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Bisphenol A exposure disrupts aspartate transport in HepG2 cells

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Abstract

The liver is the organ responsible for bisphenol A (BPA) metabolism, an environmental chemical agent. Exposure to this toxin is associated with liver abnormalities and dysfunction. An important role played by excitatory amino acid transporters (EAATs) of the *slc1* gene family has been reported in liver injuries. To gain insight into a plausible effect of BPA exposure in the liver glutamate/aspartate transport, using the human hepatoblastoma cell line HepG2, we report a BPA-dependent dynamic regulation of SLC1A3 and SLC1A2. Through the use of radioactive [^{3}H]-D-aspartate uptake experiments and immunochemical approaches, we characterized time and dose-dependent regulation of the protein levels and function of these transporters after acute exposure to BPA. An increase in nuclear Yin Yang 1 was found. These results suggest an important involvement of the EAATs in liver physiology and its disruption after acute BPA exposure.

KEYWORDS

bisphenol A, gene expression regulation, liver SLC1 transporters

1 | INTRODUCTION

Bisphenol A (BPA; 4,4′-dihydroxy-2,2-diphenylpropane; CAS 80-05-7) is an environmental chemical used in the manufacturing of many consumer products including plasticware.[1] BPA is released through a temperature and pH-dependent hydrolysis.[2] The average daily exposure of an adult to BPA is estimated to be in the range of 0.1–5 μg/kg body weight.[3] Several studies have been focused on BPA as an endocrine disruptor due to its xenoestrogenic properties.[4] However, the effects of BPA are not restricted to reproductive disruption. The liver is the primary organ responsible for BPA metabolism to its corresponding glucuronide and sulfate conjugates, [5,6] and recently, both in vivo and in vitro data suggest that BPA exerts a wide variety of metabolic effects in the liver. Lang et al[7] demonstrated that BPA is linked to the appearance of serum markers of liver damage which, in fact, are predictive of metabolic diseases. BPA can induce oxidative damage in the liver after a long and repeated exposure.[8] Both, high and low BPA doses result in proliferative effects on bile ducts and liver tumors.[9,10] The prooxidant effect of BPA has been demonstrated in isolated rat hepatocytes and HepG2 cells.[11-14] Moreover, BPA promotes lipid accumulation, lipoperoxidation, and the release of proinflammatory cytokines in the liver.[13] These data indicate that BPA exposure contributes to different liver pathologies such as steatosis, cholestasis, and cancer.[15]

Glutamate, the most abundant intracellular hepatic amino acid, participates in a plethora of liver functions. The extracellular levels of this amino acid are tightly regulated by two subtypes of plasma membrane glutamate transporters, originally described in rodent glia cells: the glutamate/aspartate transporter (GLAST) and the glutamate transporter 1, encoded by the genes *slc1a3* and *slc1a2*, respectively. Interestingly, its human counterparts, SLC1A3 (excitatory amino acid transporter 1 [EAAT1]) and SLC1A2 (EAAT2), respectively, have been implicated in human liver diseases. In cholestasis, the activity and expression of SLC1A2-mediated glutamate transport is altered.[16] More recently, SLC1A3 has been defined as a key molecule in cell proliferation.[17] In the liver, glutamate is fundamental for hepatic amino acid metabolism, and it is the crossroad in others metabolic pathways such as ammonia detoxification, protein

synthesis, and energy metabolism.[18-21] Although glutamate transporter gene expression regulation in the liver is not yet fully understood, it is possible that the described regulatory mechanisms in brain cells are operative in the liver, meaning cell-surface expression, translational, and transcriptional control.[22-24]

BPA modifies the expression of proteins that participate in glutamate transporters regulation in the central nervous system. SLC1A2 and SLC1A3 promoters contain consensus DNA-binding sites for the transcription factor Yin Yang 1 (YY1), and previous studies have revealed an important role of YY1 in glutamate transporters gene expression regulation.[25-27] BPA modulates YY1 through the inhibition of its expression.[28] It has also been reported that BPA modulates calcium homeostasis involving the Ca^{2+}-dependent chloride channels and also through the increase of [Ca^{2+}]-dependent Ser/Thr kinases activity, [29,30] among which protein kinase C (PKC) represents one of the most widely studied kinase.[31]

As BPA is associated with liver abnormalities, we investigated the expression and function of glutamate transporters of the *slc1A* gene family in HepG2 cells after BPA treatment. These cells were chosen as this pollutant is metabolized in this system.[32] In the current study, we demonstrate that both SLC1A3 and SLC1A2 transporters are expressed and active in HepG2 cells and are modified by BPA exposure. A dynamic modification of glutamate transporter systems is triggered as part of the liver response to BPA.

2 | METHODS

2.1 | Materials

BPA, 2,2-bis(4-hydroxyphenyl) propane, 4,4′8;-isopropylidenediphenol ≥ 99% (239658), dimethyl sulfoxide (DMSO) ≥ 99% (#M81802), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; # M2128) were obtained from Sigma Aldrich; ^{3}[H]-D-aspartate was obtained from PerkinElmer (Boston, MA). Cell culture reagents were from Thermo Fisher Scientific (Carlsbad, CA), and plasticware was purchased from Corning (New York, NY). Anti-SLC1A3 antibody was purchased from Abcam (ab416; 1:1000) (Cambridge, UK); anti-SLC1A2 from Alomone (AGC-022; 1:1000) (Jerusalem, Israel); anti-YY1 (C-20) (sc-281; 1:1000) and anti-SLC1A1 (sc-7761; 1:500) from Santa Cruz Biotechnology (Dallas, TX) and monoclonal anti-actin (1:250) antibody was kindly donated by Manuel Hernandez (Cinvestav, Mexico). Horseradish peroxidase-linked anti-rabbit anti-goat and the enhanced chemiluminescence reagent were obtained from Amersham Biosciences (Buckinghamshire, UK). Bradford and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents were obtained from Bio-Rad (Hercules, CA). Threo-β-benzyloxyaspartate (TBOA) (100 μM) dihydrokainic acid (DHK) (100 μM), tetradecanoylphorbol-13-acetate (TPA) (100 nM), and bisindolylmaleimide 1 (Bis 1) (1 μM) were purchased from Tocris Cookson (St Louis, MO).

