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Hexachlorobenzene modulates the crosstalk between the aryl hydrocarbon receptor and transforming growth factor-β1 signaling, enhancing human breast cancer cell migration and invasion

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ABSTRACT

Given the number of women affected by breast cancer, considerable interest has been raised in understanding the relationships between environmental chemicals and disease onset. Hexachlorobenzene (HCB) is a dioxin-like compound that is widely distributed in the environment and is a weak ligand of the aryl hydrocarbon receptor (AhR). We previously demonstrated that HCB acts as an endocrine disruptor capable of stimulating cell proliferation, migration, invasion, and metastasis in different breast cancer models. In addition, increasing evidence indicates that transforming growth factor-β1 (TGF-β1) can contribute to tumor maintenance and progression. In this context, this work investigated the effect of HCB (0.005, 0.05, 0.5, and 5 μM) on TGF-β1 signaling and AhR/TGF-β1 crosstalk in the human breast cancer cell line MDA-MB-231 and analyzed whether TGF-β1 pathways are involved in HCB-induced cell migration and invasion. RT-qPCR results indicated that HCB reduces AhR mRNA expression through TGF-β1 signaling but enhances TGF-β1 mRNA levels involving AhR signaling. Western blot analysis demonstrated that HCB could increase TGF-β1 protein levels and activation, as well as Smad3, JNK, and p38 phosphorylation. In addition, low and high doses of HCB were determined to exert differential effects on AhR protein levels, localization, and activation, with a high dose (5 μM) inducing AhR nuclear translocation and AhR-dependent CYP1A1 expression. These findings also revealed that c-Src and AhR are involved in HCB-mediated activation of Smad3. HCB enhances cell migration (scratch motility assay) and invasion (Transwell assay) through the Smad, JNK, and p38 pathways, while ERK1/2 is only involved in HCB-induced cell migration. These results demonstrate that HCB modulates the crosstalk between AhR and TGF-β1 and consequently exacerbates a pro-migratory phenotype in MDA-MB-231 cells, which contributes to a high degree of malignancy. Taken together, our findings help to characterize the molecular mechanism underlying the effects of HCB on breast cancer progression.

Abbreviations: AhR, aryl hydrocarbon receptor; ANF, α-naphthoflavone; EMT, epithelial-mesenchymal transition; ERα, estrogen receptor α; ERK1/2, extracellular signal related kinase 1 and 2; HCB, hexachlorobenzene; JNK, c-jun n-terminal kinase; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TGF-β1, transforming growth factor-β1; TβRI, type I TGF-β receptor; TβRII, type II TGF-β receptor.

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1. Introduction

Breast cancer is by far the most frequently diagnosed cancer in women. However, given the low rate of breast cancer etiology being attributed to hereditary origins (WHO, 2015), other causative factors have been postulated, including environmental exposure to persistent organic pollutants. Hexachlorobenzene (HCB) is a widespread environmental pollutant that has detrimental biological effects and the International Agency for Research on Cancer has classified it as a probable human carcinogen (ATSDR, 2002). HCB was used as a fungicide until the 1970's when such use was prohibited. However, considerable amounts are still being generated as waste by-products of industrial processes and released into the environment. Moreover, HCB has been detected in mother's (Der Parsehian, 2008) and cow's milk for human consumption (Maitre et al., 1994). The role of endocrine disruptors in cancer development and progression has been studied over the last few decades. Animal exposure to HCB elicits a number of effects such as thyroid disruption (Chiappini et al., 2009) and co-carcinogenesis in mammary tumors (Randi et al., 2006).

HCB is a "dioxin-like" compound that weakly binds to the aryl hydrocarbon receptor (AhR), a transcription factor that directs the expression of many detoxification genes (Nguyen and Bradfield, 2008). Many of the biological effects of AhR ligands involve multiple interactions between AhR and other signaling pathways. It has been proposed that, upon ligand binding, two cellular signaling events are initiated. Specifically, binding to the AhR can trigger membrane actions, where c-Src release from its cytosolic AhR multiprotein complex and phosphorylates a variety of growth factor receptors (Park et al., 2007). Furthermore, binding to AhR can trigger nuclear actions that modulate the expression of genes involved in the regulation of cell proliferation, differentiation, and/or apoptosis (Matsumura, 1994). The AhR has been shown to exhibit growth-inhibitory activity in MCF-7 breast cancer cells and to promote cell cycle progression in hepatoma cells (Abdelrahim et al., 2003). These results suggest that the AhR could act either as a tumor suppressor or an oncoprotein according to the cell context. Trombino et al. (2000) found higher AhR levels in mammary tumors compared to the corresponding normal mammary tissues. In addition, estrogen receptor α (ERα) loss is tightly associated with AhR overexpression in breast cancer (Bekki et al., 2015).

