Activation of Autophagic Flux against Xenoestrogen Bisphenol-A-induced Hippocampal Neurodegeneration via AMP kinase (AMPK)/Mammalian Target of Rapamycin (mTOR) Pathways*

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Background: The effects of xenoestrogen bisphenol-A on autophagy, and association with oxidative stress and apoptosis are still elusive. The human health hazards related to persisting use of bisphenol-A (BPA) are well documented. BPA-induced neurotoxicity is an early cell’s tolerance response. The effects of xenoestrogen bisphenol-A on autophagy, and association with oxidative stress and apoptosis are still elusive.

Results: BPA exposure during the early postnatal period enhanced the expression and the levels of autophagy marker of xenoestrogen exposure. We observed that BPA exposure down-regulated the mammalian target of rapamycin (mTOR) pathway by phosphorylation of raptor as a transient activation of autophagy protects against bisphenol-A-induced neurodegeneration via AMPK activation and decreased phosphorylation of ULK1 (Ser-757), and silenced mitophagy. These results suggest implication of autophagy in mitochondrial loss, bioenergetic deficits, and increased generation of reactive oxygen species and apoptosis were mitigated by a pharmacological activator of autophagy (rapamycin), Pharmacological (wortmannin and bafilomycin A1) and genetic (beclin siRNA) inhibition of autophagy aggravated BPA neurotoxicity. Activation of autophagy against BPA resulted in increased intracellular reactive oxygen species and apoptotic cell death. BPA inhibited autophagy and enhanced ROS generation by impairing the antioxidant system, including SOD, CAT, and GSH. During ROS generation, intracellular mitochondrial ΔΨm decreased, and collapse of ΔΨm was silenced by rapamycin. BPA exposure decreased phosphorylation of AMPK and mTOR pathways. Hence, autophagy, which prevents cell survival and demise during stress conditions, requires further assessment to be established as a biomarker of xenoestrogen exposure.

Bisphenol-A (BPA) is a potent endocrine disruptor as well as neurotoxicant (1, 2). It is released from the polycarbonate plas...
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To maintain cellular homeostasis, three types of cell death mechanism occur, namely autophagy, apoptosis, and necrosis (23, 24). Autophagy is a protective cellular cleanup process involved in the removal of unwanted and misfolded proteins inside the cells, by delivering them to the lysosomes for their degradation (25). Autophagy and apoptosis work in a coordinated manner to regulate cell survival under several conditions, such as starvation, mechanical injury, result in the generation of ROS and concomitant accumulation of damaged mitochondria and misfolded proteins inside the cells. Thus, autophagy may lead to several pathogenic conditions, such as cancer and neurological and neurodegenerative disorders, such as Parkinson and Alzheimer disease (23). Several recent studies have found that several environmental toxicants, such as arsenic (29), cadmium, chromium (30, 31), dibenzofuran (32), paraquat (33), and ethanol (34, 35), cause alterations in the basal levels of autophagy, leading to cellular toxicity. Autophagy acts as a cardinal process and interconnects several cell survival pathways (viz. AMP kinase (AMPK), mammalian target of rapamycin (mTOR), and PI3K/Akt) (36, 37). Stress leads to a decline in the ATP levels and also accretion of cellular AMP. Thus, AMPK acts as an intracellular energy sensor, which activates under low nutrient or energy-deprived conditions (38). AMPK restrains cell growth and metabolism through phosphorylation of acetyl-CoA carboxylase (ACC) and raptor (37, 39 – 41) during stress conditions. AMPK is involved in several functions like autophagy, apoptosis, and cell migration (37, 42, 43). The activity of mTOR (a serine/threonine protein kinase and master regulator of autophagy) is activated under nutrient-enriched conditions and inhibited under starvation conditions, thereby leading to inhibition and activation of autophagy, respectively (44). Herein, we studied the effects of BPA exposure on autophagy in vitro hippocampal neural stem cells (NSC)-derived neurons and in the hippocampus region (crucial region for learning and memory regulation) of the rat brain. We elucidated the molecular mechanism(s) underlying the AMPK pathway activation and mTOR down-regulation in response to BPA exposure. The decline in ATP levels after BPA exposure activates AMPK to preserve the cellular energy pool by inhibiting the anabolic processes while turning on the catabolic pathways. Moreover, AMPK balances energy levels by enhancing autophagy and inhibits mTORC1 by phosphorylation of raptor (37, 39, 41). In addition, autophagy induced against BPA also results in the phosphorylation of an AMPK substrate, ACC. On the contrary, BPA-induced energy depletion leads to the reduction in the phosphorylation of ULK1 at Ser-757. Therefore, a concerted coordination is maintained among the three kinase complexes to regulate the autophagy induction and cell survival during BPA exposure. Interestingly, inhibition of autophagy through the genetic and pharmacological approaches aggravated BPA induced neurotoxicity and enhanced ROS generation and apoptosis. Thus, our studies delineate that autophagy acts as a transient cellular protective response against BPA-induced neurotoxicity.
The doses of BPA (40 and 400 μg/kg body weight) were selected on the basis of earlier studies, where BPA induced neurobehavioral and neurochemical alterations in the rat brain (10–12). Rat pups were sacrificed at PND 21, and the effects of BPA on autophagy were studied by quantitative RT-PCR, Western blot, and transmission electron microscopy analysis. To study the effects of BPA on cleaved caspase-3 levels in the hippocampus of the rat brain, a group of pups were treated with 400 μg/kg body weight BPA and with BPA along with rapamycin (rapamycin; 1 and 2 mg/kg body weight, intraperitoneally).

Hippocampal NSC-derived Neuronal Culture and BPA Treatment—Hippocampal NSC were cultured and differentiated in neurons as described earlier (45). Neuronal cells grown in flasks were treated with different concentrations of BPA dissolved in DMSO (0, 25, 50, 100, 200, and 400 μM) for 24 h. The non-cytotoxic concentration of BPA was determined by trypsin blue and propidium iodide (PI) uptake analysis. We found that BPA was non-cytotoxic at concentrations up to 100 μM. To study the levels of LC3-II protein, cells were grown in the presence of non-cytotoxic concentrations of BPA (0, 25, 50, and 100 μM) for 3, 6, and 12 h. Further, the cells were treated in the presence/absence of pharmacological inducer (rapamycin; 100 nM) and inhibitor of autophagy (wortmannin; 10 μM). To induce starvation conditions in NSC-derived hippocampal neurons, neuronal medium was replaced with Hank’s balanced salt solution (HBSS). After respective treatments, cells were analyzed for autophagy by Western blotting, flow cytometry, and immunofluorescence studies.

