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Exposure to BDE-153 induces autophagy in HepG2 cells

Lilian Cristina Pereira[a,b,*], Filipe Valente Duarte[c], Ana Teresa Inácio Ferreira Varela[c], Anabela Pinto Rolo[c,d], Carlos Manuel Marques Palmeira[c,d], Daniel Junqueira Dorta[e]

[a] Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Universidade de São Paulo, Av. Bandeirantes, 3900, CEP:14040901, Bairro Monte Alegre, Ribeirão Preto, São Paulo, Brazil
[b] Universidade Estadual Paulista (Unesp), Faculdade de Ciências Agronômicas, Botucatu, Departamento Bioprocessos e Biotecnologia, Fazenda Experimental de Lageado, Rua José Barbosa de Barros, no. 1780, CEP: 18.610-307, Bairro Jardim Paraíso, Botucatu, São Paulo, Brazil
[c] Centro de Neurociências e Biologia Celular, Universidade de Coimbra, Rua Larga, Faculdade de Medicina, Pólo 1, 3004-504 Coimbra, Portugal
[d] Universidade de Coimbra, Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Apartado 3046, 3001-401 Coimbra, Portugal
[e] Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Departamento de Química, Universidade de São Paulo, Av. Bandeirantes, 3900, CEP:14040901, Bairro Monte Alegre, Ribeirão Preto, São Paulo, Brazil

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ABSTRACT

Autophagy is a pro-survival process that occurs under stressful "life-threatening" conditions. This process clears the cells of damaged organelles, long-lived proteins, and/or misfolded proteins. Under stressful conditions, activation of the autophagic process leads to cell death and acts as a protective mechanism against xenobiotic, which is the most widely accepted mechanism in the literature. Exposure to flame retardants and other pollutants is associated with several diseases, during which cell death and mitochondrial damage takes place. Although a body of research has aimed to understand the toxicity mechanism of flame retardants better, risk evaluation and the consequences of exposure to these toxicants have been poorly described. In this work, we have found that the BDE-153 congener (representant of flame retardants) induces autophagy after 24 and 48 h (0.1–25 μM). The autophagic process is associated with accumulation of lysosomes, and process triggering is evident from the levels of autophagy-related proteins such as p62 and LC3. Mitophagy (an autophagic process that specifically involves damaged mitochondria) may be involved, as judged from the decreased amount of mitochondrial DNA. Taken together, our results point out that induction of autophagy upon cell should contribute to better understanding of the consequences of human exposure to this class of environmental contaminants.

1. Introduction

Autophagy is a conserved catabolic process that degrades cytoplasmic constituents and organelles through the lysosome (Kaur and Debnath, 2015). At the beginning of the autophagic mechanism, the subcellular membranes rearrange themselves in order to sequester or engulf cytoplasmic material (cytosolic proteins and/or organelles) into a double-membrane vesicle named autophagosome, which is then transported and fused with the lysosome for proteolysis (Maiuri et al., 2007; Kim and Lee, 2014). Induction of this phenomenon represents an attempt of the cell to cope with stress and avoid cell death (Maiuri et al., 2007) or a cellular response to altered metabolism starvation, nutrient-replete conditions, and the presence of xenobiotics (Kaur and Debnath, 2015; Kim and Lee, 2014; Mizushima et al., 2010) such as emerging contaminants (Chiarelli and Roccheri, 2012; Ptak et al., 2012).

Knowledge about the mechanisms that underpin the toxicity of emerging contaminants gained over the past 30 years has provided a universal framework for the study of several compounds. Polybrominated diphenyl ethers (PBDEs) are a group of flame retardants that are widely used in industries and yet are classified as emerging contaminants. These toxic compounds affect human reproduction, development, and hormonal homeostasis as mentioned in some reviews (Darnerud, 2003; Dorta et al., 2013). The number of possible PBDE congeners can be as high as 209. Among them, 2,2′,4,4′,5,5′-hexabromodiphenyl ether (BDE-153) is frequently detected in the environment and in human tissues (Zhang et al., 2014).

Even though BDE-153 has attracted much attention and raised great concern in recent years, its mechanism of toxicity still needs clarification. Preliminary results obtained by our research group have shown

* Corresponding author at: Universidade Estadual Paulista (Unesp), Faculdade de Ciências Agronômicas, Botucatu, Departamento Bioprocessos e Biotecnologia, Fazenda Experimental de Lageado, Rua José Barbosa de Barros, no. 1780, CEP: 18.610-307, Bairro Jardim Paraíso, Botucatu, São Paulo, Brazil.
E-mail address: liliancp@fca.unesp.br (L.C. Pereira).

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that PBDEs can induce mitochondrial damage by causing mitochondrial swell, dissipating the mitochondrial membrane potential, and disrupting the electron transport chain. Furthermore, these compounds can induce cell death (Pazin et al., 2015; Pereira et al., 2013; Pereira et al., 2014; Souza et al., 2013). To address these issues, in this study we have determined cellular toxic effects in liver cells exposed to BDE-153. More specifically, we have assessed how exposure to BDE-153 induces autophagic processes in human HepG2 hepatoblastoma cells.