Transforming growth factor-β1 (TGF-β1) has been hypothesized to participate in breast cancer development (Moses and Barcellos-Hoff, 2011). TGF-β1 has an anti-proliferative effect on most epithelial cells and in the early-stages of tumorigenesis, whereas it accelerates cancer progression and metastasis in the late stages of cancer (Humbert et al., 2010). High TGF-β1 levels in tumor cells correlate with a higher risk of metastasis and poor prognosis (Dumont and Arteaga, 2003). TGF-β1 is secreted into the extracellular medium and forms a latent complex with accessory proteins. After activation, it binds to the type II TGF-β receptor (TβRII), which phosphorylates the type I TGF-β receptor (TβRI) that in turn phosphorylates Smad2 and Smad3. Phosphorylated Smad2/3 forms a complex with Smad4 and is subsequently transferred to the nucleus where it regulates gene transcription (Massagué et al., 2005). In addition, it is well known that TGF-β1 also induces other pathways, which include activation of extracellular signal related kinase 1 and 2 (ERK1/2), c-jun N-terminal kinase (JNK), and p38 MAPK (Derynck and Zhang, 2003).

Crosstalk between TGF-β1 and AhR signaling has been shown to have common targets involved in processes such as cell cycle control (Haarmann-Stemmann et al., 2009). Analyses in cell systems and in mouse models lacking AhR expression have demonstrated that AhR represses TGF-β1 activity and that certain of the phenotype identified in AhR-depleted mice may involve the up-regulation of this cytokine (Gómez-Durán et al., 2009). Moreover, TGF-β1 triggers cell type-specific effects on AhR by inhibiting receptor expression and activation in lung cancer cells, while the receptor function is enhanced in hepatoma cells (Döhr et al., 1997; Wolff et al., 2001). Although AhR is not as well-characterized as TGF-β1 regarding its contribution to human disease, it is reasonable to assume that low or high AhR expression could differentially affect disease progression and involve TGF-β1 signaling.

We previously demonstrated that HCB is a mammary tumor co-carcinogen in rats (Randi et al., 2006) and an inducer of proliferation in MCF-7 cells that acts in an ERα-dependent manner (García et al., 2010). Evidence in the human breast cancer cell line MDA-MB-231 has also shown that HCB can induce cell migration and invasion through c-Src, epidermal growth factor receptor, and AhR (Pontillo et al., 2011, 2013). Therefore, the aim of the present study was to examine the effect of HCB on the crosstalk between AhR and TGF-β1 pathways in MDA-MB-231 cells and analyze the role of TGF-β1 in HCB-induced cell migration and invasion.

2. Materials and methods

2.1. Chemicals

HCB (>99% purity, commercial grade) was obtained from Aldrich-Chemie GmbH & Co. (Steinheim, Germany). Anti-TGF-β1 and anti-AhR antibodies were purchased from Abcam, Ltd. (Cambridge, UK). Anti-Smad3, anti-phospho-Smad3, anti-JNK, anti-phospho-JNK, anti-p38, and anti-phospho-p38 were obtained from Cell Signaling Technology, Inc. (MA, USA). Anti-β-Actin antibody, TGF-β1, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), antibiotic–antimycotic, dimethyl sulphoxide (DMSO), trypsin, glutamine, and the inhibitors α-naphthoflavone (ANF), SB431542, SB203580, SP600125, and PD98059 were purchased from Sigma-Aldrich Chemical, Co. (St. Louis, MO, USA). The 4-Amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-d]pyrimidine (PP2) inhibitor was obtained from Calbiochem (La Jolla, CA, USA). The enhanced chemiluminescence kit (ECL) and Alexa 488 anti-mouse IgG were obtained from GE Healthcare Life Sciences (Buckinghamshire, UK). The Alexa 488 anti-rabbit IgG was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). RPMI-1640 culture medium was obtained from HyClone Laboratories, Inc. (Logan, UT, USA). The random primers were purchased from Biodynamics (Argentina). Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) and cofactors for reverse transcription were obtained from Promega Corporation (Madison, WI, USA). The kit Hot FirepolEvaGreen qPCR Mix Plus (ROX) was purchased from Solis Biodyne (Estonia), and the specific oligonucleotides for human AhR and TGF-β1 were obtained from Invitrogen Life Technologies (UK). Matrigel was purchased from Becton Dickinson Biosciences (San José, CA, USA). All of the other reagents that were used were of analytical grade.

2.2. Cell culture and treatment

The MDA-MB-231 (-ERα) cell line (American Type Culture Collection) was derived from a metastatic site (pleural effusion) of a human breast adenocarcinoma. This cell line represents a less differentiated tumor phenotype, with a great degree of malignancy. The cells were cultured at 37 °C in a 5% CO_2 incubator with RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1% antibiotic–antimycotic mixture (10,000 Units/ml penicillin, 10 mg/ml streptomycin sulfate, and 25 μg/ml amphotericin B), and 1% glutamine. After 24 h of starvation, the cells at 70–80% confluence were exposed to HCB dissolved in ethanol (EtOH). For time-course studies, the cells were treated with HCB (0.05 μM) or vehicle in RPMI supplemented with 5% FBS for 5, 15, and 30 min, as well as 2,

