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The In Vitro Immune Modulatory Effect of Bisphenol A on Fish Macrophages via Estrogen Receptor α and Nuclear Factor-κB Signaling

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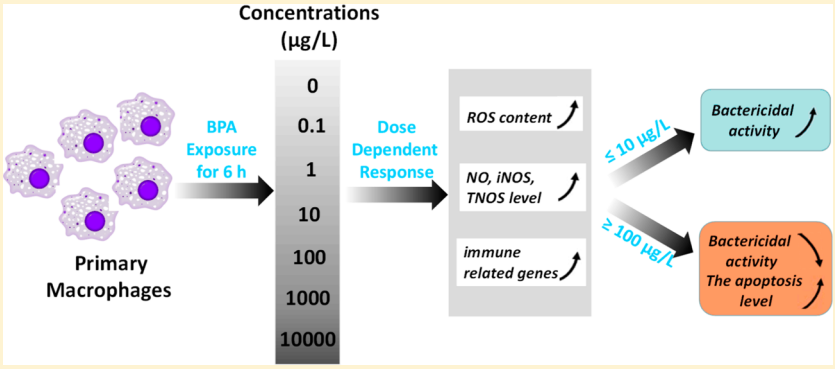
Supporting Information



ABSTRACT: Bisphenol A (BPA) is a well-known environmental endocrine-disrupting chemical. Employing primary macrophages from head kidney of red common carp (*Cyprinus carpio*), the present study aimed to evaluate the immune modulatory effect of BPA and to explore its potential action mechanism associated with estrogen receptor (ER) and nuclear factor-κB (NF-κB) pathways. A dynamic response process was observed in macrophages upon various concentrations of BPA exposure, which significantly enhanced the antibacterial activity of macrophages at 0.1, 1, or 10 μg/L, but instead induced the apoptosis at 100, 1000, and 10 000 μg/L. A potential pro-inflammatory effect of BPA exposure was suggested, judging from the increased production of nitrite oxide and reactive oxygen species (ROS), the induction of *interleukin-1β* mRNA and protein, as well as *NF-κB* and other *NF-κB*-associated immune gene expression. Following BPA coexposure with the ER or NF-κB antagonist, the induction of ROS, *ERα*, and *NF-κB*-associated immune gene expression was significantly inhibited, implying interaction between those two pathways. This study thus indicated that low doses of BPA exposure alone could significantly disturb the immune response of fish primary macrophages in vitro, and for the first time revealed the synergistic action of ERα and NF-κB transcription factors in the BPA effect.

INTRODUCTION

Bisphenol A (BPA) is a well-known endocrine disrupting chemical (EDC), a constituent of certain plastic products widely used in plastic food and beverage containers, metal can linings, thermal receipt paper, dental sealants, and household paper products. It is listed as one of the highest volume chemicals produced globally, with more than 6 billion pounds of production annually.[1] According to the reported data from the Toxics Release Inventory, U.S.A., the total release of BPA to the environment was about 2.5 million kg in 2007, with the direct release of 13 772 kg to water.[2] As a consequence of its ubiquitous existence in the environment and extensive application in our daily lives, BPA has raised considerable public health concerns since it is widely detected at low nanomolar levels in a variety of human samples such as urine, blood and breast milk.[3,4] Meanwhile, a large number of studies have detected BPA contamination in various aquatic environments at μg/L to even low mg/L or mg/kg and, therefore, its detrimental effects on aquatic animals have also raised great concern about the healthy and sustainable development of aquatic organisms.[5−7]

The immune system is crucial to the survival of organisms against pathogens and various environmental stresses. However, due to regulation by sex steroids, it is also a potential target for environmental exogenous hormones.[8,9] BPA has a relatively weak estrogenic potency compared to natural estradiol (E_2) based on its binding affinity or activating capacity

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to estrogenic receptors (ERs) from in vitro assays.[10,11] However, the in vivo studies indicate that BPA can promote estrogen-like activities that are similar to or stronger than E_2.[12] Consequently, as a xenoestrogen, BPA has the ability to mimic the effects of natural estrogens or compete with endogenous estrogens binding to ERs and disrupting the immune defense of organisms. Previous studies have reported the potential effect of BPA in modulating the immune response, however, the data obtained using mammalian models are paradoxical concerning the immune suppressing or immune stimulating effect of BPA exposure.[13−15] In contrast, there are few studies exploring the potential immunotoxicity of BPA to aquatic animals based on an environmentally relevant scenario.