2.2 | Cell culture and stimulation protocol

HepG2 cells were cultured in DMEM containing 4.5 g/L glucose supplemented with 10% fetal calf serum, 1% nonessential amino acids, 1% glutamine, and 1% penicillin-streptomycin. All cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO_2. Confluent monolayers were exposed to the indicated BPA concentrations for different time periods, and samples were processed as detailed below. Before any treatment, confluent monolayers were switched to solution A containing 25 mM HEPES-Tris, 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 33.3 mM glucose, and 1 mM NaH_2PO_4 at pH 7.4.

2.3 | Evaluation of cell metabolic activity

Mitochondrial activity was used to assess cell viability as a function of redox potential. The MTT assay was evaluated according to the manufacturer's instructions. Briefly, exponentially growing cells were seeded into 96-well plates. Upon reaching 70% to 80% confluence, cells were treated as indicated. Then, the cell viability was detected by incubating the cells in a medium containing 1 mg/mL MTT for 4 hours. One hundred mocroliters of DMSO was then added to solubilize the formazan and shaken for 10 minutes in the dark. The absorbance was recorded at 570 nm.

2.4 | Cell immunostaining

HepG2 cells were seeded on coverslips. The culture media was removed and cells were rinsed once with phosphate-buffered saline (PBS) and fixed for 10 minutes in 4% paraformaldehyde. Cells were rinsed with PBS three times, incubating five minutes at room temperature after each wash. Nonspecific binding was prevented by incubation with 4% goat serum in PBS-Tween 0.1 for 1 hour at room temperature. Cells were exposed to a 1:250 dilution of the primary antibody anti-SLC1A3, anti-SLC1A2, or anti-SLC1A1 in 2% goat serum in PBS-Tween 0.1 for 2 hour at 37°C, followed by the incubation with the respective fluorescein-labeled goat anti-rabbit or anti-goat anti-sera in 2% goat serum in PBS-Tween 0.1 (1:1000) for 2 hours at room temperature. Preparations were mounted with Fluoroshield/DAPI. Cell preparations were examined under a fluorescence microscopy (Zeiss Axioskop 40 immunofluorescence microscope and the AxioVision software; Carl Zeiss Inc, Thornwood, NY).

2.5 | SDS-PAGE and Western blots

Confluent monolayers were harvested with PBS (10 mM K_2HPO_4/KH_2PO_4, 150 mM NaCl, pH 7.4) containing phosphatase inhibitors (10 mM NaF, 1 mM Na_2MoO_4, and 1 mM Na_3VO_4). The membrane fraction was obtained according to Hu et al[33] with modifications.

The cells were homogenized in 10 volumes of ice-cold water containing 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF) and were subjected to centrifugation (14 000 rpm × 20 minutes, 4°C). The pellet was collected, resuspended in 1% SDS in 10 mM NaPi and briefly sonicated before centrifugation (6000 rpm × 20 minutes, 15°C). The nuclear fraction was prepared from cells collected by scraping in PBS and pelleted by centrifugation and gently resuspended in buffer A (10 mM HEPES pH 7.9; 10 mM KCl; 1 mM EGTA; 1 mM DTT; 1 mM PMSF; protease inhibitors) by pipetting up and down several times and incubated on ice for 15 minutes. A fixed amount of 25 μL of 10% NP40 was added and the vortexing was carried out for 10 seconds at highest setting. After the pellet was centrifuged for 1 minute at 10 000 rpm at 4°C, the nuclear pellet was resuspended in 90 to 100 μL buffer C (20 mM HEPES pH 7.9; 0.4M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF; protease inhibitors [complete]) for 30 minutes with vortexing at 4°C, and after centrifugating for 5 minutes at 9000 rpm at 4°C, the supernatant was transferred (nuclear fraction) to a clean microcentrifuge tube. Cell lysates of membrane or nuclear fractions were denaturized in the Laemmli sample buffer, and an equal amount of proteins (50 μg of total protein as determined by the Bradford method) were resolved through a 10% SDS-PAGE and then electroblotted to nitrocellulose membranes. Blots were stained with Ponceau S stain to confirm that protein content was equal in all lanes. Membranes were soaked in PBS to remove the Ponceau S and incubated in TBS containing 5% dried skimmed milk and 0.1% Tween 20 for 60 minutes to block the excess of nonspecific protein binding sites. Membranes were then incubated overnight at 4°C with the particular primary antibodies indicated in each figure, followed by secondary antibodies. Immunoreactivity polypeptides were detected by chemiluminescence. Densitometry analyses were performed with ImageJ 1.44 (National Institute of Health, Bethesda, MD), and the mean ± standard deviation (SD) was calculated with Prism, GraphPad Software (San Diego, CA).

2.6 | [^{3}H]-D-aspartate uptake activity

Confluent monolayers were washed three times to remove all nonadhering cells with 0.25 mL of solution A. The [^{3}H]-D-aspartate uptake was initiated at $t = 0$ by the addition of 0.25 mL solution A containing 0.4 μCi/mL of ^{3}[H]-D-aspartate (100 μM final aspartate concentration). The reaction was stopped by aspirating the radioactive medium and washing each well within 15 seconds with 0.25 mL aliquots of an ice-cold solution A. For the determination of the kinetic parameters, the Asp concentration was modified to a final 10 μM, 25 μM, 50 μM, 100 μM, 1 mM, and 2 mM concentrations with unlabeled Asp. The uptake was stopped as described above. The cells in the wells were then solubilized for 2 hours at 37°C to 0.25 mL NaOH 0.1M and an aliquot of the radioactivity present was quantified in a PerkinElmer scintillation counter in the presence of a scintillation cocktail. Experiments were carried at least three times with a minimum of quadruplicate determinations.

