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Low-dose bisphenol A induces RIPK1-mediated necroptosis in SH-SY5Y cells: Effects on TNF-α and acetylcholinesterase

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Abstract

Bisphenol A (BPA) is an endocrine disruptor chemical, which is commonly used in everyday products. Adverse effects of its exposure are reported even at picomolar doses. Effects of picomolar and nanomolar concentrations of BPA on cytotoxicity, nitric oxide (NO) levels, acetylcholinesterase (AChE) gene expression and activity, and tumor necrosis factor-α (TNF-α) and caspase-8 levels were determined in SH-SY5Y cells. The current study reveals that low-dose BPA treatment induced cytotoxicity, NO, and caspase-8 levels in SH-SY5Y cells. We also evaluated the mechanism underlying BPA-induced cell death. Ours is the first report that receptor-interacting serine/threonine-protein kinase 1–mediated necroptosis is induced by nanomolar BPA treatment in SH-SY5Y cells. This effect is mediated by altered AChE and decreased TNF-α levels, which result in an apoptosis-necroptosis switch. Moreover, our study reveals that BPA is an activator of AChE.

KEYWORDS
acetylcholinesterase, bisphenol A, necroptosis, TNF-α

1 | INTRODUCTION

Bisphenol A (BPA; 4,40-isopropylidenediphenol) is a widely used industrial chemical and one of the most studied endocrine disruptor chemicals (EDCs). BPA mimics estrogen and is shown to interact with estrogen receptor α and β both as an agonist and antagonist.[1,2] Although considered a weak estrogen, BPA shows a broad spectrum of actions on endocrine, reproductive, nervous, and immune systems; these effects of BPA may be triggered via the genomic or nongenomic pathways.[3]

BPA is released from plastic containers into food and beverages, which increase the risk of potential human exposure. Reports show that BPA also exerts its effects below the lowest-observable-adverse effect-level (LOAEL) in a nonmonotonic dose-dependent manner even at picomolar doses.[4,5]

Low-dose BPA exposure results in structural and behavioral changes in the brain, such as decreased dendritic spine density, impaired memory, altered hypothalamic pituitary adrenal (HPA) axis, altered levels of neuronal nitric oxide synthase (nNOS) and steroid receptor, induced aggressiveness, and anxiety.[6–12] In vivo and in vitro growth-promoting effects of BPA on SK-N-SH cells have also been reported.[13,14]

Nitric oxide (NO), a gaseous small molecule, acts as a neurotransmitter and is produced predominantly by nNOS in the central nervous system.[15] A study in mice showed that BPA alters the nNOS system in different nuclei of the brain, particularly in medial preoptic nucleus and bed nucleus of the stria terminalis.[16]

Cholinesterases are the kin enzymes with different functions. Acetylcholinesterase (AChE; EC 3.1.1.7) is a key enzyme in the cholinergic system and terminates neurotransmission by hydrolyzing the neurotransmitter acetylcholine. Conversely, butyrylcholinesterase (BChE; EC 3.1.1.8), which preferentially hydrolyses butyrylcholine, has no known physiological function.[17,18] Recent studies have shown a potential role of AChE in different forms of cell death, such as apoptosis and necroptosis.[19,20] Animal studies on BPA and AChE have mainly focused on enzymatic activity and described nonmonotonic response, increased AChE activity at low doses (10 and 25 mg/kg), and decreased AChE activity at high doses (50 mg/kg).[21–23]

Apoptosis is the regulated form of cell death and plays a major role in the development and pathology of several diseases, such as diabetes,

the Alzheimer disease, and cancer. Increased AChE levels and enzymatic activity are associated with apoptosis in different cell lines.[24] Necroptosis is a caspase-independent and tightly regulated form of necrosis, which has been recently established.[25,26] Necroptosis can be blocked by the receptor-interacting serine/threonine-protein kinase 1 (RIPK1) inhibitor necrostatin-1 (Nec-1).[26] BPA is reported to induce apoptosis and necrosis in various cell lines and tissues.[27–31]

A number of studies have identified a positive correlation of plasma and urine BPA levels with inflammatory markers.[32,33] Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine that exerts neuromodulatory effects.[34] Acetylcholine, which is a AChE substrate, and BPA are both reported to suppress TNF-α levels by inactivating NF-κβ.[35–37]

