Bisphenol A stabilizes Nrf2 via Ca$^{2+}$ influx by direct activation of the IP$_3$ receptor

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ABSTRACT — Bisphenol A (BPA) is an endocrine-disrupting chemical used in polycarbonate and epoxy resins. Previously, we found that BPA stabilized the protein levels of nuclear factor erythroid 2-related factor 2 (Nrf2) by inducing Ca$^{2+}$ efflux into the cytosol, followed by nitric oxide synthase activation, resulting in the enhanced nitrosylation of Keap1, which is a negative regulator of Nrf2. However, the mechanisms behind the stimulation of Ca$^{2+}$ efflux by BPA remain unknown. In the present study, we found that BPA stimulated Ca$^{2+}$ efflux into the cytosol from the ER, but not from outside of cells through the plasma membrane in Hep3B cells. Ca$^{2+}$ efflux and Nrf2 stabilization by BPA were inhibited by an inhibitor of the inositol 1,4,5-trisphosphate (IP$_3$) receptor, 2-aminoethoxydiphenylborane, in the endoplasmic reticulum. IP$_3$ is produced by activation of phospholipase C (PLC) from a membrane lipid, phosphatidylinositol 4,5-bisphosphate (PIP$_2$). The induction of Nrf2 by BPA was not inhibited by a PLC inhibitor, U-73122, suggesting that BPA does not induce the production of IP$_3$ via PLC activation. We found that BPA bound directly to the IP$_3$ binding core domain of the IP$_3$ receptor, and BPA competed with IP$_3$ on this site. In addition, overexpression of this domain of the IP$_3$ receptor in Hep3B cells inhibited the stabilization of Nrf2 by BPA. These results clarified that the IP$_3$ receptor is a new target of BPA, and that BPA induces Ca$^{2+}$ efflux from the endoplasmic reticulum via activation of the IP$_3$ receptor.

Key words: Bisphenol A (BPA), Nrf2, IP$_3$ receptor

INTRODUCTION

Bisphenol A (2,2-bis(4-hydroxyphenyl)propane; BPA) is an endocrine-disrupting chemical used in polycarbonate and epoxy resins and it is widely present in the environment (Vandenbarg et al., 2009). BPA causes several biological events by interacting with important signaling proteins, including the estrogen receptor (Gould et al., 1998), estrogen-related receptor gamma (Liu et al., 2014), thyroid hormone receptor (Zoeller et al., 2005), glucocorticoid receptor (Prasanth et al., 2010), and pregnane X receptor (Sui et al., 2012). In addition to the endocrine-disrupting activities of BPA, it has been suggested that BPA can aggravate several diseases, including cancer and neural disorders in estrogen receptor-independent pathways (Gao et al., 2015). We found that HIF-1alpha (Kubo et al., 2004), gamma-secretase (Baba et al., 2009), and protein disulfide isomerase (PDI) (Hiroi et al., 2006) are also target proteins of BPA. Recently, we found that BPA stabilized the nuclear factor erythroid 2-related factor 2 (Nrf2), leading to the induction of drug-metabolizing enzymes and transporters, such as UGT2B1 and MDR1 in rat livers and human hepatoma (Hep3B) cells (Nakamura et al., 2018). Nrf2 has antioxidant and detoxifying effects and it enhances the resistance of cancer cells to chemotherapeutic drugs and tumor progression (Taguchi and Yamamoto, 2017). Nrf2 is trapped by Kelch-like ECH-associated protein (Keap1), an adaptor protein of ubiquitin ligase, and is ubiquitinated, leading to proteasome-mediated degradation (Hayes and Dinkova-Kostova, 2014; Taguchi et al., 2011). Binding of active radical species such as reactive oxygen species, nitric oxide (NO), and electrophiles to the thiol groups of cysteine residues in Keap1 induces the release of Nrf2 from Keap1, and the
stabilized Nrf2 can translocate into the nucleus and activate the transcription of several genes, including antioxidant and drug-metabolizing enzymes (Takaya et al., 2012). We found that BPA increased cellular NO levels by enhancing of Ca\textsuperscript{2+} efflux into the cytosol and induced Keap1-nitrosylation (Nakamura et al., 2018). Endothelial NO synthase (eNOS) and neuronal NOS (nNOS) are activated by the small cytosolic Ca\textsuperscript{2+}-binding protein, calmodulin, at elevated cellular Ca\textsuperscript{2+} concentrations (Stuehr and Haque, 2019). In many different cell types, it has been found that BPA can increase intracellular Ca\textsuperscript{2+} concentrations (Tanabe et al., 2006; Ehrenmann et al., 2017; Gao et al., 2013; Wozniak et al., 2005), that is via activation of estrogen receptor \alpha or \beta, because BPA can act as a xenoestrogen. BPA also induces cellular Ca\textsuperscript{2+} concentrations by the activation of G protein-coupled estrogen receptor GPER/GPR30 located in the plasma membrane (Cao et al., 2017). However, the mechanisms behind the Ca\textsuperscript{2+} efflux induced by BPA in Hep3B cells and BPA targeting proteins for BPA-induced Nrf2 stabilization are still unknown.