2.7 | Statistical analysis

Data are expressed as the mean ± SD. A one-way analysis of variance was performed to determine significant differences between conditions. When this analysis indicated significance ($P < .05$), Dunnett's multiple comparison analysis was used to determine which conditions were significantly different from the control and a Tukey multiple comparison analysis was used to determine which conditions were significantly different from each other with the Prism 5, GraphPad Software. Data from the aspartate uptake experiments were analyzed by nonlinear regression using the curve-fitting program (GraphPad Software). The results were expressed as mean ± SD.

3 | RESULTS

3.1 | Expression of GLASTs of the *slc1A* gene family in HepG2 cell line

The liver expression of the glutamate transporter SLC1A2 has been reported for in vitro and in vivo models. It has also been reported that this transporter is involved in certain liver diseases.[16] In contrast, the expression of SLC1A3 in hepatic human cells has not yet been fully demonstrated. With this in mind, we first decided to ask ourselves if these transporter proteins are present in HepG2 cells. The results are shown in Figure 1, both, SLC1A3 and SLC1A2 are present in these cells, albeit at a lower concentration than in rat brain tissue, note that SLC1A13 is slightly expressed in the plasma membrane (Figure 1A,B).

To demonstrate a functional expression of these transporters, an [^{3}H]-D-aspartate uptake assay was used. It has been reported that SLC1 transporters (EAATs) are capable to transport L-glutamate, L-aspartate, and D-aspartate with high affinities.[34] The main advantage to use [^{3}H]-D-aspartate is that this amino acid is not metabolized in HepG2 cells, and therefore, its efflux rate is very low under the assay conditions, improving both the reproducibility and the sensitivity of these assays.[35] As depicted in Figure 1C, approximately 50% of the uptake is blocked by the inclusion of the selective SLC1A2 blocker dihidrokainate (DHK), suggesting the functional expression of SLC1A3, in line with the immunochemical detection of both transporters (Figure 1A). Note that preincubation with the nonselective EAAT blocker TBOA or the replacement of NaCl with choline chloride reduces the control uptake by 75%, demonstrating that in HepG2 cells, the majority of glutamate/aspartate uptake is Na^+-dependent, carried out by SLC1A2 (25%) and most possibly by SLC1A3, as the expression of the SLC1A1 (EAAC1 or EAAT3) is minimal (Figure 1). Moreover, the K_M value of 123.3 μM favors the notion of a substantial contribution of EAAT1 (Figure 1D).

Previous results have demonstrated that these transporters are regulated in short and long term by its substrate, namely glutamate.[36,37] To gain insight into a plausible glutamate-triggered regulation in liver excitatory amino acids transporters, we first explored the HepG2 cells viability after a long-term (12-24 hours) exposure to

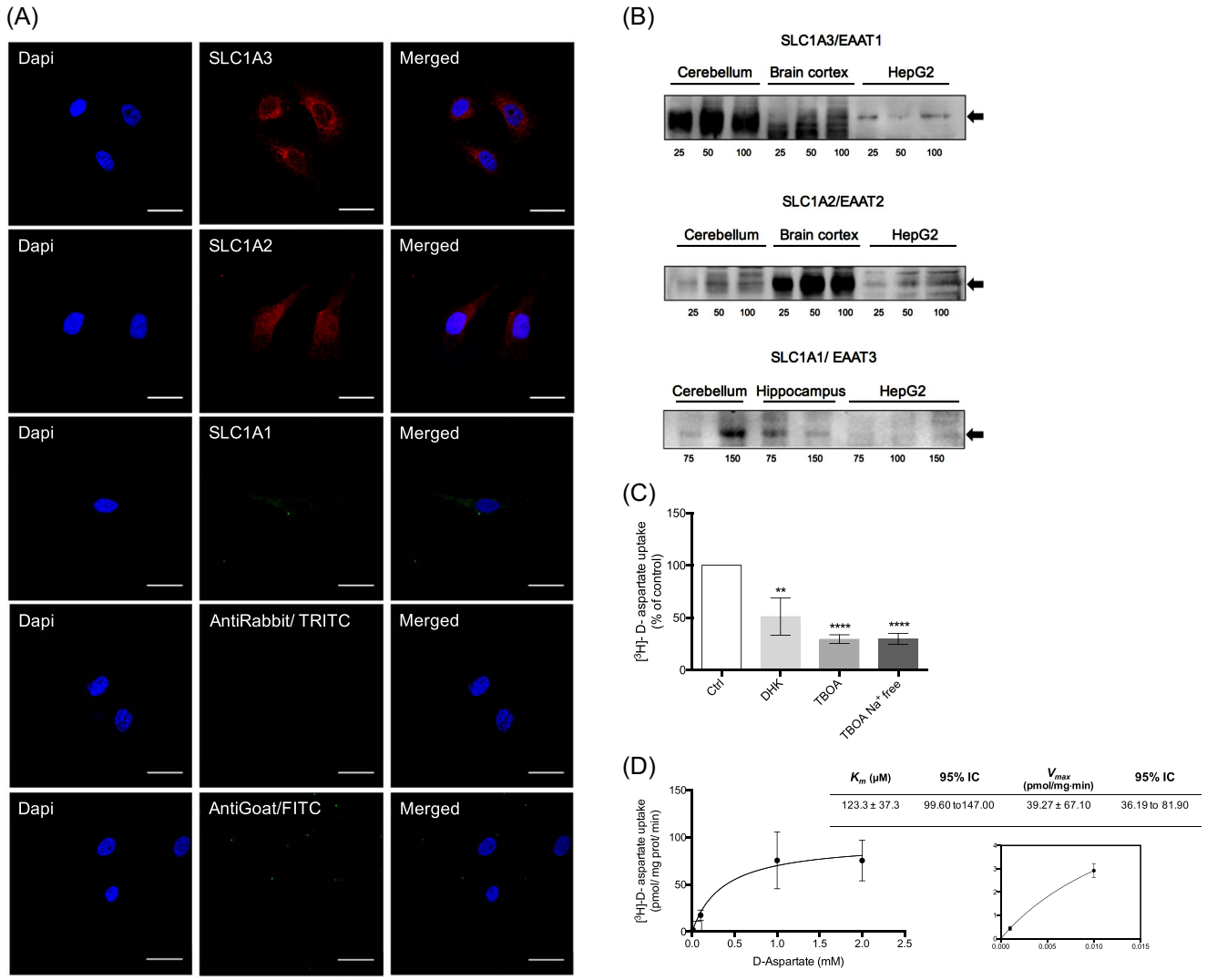
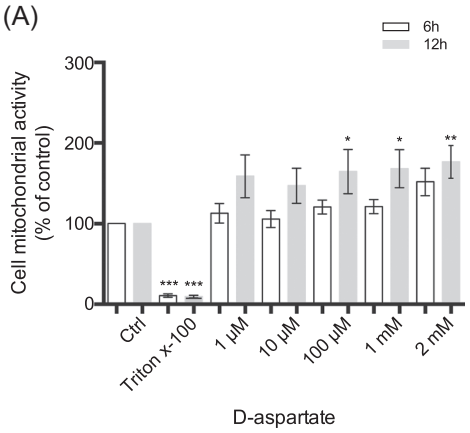