In the current study, effects of low-dose BPA on cytotoxicity, NO levels, AChE gene expression and activity, and TNF-α and caspase-8 levels were determined in SH-SY5Y cells. Cell death mechanisms were also investigated, and we found that RIPK1-dependent necroptosis was induced in low-dose BPA-treated SH-SY5Y cells.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Chemicals used in the study were high analytical grade and purchased from Sigma-Aldrich Chemical Co, (St Louis, MO), Merck (Quebec, Canada), and Calbiochem (Los Angeles, CA).

Cell culture materials were purchased from Biowest (Nuaillé, France) and Thermo Fisher Scientific (Waltham, MA).

2.2 | Cell culture

SH-SY5Y cells (ATCC) were cultured in the Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% antibiotic cocktail. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

2.3 | Cell viability assessment by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Cell viability was determined by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described.[38]

2.4 | Lactate dehydrogenase assay

For assessment of cell viability, we also used the lactate dehydrogenase (LDH) assay as previously described.[39] Maximum LDH activity (positive control) is calculated in RIPA lysed cells.

2.5 | Griess reaction method

NO production was measured according to the method described by Kuan, and data were normalized to cell number.[40]

2.6 | AChE activity

AChE activity was determined by the colorimetric Ellman method at 412 nm.[39,41]

IC_{50} values of BPA for AChE were calculated by the addition of different concentrations of BPA (0 to 4 mM) to the assay mixture at a constant substrate concentration (0.5 mM).

To analyze AChE enzyme activation, human recombinant AChE was exposed to different concentrations of BPA (1 pM and 1 nM) for 2 and 4 hours at 37°C.

Specific AChE activities in SH-SY5Y cells treated with 1 pM and 1 nM BPA were determined according to the method described by Li et al.[42]

Tetraisopropyl pyrophosphoramide (iso-OMPA; 100 μM) as a specific BChE inhibitor was added into the reaction mixture for inhibiting the BChE activity in samples.

The Santa Cruz BCA protein assay kit (Santa Cruz, CA) was used to calculate the amount of protein in the samples.

2.7 | Annexin V/propidium iodide staining

The percentages of viability, necrosis, late apoptosis, and apoptosis were evaluated by the Tali image-based cytometer (Invitrogen, Carlsbad, CA) using Tali Apoptosis Kit-Annexin V Fluor 488 and propidium iodide (PI) following the manufacturer's instructions.[43] The cells were treated with various concentrations (1 pM to 1 μM) of BPA and 50 μM Nec-1 in dimethyl sulfoxide (DMSO),

2.8 | RNA isolation, complementary DNA synthesis, and reverse transcription polymerase chain reaction

RNA was isolated using RNAGEM Tissue (ZyGEM, Charlottesville, VA), according to the manufacturer's recommendations. Purity and concentration of RNA were determined using a nanodrop spectrophotometer. Total RNA was converted to complementary DNA (cDNA) using the ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA) on a Veriti 96-well thermo cycler (Applied Biosystems, Foster City, CA) with cDNA synthesis reaction for an hour at 42°C and inactivation of enzyme at 80°C for 5 minutes.

Gene expression was evaluated by real-time reverse transcription polymerase chain reaction (RT-PCR), which was performed on a ViiA 7 by Life Technologies Real-Time PCR system and software (Applied Biosystems) according to the manufacturer's instructions. RT conditions were as follows: 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C, 1 minute at 60°C, and 1 minute at 72°C. All the primers used for PCR are listed in Table 1.

2.9 | Caspase-8 assay

Caspase-8 levels in SH-SY5Y cells exposed to different concentrations of BPA were determined using the Abcam ab119507-Caspase-8 Human ELISA Kit (Cambridge, United Kingdom) on a BioTek Power Wave XS (Winooski, Vermont, VT) microplate reader according to the manufacturer's instructions.

