHODS

Macrophage Isolation and Culture. Primary macrophages were isolated from the head kidney of red common carps (*Cyprinus carpio*) weighing 300−500 g, as described by Qiu et al.[35] The head kidney was aseptically extracted from healthy fish and washed two to three times with cell isolation and washing medium containing Hanks' balanced salt solution (Gibco BRL), heparin (10 U/mL), and penicillin and streptomycin (1%). The tissue was cut into pieces using sterile stainless-steel scissors and forceps, filtered through a 100 pm nylon mesh, and homogenized in phenol red-free Leibowitzs-15 medium (Gibco BRL) supplemented with heparin (10 U/mL) and penicillin and streptomycin. The tissue suspension was centrifuged three times at 1000 rpm for 5 min and resuspended in culture medium. The cells were counted using a hemocytometer and the concentration of the cell suspension was adjusted to 1×10^7 cells/mL. The cells were then plated in 96-well micro plates (Corning) at 100 μL/well or incubated in 25 cm^2 cell culture flasks (Corning) at 3 mL/flask. After overnight incubation at 26 °C, monolayers of adherent cells (approximately 1% of the seeded cells) in the microplates and culture flasks were washed twice with culture medium to remove unattached cells, and these were subsequently used for the chemical exposure experiments.

Lethal Concentrations. A stock solution of BPS was prepared by initially dissolving BPS in dimethyl sulfoxide (DMSO) and then adding culture medium, giving a BPS concentration of 10 g/L (DMSO at 20% v/v). Time-dependent lethal concentrations of BPS were determined at 6, 12, and 24 h after exposure of cultured fish primary macrophages (fpMQs) to BPS (Sigma-Aldrich, Saint Louis, MO) solutions at concentrations of 0, 0.1, 1, 2.5, 5, 10, 25, 50, 75, and 100 mg/L. Cytotoxicity was measured using a CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega). Following exposure to BPS solutions, the fpMQs were transferred to 20 μL culture medium containing 20 μL of MTS/PMS [3-(4,5-dimethylthiazol-2-yl)- 5-(3-carboxymethoxyphenyl)-2-(4-sulphenyl)-2H-tetrazolium, inner salt / phenazine methosulfate]. After incubation at 26 °C for 6 h, the absorbance of fpMQ at 490 nm was measured using a plate reader. The mortality of fpMQs was measured, and the time-dependent lethal concentrations at 50% (LC_{50}s) and 5% (LC_5s) were further confirmed by three independent experiments (Figure S1A and Table S2). The LC_5 of BPS for fpMQs was estimated at 1.52 mg/L after a 6 h exposure using Probit analysis, and hence, a 6 h exposure experiment was performed using BPS concentrations below this level, showing unchanged cell viability after exposure (Figure S1B).

Macrophage Exposure Experiment. On the basis of environmentally and ecologically relevant concentration of BPA,[36] the cells were then exposed to BPS at sublethal levels of 0.1, 1, 10, 100, and 1000 μg/L. Comparative analyses of the effects of BPS and BPA (Sigma-Aldrich, Saint Louis, MO) were performed after fpMQs had been exposed to 100 μg/L BPA or BPS for 6 h. A separate ER inhibition experiment involved coexposure of fpMQs to 100 μg/L BPS or BPA together with the ER antagonist ICI 182 780 (Tocris Bioscience) at 1 μM. A blank control group exposed only to culture medium and a vehicle control group (0.002% DMSO in culture medium) were also included in these experiments. The exposure solution in this study was phenol red-free culture medium, which was replaced completely every 24 h. Fresh stock solution was prepared every week, and at least three replicates for each treatment group were carried out.

Biochemical Assays. After 6 h of treatment, cells were washed and separately collected using cell scrapers for the subsequent bioassays. The protein level of each sample was determined using a Bradford assay (Beyotime Institute of Biotechnology). Lysozyme activity, total antioxidant capacity (T-AOC), hydroxyl radical formation, the lipid peroxidation level, total nitric oxide (NO) level, and the activities of total NO synthase (TNOS) and induced NO synthase (iNOS) were measured following the protocols of commercially available kits (Nanjing Jiancheng Bioengineering Institute). Intracellular ROS content was determined by measuring the oxidative conversion of 2′,7′-dichlorofluorescein diacetate (Beyotime Institute of Biotechnology) to fluorescent dichlorofluorescein (excitation of 488 nm and detection of 525 nm).[37] ROS positive cells was measured by counting fluorescent cells.