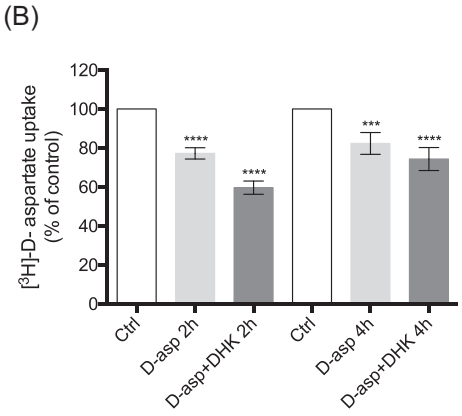
FIGURE 1 Glutamate-aspartate transporters functional expression in HepG2 cells. A, SLC1A3, SLC1A2, and SLC1A1 immunoreactivity in the HepG2 cell line. Cell staining with the proper primary antibodies (red); anti-SLC1A1 antibody (green); DAPI counter-stained nucleus (blue); and merged images are shown. The two bottom rows of this panel correspond to the negative controls (without primary antibodies). Scale bar = 10 μm. B, Membrane protein expression of SLC1A3, SLC1A2, and SLC1A1 from rat adult cerebellum, rat adult cerebral cortex tissue or hippocampus, and HepG2 cells; 25, 50, and 100 μg of protein. Predicted band size corresponds to 67/60 kDa. C, HepG2 cells were pretreated with the EAAT2 blocker dihydrokainic acid (DHK; 100 μM) or EAAT blocker DL-threo-β-benzyloxyaspartic acid (TBOA; 100 μM) for 30 minutes in assay buffer or assay buffer with choline chloride (Na+ free), [^{3}H]-D-aspartate was measured after 6 minutes. D, Saturation isotherm for [^{3}H]-D-aspartate uptake in HepG2 cells. The data shown correspond to the mean ± SD of three independent experiments performed in quadruplicate. Statistical analysis was performed using a one-way ANOVA, followed by Dunnett's multiple comparison test. *P < .05, **P < .01. A robust nonlinear regression was used to fit a model to our data and estimate the kinetic parameters. Representative images of three independent experiments. ANOVA, analysis of variance; EAAT, excitatory amino acid transporter; SD, standard deviation

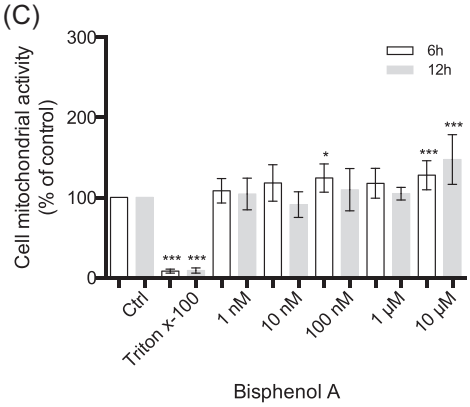
the glutamate analog, D-aspartate. No reduction in cell mitochondrial activity was detected upon the treatment with different D-aspartate concentrations. Furthermore, a significant increase in cell metabolic activity was detected with 1 and 2 mM aspartate (Figure 2A). These results enabled us to examine the effect of a 1 mM aspartate exposure for 30 minutes on the [^{3}H]-D-aspartate uptake activity after 2 and 4 hour-postexposure.[38] A reduction in the uptake activity was observed, note that after 2 hours approximately a 20% reduction in the activity was detected. The inclusion of the SLC1A2 blocker led to a further 20% reduction, suggesting that a SLC1A3-dependent uptake indeed is present in HepG2 cells (Figure 2B). After 4 hours, the contribution of SLC1A2 to the uptake was observed to be of only 10%, supporting the idea of a dynamic modulation of the expression of both transporters in these cells (Figure 2B).

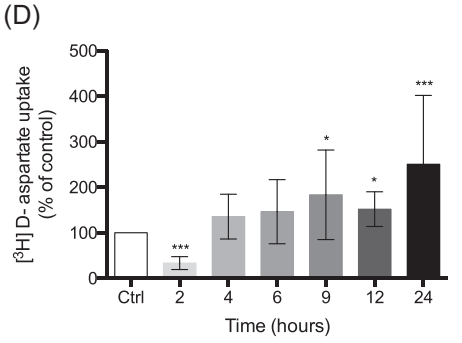
3.2 | BPA exposure impairs [^{3}H]-D-aspartate uptake

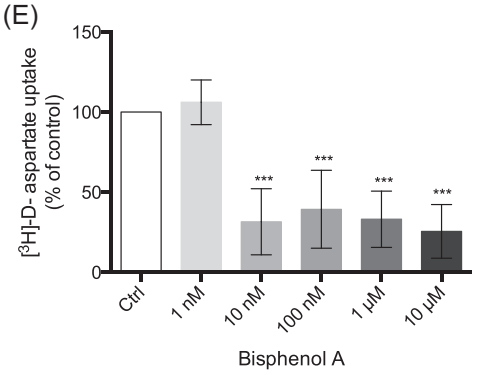
Taking into consideration the extended use of BPA and its hepatotoxic character, we decided to evaluate the effect of the exposure to