TABLE 1 Primer sequences used in RT-PCR

Target gene	Primer sequence
AChE (F)	5′-CCTGTCCTCGTCTGGATCTATG-3′
AChE (R)	5′-AAGAAGCGGCCATCGTACAC-3′
GAPDH (F)	5′-GTCGTATTGGGCGCCTGGTCAC-3′
GAPDH (R)	5′-GCCAGCATCGCCCCACTTGATT-3′
nNOS (F)	5′-GAAGGAGCGGGTCAGTAAGC-3′
nNOS (R)	5′-CCCCACGAATCAGGTCAGAGA-3′

Abbreviations: AChE, acetylcholinesterase; GAPDH, glyceraldehyde phosphate dehydrogenase; nNOS, neuronal nitric oxide synthase; RT-PCR, reverse transcription polymerase chain reaction.

2.10 | TNF-α assay

TNF-α levels in SH-SY5Y cells exposed to different concentrations of BPA were determined using the LEGEND MAX Human TNF-α ELISA Kit with Pre-coated Plates (BioLegend, San Diego, CA) on a BioTek Power Wave XS microplate reader according to the manufacturer's instructions.

2.11 | Statistical analysis

The results are expressed as mean ± SD. Statistical analysis was performed using the GraphPad Prism software (La Jolla, California, CA) using analysis of variance followed by the Bonferroni test, and *P* values less than 0.05 were considered statistically significant.

3 | RESULTS

3.1 | Effects of low-dose BPA on MTT activity, LDH activity, and NO production

BPA is an EDC and reported to have a nonmonotonic dose effect on various cell lines.[4] Treatment with 1 pM and 1 nM BPA significantly decreased MTT reduction (Figure 1A). For the LDH assay, which indicates disrupted cell membrane, only 1 nM BPA showed a significant decrease (Figure 1B). NO is an essential neurotransmitter, and BPA is reported to increase NO production.[22] NO levels in SH-SY5Y cells were increased by 1 pM and 1 nM BPA treatment (Figure 1C). MTT activity was inversely correlated to NO levels at various BPA concentrations (1, 10, and 100 nM, and 1, 10, and 100 μM) (Figure 1D)