Fish and mammalian immune systems share a number of similarities. There is evidence that ERs are expressed in piscine immune cells and participate in the regulation of immune response.[16] Thus, xenoestrogens may target fish immune system and modulate their immunocompetence.[17,18] Previous studies using fish models imply that the effect of BPA on the immune response is more likely prone to be pro-inflammatory at low doses.[14] The exposure to BPA at μg/L or low mg/L can lead to functional changes of fish lymphocytes and macrophages, such as increasing the cellular production of superoxide anions, enhancing cell proliferation and elevating leukocyte counts in vivo.[14,19,20] Moreover, our recent in vivo study indicates that BPA exposure significantly impacts the immune response of zebrafish at environmentally relevant low concentrations.[21] Several inflammatory mediators including nitric oxide (NO), cytokines and chemokines in zebrafish embryos are significantly induced upon exposure to BPA at μg/L to low mg/L, which is similar to the patterns induced by lipopolysaccharide stimulation, indicating that BPA exposure at low doses alone could significantly induce the immune response.

Macrophages are responsible for both nonspecific defense and specific defense. Fish macrophages are morphologically, histochemically, and functionally similar to their mammalian counterparts.[22] They display phagocytosis, produce oxygen radicals, synthesize cytokines/chemokines, and also have bactericidal activity. Fish macrophages express ERs and are regulated by estrogens.[23] Common carp (*Cyprinus carpio*) is one of the most important aquaculture species in the word. Widespread in Asia, red common carp is popular for food and also for decorative purposes. In the present study, the primary macrophages from head kidney of red common carp were isolated and cultured, and effects of BPA were investigated on macrophages upon a series of concentrations of BPA exposure. We determined their antibacterial function, oxygen radical production, as well as gene expression of an antimicrobial peptide hepcidin[24] and several cytokines including pro-inflammatory *interleukin-1β* (*IL-1β*), *interleukin-6 subfamily like cytokine (M17)*, and anti-inflammatory *IL-10*, which were important immune parameters associated with the activation of macrophages. Besides, the apoptosis level of macrophages was assayed. It has been reported recently that BPA affected cytokine expression in human macrophages through ER-dependent mechanism associated with extracellular regulated protein kinases/nuclear factor-κB (NF-κB) signaling.[25] Similarly, we investigated the potential roles of ERα and NF-κB transcription factors in BPA effects on fish macrophages. Our study, thereby, further testified that BPA exposure at low doses significantly modulated the immune response of primary macrophages in vitro, and for the first time revealed the synergistic action of ERα and NF-κB transcription factors in the BPA immune modulating effect.

MATERIALS AND METHODS

Chemicals. BPA (CAS number 80−05−7, 99+%) was purchased from Sigma-Aldrich (St. Louis, MO). The stock solution was prepared by dissolving the chemical in dimethyl sulfoxide (DMSO) to a concentration at 10 g/L. Fresh stock solution was made every week. All other chemicals used were of analytical grade and purchased from Sigma−Aldrich (St. Louis, MO) and Sangon (Shanghai, China).

Primary Macrophages. Red common carp weighing 300−500 g, were purchased from a fish farm in Qingpu District of Shanghai, China. Fish were acclimated to laboratory conditions in dechlorinated tap water at room temperature for at least 2 weeks and then healthy fish were chosen for the experiments. The method for isolation of primary macrophages from the head kidney of the carp was modified based on previous studies[26,27] and the experimental details were shown in Supporting Information (SI). The cells were then adjusted to a density of 1×10^7 cells/mL and plated in 96-well microplates (Corning) at 100 μL/well or in 25 cm^2 cell culture flasks (Corning) at 3 mL/flask. After overnight incubation at 26 °C, monolayers of adherent cells (about 1% of the seeded cells) in the microplates and culture flasks were washed twice to remove unattached cells, and then they were used for the chemical exposure. The viability of cells was assessed using the trypan blue (Sigma-Analytical) exclusion test. Primary macrophages were identified using nonspecific esterase staining and Wright-Giemsa staining methods (SI Figure S1).[28]

Experimental Design. Primary macrophages were exposed to BPA solutions at 0.1, 1, 10, 100, 1000, and 10 000 μg/L in phenol red-free medium for 6 h. After treatment, cells were washed three times and separately collected for the subsequent bioassays. An ER inhibition experiment was designed separately using coexposure of macrophages for 6 h to 5 mg/L of BPA with an ER antagonist ICI 182,780 (Tocris Bioscience, United Kingdom) at 50 μM.[11] Acute exposure of primary macrophages to 1000 μg/L of BPA for 48 h was performed, the concentration chosen based on the response of cells to 6 h exposure. The time-course response of primary macrophages was then measured at 6, 24, and 48 h. Two nuclear receptor inhibition groups were designed using coexposure either to 1000 μg/L of BPA with ICI 182,780 at 50 μM, or to 1000 μg/L of BPA with an NF-κB antagonist pyrrolidine dithiocarbamate (PDTC, Sigma) at 50 μM.[29] A blank control group and a vehicle control group (0.002% DMSO in culture medium) were set up in parallel with these experiments and at least three replicates were set for each treatment group. The exposure solution was replaced completely every 24 h.