In the present study, we found that inositol 1,4,5-trisphosphate (IP\textsubscript{3}) receptor, intracellular channels that release Ca\textsuperscript{2+} from the endoplasmic reticulum (ER), is a new target protein of BPA. Activation of IP\textsubscript{3} receptor is typically stimulated by binding of a ligand to a G protein coupled receptor in the plasma membrane, subsequent activation of phospholipase C (PLC), which produces IP\textsubscript{3} and diacylglycerol from membrane phospholipids such as phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) (Berridge, 2009). We found that BPA did not induce IP\textsubscript{3} production via PLC, but directly bound to the IP\textsubscript{3} receptor and activated it to influx of Ca\textsuperscript{2+} from the ER, which is required for the Nrf2 stabilization.

MATERIALS AND METHODS

Materials

Dulbecco’s modified Eagle’s medium (DMEM), BPA, anti-DYKDDDDK (FLAG) antibody, LY294002, 2-aminoethoxydiphenylborane (2-APB), and NOC7 were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). DMEM (high glucose, no glutamine, no calcium) was obtained from Thermo Fisher Scientific (Waltham, MA, USA). U73122 and D-myo-inositol 1,4,5 triphosphate, sodium salt (IP\textsubscript{3}) were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). Fetal bovine serum (FBS) was from Sigma Chemical Company (St. Louis, MO, USA). Fluo-4AM was from AAT Bioquest (Sunnyvale, CA, USA). The antibodies against Nrf2 or Keap1 were prepared as described previously (Baba et al., 2013). An antibody against BiP was obtained from GeneTex (Irvine, CA, USA).

Cell culture and treatment of cells with BPA

The human hepatoma cell line Hep3B was from the Cell Resource Center for Biomedical Research at the Institute of Development, Aging, and Cancer of Tohoku University (Miyagi, Japan). Cells were maintained in DMEM containing 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL), and maintained at 37°C in 5% CO\textsubscript{2} and 95% air. BPA at concentrations of 1-50 μM was added to the cells for 6 hr in accordance with the previous reports (Yoneda et al., 2003).

Measurement of NO in Hep3B cells and culture medium

After treatment of Hep3B cells with BPA for 6 hr, the cells were washed with phosphate-buffered saline (PBS) and lysed in HENC buffer (250 mM HEPS, 1 mM EDTA, 0.1 mM neocuproine, 0.4 CHAPS, pH 7.5) for 30 min. The cell lysates were centrifugated and tenfold diluted with PBS. 5 mM 2,3-diamino naphthalene (DAN; Tokyo Kagaku Kougyou, Saitama, Japan) was added to the sample, and stored for 10 min in the dark. A 0.7 N NaOH solution was added, and nitrite (NO\textsubscript{2}-), a metabolite of NO, was measured using a plate reader (EnVision 2104 Multilabel reader, Perkin Elmer, Waltham, MA, USA) with an excitation wavelength of 380 nm and emission wavelength of 450 nm. The amount of produced NO\textsubscript{2}- was determined using a calibration curve prepared with NaNO\textsubscript{2}.

