Benzo[ghi]perylene Induces Morphological and Genotoxic Effects in Human Bronchial Cells Independently of The AhR Pathway

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Research Article

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Abstract

Polycyclic aromatic hydrocarbons (PAH) are organic compounds found in the contaminated atmosphere of Mexico City, where the PAH present with highest concentration is benzo[*ghi*]perylene [B[*ghi*]p]. We recently demonstrated that double-stranded breaks in DNA appear after 3h of exposure, whereas cellular changes and activation of aryl hydrocarbon receptor (AHR) pathway occur after 48h in bronchial cells under exposure to B[*ghi*]p, these findings have led us to explore if the AHR pathway participates in morphological changes and genotoxic effects due to B[*ghi*]p in human NL-20 bronchial cells. Cells of the NL-20 human bronchial line were exposed to B[*ghi*]p in the presence, or absence, of the AHR receptor antagonist, CH-223191. Cell viability was quantified by the MTT assay, which revealed 76 and 66% at 6h and 24h, respectively ($p < 0.001$), regardless of the presence of CH-223191. RT-qPCR showed an increase in the expression of the *AHR* and *CYP1A1* cytochrome genes only after 24h of exposure, and the expression was inhibited by CH-223191. Genotoxicity assays revealed the presence of comets, nuclear buds (NB) and DNA fragmentation in cells exposed to B[*ghi*]p after 6h and in cells exposed to B[*ghi*]p plus CH-223191 at 24h. These results verify that B[*ghi*]p induces morphological and genotoxic effects, and these effects are independent of the AHR pathway.

Introduction

The atmospheres of cities located at high latitudes –eg. Mexico City (MXCT)– contain complex mixtures of contaminants that include polycyclic aromatic hydrocarbons (PAH) (Amador-Muñoz et al. 2011) produced primarily by the incomplete combustion of fuels such as gasoline and diesel (Visciano and Perugini 2009). Automobiles, trucks and factories are the principal sources of PAH emissions in MXCT (SEDEM-GDF. 2012, RETC. 2019). In fact, Amador-Muñoz et al (2011) demonstrated that Benzo[*ghi*]perylene (B[*ghi*]p) was the most abundant PAH in the atmosphere of MXCT in the period between 1998–2002, and that it was contained in PM₁₀. Later, Amador-Muñoz et al. (2013) showed that B[*ghi*]p is the most abundant PAH contained in PM_{2.5}, mainly in the southwestern and downtown areas of MXCT.

B[*ghi*]p is a PAH of the type pericondensed. Its structure contains 6 rings and its molecular weight is 276, but it has no bay or gulf regions (Marr et al. 1999). In 1983, the IARC classified B[*ghi*]p as non-carcinogenic in animals and humans, and the most recent evaluation (2010) established that B[*ghi*]p does not produce cancer by cutaneous application in mouse models; a finding supported by other studies that used the cutaneous injection technique (Müller 1968). Intrapulmonary application in a rat model failed to produce conclusive findings, since some tumors appeared, but the results were not reproducible (Deutsch-Wenzel et al. 1983, IARC. 1983). *In vitro* studies reported that B[*ghi*]p is mutagenic in the *Salmonella typhimurium* assay in presence of an exogenous metabolic system (rat fraction S9) (Platt and Grupe. 2005). Also, B[*ghi*]p correlated with dithiothreitol activity in an *in vitro* system designed to quantify the formation of reactive oxygen species (ROS) (Cho et al. 2005). In summary, few *in vitro* and *in vivo* studies have been conducted to evaluate the toxic properties of B[*ghi*]p. Recent work performed by our research group with cells of the human NL-20 bronchial line, showed that B[*ghi*]p after 48h of

exposure produced morphological changes in cells, consisting in the formation of small vesicles throughout the cytoplasm. Also, B[ghi]p showed these cellular effects: increased damage to the cell membrane, translocation of the aryl hydrocarbon receptor (AHR) to the nucleus, and the expression of genes that included the AHR receptor and cytochrome *CYP4B1*. Except for damage to the cell membrane and vesicle formation, these effects were reversed in presence of the CH-223191 receptor antagonist which, in turn, increased oxidative stress, mainly of superoxide (Zaragoza-Ojeda et al. 2016). Very recently, Castro-Galvez et al. (2019) demonstrated that 3h of exposure to B[ghi]p in this cell line, can induce γ H2AX foci in the nuclei, despite of the increased expression of some DNA repair genes. The cell nuclei contained B[ghi]p after 3h of exposure and B[ghi]p remained there after 24h of its washed out.