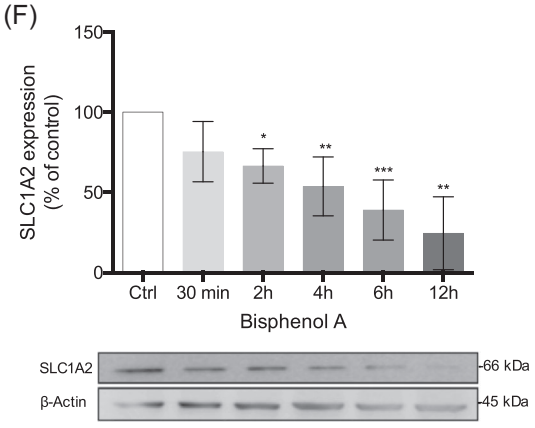


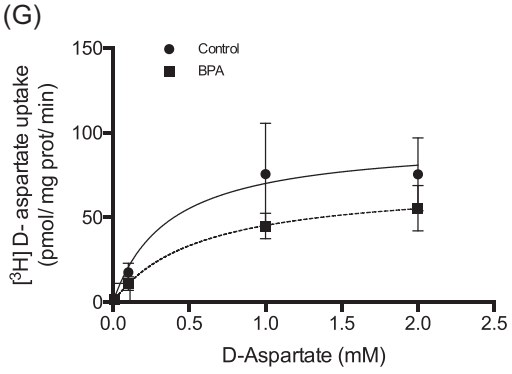




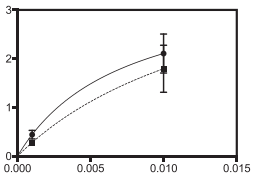


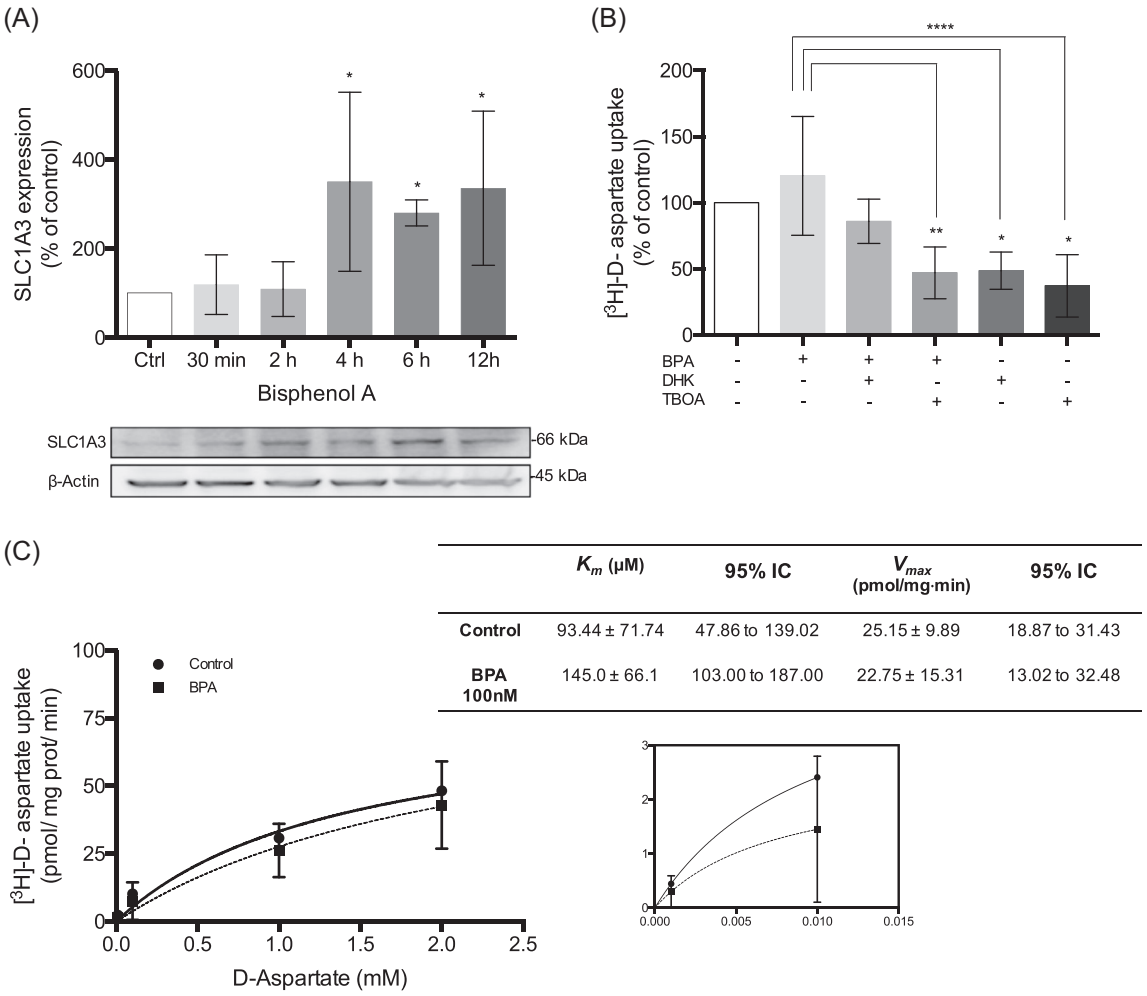






	K_m (µM)	95% IC	V_{max} (pmol/mg·min)	95% IC
Control	123.3 ± 37.3	99.60 to 147.00	39.27 ± 67.10	36.19 to 81.90
BPA 100nM	47.28 ± 33.24	26.16 to 68.40	10.34 ± 2.74	8.60 to 12.08





	K_m (μM)	95% IC	V_{max} (pmol/mg·min)	95% IC
Control	93.44 ± 71.74	47.86 to 139.02	25.15 ± 9.89	18.87 to 31.43
BPA 100nM	145.0 ± 66.1	103.00 to 187.00	22.75 ± 15.31	13.02 to 32.48