Flow Cytometry and Trypan Blue Exclusion Assay—Cell Viability—Effects of BPA on viability of hippocampal NSC-derived neurons were examined through a trypan blue and propidium iodide (PI) uptake analysis. We found that BPA was non-cytotoxic at concentrations up to 100 μM. To study the levels of LC3-II protein, cells were grown in the presence of non-cytotoxic concentrations of BPA (0, 25, 50, and 100 μM) for 3, 6, and 12 h. Further, the cells were treated in the presence/absence of pharmacological inducer (rapamycin; 100 nM) and inhibitor of autophagy (wortmannin; 10 μM). To induce starvation conditions in NSC-derived hippocampal neurons, neuronal medium was replaced with Hank’s balanced salt solution (HBSS). After respective treatments, cells were analyzed for autophagy by Western blotting, flow cytometry, and immunofluorescence studies.

Plasmids and siRNA Transfection in Neuronal Cells—The hippocampal neuronal cells were transiently co-transfected with plasmids for mammalian GFP-LC3, pmKate mitochondrial reporter gene, YFP-PARK2, and mito-CFP, and beclin, mTOR, and AMPK siRNA. The cells were then treated with BPA. Cells were visualized using a Nikon Eclipse Ti-S inverted fluorescence microscope equipped with a Nikon Digital Sight DS-Ri1 CCD camera and NIS Elements BR imaging software (Nikon, Japan).

Quantification of GFP-LC3 Puncta—For GFP-LC3 puncta counting, a person without knowledge of the experimental design blindly selected 50 cells from each group, and the number of GFP-LC3 puncta were counted individually in each cell and averaged as per well established methods (47, 48). The results were presented as mean ± S.E. of three independent experiments.

Oxidative Stress and Antioxidant Levels—ROS production in neuronal cells was assessed using 2,7-dichlorodihydrofluorescein diacetate and DHE dyes (49). Briefly, neuronal cells (1 × 10³ cells) were treated with BPA (100 μM) for 12 h, followed by incubation with 2,7-dichlorodihydrofluorescein diacetate (10 μM) for 30 min at 37 °C. Fluorescence intensity was monitored by a spectrofluorometer at excitation/emission wavelengths of 485/530 nm, respectively. To evaluate superoxide generation, DHE (5 μM) was added to control and treated cultures for 30 min, and a ratio of ethidium/DHE was analyzed. DHE reacts with the superoxide anion and forms a red fluorescent product, 2-hydroxyethidium, with maximum excitation and emission peaks at 500 and 580 nm, respectively. Further, we have assessed malondialdehyde levels using a lipid peroxidation assay kit (50). Antioxidant levels were evaluated by measuring total GSH levels in control and treated cultures following the manufacturer’s protocol. The protein levels of superoxide dismutase (SOD) and catalase were also estimated by immunoblotting.

Gene Expression Analysis by Quantitative RT-PCR—To study the expression of genes involved in autophagy, quantitative RT-PCR analysis was carried out following our earlier published method (45).

Levels of Protein Analysis by Western Immunoblot—Hippocampal tissue/cells were blotted with CellLytic MT mammalian tissue lysis/extraction kit. Membranes were blocked with LC3-II (1:1,000), beclin-1 (1:1,000), HMG1B (1:1,000), phospho-mTOR (1:1,000), beclin-1 (1:1,000), AMPK (1:1,000), phospho-ACC (1:1,000), p70S6K (1:1,000), phospho-P70S6K (1:1,000), AMPK (1:1,000), phospho-ULK1 (1:1,000), ULK1 (1:1,000), cleaved caspase-3 (1:1,000), HMGB1 (1:10,000), mTOR (1:1,000), phospho-mTOR (1:1,000), beclin-1 (1:1,000), vimentin (1:1,000), and actin (1:10,000). Similarly, the levels of PINK-1 and parkin were studied in cytoplasmic, mitochondrial, and nuclear fractions, using VDAC. Protein bands were analyzed using Scion Image for Windows (National Institutes of Health, Bethesda, MD).

Two-dimensional Gel Electrophoresis—For two-dimensional PAGE analysis, three sets of pooled protein samples were prepared from hippocampal tissue of control and treated rats using tissue lysis buffer, followed by acetone precipitation. Protein content was quantified using Bradford reagent. Samples were then resolved through two-dimensional PAGE. Isoelectric focusing was carried out using 11-cm immobilized pH gradient strips (IPG, 4–7 pH gradient strip, Bio-Rad). Isoelectric focusing was carried out at 20 °C for 30,000 V-h in a protein i12 isoelectric focusing cell (Bio-Rad). Strips were incubated with gentle shaking in Ready Prep2-D starter kit equilibration solution-I and equilibration solution-II for 10 min in each solution. Gels were run in triplicate, stained in Coomassie G-250 (0.2%), and destained. Images were acquired using a GS-800 densitometer (Bio-Rad) and analyzed using PDQuest software followed by densitometric analysis of individual spots of interest. Spots of interest were identified by peptide mass fingerprinting through matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) using a protocol described previously (51). Peptide mass fingerprinting was performed using a 4800 proteomic analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Warrington, UK) in positive reflector mode. The deduced peptide sequences were submitted to the SwissProt database or the NCBI non-redundant database. The data are presented as means ± S.E. of three samples from each group and statistically analyzed using a non-parametric t test.
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Immunofluorescence Study—Immunohistochemical localization of activated caspase-3 was carried out as described earlier (45, 52). 30-μm thick serial coronal sections encompassing the hippocampus were incubated with primary cleaved caspase-3 antibody (1:500). Slides were analyzed under a Nikon Eclipse Ti-S inverted fluorescent microscope. The quantification of cleaved caspase-3-positive cells in the hippocampus region was carried out following our earlier study.