6, and 24 h. For dose-response assays, the cells were exposed for 5 min, 15 min, and 2 h to HCB (0.005, 0.05, 0.5, and 5 μM) or vehicle in RPMI supplemented with 5% FBS. The final EtOH concentration in each treatment was 0.5% and had no influence on the parameters analyzed as shown previously (García et al., 2010). The HCB dose (5 μM) that was used was similar to that found in human serum from a highly contaminated population (To-Figueras et al., 1997), and it is reasonable to assume that the lowest HCB doses are environmentally relevant to the general population. In this respect, the HCB dose of 0.5 μM is similar to that observed in human serum samples from the general population in France (Saoudi et al., 2014). As a positive control for the activation of the AhR or TGF-β1 signaling pathways, the cells were treated with 1 nM TCDD dissolved in DMSO or 5 ng/ml TGF-β1 dissolved in water, respectively. For the inhibitor treatments, the cells were pretreated with 2 μM SB431542, which is an inhibitor of TβRI that prevents Smad phosphorylation; 10 μM PD98059 for the ERK-specific MAPK pathway; 12.5 μM SP600125 for the JNK pathway; 10 μM SB203580 for p38; 0.2 nM PP2 for c-Src, and 1 μM ANF for AhR. All of the inhibitors were dissolved in DMSO. Then, the HCB or vehicle was added to the media in the presence or absence of the inhibitors.

2.3. Western blotting

After HCB exposure, the supernatant was collected and total cellular protein lysates were obtained by scraping the cells in lysis buffer (0.1 M Tris–HCl, 1% Triton X-100, 1 mM EGTA, 0.1 mM NaF, 0.02 mg/ml leupeptin, 1 mM Na_3VO_4, 1 mM phenylmethanesulfonyl fluoride). Whole-cell lysates and an equal volume of supernatant were electrophoresed on a 10–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), prior to transfer to polyvinylidene difluoride membranes (Millipore, Bedford, MA), in a semidry transfer cell at 19 V for 1.5 h. The membranes were blocked overnight at 4 °C with 5% nonfat dry milk and 2.5% BSA in Tris buffer saline solution with Tween 20 (TBST) (10 mMTris–HCl, pH 8.0, 0.5% Tween 20, 150 mM NaCl). The membranes were incubated with anti-phospho-Smad3 (1:250), anti-phospho-JNK (1:250), or anti-phospho-p38 (1:250) antibodies. In addition, the membranes were incubated with anti-TGF-β1 (1:500), anti-AhR (1:500), anti-Smad3 (1:500), anti-JNK (1:500), anti-p38 (1:500), and anti-β-actin (1:2000) antibodies overnight at 4 °C. After the incubation, the membranes were washed five times with TBST and once with TBS (TBST without Tween 20), and then, the membranes were incubated with peroxidase-conjugated anti species-specific antibodies (1:1000) for protein detection. After washing, the blots were reacted using an ECL detection kit and quantified by scanning laser densitometry in a Fotodyne (Foto/Analyst), Gel-Pro Analyzer 3.1.

2.4. Immunofluorescence

The cells were treated with HCB (0.005, 0.05, 0.5, and 5 μM) or vehicle for 5 min, washed, and fixed with ice-cold methanol for 10 min. Then, the cells were incubated with blocking buffer (1% BSA and 1% Triton X-100 in phosphate-buffered saline, PBS) for 30 min at 37 °C and later incubated with an anti-Smad3 (1:20) rabbit monoclonal antibody or an anti-AhR(1:200) mouse monoclonal antibody in 0.2% BSA and 1% Triton X-100 in PBS for 24 h at room temperature in a humidified chamber. Finally, the cells were incubated with the secondary antibodies Alexa 488 anti-rabbit IgG (1:100) or Alexa 488 anti-mouse IgG (1:2000) for 1 h at room temperature in the dark. Hoescht staining was performed to stain the nuclei. Microscopic images were obtained using an Olympus BX50 F-3 (Olympus Optical Co., Ltd, Japan) fluorescence microscope. Random fields were chosen by counting at least 1000 cells/treatment at ×600 magnification.

2.5. Migration assay

The scratch motility assay was performed to evaluate cell migration. The cells were pretreated for 3 h with specific inhibitors of Smad, JNK, p38, or ERK1/2 pathways. The monolayer was scratched with a pipette tip and washed with PBS to remove floating cells; next, the cells were exposed to HCB (0.05 μM) or vehicle in the presence or absence of the inhibitors. The scratched area was photographed at 0 and 20 h, and the distance of wound healing in each well was evaluated (D t0 = distance at 0 h; D t20 = distance at 20 h). Finally, the migration rate was calculated by D t0 − D t20/(D t0 × 100), and the results were normalized to arbitrary units.