Ca\textsuperscript{2+} flux assay

Hep3B cells were washed with PBS and incubated with 5 μg/mL Fura-4 AM and 0.04% Pluronic F127 Probenecid in recording medium (20 mM HEPS, 115 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl\textsubscript{2}, 1.8 mM CaCl\textsubscript{2}, 13.8 mM glucose, pH 7.4) for 1 hr at 37°C. After washing with PBS, recording medium with was added to the dishes. Cells were stimulated with BPA and/or 2-APB, and the fluorescence intensity was monitored at 485/535 nm (excitation/emission) every 1 sec for 2 min with an EnVision 2104 Multilabel reader. Recording medium without CaCl\textsubscript{2} was used for measurement of changes in cellular Ca\textsuperscript{2+} that were independent of influx from outside the cells.

RT-PCR

Total RNA was isolated from Hep3B cells with Isogen (Nippon Gene, Tokyo, Japan) in accordance with the manufacturer’s instructions, and reverse transcribed.
Table 1. Primers used in this study.

| Primer numbers (human) | GenBank Accession No. | Sequences | Cycles | Annealing temperature (°C) |
|------------------------|-----------------------|-----------|--------|---------------------------|
| eNOS Forward           | NM_000603.4           | 5'-ACTACAGCTCCATTAAGAGGAG-3', 5'-AACACTTGATGGCAGCGGAA-3' | 35     | 52                        |
| eNOS Reverse           |                       | 5'-AACTTGCTCATAGATGTCGAA-3', 5'-ACGTGCTCGAGATGTGTTCAA-3' |        |                           |
| iNOS Forward           | NM_000625.4           | 5'-TGAGCCCTCCTAACAGTACT-3', 5'-ACGTGCTCGAGATGTGTTCAA-3' | 35     | 52                        |
| iNOS Reverse           |                       | 5'-AAGCAGATGAGCTCTAAAGTGGGAA-3', 5'-CCAATGTCCTTAAAGGTTGAGGAG-3' |        |                           |
| nNOS Forward           | NM_000620.4           | 5'-AACGGTGCCCAAGGCTCT-3', 5'-CCCTCTGCGATCCTTCTGCGA-3' | 35     | 52                        |
| nNOS Reverse           |                       | 5'-GAGCAGATGAGCTCTAAAGTGGGAA-3', 5'-CCAATGTCCTTAAAGGTTGAGGAG-3' |        |                           |
| β-actin Forward        | NM_001101             | 5'-CAAGAGATGCGCAAGGCTCT-3', 5'-CCCTCTGCGATCCTTCTGCGA-3' | 18     | 55                        |
| β-actin Reverse        |                       | 5'-GAGCAGATGAGCTCTAAAGTGGGAA-3', 5'-CCAATGTCCTTAAAGGTTGAGGAG-3' |        |                           |

into cDNA. PCR amplification was carried out using Go taq polymerase. Primers, GenBank accession numbers, and amplification cycles are shown in Table 1. Band intensity was quantified using the Image J program, and the relative eNOS mRNA transcript levels were normalized using β-actin.

Isolation of IP3 binding core domain of IP3 receptor (ITPR1224~604aa) cDNAs and preparation of constructs

The cDNA of human IP3 receptor (ITPR1) was isolated from Hep3B cells by PCR. The primers for human ITPR1 (Acc No. NM_001099952.2) were 5′- AA\underline{GGATCCATGAAATGGAGTGATAACAA}-3′ (forward primer for ITPR1 224~604aa ; underline, BamHI site; double underline, start codon), 5′-\underline{GAATTCTCATTTCCGATTTATTGTGGAGCA}-3′ (reverse primer for ITPR1 224~604aa; underline, EcoRI site; double underline, stop codon). PCR was performed using KOD Plus Neo. The amplified ITPR224~604aa cDNA was inserted into 3×FLAG-pcDNA4 vector using BamHI and EcoRI.