Bactericidal Activity and Phagocytic Capability of Primary Macrophages. After 6 h exposure to BPA, macrophages were washed several times with antibiotic-free culture medium to completely remove BPA residue, and then they were inoculated with freshly prepared bacterial culture to determine the bactericidal activity and the phagocytic capability against Gram-positive *Staphylococcus aureus* (CGMCC 1.363) and Gram-negative *Escherichia coli* (CGMCC 1.2389), and *Vibrio parahaemolyticus* (CGMCC 1.1615) following modified protocols described by previous studies.[30] The detailed methods are shown in SI.

Biochemical Assays. Cytotoxicity was measured using CellTiter 96 AQueous Non-Radioactive Cell Proliferation

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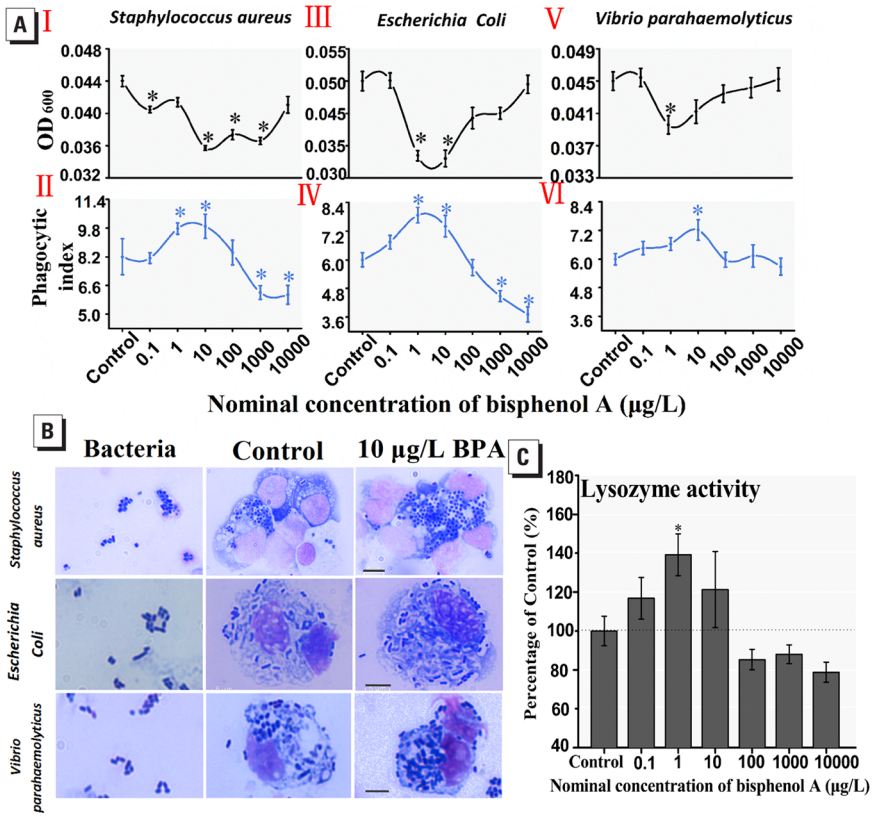

Figure 1. Changes in antibacterial activities of the primary macrophages after 6 h-exposure to bisphenol A (BPA). (A) The bactericidal activity was assayed by measuring the absorbance of extracellular bacteria at 600 nm (the black lines, $n = 6$), and the phagocytosis was measured by counting the intracellular bacteria (the blue lines, $n = 6$). (B) The representative images comparing the phagocytic activities between the control group and the 10 μg/L of BPA group (scale bar = 5 μm). (C) The lysozyme activity ($n = 3$). Values are means ± standard deviation. Significant differences versus control are indicated as $^*p < 0.05$ (ANOVA, Dunnett's test).

Assay (Promega) following the manufacturer's instructions. The protein level of each sample was determined using Bradford assay (Beyotime Institute of Biotechnology, China). Lysozyme activity, total 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 reactive oxygen species (ROS) content was determined by measuring the oxidative conversion of 2′,7′-dichlorofluorescein diacetate (Beyotime Institute of Biotechnology, China) to fluorescent dichlorofluorescein.[31]

RNA Isolation, Reverse-Transcriptase, And Quantitative Polymerase Chain Reaction. 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 the FastStart Universal SYBR Green Master (ROX) kit (Roche) and the 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 subunit S11 of the ribosomal gene 40S was selected as reference gene based on a previous study.[32] The primers specific for target genes and the endogenous *40S ribosomal protein S11* are listed in Table S1 (SI). A Ct-based relative quantification with efficiency correction normalized to *40S ribosomal protein S11* was calculated using the $2^{-\triangle\triangle Ct}$ method.