Immune-Related Gene Expression. Total RNA was extracted using an RNA prep pure tissue kit (Tiangen Biotech, China). Reverse transcriptase reactions were performed on 1 μg of total RNA using a Transcriptor First-Strand cDNA Synthesis Kit (Roche). Real-time quantitative PCR was performed using a FastStart Universal SYBR Green Master (ROX) kit (Roche) and iQ5Multicolor Real-Time PCR Detection System (Bio-Rad). Melting curve analysis and agarose gel electrophoresis were performed to validate the specificity of PCR amplicons. The endogenous 40S ribosomal protein S11 was selected as

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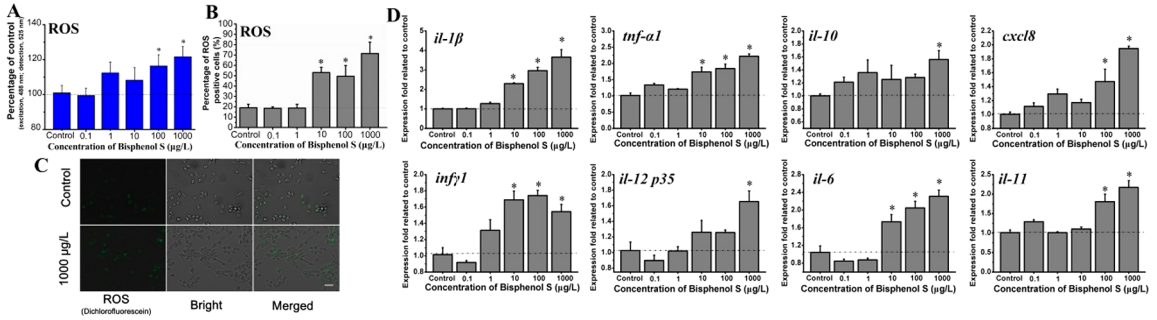

Figure 1. Increased generation of reactive oxygen species (ROS, A–C), and gene expression of cytokines and chemokines (D, n = 3) in primary macrophages after a 6 h exposure to bisphenol S (BPS). (A) Intracellular ROS content (n = 6); (B) ROS-positive cells (n = 6); (C) representative images comparing the ROS content in the control group and the 1000 μg/L BPS group (scale bar = 20 μm); (D) the mRNA levels of cytokines and chemokines. Values are the means ± standard deviation. An asterisk (*) indicates a significant difference vs the control at $p < 0.05$ (ANOVA, Tukey's test).