Conditioned Avoidance Response—The learning and memory responses were studied in control-, BPA-, rapamycin-, and BPA + rapamycin-treated rats. Rats that received a daily single intraperitoneal injection of rapamycin (0.1 mg/kg body weight) from PND 21 to 90 served as the rapamycin-treated group. Similarly, in the BPA + rapamycin group, BPA-treated rats (40 μg) from PND 14 to 90 received daily single intraperitoneal injection of rapamycin (0.1 mg/kg body weight from PND 21 to 90. We performed an assessment of two-way conditioned avoidance behavior using a shuttle box apparatus (Columbus Instruments) as described earlier (45, 53). Learning and memory in treated groups were studied as a percentage of control.

Fluoro-Jade-B Labeling for Degenerating Neurons in the Hippocampus—Fluoro-Jade B labeling was conducted in both the control and treated groups according to the manufacturer’s protocol. Fluoro-Jade B+ degenerating neurons were bilaterally counted in a total of six sections and averaged for each rat as described earlier (10, 45).

Mitophagy Study—Mitophagy was analyzed by mitochondrial mass using NAO (a mitochondria-specific dye) and mitochondrial copy number (ratio of nucleus-encoded gene mitochondrial mass using NAO (a mitochondria-specific dye), described earlier (10, 45). counting in a total of six sections and averaged for each rat as also done using MitoTracker Green FM and 200 nM Lyso-

To study the effects of BPA on mitophagy, neuronal cells were co-transfected with GFP-LC3, TOMM20, and pmKate mitochondrial reporter gene with control (Fig. 1, A and B). BPA significantly decreased viability above 100 μm concentration (Fig. 1, A and B). We found that 100 μm was a non-cytotoxic dose of BPA, and above it significantly reduced the cell viability and increased the number of PI+ cells (Fig. 1, A and B).

Further, in vivo we studied the effects of BPA on neuronal cell death in the hippocampus region using immunohistochemical analysis of activated caspase-3. Cells labeled with activated caspase-3 undergo apoptosis following BPA treatment at both 40 and 400 μg/kg body weight doses (Fig. 1C). Quantitative analysis revealed that BPA treatment dose-dependently enhanced activated caspase-3 positive cells in the hippocampus as compared with control (Fig. 1, C and D). Next, we determined the effects on neuronal degeneration of treatments with Fluoro-Jade B labeling. BPA enhanced the number of cells as compared with control and at doses as compared with control (Fig. 1, C and D). Results suggest that BPA caused neuronal degeneration in the hippocampus region of the rat brain—Because we observed that BPA caused reduced cell viability and increased neurodegeneration, we hypothesized that it could be due to alteration in cell survival process autophagy. We treated Wistar rats during PND 14–21 with two doses of BPA (40 and 400 μg/kg body weight) and examined its effects on alteration of the expression of autophagy marker genes in the hippocampus of the rat brain. We found significant increase in the mRNA expression of Lc3, Hmgb1, Beclin-1, Atg5, Atg12, Atg3, and Lamp-2 genes and decrease in the expression of p62 following BPA exposure at both of the doses studied (Fig. 2A). However, the expression of Atg7 was not significantly altered by 40 μg/kg BPA treatment. Similarly, the levels of autophagy proteins LC3-II, beclin-1, and HMGB1 in the hippocampus were significantly increased after BPA treatment at both of the doses as compared with control (Fig. 2, B and C). On the contrary, BPA treatment decreased the levels of p62 in the hippocampus region of the rat brain (Fig. 2, B and C). The increased expression and the levels of the autophagosome-associated form (i.e., LC3-II from soluble form LC3-I) suggested the increased formation of autophagosome (58).

We next carried out ultrastructural transmission electron microscopy analysis to assess whether increased expression of autophagy marker genes is also associated with enhanced formation of autophagosome. We observed increased generation of multilamellar vesicular bodies (59 autophagosomes (A), autolysosomes (AL), and degradative autophagic vacuole (AVd) in the hippocampus of BPA-treated rats as compared with con-
The presence of increased degradative autophagic vacuole rather than accumulation of autophagosomes by BPA treatment suggests that BPA neurotoxicity is relieved by the generation of an intralysosomal degradation pathway.