2.6. Transwell invasion assay

Invasive activity was quantified using a Transwell chamber (8 μm pore size) coated with 13% Matrigel (40 μl/well) in RPMI medium and then incubated for 2 h at 37 °C and 5% CO_2 for gelification. The cells were pretreated for 3 h with specific inhibitors of Smad, JNK, p38, or ERK1/2 pathways. Then, 1.5×10^4 cells per well were seeded onto the Matrigel-coated wells in serum free medium with HCB (5 μM) or vehicle in the presence or absence of the inhibitors for 72 h. RPMI with 10% SFB was added to the bottom chamber as chemoattractant. The non-invading cells were removed from the upper chamber with a moistened cotton swab. The cells that had migrated through the membrane to the lower surface were fixed with ice-cold methanol, stained with 0.5% toluidine blue, and counted under a light microscope. Each experiment was repeated at least three times, and the results were normalized to arbitrary units, with a value of 1 being assigned to the control assays.

2.7. RNA preparation and reverse transcription–quantitative PCR

MDA-MB-231 cells were pretreated for 1 h with AhR or TβRI specific inhibitors, and then, the cells were treated with HCB (0.05 or 5 μM), TGF-β1 (5 ng/ml), TCDD (1 nM), or vehicle for 2 h. Total RNA was isolated from the cells using TRI-reagent/chloroform extraction according to the manufacturer's protocol. For the first-strand cDNA synthesis, 1 μg of total RNA was reverse-transcribed using the M-MLV RT kit (Promega) with random primers. Quantitative real-time PCR (qPCR) was performed using 1 μL of the resulting cDNA, which was amplified for 40 cycles using the following protocol: 15 s at 94 °C, 20 s at the melting temperature (60 °C), and 30 s at 72 °C using the HOT FIREPol EvaGreen qPCR Mix Plus (Solis Biodyne). qPCR was performed in triplicate using the Rotor Gene Q detection system (Qiagen) and the following primers: human TGF-β1 forward, 5′-TGAACCGGCCTTTCCTGCTTCT-CATG-3′ and reverse, 5′-GCGGAAGTCAATGTACAGCTGCCGC-3′; human AhR forward, 5′-ACATCACCTACGCCAGTCGC-3′ and reverse, 5′-TCTATGCCGCTTGGAAGGAT-3′; and human CYP1A1 forward, 5′-GATTGAGCACTGTCAGGAGAAGC-3′ and reverse, 5′- ATGAGGCTC-CAGGAGATAGCAG-3′ were used. The specificity of each primer set was monitored by analyzing the dissociation curve, and the relative mRNA quantification was performed using the comparative ΔΔCt method with Actin being using as the housekeeping gene.

2.8. Statistical analysis

The data were evaluated by one-way ANOVA followed by Tukey post hoc test to identify significant differences between the

controls and treatments. The differences were considered to be significant when the p values were <0.05. The results represent the mean ± SD of at least three independent experiments.

3. Results

3.1. Effect of HCB on AhR and TGF-β1 mRNA expression

Given the link between AhR and TGF-β1 signaling (Gómez-Durán et al., 2009), we evaluated whether the pesticide could alter this functional interaction. We have previously observed that HCB exerts a biphasic effect on both cell proliferation and apoptosis. Specifically, HCB induces cell proliferation at a low dose (0.05 μM) but has no effect on proliferation at high doses. Conversely, HCB enhances apoptosis at a high dose (5 μM) but has no effect at low doses in MCF-7 cells (García et al., 2010). We selected the HCB doses for the present study by taking into account the above results. First, we have analyzed the AhR mRNA levels in MDA-MB-231 cells after HCB exposure. Our results have shown that the pesticide (0.05 and 5 μM) decreased the AhR mRNA content (30%, $p < 0.05$ and 50%, $p < 0.01$). TGF-β1 (5 ng/ml) treatment also significantly reduced AhR mRNA levels (75%, $p < 0.001$) (Fig. 1A). To assess whether this HCB action is TGF-β1-dependent, the cells were pretreated with the TβRI specific inhibitor SB431542. As shown in Fig. 1A, the inhibitor prevented the HCB-induced decrease in AhR mRNA levels and even rendered an increase with the highest HCB dose (150%, $p < 0.001$) (Fig. 1A).

Conversely, studies on TGF-β1 mRNA levels after HCB-treatment, in the presence or absence of the AhR antagonist ANF, showed that the pesticide (0.05 μM) enhances TGF-β1 mRNA levels (258%, $p < 0.01$) through interaction with the AhR signaling pathway (Fig. 1B). In addition, we confirmed that TCDD (1 nM), a strong AhR ligand, greatly increased TGF-β1 mRNA levels (690%, $p < 0.001$) (Fig. 1B).

3.2. HCB alters AhR protein expression and localization

HCB action on AhR protein expression was assessed by western blot at 2 and 6 h of exposure, with results showing a significant enhancement in AhR protein levels at 2 and 6 h following treatment with 0.05 μM of HCB (78%, $p < 0.01$ and 57%, $p < 0.05$), but no effect at 5 μM of HCB was observed (Fig. 2A and B). However, TGF-β1 (5 ng/ml) treatment did not alter AhR protein expression (Fig. 2A and B).