FIGURE 3 Bisphenol A (BPA) acute exposure increases SLC1A3 expression in HepG2 cells. A, Effect of BPA acute exposure on SLC1A3 protein expression in HepG2 cells 30 minutes, 2, 4, 6, and 12 hours of posttreatment. The cells were treated with BPA (100 nM) for 30 minutes. Anti-actin Western blots were used as loading controls. A representative blot is presented on bottom of the graph. B, Cells were treated with 100 nM BPA for 30 minutes; SLC1A2 blocker dihydrokainic acid (DHK; 100 μM) or SLC1A blocker DL-threo-β-benzyloxyaspartic acid (TBOA; 100 μM) was placed 30 minutes before. [^{3}H]-D-aspartate was measured after 6 minutes, 6 hours posttreatment. C, Saturation isotherms for [^{3}H]-D-aspartate uptake in HepG2 cells 6 hours posttreatment with BPA (100 nM) for 30 minutes. Data represents the mean ± standard deviation of three independent experiments performed in quadruplicate. Statistical analysis was performed using a one-way analysis of variance, followed by Dunnett's or Tukey's multiple comparison test. *P < .05, **P < .01. A robust nonlinear regression was used to fit a model to our data and estimate the kinetic parameters

FIGURE 2 D-Aspartate and bisphenol A (BPA) acute exposure impair excitatory amino acid transporters activity in HepG2 cells. A, Cell mitochondrial activity after analysis of intracellular purple formazan levels in HepG2 cells treated with D-aspartate (1 μM-2 mM) or Triton X-100. B, D-Aspartate downregulation of [^{3}H]-D-aspartate uptake. Cells were pretreated with 1 mM D-aspartate, SLC1A2 blocker dihydrokainic acid (DHK; 100 μM), or both for 30 minutes. [^{3}H]-D-aspartate uptake was measured 2 or 4 hours posttreatment. C, Cell mitochondrial activity after analysis of intracellular purple formazan levels in HepG2 cells treated with BPA (1 nM-10 μM) or Triton X-100. D, HepG2 cells were treated with 100 nM BPA for 30 minutes. 2, 4, 6, 9, 12, and 24 hours posttreatment [^{3}H]-D-aspartate uptake was measured. E, Cells were treated with increasing concentrations of BPA (1 nM-10 μM) for 30 minutes. [^{3}H]-D-aspartate was measured after 6 minutes and 2 hours posttreatment. F, Effect of BPA acute exposure on SLC1A2 protein expression in HepG2 cells 30 minutes, 2, 4, 6, and 12 hours posttreatment. The cells were treated with BPA (100 nM) for 30 min. Anti-actin Western blots were used as loading controls. A representative blot is presented on bottom of the graph. G, Saturation isotherms for [^{3}H]-D-aspartate uptake in HepG2 cells 2 hours posttreatment with BPA (100 nM) for 30 minutes. The data shown are the mean ± standard deviation of three independent experiments performed in quadruplicate. Statistical analysis was performed using a one-way analysis of variance, followed by Dunnett's multiple comparison test. *P < .05, **P < .01. A robust nonlinear regression was used to fit a model to our data and estimate the kinetic parameters