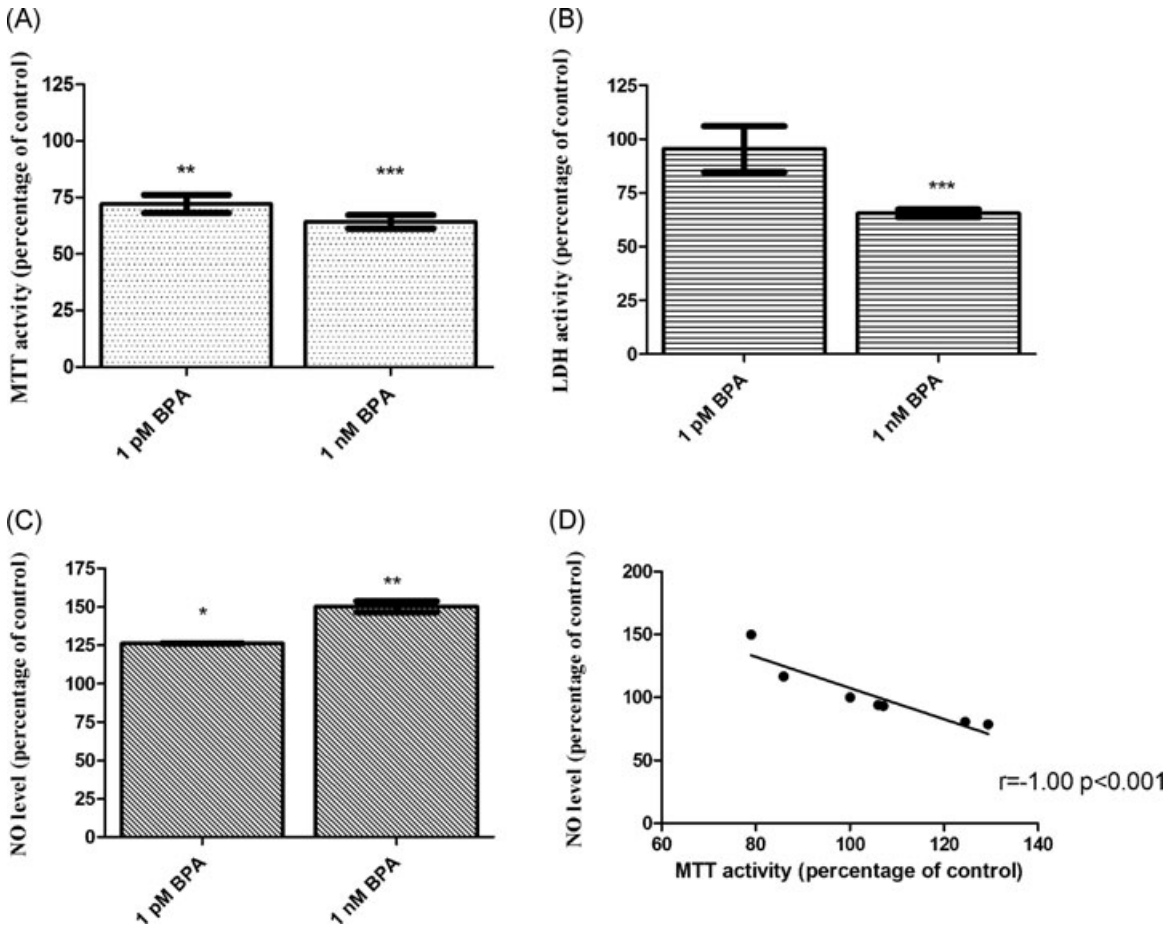

FIGURE 1 MTT activity (A), LDH activity (B), and NO production (C) in SH-SY5Y cells exposed to 1 pM and 1 nM BPA after 48 hours. Correlation of MTT activity to NO levels (D) at various BPA concentrations (1, 10, and 100 nM, and 1, 10, and 100 μM). BPA, Bisphenol A; LDH, lactate dehydrogenase; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide

3.2 | Effects on cell death mechanisms

BPA is commonly reported to induce apoptosis.[44–47] To evaluate the mechanism of BPA-induced death in SH-SY5Y cells, Annexin V/PI staining was used to discriminate apoptotic and necrotic cells, using a Tali image-based cytometer. Although apoptotic marker phosphatidyl serine is stained with Annexin V, binding of cell-impermeant PI indicates necrosis.

The population of apoptotic cells showed a significant increase at 12 and 24 hours with 1 pM and 1 nM BPA treatment, respectively. Apoptosis is replaced by necrosis after 48 hours as seen in Figure 2A.

Necroptosis is the regulated form of necrosis and requires RIPK1 activity.[48] To investigate whether the BPA-induced necrosis in SH-SY5Y cells was programmed, the effect of Nec-1 (RIPK1 inhibitor) was also evaluated. Addition of Nec-1 significantly switched necrotic cell death to apoptosis, as seen in Figure 2B, pointing to the involvement of necroptotic machineries.

3.3 | Effects on TNF-α and caspase-8

Major players of cell death machineries are death receptors (such as TNF) and caspases. TNF-α is a pleiotropic cytokine which contributes to homeostasis by modulating cell survival and death responses. TNF-α has been shown to activate the transcription factor NF-κB and to induce apoptosis and necroptosis machineries.[49] Caspase-8 is an initiator of apoptosis, but when caspase-8 is inhibited necroptosis signaling it is initiated.[50] SH-SY5Y cells were incubated with 1 pM and 1 nM BPA for 48 hours. No significant difference was observed at 12 hours. But, TNF-α levels were decreased (Figure 3A), whereas caspase-8 levels were increased significantly at 24 and 48 hours (Figure 3B). Increased caspase-8 activity is consistent with increased apoptotic cell population at 24 hours. On the other hand, at 48 hours although caspase-8 is increased, apoptotic cell population is decreased. This finding may be attributed to a possible blockage in the downstream molecules of caspase-8.

3.4 | Effects on AChE enzyme

AChE is a major cholinergic enzyme, and studies on BPA and AChE have mainly focused on enzymatic activity till date.[21–23] In this study, effects of BPA on AChE inhibition, specific activity, AChE activation, and AChE messenger RNA (mRNA) levels were evaluated. Assessment of the enzymatic activity showed that AChE activity was inhibited by BPA, with an IC_{50} value of 2.28 ± 0.29 mM (Figure 4A).