Preparation of BPA affinity resin and binding assay with IP3 binding core domain

BPA-Sepharose resin was prepared by coupling BPA amine derivative to cyanogen bromide-activated Sepharose 4B (Amersham Biosciences, Piscataway, NJ, USA) as described previously (Hiroi et al., 2006). HEK293T cells were transfected with ITPR224~604aa/pcDNA, and after 2 days, the cells were lysed in Lysis buffer (50 mM Tris-HCl [pH 8.0], containing 150 mM NaCl, 0.5% NP40, and 0.05% PIC). After centrifugation, the lysate was mixed with BPA affinity resin and incubated at 4°C for 1 hr. The resin was washed with wash buffer (50 mM Tris-HCl [pH 8.0], containing 150 mM NaCl, 0.05% NP40) five times, and further incubated with or without 200 µM IP3 at 4°C for 1 hr. After washing the resin with wash buffer, bound proteins were eluted with SDS-sample buffer at 95°C for 5 min and analyzed by SDS-PAGE and western blotting.

Statistical analysis

Statistical analysis for single comparisons between means was carried out with the Student’s t test, and p < 0.05 was considered statistically significant. For multiple comparisons, one-way ANOVA followed by a Bonferroni/Dunn post-hoc test was used.

RESULTS

BPA stabilized Nrf2 protein levels, and increased the levels of NO and Ca2+ in Hep3B cells

BPA was added to Hep3B cells at concentrations of 1-50 µM, and Nrf2 levels were investigated by western blotting. Nrf2 levels tended to be increased by 5 µM BPA (Fig. 1A) and significantly increased by 10 and 50 µM BPA (Figs. 1A and B). Nrf2 bands often appear as two bands close together as indicated by bracket (Nakamura et al., 2018; Kemmerer et al., 2015). The upper band of the two appears to be due to phosphorylation (Apopa et al., 2008). Previously, we found that stabilization of Nrf2 by BPA was inhibited by NOS inhibitor and Keap1 was nitrosylated by BPA, indicating the Nrf2 stabilization was dependent on NOS activity (Nakamura et al., 2018). Hence, we next investigated changes in the levels of three major NOS mRNAs, eNOS, nNOS, and inducible NOS (iNOS). Expression of only eNOS mRNA, but not nNOS and iNOS was detected in Hep3B cells, and eNOS mRNA was increased by the addition of 50 µM or 100 µM BPA (Fig. 1C). Furthermore, the treatment of Hep3B cells with BPA increased cellular Ca2+ concentrations (Fig. 1E). Generally, eNOS is activated by Ca2+-dependent calmod-
ulin, and we showed previously that a calcium ionophore stabilized Nrf2 as well as BPA. These results suggested that the increase in cellular NO levels and subsequent stabilization of Nrf2 by BPA was due to the activation of eNOS by increased cellular Ca\(^{2+}\) concentration and induction of eNOS mRNA levels.

**Nrf2 stabilization by an increase in cellular Ca\(^{2+}\) concentration by BPA was not due to Ca\(^{2+}\) influx from outside of the cells**

To elucidate whether the increase in the cytosolic Ca\(^{2+}\) concentration by BPA is due to Ca\(^{2+}\) influx from outside of the cells, BPA was added to the cells in Ca\(^{2+}\)-free medium and changes in Nrf2 levels and cellular Ca\(^{2+}\) levels were investigated. As a result, Nrf2 was still stabilized by BPA in Ca\(^{2+}\)-free medium (Fig. 2A). In addition, BPA also increased cellular NO levels (Fig. 2B) and Ca\(^{2+}\) concentration (Fig. 2C) in Ca\(^{2+}\)-free medium. These results suggested that increased cytosolic Ca\(^{2+}\) concentrations following BPA treatment were due to the influx from cellular Ca\(^{2+}\) stores such as the ER and mitochondria, but not from outside of the cells.
Increase in Ca\textsuperscript{2+} concentrations and Nrf2 stabilization by BPA was caused by activation of IP\textsubscript{3} receptor in the ER