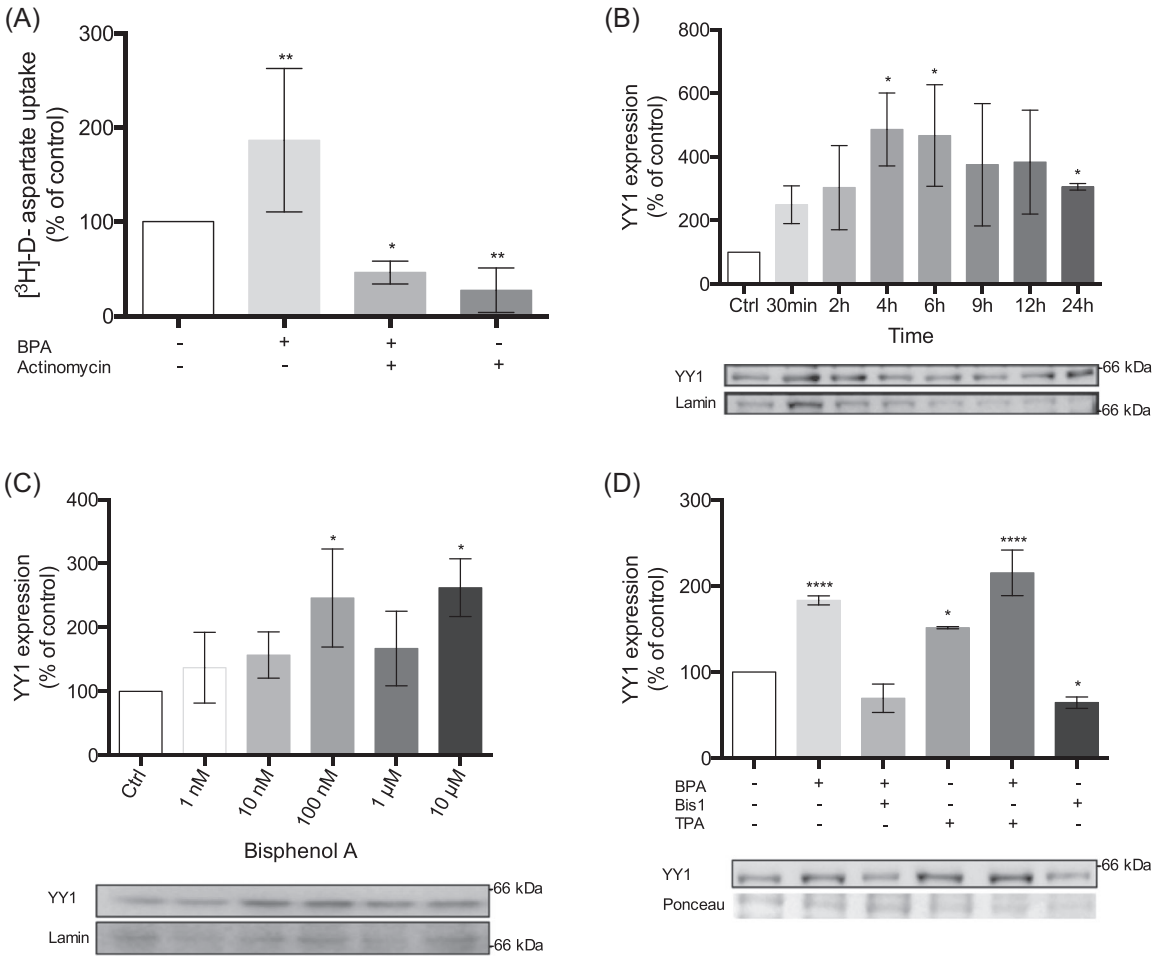


FIGURE 4 Bisphenol A (BPA) regulates *slc1A* transporters at the transcriptional level. A, HepG2 cells were treated with 100 nM BPA and/or actinomycin 10 μg/mL. [^{3}H]-D-aspartate uptake was measured, 24 hours posttreatment. B, Yin Yang 1 (YY1) nuclear expression in HepG2 cells 30 minutes, 2 hours, 4 hours, or 6 hours posttreatment. Cells were treated with 100 nM BPA for 30 minutes. C, YY1 nuclear expression 6 hours posttreatment. Cells were treated with increasing BPA concentrations (1 nM-10 μM) for 30 minutes and YY1 nuclear levels were measured as in the previous panel. D, YY1 nuclear expression 6 hours posttreatment with BPA (100 nM) for 30 minutes. Protein kinase C (PKC) blocker Bisindolylmaleimide I (Bis I; 100 μM) or PKC activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA; 100 μm) were placed 30 minutes before. Anti-lamin Western blots were used as loading controls. A representative blot is presented on bottom of the graph. The data shown correspond to the mean ± standard deviation of three independent experiments. Statistical analysis was performed using a one-way analysis of variance, followed by Dunnett's multiple comparison test. $^*P < .05$, $^{**}P < .01$

this xenobiotic in the expression and activity of excitatory amino acid plasma membrane transporters. BPA metabolism in humans occurs very quickly, and an efficient conjugation with glucuronic acid results in a small amount of free BPA, a xenoestrogen compound. However, the period that the unconjugated compound remains in humans is less in concentration and time (half-life ~89 min).[39] In addition, we were interested in concentrations in nanomolar range because it has been reported BPA produces adverse effects at levels below the acceptable daily intake. In fact, acute exposure mimics free BPA in the liver. We first evaluated the viability of HepG2 cells following exposure to different concentrations of BPA for 12 and 24 hours. No significant changes in cell metabolic activity were recorded, and interestingly, an increase in cell activity after BPA 100 nM at 6 hours and BPA 10 μM at 12 and 24 hours was detected (Figure 2C).

The effect of the exposure to 100 nM BPA for different periods in the [^{3}H]-D-aspartate uptake activity was determined. A biphasic effect was found, first a 50% reduction in the uptake (after 2 hours) and then a recovery of the activity (Figure 2D). We decided first to concentrate in the reduction of the aspartate uptake. To this end, we exposed the cultured cells to different BPA concentrations for 30 minutes and measured the uptake activity after 2 hours. The maximal uptake reduction was present after a 10 nM BPA concentration, suggesting a nonmonotonic effect (Figure 2E). This reduction matches with a decrease in SLC1A2 levels shown in Figure 2F. The reduction in the transporter content is more pronounced as a function of time. As one would expect, a clear reduction in V_{Max} was present in BPA-treated cells (Figure 2G). It is important to mention that also a decrease in the affinity of the transporters is

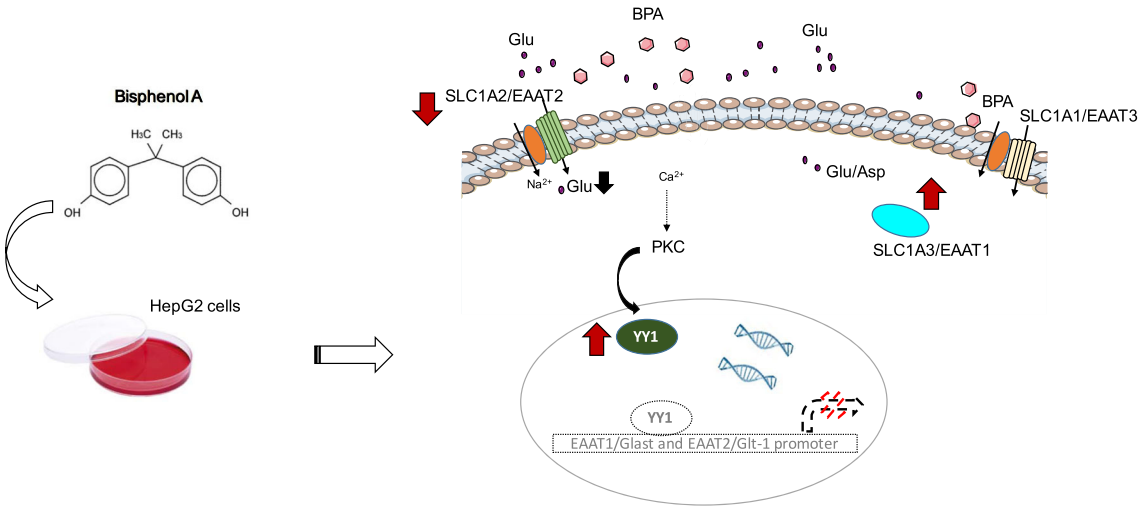


FIGURE 5 Proposed model for the effects of acute bisphenol A(BPA) exposure on SLC1A3 and SLC1A2 expression in hepatoblastoma cells. See text for details

present, pointing out either an effect in both SLC1A3 and SLC1A2 or/and a BPA-mediated conformational change in these proteins.