AHR is a ligand-dependent transcription factor that belongs to the basic helix-hoop-helix family (bHLH) Per/Arnt/Sim (PAS) and binds to a wide variety of exogenous compounds, including benzo[a]pyrene (B[a]p) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Opitz et al. 2011). AHR is found predominantly in the cytoplasm in a multiprotein complex that includes two hsp90 chaperone proteins (90-kDa heat shock proteins) that help maintain the conformation of the receptor, an AIP (auxiliary interacting protein) that stabilizes the interaction between the hsp90 and the receptor, and a p23 co-chaperone protein that stabilizes the intermediary ligand-AHR complex. In the presence of a ligand, the multiprotein complex becomes uncoupled and the AHR-PAH translocates to the nucleus, where it forms a heterodimer with ARNT (aryl hydrocarbon receptor nuclear translocator) (Larigot 2018, Opitz et al. 2011). The AHR-ARNT complex then binds to XRE (xenobiotic response elements) to induce the transcription of target genes that codify enzymes that participate in activation and detoxification; namely, phase I and phase II enzymes, such as CYPs and aldo-ketoreductases, AKR (among others), respectively, and proteins and enzymes that participate in the activation of cell cycle and cell adhesion, migration and differentiation. Once released from its ligand, AHR is degraded by the proteosome pathway in the nucleus (Roberts and Whitelaw 1999). As mentioned above, our group has described that 3h of exposure to B[ghi]p in human bronchial cell line NL-20, can induce γ H2AX foci in the nuclei, and the activation of AHR after 48h of exposure. These findings led us to design the present study, in which we evaluated if morphological and genotoxic effects due to B[ghi]p in human NL-20 bronchial cells, depend on AHR pathway.

Materials And Methods

Cultures of NL-20 cells

The NL-20 cell line was obtained from the ATCC (CRL-2503). These are human epithelial bronchial cells immortalized from normal (*i.e.*, non-tumorous) bronchi. Cells were cultured in HAM-F12 culture medium supplemented with 4% fetal bovine serum, 1% L-glutamine, 1% non-essential amino acids, 5 μ g/ml of insulin, 10 ng/ml of epidermal growth factor, 1 μ g/ml of transferrin, 500 ng/ml of hydrocortisone, 100 μ g/ml of streptomycin sulfate, and 100 μ g/ml of Penicillin-G, under the following conditions: 37°C, 5% CO₂ and relative humidity. They were detached in a solution of 0.05% trypsin and 0.01% versene for 10

min at 37°C and were centrifuged at 1800 rpm for 5 min, and then counted in a hemocytometer before performing the following exposure assays to B[ghi]p.

Exposure to B[ghi]p

Previously, we performed assays at different times to exposure NL-20 cells to B[ghi]p and after did them, we decided to continue the study at 6h and 24h of exposure to B[ghi]p. The NL-20 cells were exposed following the experimental scheme: only medium; control vehicle (DMSO); 2.03 µM of B[ghi]p and in the presence of 2.03 µM of B[ghi]p + 10 µM of CH-223191 (antagonist of AHR receptor). All experiments were performed in triplicate, and results were obtained from three independent assays. The concentration of each compound was defined according to previous published assays (Zaragoza-Ojeda et al. 2016, Castro-Galvz et al. 2019)

Total RNA extraction

Cells from the experimental scheme were lysed by adding 100 µL of Trizol per well and stored at -70°C until the samples from three biological triplicates were collected. In the next step of the extraction procedure, the cellular lysate was incubated for 5 min at room temperature, 60 µL of cold chloroform were added, and the mixture was agitated in a vortex and left to ambient temperature for 10 min. It was then centrifuged at 12,000 rpm for 15 min at 4°C and the aqueous phase containing the RNA was recovered. This was transferred to a microtube with 150 µL of cold isopropanol and 1 µL of glycogen and incubated overnight at -20°C to precipitate the largest quantity of total RNA possible. This was centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was removed, and the RNA was washed twice with 300 µL of ethanol 70%, mixed gently and then centrifuged at 10,000 rpm for 5 min at 4°C. Finally, the alcohol was decanted, and the RNA was left to dry, afterwards it was re-suspended in 10 µL water-RNases free. It was treated with DNase I (1U per 1µg of RNA) for 30 min. The enzyme was inactivated with EDTA at 70°C for 10 min and the concentration and purity of the RNA was quantified in a NanoDrop. All samples had a 260/280 relation above 1.7.

cDNA synthesis

cDNA synthesis was performed with 200 ng of total RNA using the First Strand cDNA Synthesis kit (Thermo Scientific) in a thermocycler under the following conditions: at 37°C for 60 min, at 70°C for 10 min, and at 22°C for 1 min.