Because Ca\textsuperscript{2+} did not flow in from outside of the cells after BPA treatment, we investigated the possibility of Ca\textsuperscript{2+} influx from the ER. The major Ca\textsuperscript{2+} channels that release Ca\textsuperscript{2+} from the ER are the ryanodine receptor and IP\textsubscript{3} receptor. First, we investigated the effects of ryanodine receptor inhibitor, ruthenium red, on the stabilization of Nrf2 by BPA, but it did not affect Nrf2 stabilization (Fig. 3A). Next, we used 2-APB as an IP\textsubscript{3} receptor inhibitor. As a result, 2-APB inhibited the increase in Nrf2 caused by BPA (Fig. 3B). Furthermore, the increase in cellular NO levels and Ca\textsuperscript{2+} concentrations by the addition of BPA to cells was also inhibited by 2-APB (Figs. 3C and D). These results suggested the increase in cellular Ca\textsuperscript{2+} concentrations by BPA was dependent on IP\textsubscript{3} receptor in the ER. Because the IP\textsubscript{3} receptor is known to be activated by ER stress, we examined the expression levels of an ER stress marker, glucose-regulated protein 78 (GRP78/BiP), but it remained unchanged in response to BPA (Fig. 3E). These results suggested that the increase in Ca\textsuperscript{2+} concentration in response to BPA was not caused by ER stress though BPA activated the IP\textsubscript{3} receptor.

BPA did not increase IP\textsubscript{3} production, but directly bound to IP\textsubscript{3} receptor at the IP\textsubscript{3}-binding core domain

IP\textsubscript{3} is produced from PIP\textsubscript{2} by plasma membrane-bound PLC, which is activated by a G-protein coupled recep-
and BPA was not affected by the addition of IP$_3$. These results indicated that BPA bound directly to the IBC of the IP$_3$ receptor by competing with IP$_3$. Taken together, these results suggested that BPA-induced Nrf2 stabilization was due to Ca$^{2+}$ influx from the ER by activation of the IP$_3$ receptor by BPA-binding and subsequent eNOS activation (Fig. 5).

**DISCUSSION**

Previously, we found that Nrf2 stabilization caused by BPA was due to induction of Keap1 nitrosylation (Nakamura *et al.*, 2018). In the present study, we found that the IP$_3$ receptor is a new binding protein for BPA and that an increase in cytosolic Ca$^{2+}$ concentration by BPA-induced activation of IP$_3$ receptor led to an increase in cellular NO levels in Hep3B cells. As we revealed that Hep3B cells expressed eNOS, the increase in cellular NO levels by BPA was due to Ca$^{2+}$/calmodulin-dependent eNOS activity. Furthermore, we found that BPA increased eNOS mRNA levels, which might additively increase cellular NO levels. The increase in eNOS mRNA levels in response to BPA was also observed in human umbilical vein endothelial cells (Andersson and Brittebo, 2012), human cardiomyocytes (Klint *et al.*, 2017), and mice aortic rings (Saura *et al.*, 2014), but the underlying mecha-
nisms are unknown.

The present results indicated that BPA does not activate Ca\textsuperscript{2+} channels in the plasma membrane in Hep3B cells. However, several reports indicated that BPA can affect the expression levels of voltage-gated Ca\textsuperscript{2+} channels (Kim et al., 2013b), transient receptor potential (TRP) channels (Derouiche et al., 2013), and calcium release-activated calcium channel protein 1 (Orai1) (Wang et al., 2015), suggesting that longer-term exposure to BPA may affect cytosolic Ca\textsuperscript{2+} concentrations via these channels. In addition, it has been reported that BPA can induce Ca\textsuperscript{2+} influx by activation of estrogen receptor α or β in multiple pathways, including a non-genomic pathway in different cell types (Tanabe et al., 2006; Ehrenmann et al., 2017; Gao et al., 2013; Wozniak et al., 2005), but we confirmed that longer-term exposure to BPA may affect cytosolic Ca\textsuperscript{2+} concentrations via these channels. In addition, it has been reported that BPA can induce Ca\textsuperscript{2+} influx by activation of estrogen receptor α or β in multiple pathways, including a non-genomic pathway in different cell types (Tanabe et al., 2006; Ehrenmann et al., 2017; Gao et al., 2013; Wozniak et al., 2005), but we confirmed that longer-term exposure to BPA may affect cytosolic Ca\textsuperscript{2+} concentrations via these channels.