Next, we focused in the BPA time-dependent increase in aspartate uptake depicted in Figure 2D. These results, together with the sharp decrease in SLC1A2 (Figure 2F), suggested that the recovery of the uptake activity after 4 hours of BPA might be due to an increase in SLC1A3 expression. Therefore, we measured the protein levels of this transporter as a function of BPA exposure. The results are shown in Figure 3A. A sustained increase in SLC1A3 immunoreactivity was found. To find a functional correlate of these observations, we determined the [^{3}H]-D-aspartate uptake activity 6 hours after the cells were exposed for 30 minutes to 100 nm BPA. A slight increase in uptake is detected; nevertheless, this augmentation is blocked by DHK demonstrating that the increase in SLC1A3 protein levels does not result in functional SLC1A3 transporters at the plasma membrane (Figure 3B). In line with this interpretation, no significant changes are present in the uptake kinetic parameters (Figure 3B,C).

3.3 | Transcriptional regulation of *slc1A* in HepG2 cells after BPA exposure

Previous studies from our group and from some others highlight the involvement of the transcription factor YY1 as a critical regulator in the expression and activity of *slc1A3* and *slc1A2*.[25,40] YY1 overexpression may also be correlated with the presence of certain liver pathologies; it is suggested that the metabolic system in which glutamate participates could be modified as a direct or indirect response to liver injury. According to our results, BPA exposure impairs SLC1A2 and SLC1A3 transporter activity at the long term. In view of this fact, we asked ourselves if the BPA effect is a transcriptional effect. Cells were pretreated with 100 nM BPA, 10 µg/mL actinomycin D or both. As depicted in Figure 4A, the BPA effect was sensitive to the actinomycin D treatment. These results demonstrate that the effect of acute exposure of BPA is a downregulation of the *slc1A* transporters transcription. These results prompted us to evaluate YY1 expression in HepG2 cells exposed to 100 nM BPA for different periods. A time-dependent increase in YY1 protein levels is presented after 30 minutes, 2 hours, 4 hours, 9 hours, 12 hours, and 24 hours post-BPA (Figure 4B). Note that BPA induces a hormetic response in YY1 protein levels (Figure 4C). Taking into consideration that YY1 acts as both as a transcriptional activator and as a repressor and that PKC activation is essential for the YY1 DNA-binding signal transduction cascade that regulates *slc1A3* transcription, we pretreated the cells with the PKC inhibitor Bis 1. In this scenario, BPA is no longer able to increase YY1 protein levels. As expected, a PKC, such as TPA, mimics the BPA effect. Interestingly enough, the BPA and the TPA effects are not statistically different, suggesting that YY1 overexpression is PKC-mediated (Figure 4D).

4 | DISCUSSION

Glutamate in the liver has an important metabolic role as intermediary in ammonia detoxification, gluconeogenesis, acid-base balance, and so on. The control of hepatic glutamate/aspartate transport might possibly modulate its availability and by these means regulate related intrahepatic metabolic processes.[18,20,21,41]

The current study demonstrates that HepG2 cells express functional SLC1A2 and SLC1A3 transporters. These results are in sharp contrast to the reported SLC1A2 (C-terminal splice variant) exclusive expression in mouse liver.[33] A plausible explanation for this discrepancy is the transformed character of the HepG2 cell line. It might well be possible that cell cycle disruption triggers SLC1A3 expression.

In radial glia cells, glutamate exposure downregulates the activity as well as the expression levels of SLC1A3 in a transporter-dependent manner.[38,42] Interestingly enough, as depicted Figure 2B, in HepG2

cells D-aspartate downregulates the aspartate uptake activity, albeit part of the transport is mediated by SLC1A2 as the its blocker DHK further reduces the uptake, once again demonstrating the functional expression of SLC1A3 in human liver-transformed cells.

BPA disrupts liver metabolic processes. BPA glucuronide is the major metabolite detected in blood and urine, with an estimated half-life in humans of 2 hours, whereas sulfated conjugates are minor metabolites.[39,43-46] A reduced portion of the absorbed BPA is not metabolized remaining in the form of free BPA-producing noxious effects acting as a xenoestrogen.[47]

Therefore, we decided to use a BPA concentration range from 10 nM to 10 μM. In this scenario, a BPA-dependent differential expression of glutamate transporters was detected: SLC1A2 was downregulated in a time-dependent manner, whereas SLC1A3 was upregulated. Although there is a good correspondence between SLC1A2 decreased activity and reduced protein expression under BPA exposure, no correlation was found for SLC1A3. The possibility that the mature protein is not correctly inserted in the plasma membrane which remains to be determined. Further experiments, beyond the scope of this communication, are needed to clarify this issue.

A pivotal role of YY1 in *slc1A* transcriptional control has been documented.[25,27,31,48,49] Moreover, the fact that BPA modulates SLC1A2 and SLC1A3 levels and that this effect is completely inhibited by actinomycin D demonstrated that the effect of this xenoestrogen is mediated at the transcriptional level.

Of relevance is to mention that *slc1A2* and *slc1A3* promoters contain YY1 DNA consensus-binding sites and that overexpression of this transcription factor reduces [^{3}H]-D-aspartate transport and their respective mRNA levels.[25,27] Interestingly, YY1 upregulation expression has been reported in liver pathogenic conditions.[49] In line with these data, in HepG2 cells, BPA regulates YY1. It is tempting to speculate that indeed YY1 is mediating the differential effect of BPA in *slc1A2* and *slc1A3* transcription. Experiments currently in progress in our group are aimed to reveal this issue.

In conclusion, we report here the functional expression of SLC1A3 in a liver-derived transformed cell line. Furthermore, the exposure to low BPA doses is linked to a biphasic effect on the uptake of the glutamate analog, aspartate, that is most likely involved in liver dysfunction. Our findings are summarized in Figure 5.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

C. J.-T. performed the experiments, analyzed the data and wrote the first draft paper. L. C. H.-K., M. N., and A. O. were involved in revising the manuscript. A. O. designed, coordinated the research, provided financial support, and wrote the final version of the manuscript.

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