Expression of the AHR, CYP1A1 and HOX-1 genes by RT-qPCR

Gene expression was quantified in real-time with SYBR green as the principle fluorochrome and ROX as the passive reference colorant, as specified in the Maxima SYBER Green qPCR Master Mix Kit (Thermo Scientific). The specific primers for each gene were used (Table 1) and the reactions were carried out in a Stratagene Mx3005P thermocycler (Agilent) using the following program: at 95°C for 10 min, 40 cycles at 95°C for 15 seconds at the melting temperature of each primer for 1 min, and finalizing with one cycle at 95°C for 1 min, at 58°C for 30 sec, and at 95°C for 30 sec. Expression levels were analyzed by applying

the $2^{-\Delta\Delta C_t}$ mathematical model (Pfaffl 2001; Livak and Schmittgen 2001) with GAPDH as the endogenous gene.

Table 1
Forward (F) and Reverse (R) sequences of the *GAPDH*, *AHR*, *CYP1A1* and *HOX-1* genes showing size in base pairs (bp) of each amplified product and the optimal T_M for RT-qPCR.

Genes	Oligonucleotides sequences		Amplicon, size, bp	MT
GAPDH	F	CGGAGTCAACGGATTTGGTC	96	58° C
	R	ATGAAGGGGTCATTGATGGCA		
AHR	F	GCCGGTGCAGAAAACAGTAA	80	58° C
	R	TAAGTCGGTCTCTATGCCGC		
CYP1A1	F	CCTCAACCTCCTGCTACCTG	71	58° C
	R	CTCCTGCAACGTGCTTATCAG		
HOX-1	F	CAAAGTGCAAGATTCTGCCC	94	58° C
	R	TGTAAGGACCCATCGGAGA		

Expression of the AHR protein by immunocytochemistry

6×10⁴ cells were cultured 24 h before exposure onto coverslips pre-treated with poly-L-lysine [1:10] into 35mm Petri dishes. At the end of the experimental scheme, the cells were washed with 1 mL of PBS and they were fixed with absolute methanol for 5 min. The fixative was then removed, and the cells were washed 3 times with PBS for 5 min each. 1 mL of 0.2% Triton in PBS was used per sample for 30 min to permeabilize the cells. Subsequently, 3 washes were performed with 1 mL of PBS and background noise was blocked with 1 mL of 50mM glycine. Glycine was then removed and 100 µL of 0.1% BSA in PBS supplemented with 10% goat serum was added for 1 h. Samples were washed twice with 1 mL of PBS. 35 µL of diluted primary antibody (AHR, ab 2770, 1:100) was added and incubated for 30 min at room temperature (RT) followed by incubation for 24 h at 4°C in a humid chamber. The next day, 3 washes of 10 min each were performed with 1 mL of 0.05% tween in PBS. 14 µL of the following secondary antibody was added over 90 min: anti-mouse Alexa Flour 488 (1:200) (ab 150117) diluted in PBS as indicated. Subsequently, excess of antibody was removed with 3 washes of 0.05% Tween in PBS for 10 min each and 1 wash with PBS for 10 min. The samples were incubated with 100 µL DAPI [10 µg/mL] per sample for 5 min, mounted with mounting medium Vectashield and kept in dark at 4°C for further analysis with the fluorescence microscope Leica DMI8.

MTT viability assay

Following the protocol by Tolosa et al. (2015) for this assay, 15,000 cells were cultured per well in 96-well plates 24h before the experiment. Exposure times were 6h and 24h. Cells were exposed to the experimental scheme conditions in triplicate, but 3 hours before ending each time, 20 µL of MTT at a

concentration of 5 mg/mL (in PBS) were added to each well. At the end of the exposure times, the medium was removed carefully before adding 100 μ L of acidic isopropanol to each well for 5 min under darkness. The amount of color was quantified in an ELISA plate at a wavelength of 540 nm as principal absorbance, and a wavelength of 620 nm to measure background absorbance. The percentage of reduced MTT was calculated using the absorbance obtained from the unexposed control-vehicle that was considered 100%.