2. Materials and methods

2.1. Cell culture

HepG2 cells (ATCC, no. HB8065) were cultured in "Dulbecco's Minimum Essential Medium" (DMEM from Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies, USA), 100 μg/mL streptomycin, and 100 IU/mL penicillin G (antibiotic and antimycotic solution from Sigma-Aldrich, USA) in a controlled atmosphere containing 5% CO_2 at 37 °C in a humidified incubator. The cells were allowed to reach a confluence of about 80–85% in the culture flask, and then an adequate number of cells were plated for 24 h to ensure good adhesion. After 24 h of incubation, the cells were treated with BDE-153 for exposure periods of 24 and 48 h at three concentrations (0.1, 5, and 25 μM), selected from preliminary toxicity tests by various protocols. BDE-153 (CAS: 68631-49-2) were purchased from Accustandard (New Haven, CT, USA) with purity of the 100%.

2.2. Distribution of lysosomes monitored by LysoTracker

HepG2 cells were seeded on glass coverslips in six-well plates as described above. After treatment with BDE-153, the cells were gently washed with phosphate buffered saline (PBS) and incubated for 30 min with 100 nM LysoTracker Red (Invitrogen) to mark acid cell granule, plus 1 μM Hoechst 33,342 (Sigma-Aldrich, USA) to mark cell nucleus in culture medium without phenol red. The cells were then washed, inspected, and photographed with the aid of a Nikon Eclipse TS100 fluorescence microscope. The cells was compared, and the punctuate LysoTracker pattern events in the photographed cells were counted and expressed as percentage with respect to the negative control (n = 3) and used to investigate the degree of autophagy in cells (Rodriguezenriquez et al., 2006; Chikte et al., 2014).

2.3. Immunocytochemistry for LC3-II protein

HepG2 cells were seeded on glass coverslips in six-well plates, as described above, and simultaneously treated with BDE-153 and 50 mM ammonium chloride (to inhibit vesicle degradation). As a positive control for induction of autophagy, HepG2 cells were incubated in "starvation" medium (Hank's Balanced Salt Solution supplemented with 10 mM HEPES, pH 7.4). After treatment, the cells were fixed with pre-warmed formaldehyde (3.7% in PBS) for 1 h and incubated with 1% BSA, 10% "non-lactating" milk, and 0.1% Tween in PBS for 1 h to permeabilize the cells and block possible non-specific binding sites. The cells were then incubated with the anti-LC3 polyclonal antibody, (5 μg/mL, Life technologies, Ref L10382) overnight at 4 °C. After primary antibody incubation, the cells were washed and incubated with secondary antibody conjugated with Alexa Fluor® 594 goat anti-rabbit IgG (Invitrogen, dilution 1: 2000) for 1 h, at room temperature. To stain the DNA viability the cells were incubated with 1 μM Hoechst 33342 (Sigma Aldrich, USA). The cells were analyzed and photographed with a Nikon Eclipse TS100 fluorescence microscope and then classified as autophagy-negative cells (which presented predominantly diffuse LC3-II fluorescence) or autophagy-positive cells (which presented the punctuate LC3-II pattern) and the punctuate LC3-II pattern events in the photographed cells were counted and expressed as percentage with respect to the negative control (n = 3) (Mizushima and Yoshimori, 2007). In addition, wortamannin, a classical inhibitor of the autophagy process, was used to confirm that LC3 labeling in a punctate manner decreased when the autophagic process was inhibited.

2.4. Western Blotting analysis to LC3 and p62 proteins

HepG2 cells were seeded on culture dishes, as described above, and simultaneously treated with BDE-153 and 50 mM ammonium chloride (to inhibit vesicle degradation). After exposure to 24 and 48 h cells were collected and washed with cold PBS and homogenized in RIPA lysis buffer (50 mM Tris/HCl, pH: 7.4; 150 mM NaCl; 1 mM EDTA; 1% triton; 1% deoxycholate; 0.1% sodium dodecyl sulfate (SDS), and SIGMAFast protease inhibitor tablet cocktail (Reference S8820 with dilution 1:1000)), and they were subsequently sonicated for 10 s and centrifuged at 13,000g and 4 °C, for 10 min. Protein concentration was determined by the bicinchoninic acid assay; bovine serum albumin was the standard. And aliquots of equivalent amounts the cell lysates were fractionated in 8–15% sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinylidene difluoride membranes, and incubated with anti-LC3 antibody (dilution 1:500, Sigma Aldrich Ref L7543), anti-tubulin antibody (dilution 1:5000, Sigma Aldrich Ref T6199), anti-p62 antibody (dilution 1:1000, Abcam Ref AB56416), and adequately secondary antibody (dilution 1:5000) conjugated with biotin (Life technologies) chosen as autophagic markers (Klionsky et al., 2016). The membranes were incubated with the Qdot detection system (Life technologies). The fraction prepared from starvation medium was used as positive control. The tubulin content was used as loading control for the total protein content. There were no differences between groups.