In addition, AhR intracellular localization was examined by immunofluorescence assay following 30 min and 2 h of HCB treatment. As seen in Fig. 2C and D, AhR localization is altered following 2 h of HCB exposure. The pesticide (5 μM) increases AhR translocation to the nucleus (48%, $p < 0.05$), but not effect was observed with 0.05 μM of HCB. To confirm whether this change in AhR localization involves the activation of the AhR nuclear pathway, we evaluated CYP1A1 mRNA levels, an AhR target gene. As expected, our results showed that 5 μM of HCB enhances CYP1A1 mRNA expression (95%, $p < 0.05$) (Fig. 2E).

3.3. HCB induces TGF-β1 expression and activation

Because 0.05 μM of HCB was found to enhance TGF-β1 mRNA expression in MDA-MB-231 cells, studies were also conducted to evaluate TGF-β1 protein levels. In addition, because TGF-β1 is activated during mammary carcinogenesis (Moses and Barcellos-Hoff, 2011), a potential role was considered for HCB in modulating TGF-β1 activation. The cells were treated with HCB (0.05 μM) or EtOH for 2, 6, and 24 h for western blot analyses. Additionally, because TGF-β1 is synthesized and secreted as an inactive precursor that is cleaved in the extracellular matrix into mature TGF-β1, conditioned medium from MDA-MB-231 cells was used to measure TGF-β1 activation. To this end, the 12.5 kDa active isoform of TGF-β1 was evaluated. In addition, TGF-β1 precursor expression was analyzed in whole-cell lysates by evaluating the 44 kDa inactive isoform of TGF-β1. The results showed that HCB exposure for 2 and 6 h significantly increased TGF-β1 levels in whole cell lysates (155%, $p < 0.05$ and 170%, $p < 0.01$, respectively) compared to EtOH-treated cells. Additionally, the pesticide activated TGF-β1 after 2, 6, and 24 h of treatment (180%, $p < 0.01$, 105%, $p < 0.05$ and 160%, $p < 0.01$, respectively) (Fig. 3A).

For the dose-response studies, MDA-MB-231 cells were exposed to HCB (0.005, 0.05, 0.5, and 5 μM) for 2 h. In the cell lysates, HCB (0.05, 0.5, and 5 μM) was found to increase TGF-β1 expression in a dose-dependent manner (105%, $p < 0.05$, 215%, $p < 0.001$ and 250%, $p < 0.001$, respectively) (Fig. 3B). Furthermore, the TGF-β1 active form was enhanced by HCB at all doses examined (370%, $p < 0.001$, 285%, $p < 0.01$, 360%, $p < 0.001$ and 245%, $p < 0.01$, respectively) (Fig. 3B). Taken together, these results

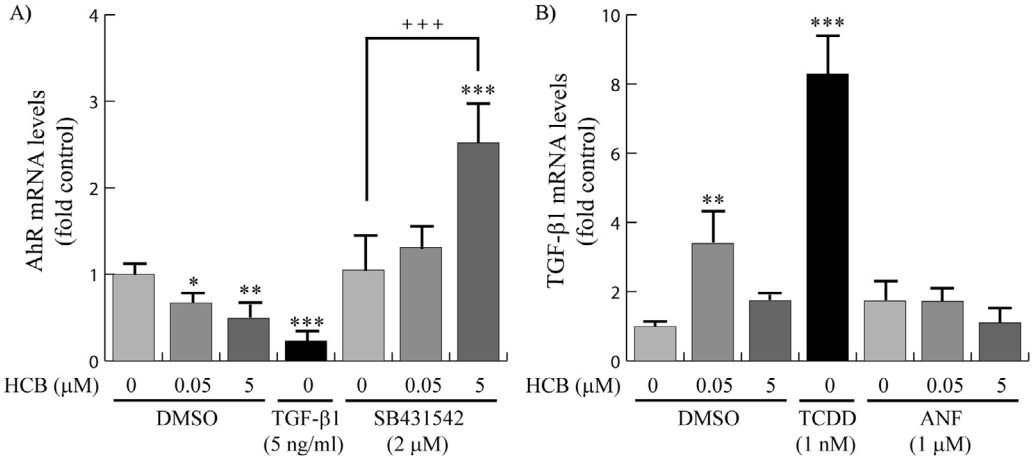

Fig. 1. HCB alters AhR and TGF-β1 mRNA levels in MDA-MB-231 cells. (A) AhR mRNA and (B) TGF-β1 mRNA levels in MDA-MB-231. The cells were pretreated with the TβRI (SB431542, 2 μM) or AhR (ANF, 1 μM) specific inhibitors for 1 h. Then, the cells were exposed to HCB (0.05 and 5 μM) or EtOH for 2 h, in the presence or absence of the inhibitors. For positive control of the activation of the AhR or TGF-β1 signaling pathway, the cells were treated with TCDD (1 nM) or TGF-β1 (5 ng/ml), respectively. Total RNA was purified and analyzed for mRNA expression by RT-qPCR using specific primers. β-Actin expression was used as a control to normalize the data. The values are mean ± SD of three independent experiments. Asterisks indicate significant differences vs. control without inhibitors (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$), and crosses indicate significant differences vs. control with inhibitors (+++$p < 0.001$), ANOVA and Tukey post hoc test.