Cell morphology

Cells were cultured and were left to adhere on sterile coverslips before being exposed to the experimental scheme. At the end of the exposure times, they were fixed in absolute methanol for 5 min and left to dry before staining with 500 μ L of Giemsa for 10 min. Next, they were washed to remove the excess colorant and left to dry. Finally, they were mounted with resin on slides and observed under an optical microscope at 100x. Photomicrographs were registered for any experimental scheme to reveal morphological changes.

Nuclear electrophoresis analysis: COMET assay and nuclear buds

At the end of the experimental scheme, cells were detached using trypsin and saline solution (1:9) in an incubator at 37°C for 15 min. To concentrate, cells were centrifuged at 1800 rpm for 5 min, the supernatant was discarded, and cells were re-suspended in 100 μ L of complemented HAM-F12 culture medium. Next, 50 μ L were extracted and mixed with 90 μ L of low-fusion point agarose at 0.5% at 38°C. Prior to this, agarose gels at 1% had been prepared in PBS on slides to empty the second layer of agarose with the cells, which was left to gel at 4°C for 7 min. To obtain nuclei, the gels were left in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, Triton 1% X-100 and DMSO 10%, pH 10) for 24h at 4°C.

In darkness, the gels were placed in a horizontal electrophoresis chamber with an alkaline buffer at 4°C (300 mM NaOH, 1 mM EDTA pH 13) for 20 min to allow the DNA to unroll, and then for another 20 min at 25 V to start the migration of the DNA fragments towards the anode. At that point, the gels were neutralized by 3 washings in neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min each. The gels were then fixed in absolute ethanol at 4°C for 10 min and stained with 50 μ L of Gel Red (10%) for analysis under fluorescence microscopy. The parameters considered to measure the damage in 100 nuclei per treatment were nuclei with comets and buds, expressed as the percentage of comets and of nuclei with buds.

Detection of DNA fragmentation induced by B[ghi]p with the TUNEL assay

To conduct this assay, we used the DeadEnd Colorimetric TUNEL System kit (Promega), exposing 45,000 cells from each experimental scheme to B[ghi]p. After removing the culture medium, the cells were washed in 1 mL of saline solution to separate them with trypsin for 10 min. To concentrate cells, they were centrifuged at 1800 rpm for 5 min. The solution was decanted and 100 μ L of saline solution were added for a wash with centrifugation at 1800 rpm for 5 min. The solution was decanted again, and cells

were re-suspended in 100 μ L of saline solution. On previously prepared slides coated with silane 0.1%, smears were taken for each experimental scheme and left to dry at 37°C. These samples were then fixed with paraformaldehyde at 4% for 25 min and the presence of DNA fragmentation was revealed, following the instructions of the kit. The slides from three independent experiments were evaluated in an optical microscope, and the nuclei with DNA fragmentation were quantified in 100 cells and expressed as percentages of nuclei with DNA fragmentation.

Statistical analysis

The differences in the levels of expression of *AHR*, *CYP1A1* and *HOX-1*, and the viability test were evaluated by ANOVA analysis. The presence of buds, the number of nuclei with DNA fragmentation, as determined by TUNEL, and the number of comets induced, were all evaluated by Ji-squared test. Differences were considered significant when the p value was < 0.05.

Results

The effect of B[ghi]p on cell viability is independent of the activation of the AHR pathway

The viability of cells exposed to B[ghi]p decreased ($p < 0.001$) to 76% and 66% at 6h and 24h of exposure, respectively (Fig. 1A). Both percentages of viability were above of the 50% inhibitory concentration (CI_{50}). The effects of the reduction of viability due to exposure to B[ghi]p were not reversed by the AHR antagonist CH-223191 (Fig. 1A), this indicates that the reduction of the viability of the cells exposed to B[ghi]p after 6h and 24h of exposure in the presence of the CH-223191 antagonist occurs independently of AHR pathway activation.