2.5. Mitochondrial copy number (Cytochrome b/Pyruvate Kinase ratio)

For mitochondrial DNA (mtDNA) analysis, total DNA was extracted by using a specialized kit (Qiagen GmbH) and quantified in a Nanodrop instrument (Thermo Scientific). The mtDNA copy number was assessed by qPCR on the basis mtDNA was amplified by using primers specific for the mitochondrial encoded gene (Cytochrome b) and normalized against a nuclear encoded gene (Pyruvate Kinase) (Gomes et al., 2012) and compared with the control group (n = 3).

2.6. Statistical analysis

Results are presented as mean ± S.E.M. of the number of different experiments (three or four independent experiments). Solvent controls were included within each experimental determination. The results of the experiments were analyzed by one-way ANOVA followed by Dunnet test for comparison of the several treated groups to their control, and the differences were considered significant when the p values were < 0.05.

3. Results

Autophagosome formation is a marker of late autophagy related with accumulation of acidic vacuolar compartments (Nakatogawa et al., 2009; Mizushima, 2007). LysoTracker is an acidotropic, fluorescent cell-permeant dye that is commonly used to detect lysosomal activity and monitor autophagic processes. Here, after exposure to BDE-153, HepG2 cells displayed increased cellular staining with the lysosomal dye LysoTracker Red (Fig. 1A). Increased punctual marking in lysosomes (acidic granules/lysosomes) occurred at all the evaluated concentrations as compared to the control, for both periods of exposure studied herein (24 and 48 h) showed in Fig. 1B. Culture medium deprived of nutrients (starvation medium) served as a positive control for the induction of autophagy. Intense staining by the LysoTracker Red probe was observed in this control.

In the cell culture, HepG2 cells exposed to BDE-153 or to a condition of starvation had increased LC3-II content. In other words, BDE-153