In this study, we found the BPA activated the IP\textsubscript{3} receptor in the ER. The IP\textsubscript{3} receptor is the most widely expressed Ca\textsuperscript{2+} channels in many cell types and has three subtypes. IP\textsubscript{3} receptors type 1 and 2 are predominantly expressed in hepatocytes (Hirata et al., 2002), whereas type 3 is absent in normal hepatocytes but it expressed in hepatocellular carcinoma and associated with the survival of cells (Guerra et al., 2019). IP\textsubscript{3} is the only endogenous ligand of IP\textsubscript{3} receptor and is produced by PLC, which is activated by stimulation mediated by many receptors in the plasma membrane, including G-protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) (Rossi and Taylor, 2018). Although PLC activity was stimulated by G-protein coupled estrogen receptor GPER/GPR30, which can be activated by BPA in hepatocytes (Girgert et al., 2019; Teng et al., 2015), we found that PLC activation was not involved in BPA-induced Nrf2 stabilization, and that BPA bound directly to the IP\textsubscript{3} receptor at the IBC domain. IP\textsubscript{3} binds to opposing sides of the two clam-like α- and β-domains in the IBC to activate the IP\textsubscript{3} receptor, and the 4,5-bisphosphate and 6-hydrox-
yl groups of IP₃ are important for its binding (Rossi et al., 2010). In addition, the dissociation constant of IP₃ receptor type 1, 2 and 3 was estimated to be 49.5 nM, 14.0 nM, and 163.0 nM, respectively (Iwai et al., 2007). We found that the BPA competed with IP₃ on the IBC of the IP₃ receptor, but further study is necessary to determine the binding site and the affinity of the IBC for BPA-binding. Although the dose of BPA in this study is much higher than concentration of BPA in human serum (up to 10 ng/mL = 44 nM), it has been reported that the highest concentration of workers’ urine BPA in thermal paper manufacturing was 1500 ng/mL (6.6 μM) in Finland (Heinälä et al., 2017). In adults, BPA is metabolized to non-estrogenic monoglucuronide and glucuronide/sulfate conjugates in the liver (Yokota et al., 1999). An expression of UDP-glucuronosyl transferase (UGT) is induced by Nrf2 activation, and our previous study confirmed the induction of UGT2B1 by BPA exposure in rat liver. Although conjugated BPA levels are lower than free BPA levels in both the adult and fetal liver tissues (Nahar et al., 2013), further investigation is necessary to determine whether the conjugated BPA can bind to IP₃ receptor as well as BPA.

In the present study, we found that Nrf2 was stabilized by Ca²⁺ influx via activation of the IP₃ receptor. Kim et al. (2013a) also reported that increases in cellular Ca²⁺ concentrations promoted the translocation of the complex of Nrf2 with IQGAP1, which is an Nrf2 stabilizing protein, from the cytosol into nucleus, but the underlying mechanism is still unknown. In addition, enhanced Ca²⁺ concentrations can activate the ERK pathway (Dolmetsch et al., 2001), and phosphorylation of Nrf2 by ERK stabilizes and activates Nrf2 (Yuan et al., 2006). Therefore, Ca²⁺ influx via the IP₃ receptor will activate these pathways to stabilize Nrf2 in addition of NOS-mediated Keap1 nitrosylation. Furthermore, the findings of this study raise a possibility that cellular PLC-activating signaling or direct activation of the IP₃ receptor by modification through phosphorylation or oxidation (Berridge, 2016) may induce stabilization of the Nrf2 protein. Activation of Nrf2 is observed in several types of cancer cells, and it promotes cancer progression due to its antioxidant and chemical resistance effects (Taguchi and Yamamoto, 2017). In addition, it has also been shown that hyperactivity of the IP₃ receptor drives the onset and progression of cancer (Berridge, 2016). Therefore, stabilization of Nrf2 via IP₃ receptor activation may be one of the causes of IP₃ receptor-related cancer progression, and further investigation on the relationship between BPA-induced activation of this pathway and cancer progression may explain the effects of BPA exposure on estrogen receptor-independent cancer progression and development.

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Conflict of interest----The authors declare that there is no conflict of interest.

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