The expression of AHR pathway is verified at 24 of exposure of B[ghi]p

The transcriptional activation of the AHR pathway was evaluated. B[ghi]p

induces a significant increase ($p < 0.05$) in the expression of the *AHR* (Fig. 1B) and *CYP1A1* (Fig. 1C) genes, but only after 24h of exposure, compared to control vehicle (DMSO). However, the high expression of *AHR* and *CYP1A1* after 24h of exposure returned to basal levels when cells were exposed to B[ghi]p plus the AHR antagonist CH-223191 (Figs. 1B and 1C, respectively). These results proved that B[ghi]p at 24h activates the AHR pathway. Previously, Zaragoza-Ojeda et al. (2016) demonstrated that B[ghi]p induces oxidative stress at 48h of exposure. Therefore, we decided to evaluate the expression of the *HOX-1* gene due to exposure to B[ghi]p. Figure 1D shows an increase of expression of this gene ($p < 0.05$), in 6h and 24h of exposure, which indirectly indicates the induction of oxidative stress.

Translocation of the AHR receptor after B[ghi]p exposure

Immunocytochemistry illustrates that, after 6h of exposure to B[ghi]p, there is a weak signal of protein (Figs. 2H and 2K), and it remains mainly in cytoplasm (Fig. 3I), also in presence of the antagonist CH-

223191 (Figs. 2I and 2L). At 24h of exposure to B[ghi]p, the AHR protein is translocated mainly to the nucleus (Figs. 2T and 2W, cyan color in merge, Fig. 2W) and in presence of AHR antagonist CH223191, the protein is weakly detected (Figs. 2U and 2X). These results verify B[ghi]p activates the pathway because the protein is translocated to the nucleus

Morphological changes in cells exposed to B[ghi]p

To characterize the morphological changes produced by B[ghi]p, at 6h and 24h, the analysis of photomicrographs shows the presence of intracytoplasmic vesicles (IVs) in NL-20 cells exposed to both B[ghi]p (Figs. 3C and 3G) and in B[ghi]p plus antagonist CH-223191 (Figs. 3D and 3H). At 6h, there is not AHR gene expression (Fig. 1B) and cells exposed to B[ghi]p alone have IVs (Fig. 3C): it proves this morphological modification is independent of any interaction of B[ghi]p to any receptor. The presence of IVs also remains after 24 of exposure to B[ghi]p (Fig. 3G), even in presence of the antagonist CH-223191 (Fig. 3H). The IVs are formed independent of the AHR pathway.

Genotoxic effects due to B[ghi]p

To evaluate the genotoxic effects that B[ghi]p might have on the nuclei of bronchial cells, a COMET assay, quantification of nuclear buds (NB), and DNA fragmentation determined by the TUNEL assay were performed. COMET analysis showed low, non-significant differences in formation of comets induced by B[ghi]p between 6h and 24h of exposure, even in presence of the antagonist CH-223191 ($p > 0.05$) (Fig. 4A), so we did not do any other measurements (v.g. tail moment of the comet/width of the tail). Regardless, the number of comets induced were significantly higher ($p < 0.05$) in cells exposed to B[ghi]p compared to those observed in the negative control and medium (Fig. 4A). In contrast, there was a significant increase of NB in cells exposed to both conditions: B[ghi]p alone and in presence of the AHR antagonist CH-223191 at 6h and 24h ($p < 0.05$) (Fig. 4B). These results show that induction of comets and NB occurs independently of the AHR receptor pathway.

Finally, B[ghi]p induced DNA fragmentation in 70% of the exposed cells, compared to only 3% of controls ($p < 0.05$) at 6h of exposure. CH-223191 cannot lower the DNA fragmentation as it is observed in controls (Fig. 4C). However, at 24h, there was reduced percentage of cells with DNA fragmentation (18%). The presence of the CH-223191 (Fig. 4C), reduced cells with DNA fragmentation at 15%, but these differences were not significant ($p > 0.05$). This confirm that DNA fragmentation is not dependent of the AHR pathway.

Discussion

Cell viability and morphological changes

Although the AHR pathway was not activated at 6h of exposure to B[ghi]p, surprising observations were performed and included the decrease in cell viability accompanied by morphological changes in the NL-20 bronchial cells. These results are similar with those of a study conducted with A549 pulmonary

adenocarcinoma cells exposed to 2 μ M of B[ghi]p for 14h, which reported 68% viability (Genies et al. 2016). Those findings indicate that B[ghi]p is cytotoxic and, perhaps, that the integrity of the mitochondria of the exposed cells is compromised by a decrease in the amount of metabolically-active cells, accompanied by greater oxidative stress. The particulate material (PM) and fuel emissions associated with the PAH have been shown to interfere with the function of mitochondria in both electron transport and the transitory permeability of the aperture of the mitochondrial pore (Xia et al. 2007). The mitochondrial toxic effect of B[ghi]p is currently under study in our laboratory.