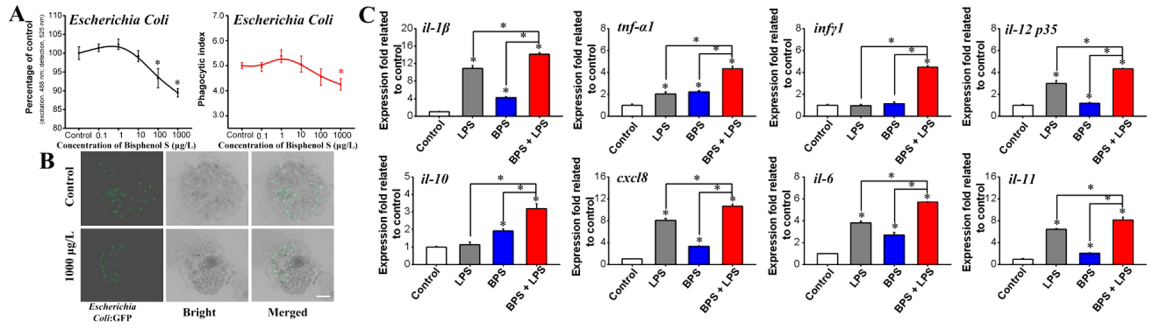

Figure 2. Changes in the antibacterial activities of primary macrophages after a 6 h exposure to bisphenol S (BPS). (A) The bactericidal activity (the black lines, n = 6) and phagocytic index (the red lines, n = 6). (B) Representative images comparing the phagocytic activities of the control and 1000 μg/L BPS groups (scale bar = 5 μm). (C) The effect of BPS on cytokine gene induction was assessed in fish primary macrophages after the simulation of 6 h BPS (100 μg/L) treated cells with 50 mg/L lipopolysaccharide (LPS) for 2 h. A blank control group cultured in BPS-free medium for 8 h, an LPS control group cultured for 6 h in BPS-free medium followed by 2 h of LPS stimulation, and a BPS control group (cells exposed to BPS for 6 h and then cultured in BPS-free medium for 2 h) were used parallel to these experiments. Values are the means ± standard deviation. An asterisk (*) indicates a significant difference vs the control at $p < 0.05$ (ANOVA, Tukey's test).

reference gene based on a previous study.[38] The primers specific for target genes and endogenous 40S ribosomal protein S11 are listed in Table S1. A Ct-based relative quantification with efficiency correction normalized to the reference gene was calculated using the $2^{-}\Delta\Delta^{Ct}$ method.

Phagocytic Activity. The phagocytic activity of macrophages against pathogenic Gram-positive *Staphylococcus aureus* (CGMCC 1.363), Gram-negative *Vibrio parahemolyticus* (CGMCC 1.1615), and green fluorescent protein (GFP)-expressing *Escherichia coli* (CGMCC 1.2389) were evaluated following the modified protocols[35,39] described in the Supporting Information. The bactericidal activity was assessed by measuring the fluorescence of extracellular *Escherichia coli* under a fluorescence microscope (excitation of 488 nm and detection of 525 nm). Phagocytic index was equal to the percentage of macrophages containing at least one bacterium multiplied by the mean number of bacteria per positive cell.

Lipopolysaccharide Challenge. Lipopolysaccharide (LPS: the major component of the outer membrane of Gram-negative bacteria and a known endotoxin) from *Pseudomonas aeruginosa* 10 was employed in the present study to activate fpMQs after exposure to BPS to assess the effect of BPS on the activation potential of macrophages. Briefly, fpMQs were washed three times after exposure to 100 μg/L BPS for 6 h and re-exposed in 50 mg/L LPS-supplemented culture medium for 2 h. The cells were collected for further analysis of the expression levels of cytokine- and chemokine-related genes. A blank control group (incubated in culture medium for 8 h), an LPS control group (incubated in LPS-free medium for 6 h and then LPS-supplemented medium for 2 h), and a BPS control group (incubated in BPS-supplemented medium for 6 h and then in BPS-free medium for 2 h) were set up in these experiments.

Apoptosis Level. The level of apoptosis was determined by measuring the labeling of DNA strand breaks using an in situ cell death detection kit (TUNEL Technology, Roche, Switzerland) and caspase-3 activity (Beyotime Institute of Biotechnology, China). Qualitative analysis of the DNA damage was conducted using an apoptotic DNA ladder kit (Applygen Technologies Inc., Beijing, China) following agarose gel electrophoresis. Approximately 3 μg of DNA for each sample was loaded onto a 2% agarose gel, electrophoresed at 60 V for 30 min and stained with ethidium bromide.

Statistical Analysis. The data were checked for normality and homogeneity of variance using Kolmogorov–Smirnov one-sample test and Levene's test. One-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference test were performed using SPSS Statistics 18.0 (SPSS Inc., Chicago, IL). For all the evaluated parameters, there was no significant difference between the blank and vehicle control groups, and thus, the vehicle control was used as the control group for the statistical analyses that followed. The level for statistical significance was set at $p < 0.05$ and indicated by an

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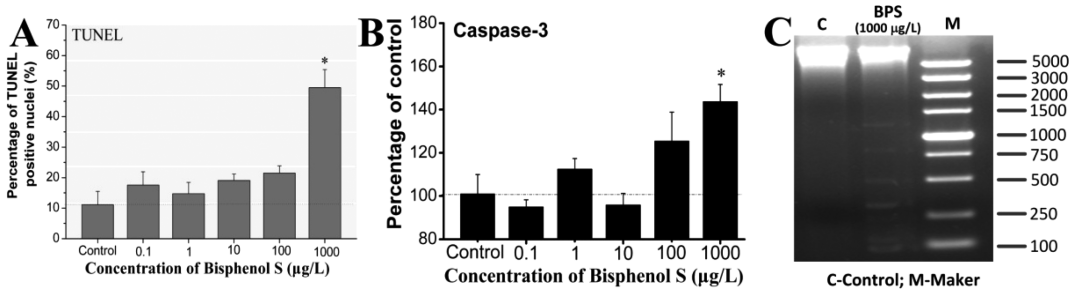