Proteomics Analysis of Autophagy-associated Proteins in the Hippocampus Region of BPA-treated Rats—The effects of BPA on autophagy in the hippocampus of BPA (40 and 400 μg/kg body weight)-treated rat pups were further studied using two-dimensional analysis in tandem with a mass spectrometry proteomics approach. A total of 50 protein spots on the gel were detected, and quantitative analysis of protein was performed (Fig. 2 and Table 1). We observed alterations in the levels of various autophagy-regulating proteins in BPA-treated rats at both the doses as compared with control (Fig. 2F and Table 1). The levels of 21 proteins were up-regulated, and six were down-regulated by BPA treatment in comparison with control (Table 1). Cathepsin D, which is a lysosomal protein required for complete
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autophagic degradation, was significantly up-regulated by BPA treatment, suggesting autophagic induction against BPA rather than accumulation of autophagosomes. Phosphatidylethanolamine-binding protein, which is involved in LC3-II lipidation, was also significantly increased due to BPA treatment. Other proteins detected were heat shock cognate protein (Hsc71, Hsc70, and Hsc60) and peroxiredoxin-2, which play a vital role in the identification and proteasomal degradation of redundant proteins as well as enhancing their lysosomal activity. Similarly, peroxiredoxin, which is involved in peroxisome-mediated autophagy, was also significantly up-regulated by BPA. Several other metabolic enzymes, such as pyruvate kinase, malate dehydrogenase and its isoforms, and α- and γ-enolase and their isoforms were also significantly up-regulated by BPA (Table 1).
BPA Induces Autophagy in the Hippocampal NSC-derived Neurons in Vitro—To examine whether BPA induces autophagy, neuronal cells were treated with various non-cytotoxic concentrations of BPA (i.e., 0, 25, 50, and 100 μM). The levels of LC3-II were significantly up-regulated in the hippocampal NSC-derived neurons by a 100 μM concentration of BPA as compared with control (Fig. 3, A and B). BPA (100 μM) significantly enhanced the levels of LC3-II during 12 h of exposure (Fig. 3, A and B). A significant increase was found in the levels of LC3-II after 3 h of BPA exposure in neuronal cultures, which further augmented at 12 h (Fig. 3, C and D). We next studied the effects of BPA on the formation or accumulation of GFP-LC3 puncta in GFP-LC3-transfected hippocampal NSC-derived neuronal cells. After transfection, we examined the formation of GFP-LC3 puncta and puncta index in neuronal cells, which is an indicator of formation of autophagosomes in the cells. We observed a significantly increased number of GFP-LC3 puncta in 100 μM BPA-treated neuronal cultures as compared with control (Fig. 3, E and F). Further, we confirmed that increased LC3-II levels observed are due to the enhanced autophagy rather than blockade at any step during autophagy. To this end, we arrested LC3-II-mediated autophagosome degradation using lysosomal protease inhibitor bafilomycin A1 (60 nM) in BPA-treated neuronal cells. BPA treatment in the presence of bafilomycin A1 potentiated LC3-II lipidation and the GFP-LC3 puncta index in neuronal cultures in comparison with cultures treated with BPA alone (Fig. 3, G–J). Moreover, for starvation, neuronal medium was replaced with HBSS (Fig. 3, G and H).
We further validated the effects of BPA on LC3-II lipidation and GFP-LC3 puncta formation in the presence/absence of pharmacological activator (rapamycin) and inhibitor (wortmannin) and genetic inhibitors (beclin siRNA) of autophagy. Beclin siRNA significantly reduced beclin protein levels (Fig. 4A). BPA along with rapamycin enhanced the levels of LC3-II.
protein as well as increased the GFP-LC3 puncta index in neuronal cultures (Fig. 4, B–E). Moreover, preincubation of neuronal cultures with wortmannin followed by BPA exposure inhibited LC3-II lipidation and also decreased the number of GFP-LC3 puncta. Further, siRNA-mediated knockdown of beclin suppressed the formation of GFP-LC3 puncta after BPA treatment (Fig. 4, B–E). Thus, these results imply that rapamycin potentiates and that wortmannin and beclin siRNA inhibit LC3-II levels and GFP-LC3 puncta formation after BPA treatment in hippocampal NSC-derived neuronal cultures.

To examine the effects of BPA on autophagic substrate, the levels of p62 were studied in the cultures of hippocampal neurons treated with BPA in the presence/absence of bafilomycin A1. BPA treatment in the presence of bafilomycin A1 increased the levels of p62 protein (Fig. 4, F–I). Moreover, the treatment of BPA alone in neuronal cultures decreased the levels of p62 protein (Fig. 4, F–I). Treatment with BPA along with rapamycin further decreased the levels of p62 (Fig. 4, F–I). On the contrary, BPA treatment along with rapamycin enhanced the levels of p62 in the presence of bafilomycin A1 (Fig. 4, F–I). These results suggest that BPA induces autophagic flux rather than inhibiting autophagic proteolysis.

HMGB1, an endogenous proautophagic protein, participates in the autophagy process, increases cell survival, and retards apoptotic cell death (61, 62). Translocation of HMGB1 from the nucleus to the cytosol induces autophagy (61, 62). We found...
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Autophagy generated against BPA promotes extranuclear HMGB1 translocation. A and B, rat hippocampal NSC-derived neurons were treated with rapamycin (100 nm), wortmannin (10 μM), and beclin siRNA followed by BPA (100 μM) for 12 h and then were immunostained with HMGB1. The figure depicts translocation of HMGB1 from the nucleus to the cytosol. The mean nuclear and cytosolic HMGB1 intensity per cell were observed by imaging cytometric analysis. The data are represented as mean ± SEM (bars) (n = 3 independent experiments). Scale bar, 20 μm. *p < 0.05.

that HMGB1 translocation takes place in conditions like starvation and in the presence of inhibitors (i.e. rapamycin in neuronal cultures). We observed that BPA treatment resulted in extranuclear HMGB1 translocation as compared with control, wortmannin and beclin siRNA (Fig. 5A). However, the cytosol after BPA treatment was observed in neuronal cultures, the results suggest that BPA also induced extranuclear translocation of HMGB1, leading to increased HMGB1 levels.

Autophagy Protects BPA-induced Toxicity and Apoptosis in Neuronal Cells and in the Hippocampus of the Rat Brain—BPA causes apoptotic cell death in neurons (8), and autophagy is reported to regulate apoptosis (23, 63). Therefore, to study the role of autophagy in the regulation of BPA-mediated apoptotic cell death, we treated neuronal cells with rapamycin as well as wortmannin. BPA treatment significantly enhanced the number of PI-positive and cleaved caspase-3-positive cells in vitro (Fig. 6, A and B) and in vivo (Fig. 6, C and D). Treatment of rapamycin along with BPA significantly mitigates BPA-mediated toxicity and apoptotic cell death. Conversely, wortmannin further enhanced cytotoxicity and apoptosis in BPA-treated cultures (Fig. 6, A and B). Concurrently, beclin knockdown resulted in significant reduction of autophagy and exacerbated BPA-mediated apoptotic cell death in neuronal cultures (Fig. 6, A and B). Scrambled siRNA sequence showed no significant effect on cell viability (data not shown). The pretreatment of catalase decreased BPA-induced cytotoxicity and apoptotic cell death even in beclin siRNA-transfected neuronal cultures after BPA treatment. These findings suggest that BPA-induced cell death was significantly exacerbated and mitigated by the inhibition and activation of autophagy, respectively (Fig. 6, A and B).

We next studied the role of autophagy in the regulation of BPA-mediated apoptotic cell death in the hippocampus of the rat brain. BPA significantly enhanced the number of caspase-3-positive cells in the dentate gyrus, hilus, and molecular layer region of the hippocampus as compared with control (Fig. 6, C and D). Rapamycin (1 and 2 mg/kg body weight, intraperitoneally) reduced the number of caspase-3-positive cells in BPA-treated rats. These results suggest that the induction of autophagy reduces BPA-mediated apoptotic cell death in the hippocampus region of the rat brain.