TUNEL Staining. The apoptosis level of primary macrophages after exposure to various concentrations of BPA was determined based on the labeling of DNA strand breaks (TUNEL technology) by fluorescein using an in situ cell death detection kit (Roche) following the manufacturer's instructions. Cells were costained with 4′,6′-diamidino-2-phenylindole to identify the nuclei. TUNEL-positive cells were analyzed with a confocal fluorescence microscope (LSM 780 NLO, ZEISS) and the percentages of TUNEL-positive cells were then calculated.

Enzyme-Linked Immunosorbent Assay for Interleukin 1β. Interleukin 1β levels in the cell homogenates were measured using a Fish Interleukin 1β ELISA Kit (MyBioSource) employing the quantitative sandwich enzyme immunoassay technique. Antibody specific for fish IL-1β was precoated onto a 96-well microplate. A series of gradient dilutions of IL-1β standard and the concentrations of IL-1β in the samples were then interpolated from the standard curve.

Statistical Analysis. All data were normalized using the Kolmogorov–Smirnov one-sample test and Levene's test. Statistical analyses were performed using SPSS Statistics 18.0 (SPSS Inc., Chicago, IL). For all the tested parameters, there was no significant difference between the blank control group and the vehicle control group, and thus the vehicle control was set as the control group for the statistical analysis that followed.

RESULTS

Bactericidal Activity, Phagocytic Capability and Lysozyme Activity. The viability of primary macrophages was not impaired after 6 h exposure to BPA based on the cytotoxicity assay (SI Figure S2). In contrast, the bactericidal activity of primary macrophages was induced in a U-shaped

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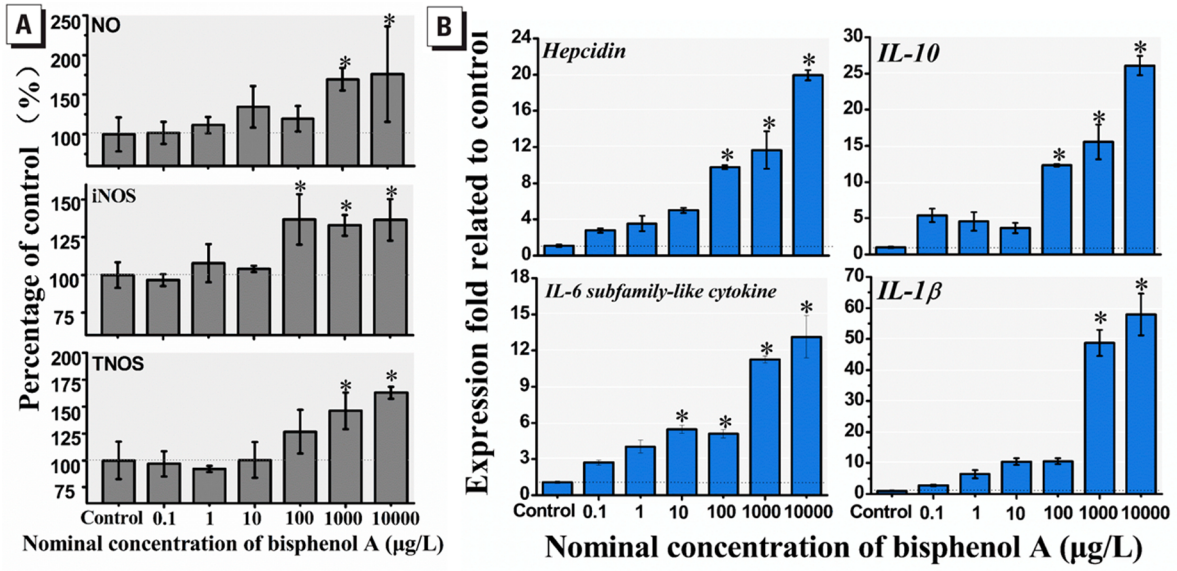

Figure 2. Induction of nitric oxide production, induced and the total nitric oxide synthase activities (A), as well as the expression of immune related genes (B) in the primary macrophages upon 6 h-exposure of bisphenol A. Values are means ± standard deviation (n = 3). Significant differences versus control are indicated as *p < 0.05 (ANOVA, Dunnett's test).

pattern judging from the decrease of bacterial density based on the OD_{600} value (Figure 1A I, III, V). Especially, in some BPA treatment groups at environmentally relevant concentrations of 1 or 10 μg/L, the bactericidal activity was significantly highly promoted compared to the control (Dunnett's test, p < 0.05). Correspondingly, the phagocytic capability of primary macrophages was changed in an inverse U-shaped pattern as shown in Figure 1A II, IV, VI. Consistent with the bactericidal activity, the calculated phagocytic index of primary macrophages was highest in the 1 or 10 μg/L BPA treatment group. The enhanced phagocytosis of cells exposed to 10 μg/L of BPA is shown in Figure 1B. Consistently, as shown in Figure 1C, the cellular lysozyme activity was also changed in an inverse U-shaped pattern after BPA exposure, and the highest induction was presented in the 1 μg/L BPA treatment group compared to the control (Dunnett's test, p < 0.05).