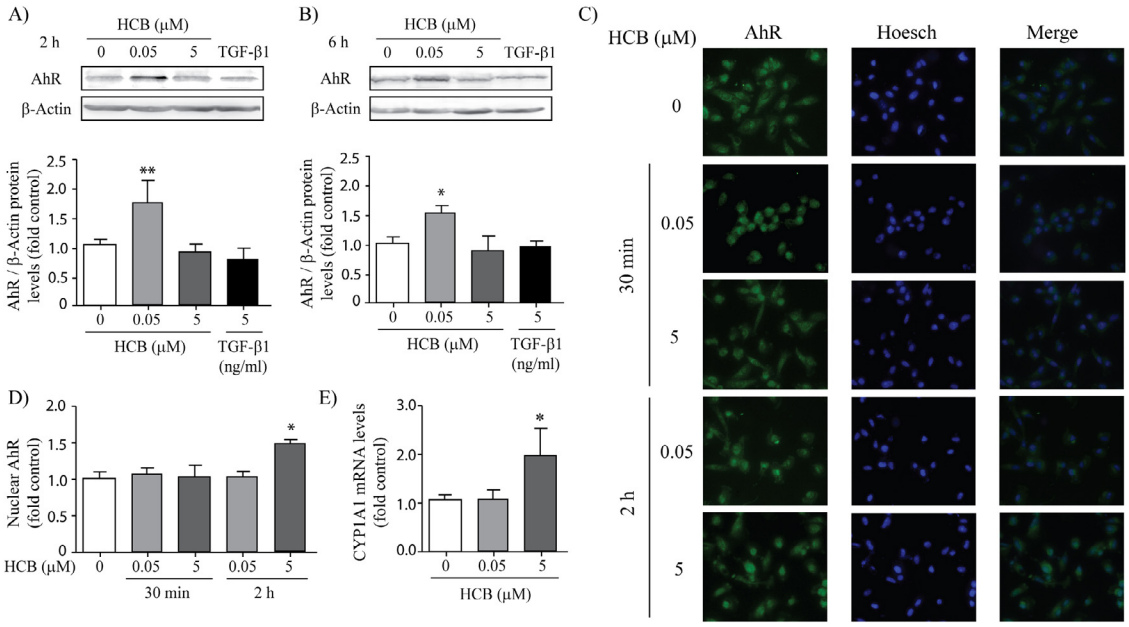

Fig. 2. HCB alters AhR protein expression and localization. (A and B) MDA-MB-231 cells were treated with HCB (0.05 and 5 μM), TGF-β1 (5 ng/ml) or vehicle for 2 and 6 h. Whole-cell lysates were used to analyze the AhR protein levels by western blot. Values were normalized by immunoblotting using anti-β-Actin antibody. A western blot from one representative experiment is shown in the upper panels. Quantification by densitometry scanning of the immunoblots is shown in the lower panels. (C–E) The cells were treated with HCB (0.05 and 5 μM) or vehicle for (C and D) 30 min and 2 h, and (E) 2 h. (C) AhR translocation to the nucleus was evaluated by immunofluorescence. After treatment, the cells were fixed with methanol, stained with AhR specific antibody and incubated with secondary antibody Alexa488. After that, Hoescht stain was performed. Magnification x600. (D) Quantification of the nuclear AhR-positive cells was normalized to arbitrary units. We chose random fields counting at least 1000 cells/treatment. (E) Total RNA was purified and analyzed for CYP1A1 mRNA expression by RT-qPCR using specific primers. β-Actin expression was used as a control to normalize the data. The values represent the mean ± SD of three independent experiments. Asterisks indicate significant differences vs. control (*$p < 0.05$, **$p < 0.01$). ANOVA and Tukey post hoc test.

indicate that HCB increases TGF-β1 protein expression and activation in MDA-MB-231 cells.

3.4. HCB stimulates TGF-β1 signaling through Smad3-dependent and -independent pathways

It is well known that activation of TGF-β receptors initiates both Smad-dependent and -independent signaling events (Parvani et al., 2011). To examine whether the pesticide stimulates the canonical TGF-β1 signaling pathway, time-dependent studies were performed incubating MDA-MB-231 cells with HCB (0.05 μM) for 5 min, 15 min, 30 min, 2 h and 24 h. Total cell lysates were electrophoresed and immunoblotted using Smad3 specific antibodies. As shown in Fig. 4A, HCB increased Smad3 phosphorylation (100%, $p < 0.01$) after 5 min of treatment.