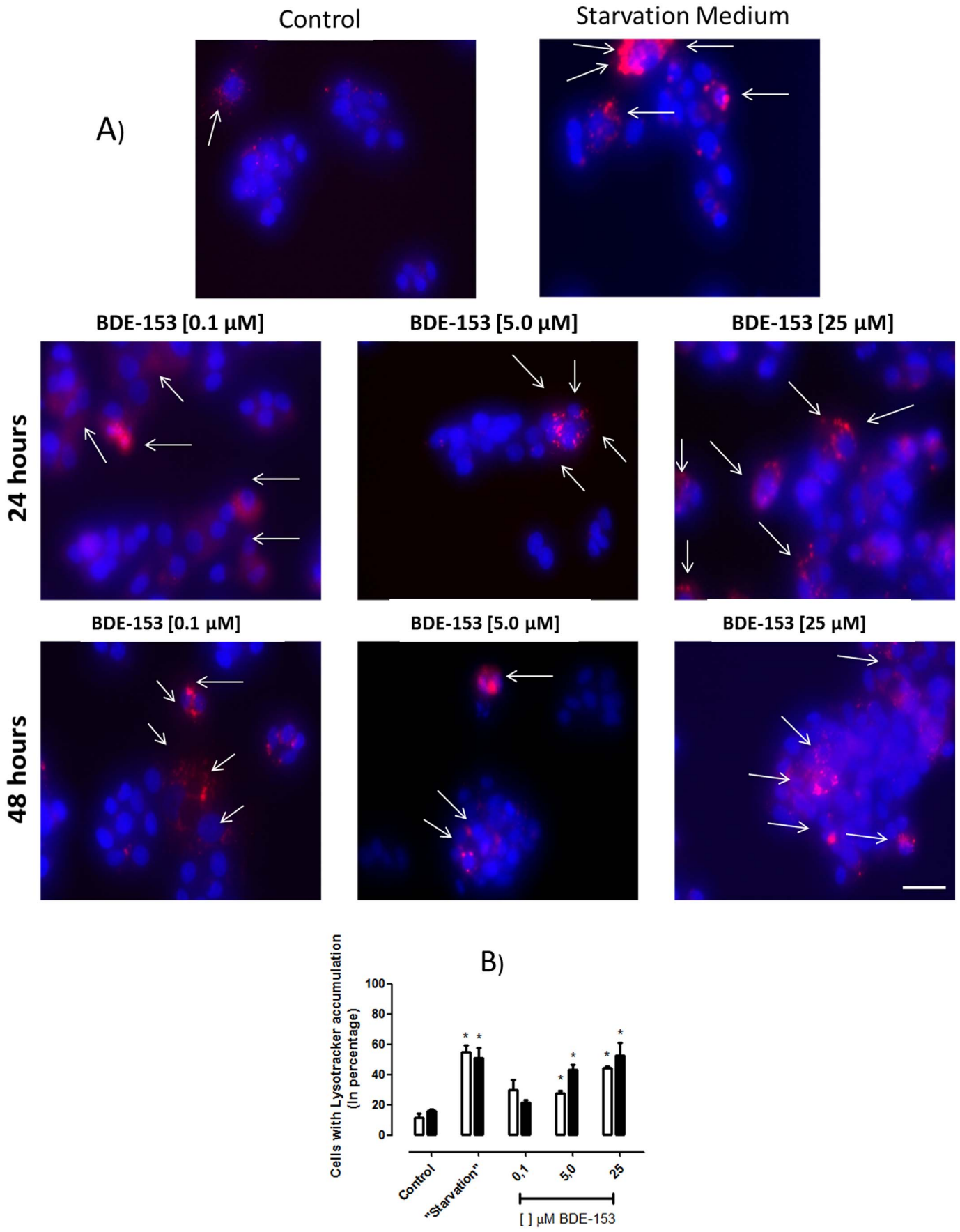

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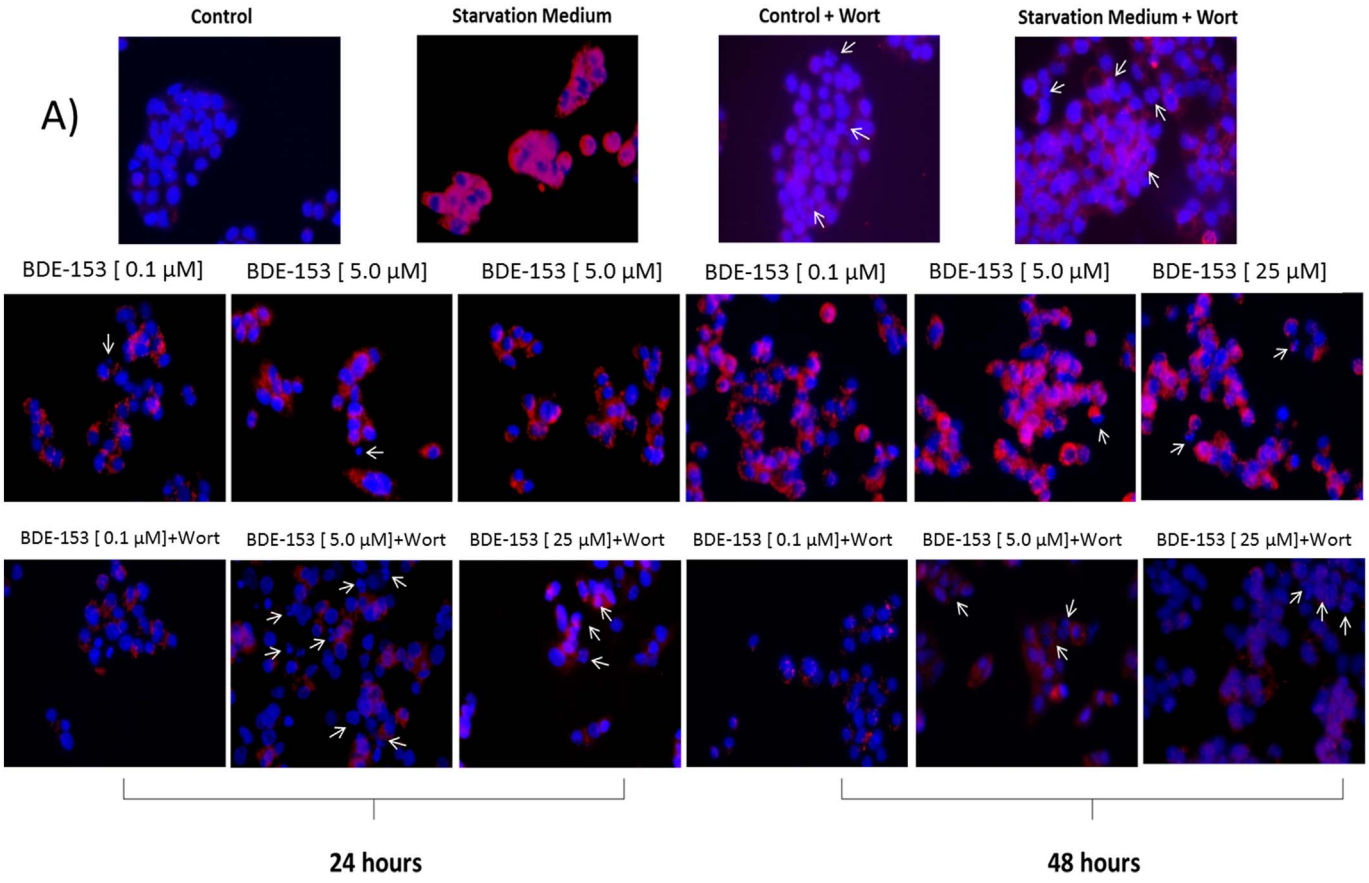
Fig. 1. LysoTracker Red accumulation in HepG2 cells. Cells were incubated with LysoTracker Red DND-99 (100 nM, 30 min) and Hoechst (1 μg/mL, 10 min) after exposure to BDE-153 for 24 h and 48 h. A) Representative images of three experiments are presented with fluorescence microscopy. The white arrows show the regions of lysosome accumulation marked by the probe. B) Photographed cells were counted and expressed as percentages with respect to the negative control. Bars represent the mean ± S.E.M. of three different cell cultures. *Significantly different from vehicle control group.