B[ghi]p induces changes in the cytoplasm that include increasing the number of vesicles at 48h of exposure to different concentrations. Vesicle formation is not inhibited by the AHR antagonist (Zaragoza-Ojeda et al. 2016), suggesting that cell changes caused by exposure to B[ghi]p is produced by an AHR-independent pathway. Our study demonstrated that the AHR pathway was not activated at 6h of exposure, but cells have vesicles characteristic of longer exposure (24h), this result also verify the AHR-independent pathway involved. Other research has shown that exposure of A549 cells to PM₁₀ generates the appearance of what are called cytoplasmic inclusions, with 2-3 μ m alin diameter (Mazzarella et al. 2007), a finding also reported by Ferecatu et al. (2010), but with PM_{2.5}. It may be related to the onset of cell death by apoptosis, as reported by Johnson et al. (2000), who induced DNA damage with staurosporine in the presence of caspase inhibitors. Or IVs can represent an alteration of another via, v.g. vesicular traffic, a finding that is currently being explored in our laboratory

The genotoxic effects of B[ghi]p after short exposure times occur independently of the AHR pathway

Previously, our group demonstrated that exposing NL-20 bronchial cells to B[ghi]p for 48h, induces morphological damage and the transcriptional activation of the AHR pathway (Zaragoza-Ojeda et al. 2016). However, the question of whether cell damage and AHR pathway activation can begin after only short exposure times to B[ghi]p remained unresolved. In an effort to clarify this, the present study evaluated genotoxic damage and AHR pathway activation at 6h and 24h of exposure to B[ghi]p, at concentrations lower than those reported earlier by our group. The most significant result of this study was that the AHR pathway was not activated at 6h of exposure, although cytotoxic and genotoxic damage and morphological changes to the human NL-20 bronchial cells did occur.

In cells of the WB-T344 epithelial line, the complex mixtures derived from the urban particles did activate the AHR receptor, and that activation contributes to the carcinogenic properties of these mixtures (Andrysik et al. 2011). There is, however, controversy in this regard. Depending on cell type, activation of this pathway can produce different effects, including the fact that in LNCaP prostate carcinoma cells, AHR pathway activation plays an important role in the non-genotoxic effects of B[ghi]p, such as inhibition of the cell cycle (Hrubá et al. 2011). It is important to note that studies with other cell lines (eg. H4IIE hepatoma), have shown that this hydrocarbon is a weak AHR inducer compared to the agonist, TCDD, since 10 μ M of B[ghi]p is required to achieve the same effect that is induced with only 100 pM of TCDD after 6h of exposure. However, the mutagenesis or genotoxicity processes and potential AHR activation are properties of the PAH that can occur independently (Machala et al. 2001), as has been shown using

benzo[*k*]fluoranthene, a potent AHR inducer, but weak genotoxic agent, that produces low levels of adducts in the DNA of alveolar epithelial cells of A549 adenocarcinoma (Libalová et al. 2014).

The increase of RNAm of the *CYP1A1* and *AHR* genes is a classic marker of the AHR pathway activation that reflects the activation of the metabolism of phase I xenobiotics (Pan et al. 2013). The present study, however, did not detect any increase in the transcription of the *AHR* and *CYP1A1* genes at 6h of exposure of the NL-20 cells to B[*ghi*]p. This indicates that longer exposure times to B[*ghi*]p are required to activate the pathway, as was demonstrated at 24h of exposure, with the resulting transcriptional increase of *AHR* and *CYP1A1*.

Genotoxic damage induced by B[*ghi*]p in cells of the NL-20 bronchial line

The PAH with bay or gulf regions have the potential to induce DNA damage; one example is B[*a*]p (MacLeod et al. 1991). The main pathway for producing toxic and genotoxic damage is activation of the AHR receptor (Andrysik et al. 2011, Libalova et al. 2014). However, the short exposure time to B[*ghi*]p used in the present study revealed cell damage without AHR pathway activation. This is similar with reports that observed DNA damage due to the B[*a*]p metabolite, benzo[*a*]pyrene-trans-7,8-dihydrodiol-9-10-epoxide (BPDE), which can bind directly to DNA independently of the metabolic process of the AHR pathway (Hockley et al. 2007).