Autophagy as a Protective Response against BPA-induced ROS Generation—Exposure with BPA causes ROS generation, resulting in neurodegeneration inside the cell (1, 48). Therefore, we studied the role of autophagy in BPA-mediated ROS generation in neuronal cultures. AMPK and mTOR siRNA significantly reduced the respective protein levels (Fig. 7A). The neuronal cultures were treated with an antioxidant enzyme catalase and an antioxidant, N-acetylcysteine, followed by BPA treatment for 24 h. Catalase significantly enhanced the levels of LC3-II and LC3-I in BPA-treated neuronal cells, in which treatment with NAC was given prior to BPA, and BPA treatment with catalase resulted in increased ROS generation in BPA-treated neuronal cultures (Fig. 7B). Pretreatment with catalase inhibited autophagy by reducing the levels of LC3-II and LC3-I in BPA-treated neuronal cultures. BPA-mediated ROS generation was increased in a time-dependent manner in neuronal cells (Fig. 7, C–E). BPA-mediated ROS generation was decreased by the autophagy inducer rapamycin and further enhanced in the presence of bafilomycin A1.

Further, we examined the increase in the levels of malondialdehyde after BPA exposure. Interestingly, BPA exposure enhanced the levels of malondialdehyde in beclin and AMPK siRNA- and catalase-treated neuronal cultures, where autophagy was reduced (Fig. 7F). Induction of autophagy by mTOR siRNA and rapamycin alleviated the BPA-induced increase in lipid peroxidation. Similarly, activation of autophagy increased the levels of total glutathione in BPA-treated neuronal cells. On the other hand, inhibition of autophagy further reduced the glutathione levels in BPA-treated cells (Fig. 7G).
BPA-induced Autophagy Is Associated with the Activation of AMPK and Down-regulation of mTOR Signaling—The AMPK and mTOR pathways are primarily involved in the autophagy process in cells. Therefore, we studied the role of the AMPK/mTOR pathways in BPA-mediated autophagy. We observed a dose-dependent decline in the ATP levels in response to BPA exposure in hippocampal NSC-derived neuronal cultures (Fig. 8A). We determined the effects of BPA on neuronal cells after silencing the AMPK and mTOR genes, through flow cytometry PI uptake apoptosis analysis (Fig. 8, B and C). BPA exposure increased the number of apoptotic cells, which was further enhanced after AMPK knockdown in neuronal cells. On the contrary, mTOR knockdown in BPA-exposed cultures significantly decreased the numbers of apoptotic cells (Fig. 8, B and C). These results suggest the involvement of AMPK activation in autophagy induction against BPA-induced cell death, whereas silencing of mTOR enhanced autophagy, leading to decreased apoptosis after BPA exposure (Fig. 8, B and C). Silencing of AMPK decreased BPA-induced phosphorylation of AMPK at Thr-172 in neuronal cells (Fig. 8, D and E). Moreover, BPA-induced GFP-LC3 puncta and LC3-II levels were also decreased after AMPK knockdown (Fig. 8, D–F).

Furthermore, BPA decreased the phosphorylation of mTOR at Ser-2481 and phosphorylation of its downstream target...
P70S6K at Thr-389 (Fig. 9, A and B). In addition, BPA also decreased the phosphorylation of ULK at Ser-757 in neuronal cells (Fig. 9, A and B). Interestingly, BPA increased the phosphorylation of AMPK, ACC at Ser-79 and raptor at Ser-792 (Fig. 9, C and D). AMPK knockdown resulted in significant down-regulation of ACC and raptor phosphorylation, but up-regulation of ULK1 at Ser-757 in BPA exposed neuronal cultures (Fig. 9, C and D). Corresponding with these findings, BPA-mediated decrease in phospho-mTOR levels was suppressed by AMPK knockdown, suggesting involvement of the mTOR as a downstream effector of the AMPK pathway in regulation of autophagy induced against BPA exposure (Fig. 9, E and F).

BPA-induced Mitochondrial Loss and Mitophagy in Neuronal Cells—Mitochondrial dysfunction is a major source of ROS generation and cell death; therefore, we hypothesized that BPA-mediated cell death could be attributed to enhanced mitochondrial dysfunction. To study the effects of BPA on mitochondrial mass in neuronal cells, we stained neuronal cells with the mitochondria-specific dye NAO. Treatment of neuronal cells with 25, 50, and 100 μM BPA for 12 h caused a reduction in mitochondrial mass dose-dependently (Fig. 10A). We next examined the ratio of mRNA for COX-II (mitochondrial DNA-encoded gene) and 18S rRNA (nucleus-encoded transcript), to study mitochondrial DNA content (54). We found significant reduction in the COX-II/18S rRNA ratio after BPA exposure in
neuronal cells, suggesting mitochondrial loss by BPA treatment (Fig. 10B). Mitochondrial dysfunction plays an important role in the generation of ROS, and dysfunctional mitochondria are removed by a process known as mitochondrial autophagy (mitophagy) (64). Mitophagy involves selective degradation of damaged mitochondria by autophagosomes, by passing them to lysosomes (64, 65) (data not shown). Co-localization of the pmKate mitochondrial reporter gene (55, 56) or pDsRed2 with GFP-LC3 is an indicator of the mitophagy process in the cells (66). We observed enhanced co-localization of the pmKate mitochondrial reporter gene (red) with GFP-LC3 (green) (Fig. 10) (Manders co-localization coefficient \(M = 0.19\) after BPA exposure as compared with control \(M = 0.05\)). In BPA + rapamycin and BPA + beclin siRNA-treated cultures, the co-localization coefficients were \(M = 0.27\) and 0.10, respectively (Fig. 10, C and D). We observed that increased autophagic activity, in response to BPA, was involved with an increase in the number of mitochondria co-localized with autophago-
somes in neuronal cells (i.e. LC3-positive mitochondria). Additionally, we observed that rapamycin treatment enhanced the number of GFP-LC3-positive autophagosomes as well as the number of mitochondria that co-localized with autophagosomes (Fig. 10, C and D). On the contrary, we also found that knockdown of the expression of beclin inhibited the number of mitochondria co-localized with autophagosomes (Fig. 10, C and D). The increase in autophagosome number was due to an increase in autophagosome formation rather than proteolytic inhibition (data not shown).