Figure 3. Induction of apoptosis in primary macrophages after 6 h of exposure to bisphenol S (BPS). (A) Percentage of TUNEL-positive nuclei (n = 6); (B) caspase 3 activity (n = 3); (C) DNA ladder formation. Values are the means ± standard error of the mean relative to the control. An asterisk (*) indicates a significant difference vs the control at $p < 0.05$ (ANOVA, Tukey's test).

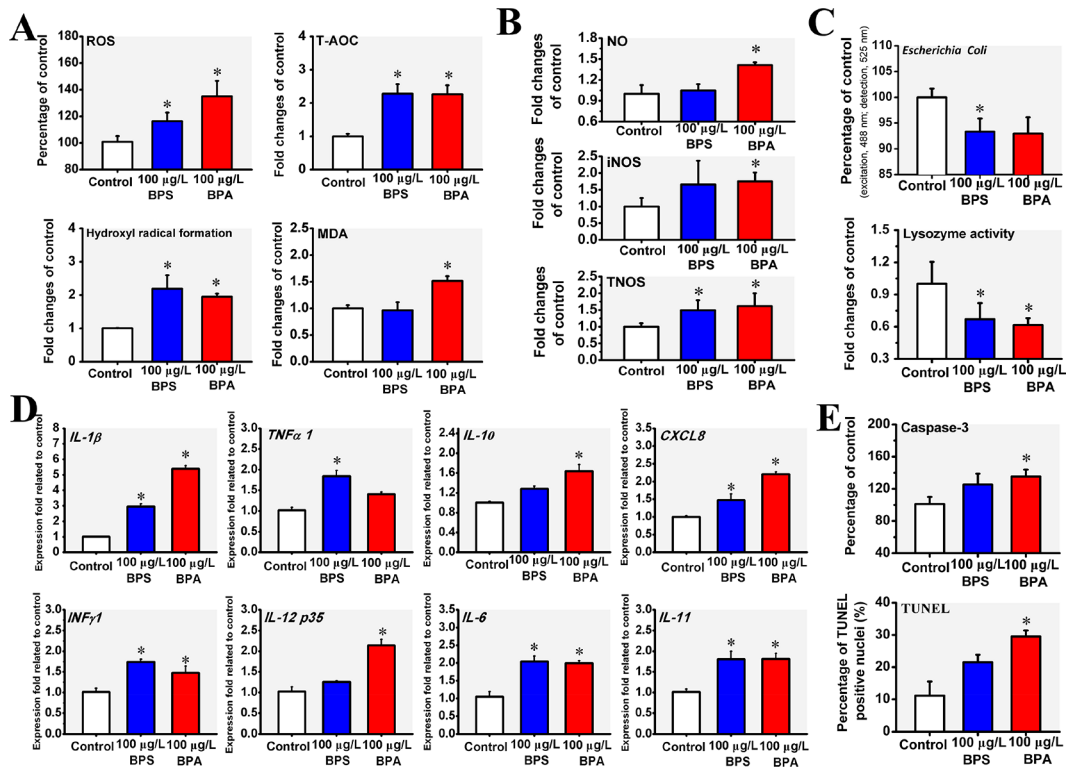

Figure 4. Comparison of the effects of bisphenol S (BPS, 100 μg/L) and bisphenol A (BPA, 100 μg/L) on primary macrophages after a 6 h exposure. (A) Reactive oxygen species (ROS), total antioxidant capacity (T-AOC), hydroxyl radical formation level, and lipid peroxidation level (MDA content); (B) nitric oxide production (NO), induced nitric oxide synthase (iNOS), and total nitric oxide synthase (TNOS) activities; (C) antibacterial activities and Lysozyme activity; (D) cytokine and chemokine gene expression; (E) caspase 3 activity and percentage of TUNEL-positive nuclei. Values are the means ± standard error of the mean relative to the control (n = 6 for ROS and n = 3 for the others). An asterisk (*) indicates a significant difference vs the control at $p < 0.05$ (ANOVA, Tukey's test).