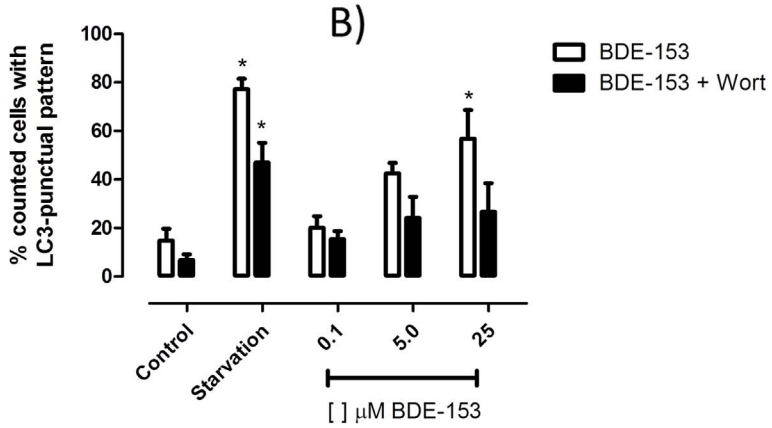


Fig. 2. Immunocytochemistry/Immunofluorescence of LC3B-stained HepG2 cells after 24 and 48 h of exposure to BDE-153, with and without 100 nM wortmannin. (A) Immunocytochemistry/Immunofluorescence was analyzed and photographed by using a Nikon Eclipse TS100 fluorescence microscope. Where wortmannin (100 nM) was used as a classic signaling pathway inhibitor of autophagy and the white arrows show cells that have fragmented nuclei; (B and C) Photographed cells were counted and expressed as percentages with respect to the negative control of the punctuated LC3-II pattern, respectively to 24 and 48 h to exposure. Incubating the cells in "starvation" medium was used as positive control. Bars represent the mean ± S.E.M. of three different cell cultures. *Significantly different from vehicle control group.

induced conversion of LC3-I to LC3-II and accumulation of this marker of autophagy in punctuate vesicular structures related to autophagosome formation, as shown in Fig. 2A. The same pattern emerged for the HepG2 cells treated with starvation medium. After activation of the autophagic process, the LC3-I protein is known to undergo cleavage and lipidation with a phosphatidylethanolamine, followed by conversion to LC3-II (Wu et al., 2006). A quantitative analysis showed that this punctuated pattern-distribution significantly increased after exposure to BDE-153 for 24 and 48 h, as shown in Fig. 2B and C, respectively. Furthermore, in HepG2 cells pre-incubated with wortmannin (a classical inhibitor of the autophagic process) and exposed to BDE-153, the punctuated pattern-distribution decreased significantly, and cells that presented nuclear fragmentation (stained with Hoechst) increased simultaneously, suggesting enhanced apoptosis upon inhibition of the