**BPA Enhances Mitophagy by Increasing the Levels of PINK1 and PARKIN and of PARK2 Mitochondrial Translocation**—Next, we studied the effect of BPA on the expression and levels of PARKIN and PINK, which are involved in mitochondrial quality control (67). Several lines of evidence indicate that damaged mitochondria lead to neurodegenerative disorders (68). It is observed that PINK1 and PARKIN recruit on the damaged mitochondria and promote their separation from mitochondrial circuitry by degradation via mitophagy (67–69). BPA treatment enhanced the expression of PINK and PARKIN (PARK2) genes and their protein levels in mitochondrial fractions dose-dependently (Fig. 11, A–C). To study the effects of BPA on PARK2 mitochondria translocation, neuronal cells were transfected with YFP-PARK2 and mito-CFP plasmids. BPA treatment caused increased PARK2 translocation to mitochondria, suggesting enhanced mitophagy (Fig. 11, D and E). We observed enhanced co-localization of PARK2 (green) with mito-CFP (red) (M = 0.29) after BPA exposure as compared
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We studied a time course response of BPA on the levels of autophagy proteins p62 and LC3-II, antioxidant enzymes catalase and SOD, and apoptotic protein cleaved caspase-3. NSC-derived neuronal cultures were exposed with BPA up to 72 h. The levels of LC3-II by BPA exposure were significantly increased only up to 12 h (Fig. 12, A and B). Further, BPA increased the levels of p62 up to 12 h, which was significantly decreased in the BPA treatment after 24 h (Fig. 12, C and D). The levels of cleaved caspase-3 and LAMP-2 after 12 h (Fig. 12, E and F), suggesting the impairment of the autophagy-pathway and regulation of apoptosis. Similarly, the time-dependent decrease in the levels of catalase and SOD (Fig. 12, A and B). These results suggest altered BPA exposure caused impairment of autophagic flux, which resulted in BPA-mediated apoptosis. Further, we also found that long term exposure of BPA enhanced hippocampal neurodegeneration (Fig. 12, E and F), leading to impaired learning and memory ability in the rats (Fig. 12G). Interestingly, activation of autophagy by administration of rapamycin caused reduced neurodegeneration and also restored learning and memory deficits induced after BPA treatment (Fig. 12, E–G).

On the basis of our experimental studies, we proposed a schematic model illustrating the plausible mechanism(s) of BPA-mediated alterations on the regulatory dynamics of autophagy (Fig. 13). BPA-induced loss in the ATP levels were compensated with a rise in the phosphorylation of AMPK, suggesting that autophagy generated against BPA-induced toxicity was accompanied by the up-regulation of AMPK. Furthermore, AMPK activation resulted in phosphorylation of AMPK substrates, such as ACC and raptor, to preserve the cellular ATP pool during BPA exposure. BPA induced autophagy by decreased phosphorylation of mTOR and its downstream target molecules, including P70S6K, and also of ULK1 at Ser-757. Interestingly, silencing the expression of AMPK enhances the phosphorylation of ULK1 at Ser-757 and mTOR, supporting the involvement of AMPK chiefly in autophagy generated against BPA. Inhibition of autophagy aggravated BPA-induced neurotoxicity by enhancing ROS generation and apoptotic cell death. Activation of autophagy by rapamycin and mTOR siRNA reduced the number of BPA-induced apoptotic cells in

with control ($M = 0.04$) (Fig. 11). In BPA + beclin siRNA- and BPA + PINK1 siRNA-treated cultures, co-localization coefficient were $M = 0.14$ and 0.08, respectively. BPA treatment also increased PARKIN levels in the mitochondrial fraction, leading to decreased levels in cytosol (Fig. 11F). Further, we also examined the effects of BPA on mitophagy in neuronal cells after AMPK silencing. BPA enhanced co-localization of GFP-LC3 plasmid with TOMM20 protein. We observed enhanced co-localization of TOMM20 protein (red) with GFP-LC3 (green) ($M = 0.31$) after BPA exposure as compared with control ($M = 0.08$). In AMPK siRNA- and BPA + AMPK siRNA-treated cultures, the co-localization coefficient was $M = 0.04$ and 0.12, respectively. We observed that BPA-induced damaged and fragmented mitochondria were taken up by autophagosomes for degradation (Fig. 11, G and H). In addition, BPA decreased the levels of mitochondrial proteins TOMM20 and COX-IV and p62, showing enhanced mitophagy, which was significantly blocked by AMPK knockdown in neuronal cells (Fig. 11, I and J). These results suggest that mitophagy was induced in response to BPA exposure, through activation of the AMPK pathway.

Time-dependent Responses of BPA on Autophagy, Apoptosis, and Antioxidant Levels in Neuronal Cells—We studied a time course response of BPA on the levels of autophagy proteins p62 and LC3-II, antioxidant enzymes catalase and SOD, and apoptotic protein cleaved caspase-3. NSC-derived neuronal cultures were exposed with BPA up to 72 h. The levels of LC3-II by BPA exposure were significantly increased only up to 12 h (Fig. 12, A and B). Further, BPA increased the levels of p62 up to 12 h, which was significantly decreased in the BPA treatment after 24 h (Fig. 12, C and D). The levels of cleaved caspase-3 and LAMP-2 after 12 h (Fig. 12, E and F), suggesting the impairment of the autophagy-pathway and regulation of apoptosis. Similarly, the time-dependent decrease in the levels of catalase and SOD (Fig. 12, A and B). These results suggest altered BPA exposure caused impairment of autophagic flux, which resulted in BPA-mediated apoptosis. Further, we also found that long term exposure of BPA enhanced hippocampal neurodegeneration (Fig. 12, E and F), leading to impaired learning and memory ability in the rats (Fig. 12G). Interestingly, activation of autophagy by administration of rapamycin caused reduced neurodegeneration and also restored learning and memory deficits induced after BPA treatment (Fig. 12, E–G).