To evaluate the possible genotoxic effect of B[*ghi*]p, the present study utilized, among other techniques, the COMET assay (Merk and Speit 1999). Because B[*ghi*]p is a PAH without bay or gulf regions, it cannot generate dihydrodiol epoxides; hence, it likely causes oxidative damage of the bases, similar to what has been suggested for anthanthrene (Desler et al. 2009). This may explain the low number of small-tailed comets reported by Platt and cols. (2008) in V79 hamster lung fibroblast cells, even at a high concentration, 50 µM of B[*ghi*]p. This damage did not increase with metabolic activation. The frequency of comets induced by exposure to B[*ghi*]p is low even at concentrations 10 times higher than the one used in the present study (2.03 µM), as reported by Rodríguez-Romero et al. (2012) in human peripheral lymphocytes, where exposure to this hydrocarbon produced around 9% of comets, a figure that increased to almost 28% with S9 metabolic activation. The low frequency of comets induced by B[*ghi*]p is due to the comet assay is essentially a method of detecting single-strand breaks (Kawaguchi et al. 2010, Heuser et al. 2002), and B[*ghi*]p, according to results reported by Castro-Galvez et al. (2019), induces double-strand breaks that were proved by the results of TUNEL assay in this study, that will be discussed below.

However, upon analyzing the nuclei after unicellular electrophoresis of the exposed cells, we observed a significant percentage of NB at 6h of exposure. Some researchers consider that these abnormalities of the nuclei are zones of amplified DNA that the cells removed during the S phase of the cell cycle (Fenech 2002). It is thought that almost all these NB originate from acentric, telomeric or interstitial fragments, perhaps representing DNA that is trapped in the nuclear membrane and remains there after nuclear division or, perhaps, excess DNA that is excluded from the nucleus but stays tightly-connected to it through a stem of nucleoplasmic material. These events have been associated with chromosomal instability (Lindberg et al. 2007, Fenech et al. 2011). Meanwhile, by marking all the chromosomes with

fluorescent probes and evaluating the genotoxic effect of zidovudine on bone marrow mesenchymal cells, Dutra et al. (2010) demonstrated that less than 50% of the NB contain positive signs for one or two chromosomes, although over 50% had no marking signal, and some even showed the presence of a centromere. This suggests that the NB may contain fragments of some chromosome, a hallmark of genetic instability and do not necessarily represent only amplification zones.

In addition, NB are sensitive biomarkers that indicate exposure to diverse compounds (Nersesyan. 2005). Examples of this are the PAH mixtures to which coke oven operators are exposed, which showed a 3.5-fold increase in the amount of NB compared to a control group. This means that they can be used as indicators of chromosomal damage to biomonitor workers exposed to PAH, and that their frequency can be correlated with exposure time (Duan et al. 2009). While the mechanism of NB formation in this system is unknown, the fact that their appearance was observed after short exposure times during which, the AHR-mediated pathway was not activated, suggests that NB can function as early markers of exposure to B[ghi]p.

DNA fragmentation

The results of exposure to B[a]p by Tung et al. (2014) and B[ghi]p found in this study demonstrate induction of DNA fragmentation. The B[a]p has a bay region that identifies it as a carcinogenic with a high capacity to generate DNA fragmentation (Ramesh et al. 2004). The B[ghi]p in contrast, has no bay or gulf region but, according to our results, is also capable of inducing DNA fragmentation (Castro-Galvez et al. 2019). Another molecule that has no such regions is anthanthrene but, according to the results of Desler et al. (2009), it can induce DNA fragmentation in cell lines regardless of distinct gain- and loss-of-function phenotypes in the DNA repair mechanisms. Using *in vitro* DNA, Platt et al. (2008) showed that the K region of B[ghi]p can produce 3,4-oxides responsible for DNA interaction with a weak binding property, such that no adducts are produced. This K region, which is present in other PAH as well – including B[ghi]p – preferentially generates deoxyguanosine (Rojas and Alexandrov 1986) and deoxyadenosine (Nair et al. 1991). In this regard, Desler et al. (2009) propose that the PAH with no bay or gulf regions are generators of oxidative stress, and that it is through this pathway that they cause oxidative damage in nitrogenous bases of DNA such as 8-oxo-dG. These PAH with K regions are very weak tumor initiators compared to the PAH that have bay or gulf regions (Berry et al. 1977).

DNA fragmentation can support the fact that B[ghi]p do not have bay or gulf regions and its oxidative effects can induce breaks of DNA double-strand. This was verified by Castro-Galvez et al. (2016) previously since this PAH can induce as earlier breaks of double-strand DNA at 3h by γ H2AX foci in the nuclei, and at 6h by TUNEL positive cells. Probably, at 24h, DNA repair enzymes could repair, and it can explain the reduction of TUNEL positive nucleus.