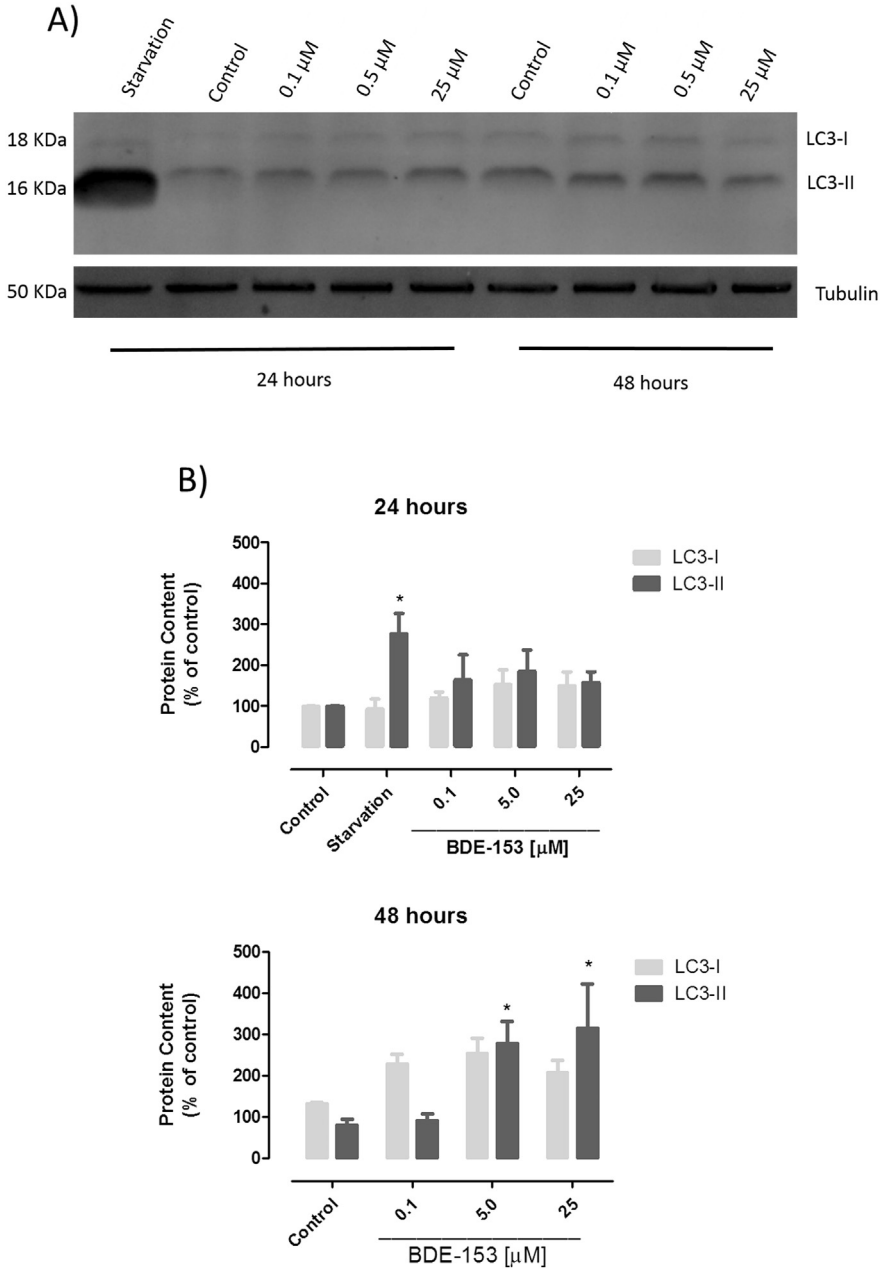


Fig. 3. Autophagy-related protein markers content in HepG2 cells exposed to BDE-153. Cell lysates obtained upon exposure to BDE-153 were used to perform Western Blot experiments and evaluate protein contents. (A) LC3 conversion and accumulation (LC3-I and LC3-II). (B) LC3/tubulin ratio. Bars represent the mean ± S.E.M. of three different cell cultures. *Represents statistically significant differences ($P < 0.05$).

autophagic process.

Western Blotting helped to confirm conversion and accumulation of the LC3 protein. Our results showed that, in addition to the increased amount of the LC3 protein, there was higher conversion of LC3-I to LC3 II (Fig. 3A and B). Particularly after 48 h, the two higher concentrations of BDE-153 led to significantly higher LC3-II protein content. The p62 protein, also known as SQTM1, has also received researchers' attention because it regulates the transport of cellular components to the autophagosome (Orrenius et al., 2013). This protein is recruited to the autophagosome and binds to LC3 therein, which makes it an important marker of autophagy. After treatment with BDE-153 and ammonium chloride for 48 h, p62 clearly accumulated in the HepG2 cells (Fig. 4A and B).

Moreover, the HepG2 cells exposed to BDE-153 exhibited decreased mitochondrial DNA copy number both at 24 and 48 h (Fig. 5). This pointed out that cells exposed to BDE-153 try to manage mitochondrial damage by selectively degrading damaged mitochondria *via* mitophagy (specific degradation of damaged mitochondria). The mitochondrial DNA copy number was assessed by qPCR on the basis of the number of DNA copies of a mitochondrial encoded gene (Cytochrome b) normalized against a nuclear encoded gene (Pyruvate Kinase).

4. Discussion

In the present research, the experimental data shows that the flame retardants, here represented by BDE-153 (0.1–25 μM) not only induces mitochondrial damage but also activates autophagy in human HepG2 hepatoblastoma cells. Increased conversion of the LC3 protein (from LC3-I to LC3-II) along with the appearance of dot-like formations of endogenous LC3 (microtubule-associated protein light chain 3) and increased accumulation of lysosomes indicate induction of autophagy. Additionally, upon inhibition of the autophagy mechanism, exposure to