Given that the Smad-independent signaling includes p38, JNK, ERK1/2, and Akt and that studies by our group have shown ERK1/2 and Akt phosphorylation in this cell line (Pontillo et al., 2011), the

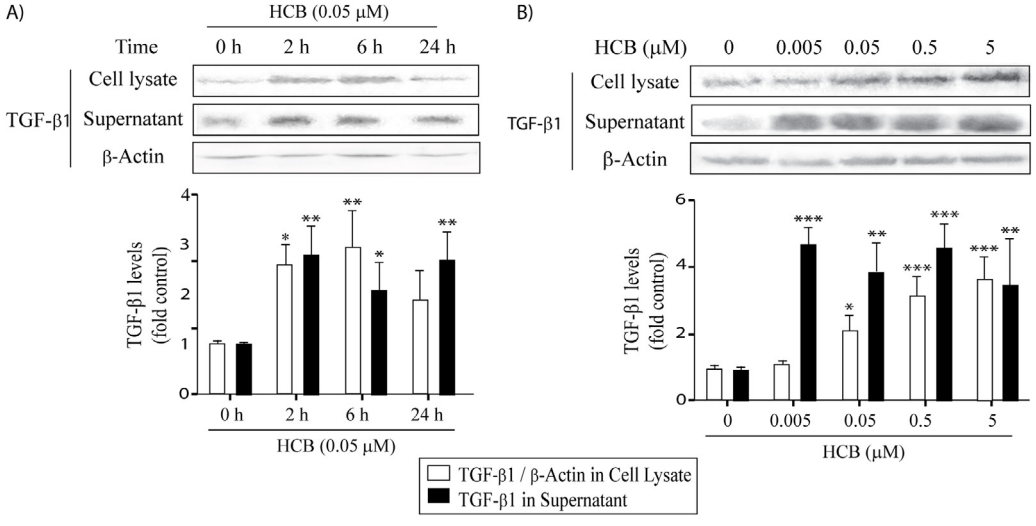

Fig. 3. HCB alters TGF-β1 expression and activation levels. MDA-MB-231 cells were treated with HCB or vehicle (A) for time-course studies (0.05 μM HCB for 2, 6, and 24 h) and (B) for dose-response studies (0.005, 0.05, 0.5, and 5 μM HCB for 2 h). MDA-MB-231 whole-cell lysates were used to analyze intracellular TGF-β1 protein levels (44 kDa inactive isoform), and MDA-MB-231–conditioned media were used to measure activated TGF-β1 protein levels (12.5 kDa active isoform) by western blot. The values were normalized by immunoblotting using anti-β-Actin antibody. A western blot from one representative experiment is shown in the upper panels. Quantification by densitometry scanning of the immunoblots is shown in the lower panels. The data are expressed as means ± SD of three independent experiments. Asterisks indicate significant differences vs. vehicle (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). ANOVA and Tukey post hoc test.