Conclusions

This work demonstrated that short-term exposure to B[ghi]p functions as a cytotoxic and genotoxic agent whose effect is independent of the AHR-mediated pathway. B[ghi]p can induce morphological changes in

cells, reduces their viability, and cause comets, formation of NB and DNA fragmentation: markers of genotoxicity. This finding opens up a broad field of study since B[*ghi*]p seems to affect cells in two distinct ways: independently of the AHR- pathway at short exposure times, and at 24h. This raises the following question: ¿what is the overall effect of prolonged exposure to B[*ghi*]p on the structure of the genome of bronchial cells? Research into these important issues is underway in our laboratory with the expectation that in the near future we will be able to provide some answers to these key questions.

Declarations

Declarations

Not applicable

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Zelmy Castro-Gálvez, perform assays of morphological changes, immunocytochemistry, cytotoxicity and genotoxicity. Also statistical analysis. Montserrat Zaragoza-Ojeda, perform the analysis of genes expression. Abrahan Hernández-Hernández, critical review to draft. Francisco Arenas-Huertero drafted the first version of the manuscript and finish the scope of the study.

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Figures

Figure 1

A. MTT Viability assay of viability of MTT NL-20 cells exposed to DMSO (black bars), B[ghi]p (grey bars) and B[ghi]p + CH-223191 (dashed bars), by 6 hours and 24 hours. Each bar shows the mean + standard error. Results are from three independent experiments. *** p<0.001, ** p<0.01. B. Gene expression of AHR (B), CYP1A1 (C) and HOX-1 (D) quantified by qPCR-RT of NL-20 cells exposed to medium (white bars), DMSO (black bars), B[ghi]p (grey bars), B[ghi]p + CH-223191 (dashed bars) by 6 hours and 24 hours. Each bar shows the mean + standard error. Results are from three independent experiments. ** p<0.01.

Figure 2

A. Immunocytochemistry of AHR in cells exposed to 6 hours. 1, 2 and 3, micrographies in bright field. 4, 5 and 6 nuclei staining by DAPI and observed by fluorescence. 7, 8 and 9 immunostaining against AHR. 10, 11 and 12 merge images from 4 and 7, 5 and 8, and 6 and 9, respectively. B. Immunocytochemistry of AHR in cells exposed to 24 hours. 1, 2 and 3, micrographies in bright field. 4, 5 and 6 nuclei staining by DAPI and observed by fluorescence. 7, 8 and 9 immunostaining against AHR. 10, 11 and 12 Merge images from 4 and 7, 5 and 8, and 6 and 9, respectively. Black and white bar of scale is equivalent to 10 μ m.

Figure 3

Morphological changes in NL-20 cells. A. Cells growing in medium; B. Cells exposed to: DMSO; C. B[ghi]p; and D. B[ghi]p + CH-223191 (antag), by 6 hours. D. Cells growing in medium; E. Cells exposed to: DMSO; F. B[ghi]p; and G. B[ghi]p + CH-223191 (antag), by 24 hours. Microscopy of bright field. Giemsa staining. The black bar of scale is equivalent to 10 μ m

Figure 4

Genotoxic effects of B[ghi]p on NL-20 cells. A. Nucleus with comets in cells growing in medium (white bars), DMSO (black bars), B[ghi]p, (grey bars), B[ghi]p + CH-223191 (dashed bars) by 6 hours and 24 hours. Each bar shows the mean + standard error. The results are from three independent experiments. *** $p < 0.001$, ** $p < 0.01$. B. Nuclear buddings in NL-20 cells growing in medium (white bars), DMSO (black bars), B[ghi]p (grey bars), B[ghi]p + CH-223191 (dashed bars) by 6 hours and 24 hours. Each bar shows the mean + standard error. The results are from three independent experiments. *** $p < 0.001$. C. DNA fragmentation in NL-20 cells revealed by TUNEL staining. Cells exposed to 10 U/mL of DNase I (vertical dashed bars/positive control), cells growing in medium (white bars), DMSO (black bars), B[ghi]p (grey bars), B[ghi]p + CH-223191 (horizontal dashed bars) by 6 hours and 24 hours. Each bar shows the mean + standard error. The results are from three independent experiments. ** $p < 0.01$.