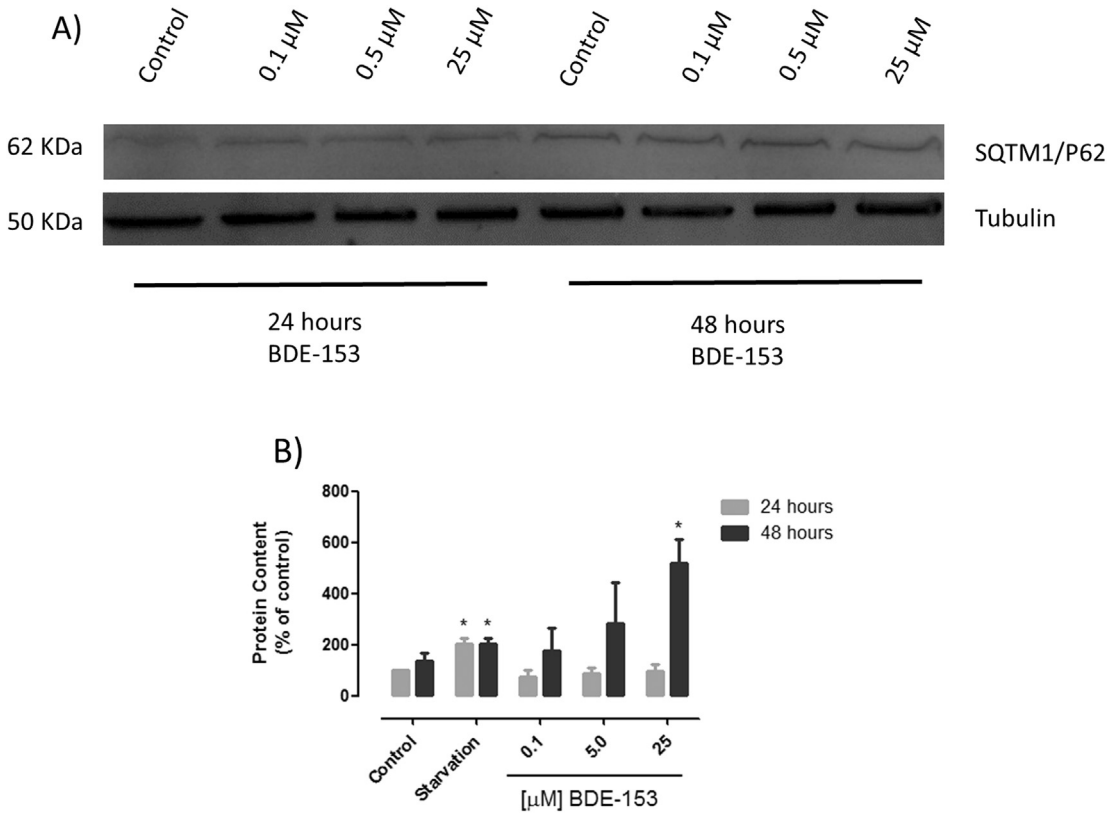


Fig. 4. Autophagy-related protein markers content in HepG2 cells exposed to BDE-153. Cell lysates obtained upon exposure to BDE-153 were used to perform Western Blot experiments and evaluate protein contents. (A) p62 protein content in HepG2 cells exposed to BDE-100; (B) p62/tubulin ratio. Bars represent the mean ± S.E.M. of three different cell cultures. *Represents statistically significant differences ($P < 0.05$).

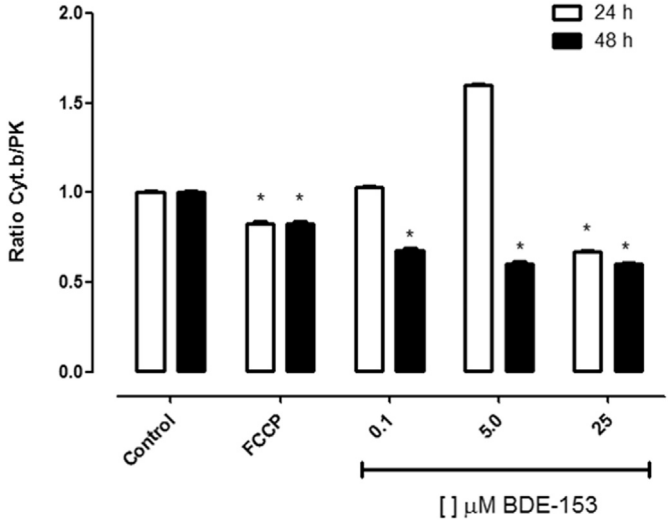


Fig. 5. Amount of mtDNA amount in HepG2 cells exposed to BDE-153. Copy numbers for each gene were calculated on the basis of standard curves. The ratio of mitochondrial gene (cytochrome b) copy number to the nuclear gene (Pyruvate Kinase) copy number was calculated and expressed as the mtDNA copy number per cell. Bars represent the mean ± S.E.M. of three different cell cultures. *Represents statistically significant differences ($P < 0.05$). FCCP 1 μM served as positive control.

BDE-153 results in increased cellular apoptotic features, such as DNA fragmentation.