Specific AChE activity of SH-SY5Y cells was significantly increased at 2 and 12 hours with 1 pM and 1 nM BPA, whereas a decrease was observed after 4 hours in SH-SY5Y cells treated with 1 pM BPA (Figure 4B). Enhanced specific AChE activity in SH-SY5Y cells after 2 hours led us to investigate whether BPA was an activator of AChE. As seen in Figure 4C, 2 and 4 hours of BPA treatment significantly increased AChE activity at various BPA doses. However, no significant effect on AChE gene expression was determined in 1 pM or 1 nM BPA-treated SH-SY5Y cells at 48 hours (Figure 4D).

3.5 | Effects on nNOS genes expression

nNOS gene expressions were determined in the SH-SY5Y cells treated with 1 pM or 1 nM BPA. Although 1 nM BPA treatment increased nNOS mRNA level to 1.2 fold, no significant effect on nNOS gene expression was determined in the SH-SY5Y cells at 48 hours.

4 | DISCUSSION

Physiological levels of hormones in picomolar to nanomolar concentration range are well established. Translation of this knowledge to EDC research is relatively new, but crucial. In vivo and in vitro effects of BPA on the endocrine, neuronal, and immune systems are reported to occur below 1×10^{-7} M concentration.[51–55]

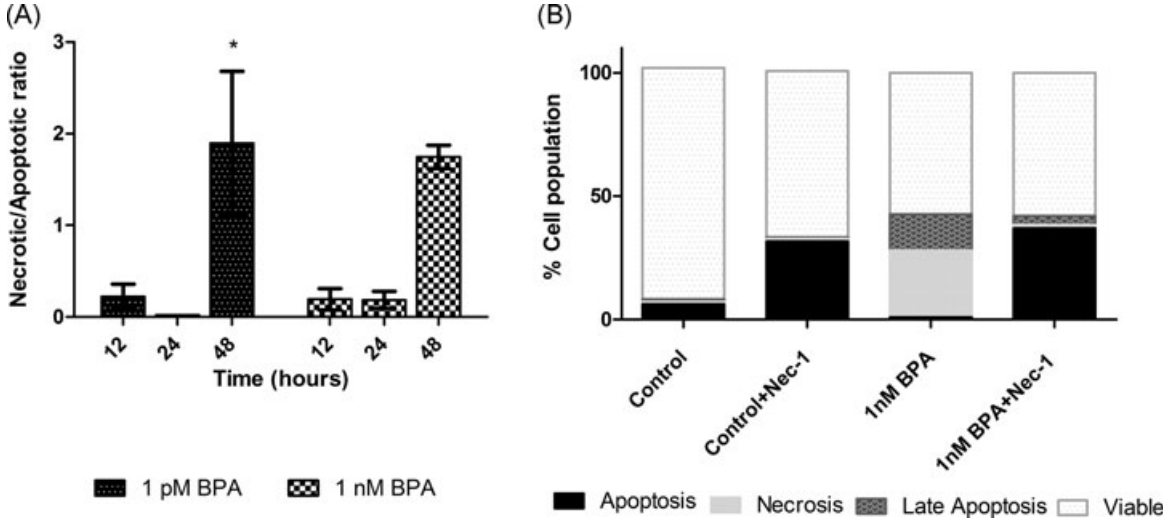

FIGURE 2 Effects of low-dose BPA on apoptotic/necrotic ratio (A) inhibition of necroptosis with 50 μM necrostatin-1 (B) in SH-SY5Y cells. Data are presented as mean ± SEM of three independent experiments with two technical replicates (*$P < 0.05$, compared with necrotic/apoptotic ratio at 24 hours). BPA, bisphenol A