NO Level, and iNOS and TNOS Activity. A roughly concentration-dependent increase of NO level, and iNOS and TNOS activity was observed in macrophages upon 6 h exposure to BPA, as shown in Figure 2A. The NO level and TNOS activity significantly increased in the 1000 and 10 000 μg/L groups. However, the iNOS activity significantly increased in the 100, 1000, and 10 000 μg/L groups (Dunnett's test, p < 0.05).

Expression of Immune Related Genes. Gene expression of a broad-spectrum antimicrobial peptide and an acute phase protein, *hepcidin*, as well as several pro-inflammatory mediators was assessed to evaluate the effects of BPA on primary macrophages after 6-h exposure. Generally, the mRNA levels of the tested genes including *hepcidin*, *IL-10*, *M17*, and *IL-1β* were dramatically induced in a roughly concentration-dependent manner after BPA exposure (Figure 2B). The mRNA levels of *hepcidin* and *IL-10* increased significantly in the 100, 1000, and 10 000 μg/L groups (Dunnett's test, p < 0.05); the mRNA level of *M17* was significantly induced by exposure to 10, 100, 1000, and 10 000 μg/L of BPA (Dunnett's test, p < 0.05); and the mRNA level of *IL-1β* in the macrophages was significantly induced by exposure to 1000 and 10 000 μg/L.

ROS Content, NF-κB Expression and the Apoptosis Level. To evaluate the damage of acute BPA exposure on the macrophages, the cellular ROS content was examined after 6 h exposure, and it was significantly induced in a concentration dependent manner in the 100, 1000, and 10 000 μg/L treatment groups compared to the control (LSD test, p < 0.05) as shown in Figure 3A. Correspondingly, a significant increase of *NF-κB* (*p65*) expression was also observed in

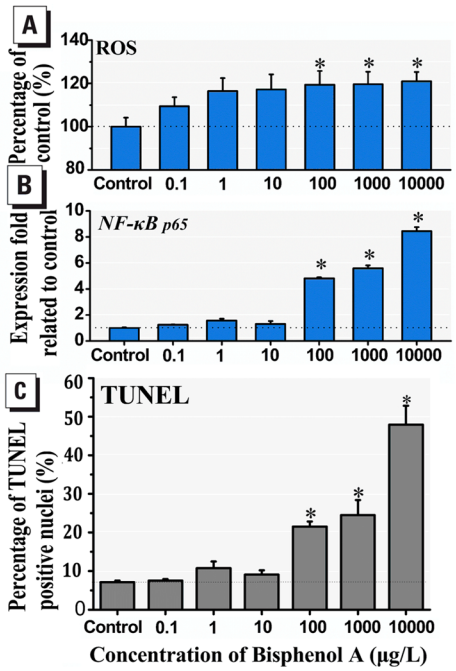

Figure 3. Bisphenol A induced the production of reactive oxygen species (ROS) (A), the expression of *NF-κB* (B) and the apoptosis (C) in primary macrophages upon 6 h-exposure. Values are means of the standard error of means relative to the control (n = 6 for ROS and n = 3 for the others). Significant differences versus control are indicated as *p < 0.05 ((ANOVA, LSD's, or Dunnett's test)).

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primary macrophages upon 6-h exposure to BPA at 100, 1000, and 10 000 μg/L (Figure 3B). A concentration-dependent increase of apoptosis level was observed in primary macrophages upon 6 h exposure to BPA, as shown in Figure 3C and Figure S3 in SI. A significant increase of the cell number of TUNEL positive nuclei was shown in the 100, 1000, and 10 000 μg/L BPA treatment groups (Dunnett's test, $p < 0.05$).

Gene Expression of ERα. To understand the potential activity of ERs in the immune response upon BPA exposure, the expression level of *ERα* was examined in primary macrophages. As shown in Figure 4A, the mRNA level of