asterisk. The values for all groups were expressed as mean ± standard deviation (SD)

RESULTS

Up-Regulation of Immune Response upon BPS Exposure. Oxidative stress and antioxidative defense parameters, as well as the expression of a number of cytokine- and chemokine-related genes, were assessed in fpMQs after a 6 h exposure to BPS. Cellular ROS content was significantly increased following exposure to a high concentration of BPS than in the control (Tukey's test, $p < 0.05$), as shown in Figure 1A–C. Correspondingly, significantly increased T-AOC, hydroxyl radical formation, and lipid peroxidation was observed in primary macrophages after a 6 h exposure to BPS at 10, 100, or 1000 μg/L (Figure S2). Increased NO levels and TNOS and iNOS activities were also observed in macrophages after a 6 h BPS exposure, as shown in Figure S3.

Similarly, the mRNA levels of cytokine and chemokine genes, including *il-1β*, *tnf-α1*, *il-10*, *cxcl8*, *infγ1*, *il-12(p35)*, *il-6*, and *il-11*, were considerably increased in an approximately concentration-dependent manner after exposure to BPS for 6 h (Figure 1D). Briefly, *il-10* and *il-12(p35)* were significantly up-regulated by exposure to 1000 μg/L BPS (Tukey's test, $p <$ 0.05), *cxcl8* and *il-11* were significantly upregulated by exposure to 100 and 1000 μg/L BPS (Tukey's test, $p < 0.05$), and *il-1β*, *tnf-α1*, *infγ1*, and *il-6* were significantly up-regulated in the 10, 100, and 1000 μg/L BPS groups (Tukey's test, $p <$ 0.05). However, the expression of cxc-chemokine, *il-12 p40*, *infγ2*, *tnfα2*, *m17* and *infα* had no significant difference between treatment group and control (Figure S4).

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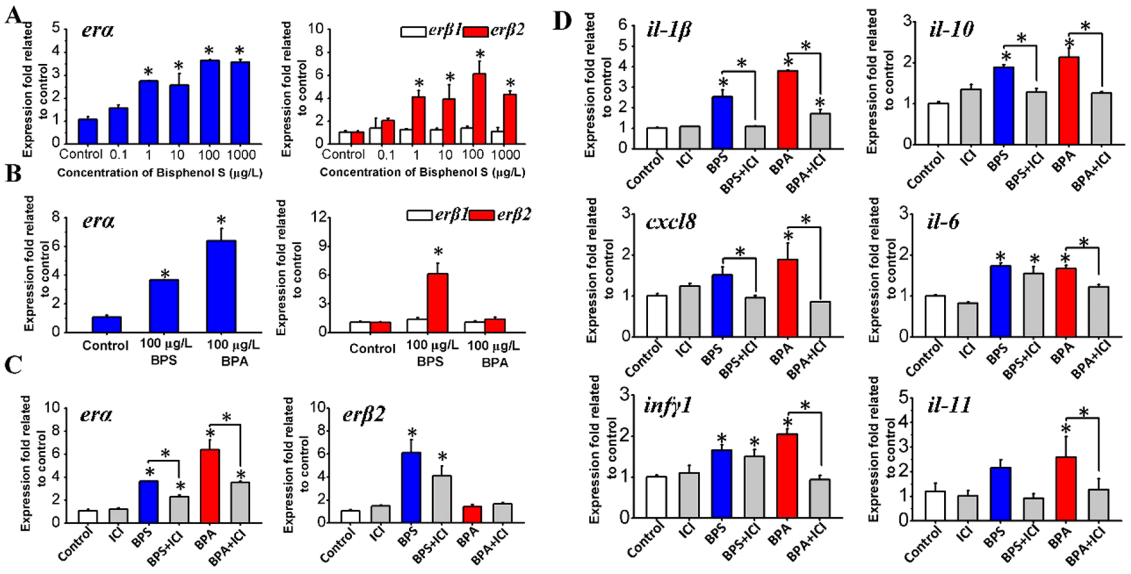