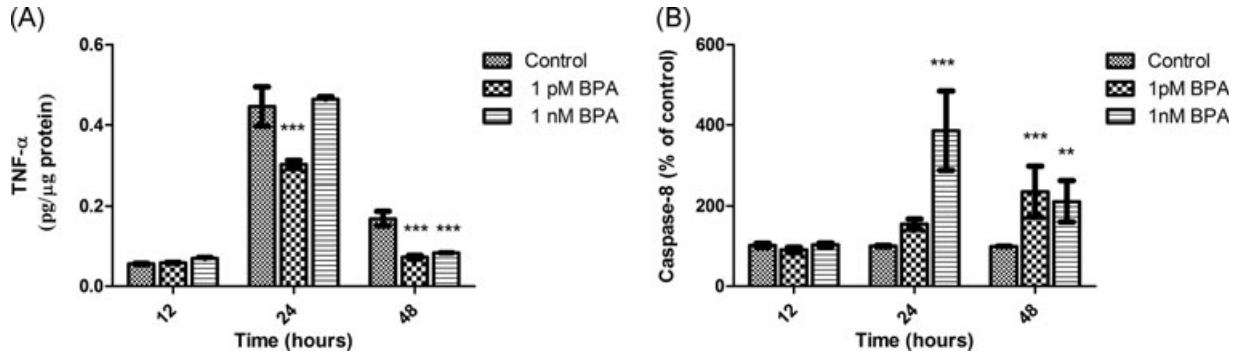

FIGURE 3 Time-dependent analysis of TNF-α levels (pg/μg) (A) and caspase-8 levels (B) in BPA-treated SH-SY5Y cells. Data are presented as mean ± SEM of three independent experiments with two technical replicates (**$P < 0.01$ and ***$P < 0.001$ compared with the corresponding parameter of control). BPA, bisphenol A; TNF-α, tumor necrosis factor-α

The aim of this study was to assess the low-dose effects of BPA in SH-SY5Y cells in terms of cytotoxicity, NO level, cell death mechanisms, and effects on AChE. BPA is well documented to be cytotoxic at different doses in various cell lines.[56–59] There is limited number of reports on cytotoxic effects of low-dose BPA. Our results showed that 1 pM and 1 nM BPA treatment decreased MTT reduction and LDH activity in SH-SY5Y cells.

NO is a signaling molecule, which also acts as a neurotransmitter. In the current study, we also demonstrated that NO levels in SH-SY5Y cells were increased by low-dose BPA treatment. However, changes in the expression of nNOS were negligible. Increased NO levels and decreased cell viability indicate a correlation ($r = -1.00$) suggesting that NO may be a component of low-dose BPA cytotoxicity.

In a healthy cell, death machineries are an essential part of homeostasis. Majority of the previous studies refer to BPA as an apoptotic rather than a necrotic agent.[56,58,60–62]

Remarkably, we observed necrosis after 48 hours, whereas apoptosis was dominant in the first 24 hours. This finding directed