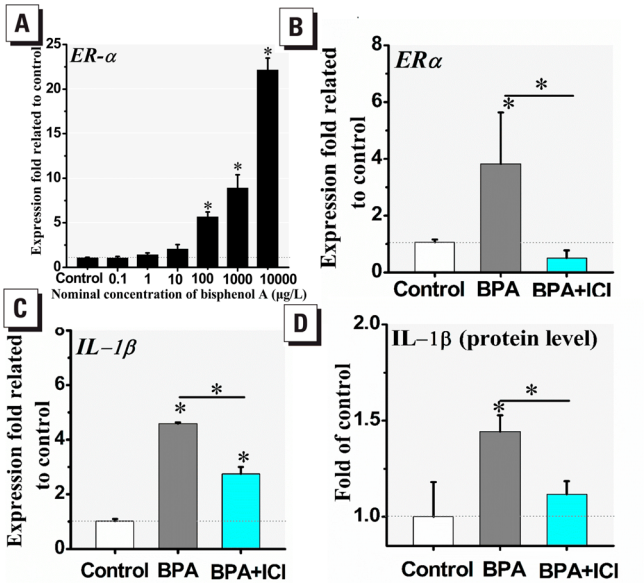

Figure 4. Bisphenol A (BPA) induced *estrogen receptor α (ERα)* expression in the primary macrophages upon 6 h exposure (A). The estrogen inhibitor ICI 182,780 (50 μM) significant decreased the induction of *ERa* (B) and *interleukin 1β (IL-1β)* expression (C) and the protein level of IL-1β (D). Values are means of the standard error of means ($n = 3$) relative to the control. Significant differences versus control or between groups are indicated as *$p < 0.05$ ((ANOVA, LSD's or Dunnett's test)).

ERα increased significantly (Dunnett's test, $p < 0.05$) as BPA concentrations were raised to 100, 1000, and 10 000 μg/L in a concentration-dependent manner. After correlation analysis between the tested parameters using Spearman's test (SI Table S2), it was notable that the elevated *ERα* expression levels in macrophages upon BPA exposure were significantly correlated with the changes of lysozyme activity, iNOS and TNOS activities, as well as tested immune related gene expression and apoptosis levels in our study, which implied an important role of *ERα* in the immune regulation upon acute BPA exposure.

The Effect of an Estrogen Receptor Antagonist. To determine the involvement of *ERα* in the immunotoxicity of BPA exposure, coexposure of the cultured macrophages to a relatively high concentration of BPA with an ER inhibitor ICI 182 780 was performed and the mRNA levels of *ERα* and *IL-1β,* as well as the protein level of IL-1β in macrophages, were detected after exposure (Figure 4B, C, and D). The results showed that coexposure to BPA with ICI 182 780 efficiently inhibited the induction of *ERα* expression. The expression of *IL-1β* and IL-1β protein level in macrophages were both induced after BPA exposure, which was in parallel with the increased gene expression of *ERα.* However, after coexposure to BPA with ICI 182 780, the induction levels of *IL-1β* mRNA and IL-1β protein in macrophages were both significantly decreased compared to the BPA exposure group (LSD test, $p < 0.05$), indicating an involvement of the ER pathway in the immune modulation upon BPA exposure.

The Time-Course Response of Macrophages upon Coexposure to BPA with ER or NF-κB Antagonist. The viability of primary macrophages was not significantly impaired during 48 h exposure to BPA and coexposure to BPA with ER or NF-κB antagonist compared to the vehicle control (SI Figure S4). As shown in Figure 5A, the cellular ROS content was significantly induced upon exposure to BPA after 6 and 24, but not after 48 h. However, upon coexposure to BPA with ICI 182 780 or PDTC, the induction level of ROS content was significantly inhibited after 6 h by the antagonist effects of the ER or *NF-κB* inhibitor. Similar to the results of ROS content, increased mRNA levels of *ERα, IL-1β, hepcidin, M17,* and *IL-10* were observed in the BPA treatment group after 6, 24, and also 48 h (Figure 5B). However, the induction levels of those genes were all significantly decreased (Dunnett's test, $p < 0.05$) by coexposure with ICI 182,780 at all the time points or PDTC after 6 and 24 h. The results indicated that ER inhibitor ICI 182 780 significantly inhibited the induction of the ROS content and the immune gene expression in macrophages upon exposure to BPA. In addition, the immune response mediator NF-κB antagonist PDTC in turn could significantly restrain the ER induction in macrophages upon BPA exposure.

DISCUSSION

Using fish primary macrophages as a model, our study presented a dynamic response process of immunological parameters in macrophages upon various concentrations of BPA exposure. With increased BPA exposure, ROS and NO levels, iNOS and TNOS activities, as well as immune gene expression such as *IL-1β, IL-10* in the macrophages was significantly induced in a concentration-dependent manner, consistent with our previous in vivo observations on zebrafish embryos upon 7 day BPA treatment.[21] Moreover, the alterations of most tested parameters were significantly correlated (SI Table S2), indicating a coordinated modulation process in macrophages responded to increased BPA exposure. Consistent with our observation on *M17* (*IL-6 like*), Liu et al.[25] have showed that BPA treatment increased pro-inflammatory cytokines *IL-6* and tumor necrosis factor-α (TNF-α) production in THP1 macrophages and primary human macrophages at BPA concentrations of 0.01 and 0.1 μM, that is, about 2 and 20 μg/L. Also, Cabas et al.[33] have reported that 17α-ethynylestradiol, an environmental estrogen, at low concentrations increase the mRNA levels of *IL-1β, IL-6,* and *TNF-α* in fish macrophages, which is similar to our observations on that BPA exposure alone has a pro-inflammatory effect in vitro. Tissue macrophages, as well as newly recruited monocytes, are subject to a hierarchy of activation states, such as phagocytosis, cellular activation and the release of cytokines, chemokines and growth factors. The activated macrophages and their released pro-inflammatory products participate in both beneficial and detrimental outcomes during the inflammatory process and lead to the pathogenesis of inflammatory and degenerative diseases.[34] Therefore, the activated and promoted status of macrophages upon BPA exposure would be adverse to organisms in the long term, and may lead to an increase of local inflammatory burden, thus