Figure 5. Roles of estrogen receptors (ERs) in mediating the effects of bisphenol S (BPS) and bisphenol A (BPA) on primary macrophages. (A) Effects on ER-α (*erα*), ER-β 1 (*erβ1*), and ER-β 2 (*erβ2*) mRNA levels after a 6 h exposure to BPS. (B) Comparison of the effects of BPS (100 μg/L) and BPA (100 μg/L) exposure on *er* mRNA levels after a 6 h exposure. (C,D) Effects of the co-exposure to an ER antagonist, ICI 182 780 (1 μM), with either BPS or BPA on *er* mRNA levels (C) and cytokine and chemokine mRNA levels (D). Values are the means ± standard error of the mean relative to the control (n = 6 for ROS and n = 3 for the others). An asterisk (*) indicates a significant difference vs the control at $p < 0.05$ (ANOVA, Tukey's test).

Altered Cell Functions after BPS Exposure. The phagocytic activity of fpMQs against GFP-expressing *E. coli* was significantly inhibited by exposure to BPS at relatively high concentrations, showing a decrease in both the fluorescence intensity of *E. coli* phagocytosed in cells and also calculated phagocytic indices (Figure 2A,B). The phagocytic activities of fpMQs against pathogenic Gram-positive *S. aureus* and Gram-negative *V. parahemolyticus* were both consistently reduced following exposure to high doses of BPS (Figure S5), and the cellular lysozyme activity was also significantly inhibited in the 100 and 1000 μg/L BPS treatment groups with respect to that in the control (Tukey's test, $p < 0.05$, Figure S6). Gene expression levels of the examined cytokines and chemokine were evaluated to assess the activation status of fqMQs exposed to BPS for 6 h followed by 2 h of LPS stimulation, as shown in Figure 2C. The expression levels of *il-1β*, *tnf-α1*, *infγ1*, *il-12(p35)*, *il-10*, *cxcl8*, *il-6*, and *il-11* were all significantly increased in cells exposed to BPS followed by LPS stimulation (BPS + LPS treatment) compared to either the blank controls or cells exposed to BPS or LPS alone. In particular, the expression of *il-10* and *infγ1* mRNAs was not induced by LPS stimulation alone but was significantly induced in the fpMQs after BPS + LPS treatment (Tukey's test, $p < 0.05$).

Induction of Apoptosis after BPS Exposure. A significant increase in the number of cells containing TUNEL-positive nuclei was observed in the 1000 μg/L BPS treatment group (Tukey's test, $p < 0.05$, Figure 3A and S7). Consistently, cellular caspase-3 activity was significantly increased in the 1000 μg/L BPS treatment group than in the control (Tukey's test, $p < 0.05$, Figure 3B). DNA ladder formation, considered a useful indicator of apoptosis, was also observed in the 1000 μg/L BPS exposure group (Figure 3C).

Comparison of the Effects of BPS and BPA. A comparative evaluation of the aforementioned immune response parameters was performed simultaneously in fpMQs exposed to 100 μg/L of either BPS or BPA. ROS, T-AOC, and hydroxyl radical formation (Figure 4A) were significantly increased in response to exposure to both BPS and BPA (Tukey's test, $p < 0.05$), whereas the lipid peroxidation level was significantly increased only by BPA treatment (Figure 4A) . Similarly, TNOS activity was significantly increased in response to both BPS and BPA treatments, whereas BPS exposure affected neither NO levels nor iNOS activity, although BPA treatment significantly increased the NO levels and iNOS activity (Tukey's test, $p < 0.05$) (Figure 4B). Generally, the expression levels of the eight examined immune-related genes were all increased after treatment with either BPS or BPA; however, whereas those of *il-1β*, *cxcl8*, *infγ1*, *il-6*, and *il-11* were significantly increased by both treatments, *tnf-α1* was significantly increased only in the BPS treatment group, and *il-10* and *il-12(p35)* were significantly increased only in response to BPA treatment (Tukey's test, $p < 0.05$) (Figure 4D). The phagocytic activity of macrophages against GFP-expressing *E. coli* was significantly altered only in the BPS treatment group, although inhibition was observed in both treatment groups (Figure 4C). In contrast, cellular lysozyme activity was significantly lower in both treatment groups (Tukey's test, $p < 0.05$) (Figure 4C). Furthermore, the caspase-3 activity of fpMQs and the number of cells with TUNEL-positive nuclei were significantly increased only by the BPA treatment (Tukey's test, $p < 0.05$) (Figure 4E), which is consistent with the results reported in our previous study[40] and also with other results obtained in the present study.