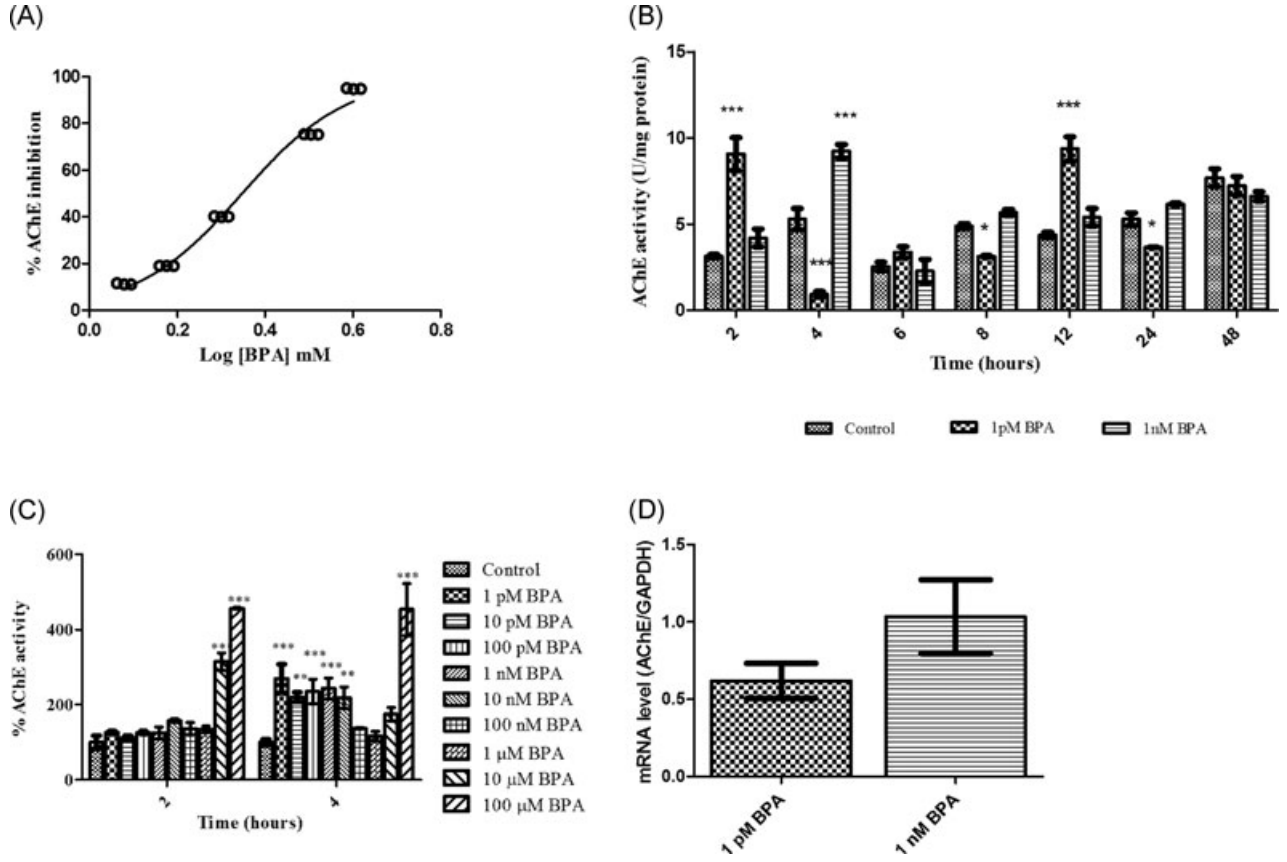

FIGURE 4 Inhibition of human recombinant AChE by BPA (A), effects on specific AChE activity in SH-SY5Y cells (B), activation of human recombinant AChE by BPA (C), and effects on AChE gene expression levels (D). AChE, acetylcholinesterase; BPA, bisphenol A; GAPDH, glyceraldehyde phosphate dehydrogenase; mRNA, messenger RNA

us to investigate the possibility of programmed necrosis known as necroptosis. In the current study, we demonstrated for the first time that 1 nM BPA treatment induces RIPK1-mediated necroptosis in SH-SY5Y cells at 48 hours; this necroptosis can be blocked by Nec-1 application. Low-dose BPA is also reported to reduce proapoptotic Bax levels resulting in a decrease in apoptosis.[63]

Caspase-8 is an initiator of apoptosis and repressor of necroptotic signaling.[50] Caspase-8 levels in low-dose BPA-treated SH-SY5Y cells were found to be increased after 24 and 48 hours. Although this finding may be opposing necroptosis, which is observed at 48 hours, a previous study also reported that RIPK-dependent necrosis may be initiated even with activated caspase-8.[64]

TNF-α is a pleiotropic cytokine, which induces apoptosis and necroptosis machineries.[65] Our findings showed that TNF-α levels in SH-SY5Y cells were decreased significantly at 48 hours with 1 pM and 1 nM BPA treatment. Previous studies showed that EDC decreases TNF-α-induced apoptosis even at nanomolar doses, which leads to an apoptotic resistance.[66,67]

AChE is reported to be proapoptotic and induced during apoptosis in various cell lines,[68–70] and it has been linked to necroptosis in ovaries only in a single study.[71] BPA-AChE interactions are also conflicting; both enhancement and reduction in AChE activity have been reported.[72,73] Our results showed that BPA is a weak inhibitor, but an activator of AChE. BPA's AChE-activating properties may possibly led to the minor changes observed in AChE mRNA levels after 48 hours as a compensatory mechanism. It is in accordance with the fact that AChE specific activity in SH-SY5Y cells was decreased at 24 and 48 hours of BPA treatment. It should also be mentioned that the trend of decreasing specific AChE activity after 24 and 48 hours of BPA treatment may be responsible for the reduction in TNF-α levels via the cholinergic anti-inflammatory pathway.[35]

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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