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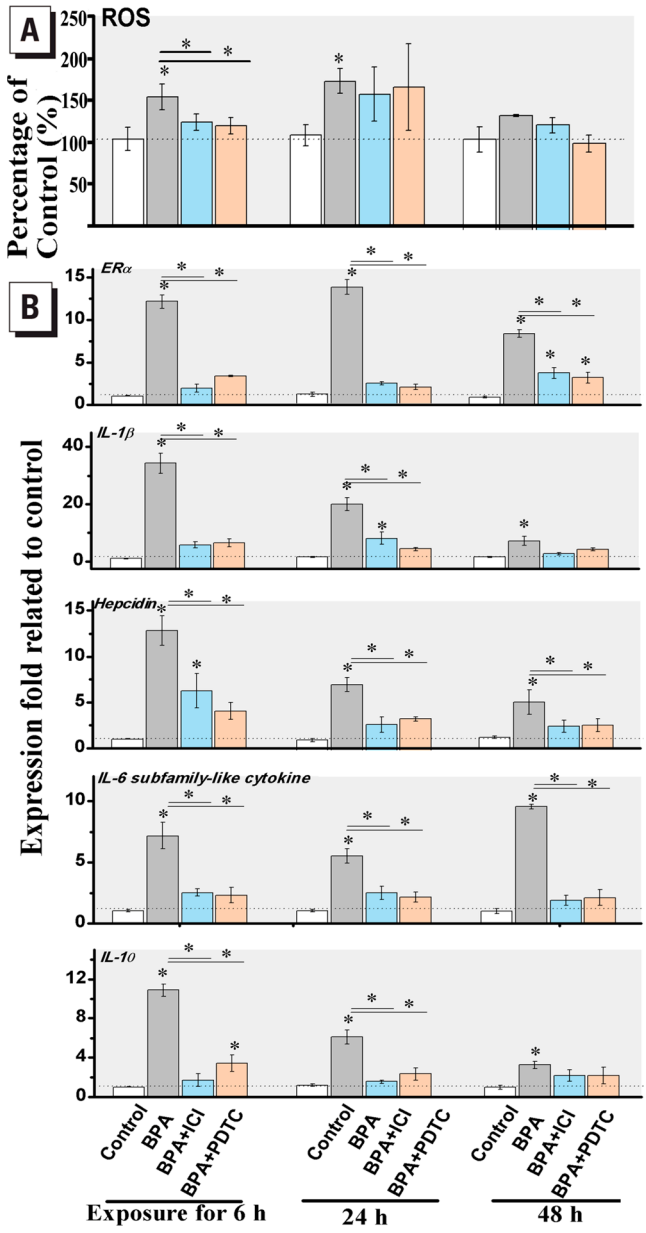

Figure 5. Production of reactive oxygen species (ROS) (A) and the expression levels of *estrogen receptor a (ERa)*, *interleukin-1β (IL-1β)*, *hepcidin*, *interleukin-6 subfamily like cytokine (M17)*, and *interleukin-10 (IL-10)* (B) in the primary macrophages upon 48 h exposure to bisphenol A (BPA, 1000 μg/L), a mixture of BPA (1000 μg/L), and ICI 182 780 (50 μM), and a mixture of BPA (1000 μg/L) and pyrrolidine dithiocarbamate (PDTC, 50 μM). Values are means of the standard error of means (n = 6 for ROS and n = 3 for the others) relative to the control. Significant differences versus control or between groups are indicated as $*p < 0.05$ (ANOVA, Dunnett's test).

affecting the acquisition or progression of inflammatory disorders.[14]

Our results indicated that the effect of BPA on bactericidal function of macrophages was a nonmonotonic dose response, in a manner that at low concentrations of 0.1, 1, and 10 μg/L it enhanced antibacterial activity, whereas at higher concentrations it impaired the antibacterial activity and promoted the apoptosis. Those observations are consistent with the findings by Iwanowicz et al. on that low physiological concentrations of E_2 (100 pM and 1 nM) significantly enhance the bactericidal activity of fish leukocytes, while pharmacological concentrations decrease bactericidal activity coincided with an enhanced ability of producing intracellular superoxide (i.e., respiratory burst activity).[35] Similarly, we observed a significant outburst of oxygen free radical production, ROS and NO, in fish macrophages upon high doses of BPA treatment, which implied occurrence of potential oxidative stress in the cells. There is mounting evidence implicating that oxidative stress is involved in the regulation of apoptotic machinery induced by various environmental stressors.[36] In addition, oxidative stress-dependent apoptosis also has been reported as an important regulatory process of carp leukocytes upon in vitro and in vivo immunostimulation.[37] Accordingly, our observation on increased apoptosis in the high doses of BPA treated macrophages thus might be associated with BPA induced oxidative stress, as well as the dramatic regulation of cell status upon the BPA challenge.