Roles of ERs in Mediating the Effects of BPS and BPA. The involvement and potential roles of ERα and ERβ in mediating the effects of BPA and BPS on immune regulation were determined simultaneously in fpMQs exposed to either BPS or BPA. As shown in Figure 5A, the expression of both *erα* and *erβ2* was significantly induced (Tukey's test, $p < 0.05$) by exposure to BPS at concentrations ≥1 μg/L. In contrast, exposure to BPA, even at the relatively high concentration of 100 μg/L, induced only *erα* expression (Figure 5B).

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Correlation analysis was performed between the evaluated parameters using Spearman's test (Table S3), which indicated that the expression levels of *erα* and *erβ2* induced in fpMQs by exposure to different concentrations of BPS were significantly correlated with changes in ROS content, T-AOC, and level of hydroxyl radical formation, as well as with cytokine gene expression and the level of apoptosis. These results are indicative of the important roles of ERα and ERβ2 in immune regulation after BPS exposure. Furthermore, as shown in Figure 5C, cotreatment with the ER antagonist ICI 182 780 significantly suppressed *erα* up-regulation (Tukey's test, $p < 0.05$) in fpMQs after either BPS or BPA treatment; however, it did not significantly block *erβ2* upregulation in response to BPS treatment. Consistent with this inhibition profile, the up-regulated expression of three examined genes, *il-1β*, *il-10*, and *cxcl8*, was significantly inhibited by cotreatment with ICI 182 780 in both BPS and BPA groups, whereas the induction of three other genes, *il-6*, *infγ1*, and *il-11*, was significantly suppressed by ICI 182 780 co-treatment only in the BPA group (Figure 5D). These results thus confirm the role of ER receptor pathways in the MOAs of BPA and BPS but also suggest that there are slight differences in the effects of BPA and BPS, as mediated by ER signaling.

DISCUSSION

Our results indicated that acute BPS exposure could evoke a dynamic concentration-dependent response in fpMQs in vitro. Alterations in most of the examined immunological end points were significantly correlated with each other, indicating a coordinated modulation of these physiological parameters for maintaining cell homeostasis upon exposure to BPS. On the basis of the approximately concentration-dependent induction of NO and ROS production, the total antioxidant capacity, and gene expression of inflammatory cytokines (such as *il-1β*, *tnf-α1*, *infγ1*, and *il-6*), the pro-inflammatory effects of BPS appeared to be elicited at low exposure doses, which is consistent with observation using a mouse macrophage cell line.[33] However, at higher BPS exposure concentrations, severe oxidative stress occurred, as indicated by the hydroxyl radical formation and lipid peroxidation at relatively high exposure concentrations, with a consequent disruption of cell homeostasis and promotion of apoptosis. Furthermore, cell functions such as phagocytic activity were significantly impaired in response to high concentrations of BPS, and cells exposed to BPS also exhibited a heightened response to endotoxin stimulation, particularly the over-expression of cytokines, including *il-1β*, *il-6*, *il-10*, and *infγ1*, upon LPS challenge. Moreover, through the simultaneous evaluation and thorough comparison of multiple end points in terms of the modulating effects and ER-involved signaling pathways, our results indicate the comparable effects of BPS and BPA at the same mass concentrations in regulating the response of immune cells and altering their immune functions. Furthermore, our findings revealed that the MOAs of BPS and BPA associated with ER pathways could be partly differentiated.