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vitro and in vivo. Mitophagy was also increased against BPA neurotoxicity, which reduced the accrual of damaged mitochondria by mitochondrial translocation of PARK2. Beclin and PINK1 siRNA blocked BPA-mediated enhanced PARK2 mitochondrial translocation and thus mitophagy. Thus, BPA-induced toxicity was mitigated through the generation of an autophagic response.

Discussion

Several clinical and experimental studies suggest that exposure of xenoestrogen BPA causes cognitive and behavioral alterations in humans and animals (1, 2, 4, 7, 10, 11, 70, 71). BPA causes ROS generation, which decreases the process of generation of new neurons (neurogenesis) and myelination in the hippocampus as well as altering cognitive functions (10, 11, 53). BPA exerts neurotoxicity through the induction of apoptosis in neurons (14). However, whether BPA-induced ROS generation and apoptosis in the brain are associated with other forms of cell death mechanism (i.e., autophagy) is still elusive. Autophagy is found to be decreased in the pathogenesis of several neurodegenerative and neurological disorders (25). A recent study has reported the role of autophagy in BPA-induced glucose metabolic disorders in the pancreas of high fat diet-fed rats (73). Herein, for the first time, we explored the role of autophagy in
BPA-mediated neurotoxicity. We examined the underlying molecular mechanism(s) of BPA-induced autophagy in the hippocampus region of the rat brain and hippocampal NSC-derived neurons in culture. We reported that autophagy flux was generated as a cellular protective response against BPA neurotoxicity through the dynamic interplay among the AMPK and mTOR pathways. Strikingly, autophagy by increasing the cellular tolerance might act as a secondary protective response against the oxidative stress (74). 

**BPA Induces Autophagic Flux**—Autophagy acts as a cardinal process in sustaining the protein machinery by the process of membrane trafficking, involving degradation and recycling excess of defective macromolecules inside the cells (75). Autophagy is greatly enhanced during developmental stages.
and also becomes activated in response to environmental stressors to maintain the cellular homeostasis (31, 76). The conversion of LC3-I to LC3-II and the formation of autophagosomes are hallmark features of autophagy (58). However, an increase in the LC3-II levels may also be due to the decrease in autophagic proteolysis (58).

We found that BPA enhanced LC3-II lipidation both in vivo and in vitro. The expression and levels of p62 were decreased by BPA treatment time-dependently. Further, we found that the mRNA expression of autophagy genes, such as \( \text{Lc3} \), \( \text{Hmgb1} \), \( \text{Beclin-1} \), \( \text{Atg5} \), \( \text{Atg12} \), \( \text{Atg3} \), and \( \text{Lamp-2} \), were significantly increased following BPA treatment. Similarly, the protein levels of LC3-II, HMGB1, and BECLIN-1 were also increased by BPA. Subsequently, using electron microscopy, we found that BPA significantly enhanced generation of multilamellar vesicular bodies and single-membrane organelle autolysosomes in the hippocampus, suggesting the induction of autophagy. In addition, with the help of the two-dimensional analysis in tandem with mass spectrometry, we found alterations in the proteomics profile of several autophagy-related proteins in the hippocampus.
pus region of the BPA-treated rat as compared with control. Next, we found that BPA enhanced the formation of GFP-LC3 puncta and up-regulated LC3-II levels in the presence of bafilomycin A1 (a persuasive inhibitor of lysosome V-ATPases) and caused accumulation of autophagosomes by impairing the fusion between autophagosome and lysosome (60). These results suggest that BPA induced autophagic flux rather than inhibiting autophagic proteolysis. Similarly, other toxicants, such as ethanol (34) and cadmium (30), were also found to increase GFP-LC3 puncta and decreased levels of p62.

Further, we studied the mechanistic aspects of autophagy generated against BPA neurotoxicity. We found that the exposure of rapamycin (an autophagy inducer) enhances the BPA-mediated increase in the levels of LC3-II and the number of GFP-LC3 puncta in neuronal cells. Conversely, wortmannin (an autophagic inhibitor) and beclin siRNA restrain BPA-induced GFP-LC3 puncta and LC3-II levels. Considering the fact that autophagy is enhanced in serum-deprived conditions, the experiments were both performed in HBSS (i.e. starvation- and nutrient-rich conditions) to avoid any discrepancy.

Autophagy as the Cell’s Self-compensatory Response against BPA-induced Cytotoxicity as Well as Oxidative Damage—Several studies have reported that BPA exposure results in enhanced oxidative stress, apoptotic cell death, and neurotoxicity (1, 5, 14, 77). Numerous compounds, including melatonin, green tea, etc. are reported to ameliorate BPA-induced neurotoxicity as well as cognitive deficit. BPA exposure may deplete the levels of antioxidants inside the cells (1, 15, 78). We also found that exposure with antioxidants like catalase and an antioxidant NAC inhibited BPA-induced LC3-II up-regulation, signifying that autophagy is generated against BPA-induced autophagy.

The paradox regarding the autophagy-mediated cell death still exists, whether autophagy is life or death. Autophagy is also responsible for cell death (runaway autophagy). It has been reported that runaway autophagy (excessive autophagy) results in breakdown of crucial cell organelles as well as biomolecules (26, 79). Thus, it is very important that autophagy should take place at a particular threshold, and beyond that, it may prove to be deleterious (26, 80). Overall, our results suggest that autophagy is generated as a transitory cellular tolerance against BPA-mediated toxicity to mitigate BPA-induced oxidative stress and cell death. Moreover, exposure with antioxidants curtails BPA-induced neurotoxicity by decreasing oxidative stress and ROS generation in neuronal cells. However, our study suggested that activation of autophagy either pharmacologically or genetically alleviated BPA-induced ROS generation, by decreasing the levels of peroxides and superoxides (13) as well as by decreasing the levels of malondialdehyde, which were increased after BPA exposure (15–21). In addition, BPA-induced decreases in total glutathione levels were also ameliorated by activation of autophagy. Further, autophagy activation also decreased caspase-3 activation and neurodegeneration in the hippocampal neurons and also caused inhibition of cognitive impairments. Conversely, inhibition of mTOR enhanced autophagy in BPA-treated neuronal cultures, thereby decreasing BPA induced ROS generation and apoptotic cell death.