Nuclear factor-κB is recognized as a key regulator of proinflammatory gene expression activated in response to diverse inflammatory stimuli and environmental stressors.[38] Previous studies demonstrate that BPA exposure can lead to differential regulation of NF-κB. BPA induces apoptosis and activates the NF-κB signaling pathway in mouse hippocampal HT-22 cells.[39] However, it has been reported that BPA affects the regulation of the immune system by reducing NO and tumor-necrosis factor-alpha via the inhibition of NF-κB transactivation in the macrophages.[40] Pro-inflammatory *IL-1β* is an NF-κB-driven gene, and *M17* and *IL-10* are presumed NF-κB pathway-associated genes.[38,41] Hepcidin genes have putative NF-κB binding motifs in the upstream region of their genes, and the results in mouse macrophages reveal the involvement of NF-κB signaling in hepcidin expression.[42] In addition, NF-κB is the first transcription factor shown to be redox-regulated, and ROS generation in macrophages is closely associated with the NF-κB pathway.[43] Our observations on the induction of ROS, *p65* expression and NF-κB-associated gene expression in macrophages upon BPA exposure implied the inducing effect of BPA on the NF-κB pathway. Moreover, the coexposure with PDTC inhibited the induction of ROS and relevant NF-κB-driven gene expression, suggesting that the general symptoms of proinflammatory response caused by BPA treatment had been dampened down by inhibition of the NF-κB pathway. Therefore, these observations confirmed the involvement of NF-κB pathway in modulating the immune response of primary macrophages upon BPA exposure.

ERs belong to the nuclear receptor superfamily and are activated by the hormone estrogen and translocated into the nucleus where they bind to target DNA and regulate gene transcription.[44] Most of the estrogenic EDCs, including BPA, interfere with the normal estrogen signaling pathway by interacting with two forms of ERs: ERα and ERβ. These two ERs play their respective, but to some extent overlapping, physiological roles in mediating estrogen signaling. The ability of BPA to induce ERα gene expression has been explored in various types of cells across diverse taxa.[45] Reports show that ERα is the primary estrogen nuclear receptor expressed and functioning in macrophage biology,[46,47] consistent with our observation on the ERα expression in fish macrophages. Moreover, our observation on ERα, but not ERβ, signaling in the immune modulating effect of BPA on macrophages was highly similar to previous reports on the immune regulating effect of E_2 on mouse macrophages[48,49] as well as fish leukocytes.[35]

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In mammalian models, the functional interaction between ER and NF-κB can serve as an important regulatory link between the endocrine and immune systems.[50] From our results, after the coexposure of macrophages to BPA with either ER or NF-κB inhibitor, the induction of cellular ROS levels, ERα expression, as well as *hepcidin* and *IL-1β*, *IL-10*, *M17* expression were almost all significantly inhibited during the 48 h time course, suggesting a potential interaction of ER and NF-κB signaling pathways in immunomodulation upon BPA treatments. It should be noted that the interaction between ER and NF-κB signaling pathways was bidirectional in macrophages upon BPA exposure: promoted cellular immune regulation and NF-κB-associated gene expression were accompanied by the induction of *ERα* expression; but the suppression of NF-κB signaling restrained not only the immune-related parameters but also ERα expression; and blocking of the ER pathway inhibited not only the induction of ERα expression but also the symptoms of the pro-inflammatory response. Therefore, our results demonstrated positive synergism between ER and NF-κB signaling pathways in the immune modulating effect of BPA on fish macrophages. In contrast to the negative crosstalk between ER and NF-κB, the positive interaction between these two pathways has rarely been reported and is proposed to be highly promoter-specific.[38,51] As to the molecular basis for the synergistic action of ER and NF-κB pathways underlined in the immunomodulatory mechanism of BPA exposure, this still remains elusive and needs to be further explored.

In short, this study revealed the immunomodulatory effect of BPA on fish macrophages in a concentration-dependent manner within a relatively low and environmentally relevant concentration range. A pro-inflammatory effect of BPA was observed, and the involvement of ERα and NF-κB pathways was suggested in its action mechanism. Our findings provide further evidence that BPA interferes with the immune regulation of fish macrophages under in vitro conditions. The data provided in the study shall strengthen our understanding of BPA immunotoxicity in fish.

■ ASSOCIATED CONTENT

Supporting Information

Additional details and description of some of the methods used as well as supporting figures and tables referred to in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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