Exposure to flame retardants and other environmental pollutants is associated with several mechanisms of toxicity to humans (Kim et al., 2015; Li et al., 2014). PBDEs are widespread environmental contaminants that emerge mainly due to runoff, production, direct discharges, usage, incorrect disposal, and accumulation in the environment (Ondarza et al., 2014; Shi et al., 2009). Although some research has shown the toxicity of this class of compounds, evaluation of the risk and consequences of exposure to these toxicants is scarce. The present study has shown that the BDE-153 congener induces autophagy after 24 and 48 h, which is associated with increased accumulation of lysosomes and with triggering of autophagic processes as monitored by evaluation of p62 and LC3-II protein levels.

Some works have already reported that brominated flame retardants can induce mitochondrial damage (Pazin et al., 2015; Pereira et al., 2013). Other study has specifically demonstrated that BDE-154 can cause mitochondrial damage (Pereira et al., 2014). BDE-154 is a congener that resembles the BDE-153 congener evaluated in this present study and is also a representative of the hexabrominated class of PBDEs. BDE-154 and -153 differ only in terms of structural conformation. In addition, lysosomes have also been considered a target for the toxicity mechanism elicited by BDE-47 (Liu et al., 2015). Indeed, several works have described the toxicity of these compounds to cell lines in culture. Examples are involvement of the mitochondrial p53 in SH-SY5Y cells exposed to BDE-47 (Zhang et al., 2013); oxidative stress and apoptosis in mouse cerebellar granule neurons elicited by the same congener (Costa et al., 2015); and effects on the regulation of growth and apoptosis in breast, ovarian, and cervical cancer exposed to BDE-209 (Li et al., 2012). There is also evidence that other types of environmental contaminants can damage mitochondria in liver (Duarte et al., 2013) and cause mitochondrial dysfunction in primary organs affected by exposure to air pollution, such as the lung (Duarte et al., 2011; Duarte et al., 2012). Some reports have shown toxic effects in other models, namely developmental toxicity in zebrafish induced by BDE-49 (McClain et al., 2012). However, the present study is the first report on the induction of autophagy after exposure of a cell line model (HepG2 in culture) to BDE-153.

Exposure of HepG2 cells to BDE-153 induces higher conversion of LC3-I to LC3-II, indicating larger autophagosome formation and suggesting increased activation of autophagy mechanisms. However, increased conversion of LC3-I into LC3-II could actually indicate not only enhanced autophagosome formation, but also impaired LC3-II degradation and recycling within lysosomes (remember that LC3 is degraded in autolysosomes), as reported for other compounds that inhibit lysosomal function (Klionsky et al., 2012). Chloroquine or ammonium chloride (which can disrupt the fusion of autophagosome with lysosome and raise lysosomal pH, to suppress the activity of lysosomal acid hydrolases, block the degradation of autolysosome, and promote the accumulation of LC3-II in the cells) provided a detailed assessment of LC3 conversion and degradation in HepG2 cells exposed to BDE-153 (Yamamoto et al., 1998). Concomitantly, increased cytosolic protein p62/sequestosome 1 (SQSTM1), which participate in the autophagic process, confirmed induction of autophagy (the ultimate autophagosome-lysosome fusion and degradation was inhibited).

Because autophagosome formation requires class III PI3-kinase activity, one of the commonest approaches to studying autophagy induction is to inhibit autophagy *in vitro* by using PI3-kinase inhibitors such as wortmannin (Blommaart et al., 1997). Here, autophagy inhibition resulted in increased apoptosis after exposure to BDE-153. Together, these findings showed that autophagy plays an important role in protecting HepG2 cells from BDE-153-induced apoptosis.

Autophagy is a pro-survival mechanism induced under stressful "life-threatening" conditions in most liver diseases (Rautou et al., 2010). This process clears the cells of damaged organelles, long-lived proteins, and/or misfolded proteins (Jiang et al., 2015). Under physiological conditions, autophagy has a number of vital roles, such as the maintenance of the amino acid pool (Mizushima et al., 2010). Under stressful conditions, activation of the autophagic process leads to cell death and acts as a protective mechanism against xenobiotic-induced toxicity, which is the most widely accepted mechanism in the literature (Orrenius et al., 2013; Shen et al., 2011). This same feature can be observed in our model, where the liver cells are more susceptible to BDE-153-induced cell death if autophagy (triggered initially as a protective mechanism) is inhibited.

Note that mitophagy (an autophagic process that specifically involves damaged mitochondria) is a specific process that may be involved in HepG2 autophagy as observed from the smaller amount of mitochondrial DNA after exposure of HepG2 cells to BDE-153 as compared to the control. This effect was somehow expected, given that our previous data had shown induction of mitochondrial damage, a severe differential damage considering the evaluated BDE counterpart (Pazin et al., 2015; Pereira et al., 2013; Pereira et al., 2014).

In conclusion, our results suggest that BDE-153 can induce autophagy which, in the model employed herein, represents a cellular protective response against BDE-153-induced toxicity after the tested exposure periods. Our present observations provide the basis for further investigations that will help to characterize the mechanisms underlying toxicity mechanisms and the triggering/modulation of autophagy in liver cells after exposure to BDE-153 in greater detail.

Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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