Autophagy Induced against BPA Involved Dynamic Interplay among the AMPK and mTORC Pathways—AMPK is attributed as one of the fundamental controller of cellular metabolism in eukaryotes, which activates during cellular ATP depletion (38). Inhibition of AMPK significantly inhibits autophagy and also decreases the viability of astrocytes during oxygen- and glucose-deprived conditions (81). Autophagy induction is lethal in neurons lacking AMPK, whereas AMPK induces autophagy by inhibiting the mTOR pathway (82). During stress conditions, AMPK activates the catabolic pathways and inhibits energy-utilizing processes inside the cell (37, 42). On the contrary, mTOR acts as a crucial target of many essential pathways, such as insulin, growth factors, and nutrient transporters, and is also involved in regulation of autophagy (83). The mTOR pathway is activated in the presence of excess nutrients, neurotrophic factors, and neurotransmitters, which increases protein synthesis and inhibits autophagy (83). BPA exposure results in neuronal migration loss, increased neurodegeneration, and decreased synaptogenesis (8, 84). In this study, we observed that BPA treatment depleted the energy content inside the cells by lowering the ATP levels in comparison to the BPA-mediated low energy levels in the sham group, and these findings were enhanced and accompanied by increases in ROS. The activation of the AMPK pathway results in phosphorylation of raptor inhibiting the mTOR pathway (82). During stress conditions, AMPK activation inhibits mTORC1 by phosphorylation of TSC and raptor, which increases the phosphorylation of raptor and ACC. Interestingly, AMPK knockdown resulted in increased phosphorylation of mTOR and also ULK1 (Ser-757), which was decreased after BPA treatment, suggesting the involvement of mTOR as an effector downstream module of AMPK pathway in BPA-mediated autophagy. Most importantly, it was observed that BPA decreased the phosphorylation of ULK1 at Ser-757. During nutrient-rich conditions, mTORC1 becomes activated and disrupts ULK1-AMPK interaction, leading to the inhibition of autophagy (37, 41, 85, 86). At the time of starvation or stress conditions, AMPK activation inhibits mTORC1 by phosphorylation of TSC and raptor, which reduces ULK1 Ser-757 phosphorylation (37, 41). Therefore, unphosphorylated ULK1 at Ser-757 is able to interact with and is activated by AMPK (41, 87). Thus, a balanced coordination among these kinases helps in regulation of the cellular decision regarding autophagy induction and cell proliferation (37, 85). We observed that BPA exposure decreased the phosphorylation of ULK1 Ser-757, which results in the inability of ULK1 to interact with mTOR. Thus, overall, it can be concluded that autophagy is induced against BPA neurotoxicity through the involvement of the AMPK and mTOR pathways.

BPA-mediated Induction of Mitophagy Blocked by AMP Inhibition—Mitochondria are indispensable organelles in regulation of the cellular homeostasis. The exclusion of defective mitochondria by delivering them to lysosomes for their degra-
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diation (i.e. through autophagy) is known as “mitophagy” (64). We observed that mitophagy is enhanced against BPA neurotoxicity, which comprises mitochondrial loss, leading with bioenergetic deficits. BPA enhanced the co-localization of GFP-LC3 with the pmKate mitochondrial reporter gene, which was further increased in the presence of rapamycin, suggesting increased mitophagy. On the contrary, beclin-1 knockdown decreased BPA-induced mitophagy, thereby decreasing the co-localization of GFP-LC3 with pmKate. Recent studies have revealed a vital role of PINK1 and PARKIN in regulation of mitophagy. Intriguingly, we also found that enhanced mitophagy against BPA was correlated with the rise in the expression and levels of PINK1 and PARKIN (PARK2). We found that BPA induced PARK2 mitochondrial translocation, as observed by enhanced YFP-PARKIN and mito-CFP co-localization. Several recent studies suggest vital involvement of AMPK in the regulation of the mitophagy process (88). We observed that AMPK knockdown resulted in defective mitophagy and increased levels of TOMM 20, COX-IV, and p62 from the basal level. Strikingly, exposure of BPA decreased the levels of TOMM 20, COX-IV, and p62 even after AMPK knockdown, unambiguously showing that BPA induced mitochondrial loss, resulting in mitophagy. Next, we also observed that BPA induced the co-localization of TOMM20 and GFP-LC3, which was reduced by the AMPK knockdown. The results suggest vital involvement of the AMPK pathway for mitophagy induced against BPA. Hence, we may speculate that mitophagy is enhanced against BPA neurotoxicity and impairs damaged mitochondria and to prevent programmed cell death. The levels of p62 were found to be increased, whereas levels of Lamp-2 were significantly decreased at later time points, suggesting an impaired autophagy-lysosome pathway and cognitive dysfunctions (90, 91). The decrease in the levels of Lamp-2 results in accumulation of p62 aggregates and neurodegeneration (91). Thus, we demonstrated that impaired autophagic flux by BPA enhances apoptosis by elevating the levels of cleaved caspase-3. Several studies report that autophagy impairment increases caspase-3 activity and cell death (72, 92). Interestingly, long term exposure of BPA caused hippocampal neurodegeneration and reduced learning and memory ability in the rats. Activation of autophagy reduced BPA-mediated neurodegeneration and also restored learning and memory deficits.

In conclusion, these results suggest that autophagy acts as a transitory survival pathway under stress conditions, but at the same time, when it occurs excessively or when it is impaired, it may be deleterious and also lead to activation of apoptosis-mediated cellular demise. Last, the autophagic responses against the widespread usage of environmental xenoestrogen require further assessment.

Author Contributions—S. A., S. K. T., and R. K. C. conceived and coordinated the study, performed experiments, analyzed data, and wrote the paper. S. A., S. K. T., B. S., A. Y., R. S. R., S. K. G., A. T., A. K., and S. S. designed, performed, and analyzed the experiments shown in Figs. 1–9. A. S., A. M., and D. P. designed, performed, and analyzed the experiments shown in Fig. 2F and Table 1. L. K. S. C performed and analyzed the experiments shown in Fig. 2, D and E. S. A., S. K. T., and V. C. performed and analyzed the experiments shown in Figs. 10–12. All authors reviewed the results and approved the final version of the manuscript.

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