The that the acute toxicity test showedLC_{50}s of BPS for fpMQs at different time points during a 24 h exposure are in the order of milligrams per liter. Previously, it has been reported that the 24 and 48 h EC_{50}s of *Daphnia magna* in response to BPS were 76 and 55 mg/L, respectively,[41] whereas the 96 h LC_{50}s of the freshwater amphibian (*Pelophylax nigromaculatus*) in response to BPS were >100 mg/L.[42] Compared with these previously reported data, our results indicate that in vitro fpMQs are more sensitive to BPS exposure than in vivo aquatic organisms. Cellular ROS and NO contribute to the immune defense function of fish head kidney macrophages in destroying invading pathogens. However, overproduction of these free radicals typically gives rise to a cellular redox imbalance, thereby inducing oxidative damage, promoting apoptosis,[43−45] and impairing cell function. Consistent with this scenario, in the present study and in our previous study,[40] we observed that exposure to high concentrations of either BPS or BPA promoted the apoptosis and inhibited the phagocytosis of fpMQs, which might be attributable to the induction of severe oxidative stress in cells. In this regard, Zhang et al.[30] recently suggested that BPA and BPS exposure has resulted in elevated oxidative stress in people living in and around e-waste dismantling facilities. Similarly, Ullah et al.[29] reported that BPS potentially induces ROS and lipid peroxidation in male rats in vitro and in vivo, consistent with our observations.

Cytokines play a variety of regulatory roles in host defense and normal or abnormal homeostatic mechanisms.[46] The over-expression of inflammatory cytokine genes in fish macrophages after BPS exposure observed in the present study is relatively similar to the previously reported immunomodulatory effect of BPA,[47−49] which could promote an excessive immune response and result in damage to the immune cells. LPS represents a pathogenic stimulus that has the ability to elicit a strong immune response and activate oxidative and cytokine pathways associated with the inflammatory responses and pathophysiology of autoimmune responses. Challenge with LPS after exposure to 100 μg/L of BPS exposure led to overexpression of several cytokines, including *il-1β*, *il-6*, *il-10*, and *infγ1*, compared with the treatments with either LPS or BPS alone, indicating the potential of acute BPS exposure to induce an over activation of fpMQs. These results further confirmed the proinflammatory effects of BPS, and also imply the potential of BPS and other bisphenols to act synergistically with pathogenic stimulation in modulating the inflammatory response in aquatic organisms. Nevertheless, to verify this assumption, it will be necessary to determine the mechanisms underlying these effects.

The comparable immunomodulatory effects of BPS and BPA at 100 μg/L were revealed in the present study based on the similar enhancing effects on oxidative stress indices and inflammatory cytokine gene expression as well as similar inhibitory effects on the function of cells against bacteria, which are consistent with previous findings on BPA.[40] However, BPS showed slightly weaker potency than BPA with respect the induction of apoptosis at 100 μg/L, and BPS did not enhance the phagocytic activities of fpMQs within the relatively low concentration range of 0.1−1 μg/L, which contrasts with the inducing effects previously reported for BPA at the same doses.[40] Moreover, our results suggest a role of ERs in BPS and BPA exposure in head kidney macrophages. In this context, ERα and ERβ2 seem to be more important than ERβ1 in response to immunomodulation of BPS. Similar research has reported that 17β-estradiol induced the expression of both *erα* and *erβ2* but not *erβ1* during immune system development in sea bass head kidney.[50] Also, the effects of BPS were, however, significantly attenuated after coexposure with an ER antagonist, confirming the role of an ER receptor pathway in its action. These findings also indicate that there are slight differences in the MOAs of BPA and BPS, as mediated by ER signaling pathways. As shown in our results, only *erα* was induced in

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Environ. Sci. Technol. 2018, 52, 831−838

response to BPA exposure, which consisted with the capacity of BPS to activate both ERα and ERβ in the HELN cell line,[51] and the ability of BPA to induce *ERα* but not *ERβ* gene expression in various types of cells.[26,40,52]

In short, this study revealed the immunomodulatory effect of BPS on head kidney macrophages in a concentration-dependent manner, and both ERα and ERβ2 pathways were involved in its action mechanism. Our study provides foundational information using a unique model system for investigating mechanisms of BPS on immunological end points. The comparable pro-inflammatory potential of BPA and BPS provides important supporting evidence that BPS is not necessarily a safer alternative to BPA.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b04226.

Additional experimental materials and methods details. Figures showing acute and sublethal toxicity of bisphenol S, primary macrophage data, induction of nitric oxide data, the expression of immune-related genes in primary macrophages, changes in the phagocytic index, changes in lysozyme activity, and representative fluorescent images. Tables showing primers used for real-time PCR, lethal concentrations, and correlation coefficients. (PDF)

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