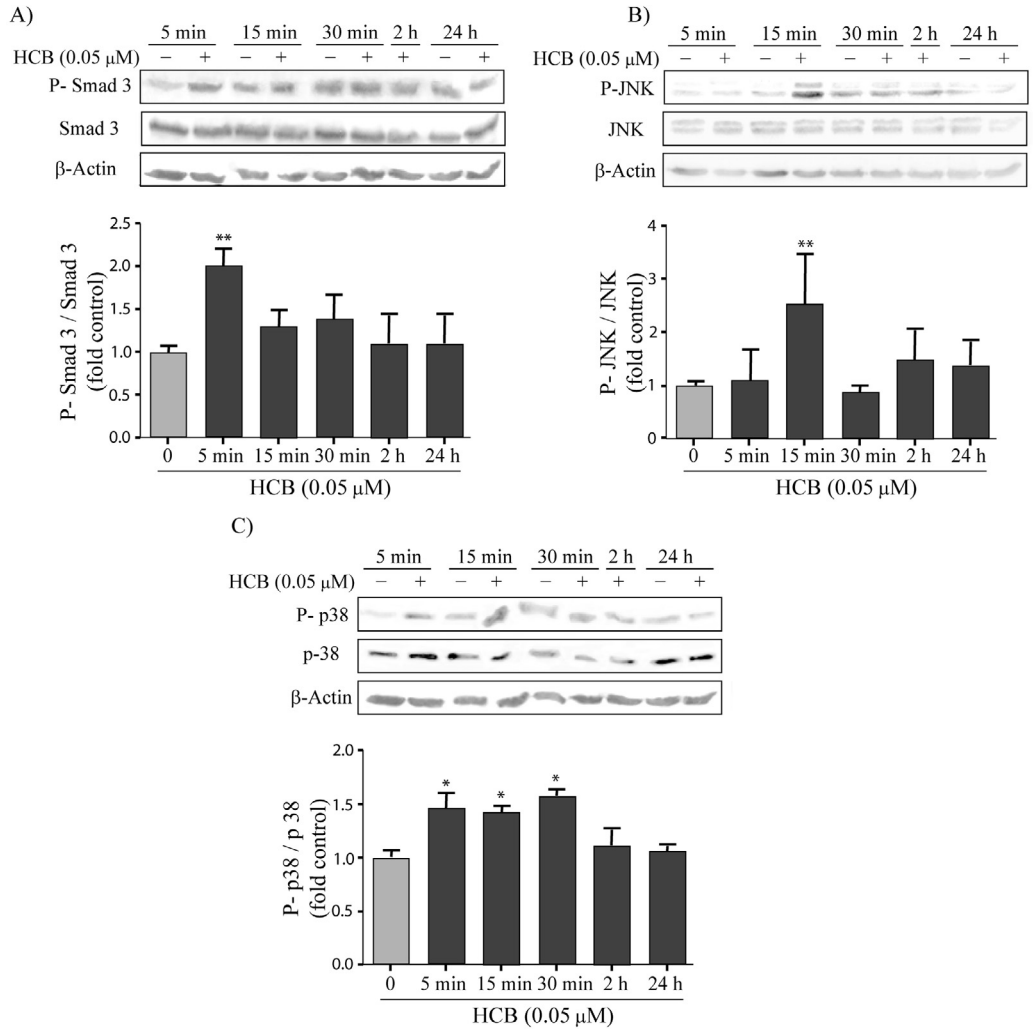

Fig. 4. Time-course of HCB effects on Smad3, JNK, and p38 phosphorylation. (A) Total and phospho-Smad3; (B) total and phospho-JNK; (C) total and phospho-p38 levels. The cells were exposed to HCB (0.05 μM) or vehicle for 5, 15, 30 min, 2, and 24 h. Whole cell lysates were prepared, and protein was resolved by 10% SDS-PAGE and blotted for p-Smad3, p-JNK, and p-38, and then, reblotted for total proteins. β-Actin protein expression was used as loading control. Western blot from one representative experiment is shown in the upper panels. Quantification the corresponding phosphorylated protein/total protein ratio by densitometry scanning of the immunoblots were normalized to arbitrary units, and the results are shown in the lower panels. The data are expressed as means ± SD of three independent experiments. Asterisks indicate significant differences vs. control (*$p < 0.05$ and **$p < 0.01$). ANOVA and Tukey post hoc test.

effects of HCB on JNK and p38 were further investigated. HCB was found to significantly enhance JNK phosphorylation (135%, $p < 0.01$) after 15 min of treatment (Fig. 4B). In addition, phospho-p38 protein levels were increased following 5, 15, and 30 min of HCB exposure and later returned to control values (Fig. 4C: 40, 38, and 50%, respectively; $p < 0.05$).

Based upon these results, we next evaluated the dose-response effect of HCB on Smad3, JNK, and p38 phosphorylation. As shown in Fig. 5A, HCB enhanced Smad3 phosphorylation in a dose-dependent manner after 5 min of exposure (53%, $p < 0.05$, 50%, $p < 0.05$, 80%, $p < 0.01$, and 162%, $p < 0.001$, respectively). HCB (0.05, 0.5, and 5 μM) also increased phospho-JNK (99%, $p < 0.01$, 136%, $p < 0.001$, and 90%, $p < 0.05$, respectively) and phospho-p38 levels (261%, $p < 0.05$, 291%, $p < 0.01$, and 259%, $p < 0.05$, respectively) after 15 min of treatment (Fig. 5B and C). These results demonstrate that the pesticide stimulates both canonical and non-canonical TGF-β1 signaling pathways following short exposure times in MDA-MB-231 cells.

Although HCB induces Smad3 phosphorylation, the mechanism underlying canonical TGF-β1 signaling activation remains unclear. HCB is a weak agonist of AhR that can then trigger AhR-dependent or -independent effects (Hahn et al., 1989). To determine whether HCB activation of the Smad3 pathway could involve AhR, MDA-MB-231 cells were pre-incubated in the presence or absence of an AhR inhibitor (1 μM ANF) and then exposed to HCB (0.05 and 5 μM) for 5 min. As shown in Fig. 5D, ANF inhibited the HCB-induced increase in Smad3 phosphorylation at both HCB concentrations.

We have previously observed that HCB enhances c-Src phosphorylation following 5 min of exposure in MDA-MB-231 (Pontillo et al., 2011), and Galliher and Schiemann (2006) have demonstrated that c-Src can phosphorylate TβRII to activate TGF-β1 signaling. Therefore, we evaluated the possible involvement of c-Src on HCB-mediated activation of Smad3. MDA-MB-231 cells were pretreated with a c-Src specific inhibitor (0.2 nM PP2) and exposed to HCB (0.05 and 5 μM) or vehicle for 5 min in the presence or absence of the inhibitor. The results showed that PP2 could prevent HCB-stimulated Smad3 phosphorylation (Fig. 5D).

Because Smad3 phosphorylation allows for the formation of the Smad3/Smad4 complex, its nuclear retention, and the activation of TGF-β1 signaling (Dai et al., 2009), we investigated if the pesticide could increase Smad3 translocation to the nucleus. MDA-MB-231 cells were exposed to HCB (0.005, 0.05, 0.5, and 5 μM) for 5 min, and Smad3 localization was evaluated by immunofluorescence. As

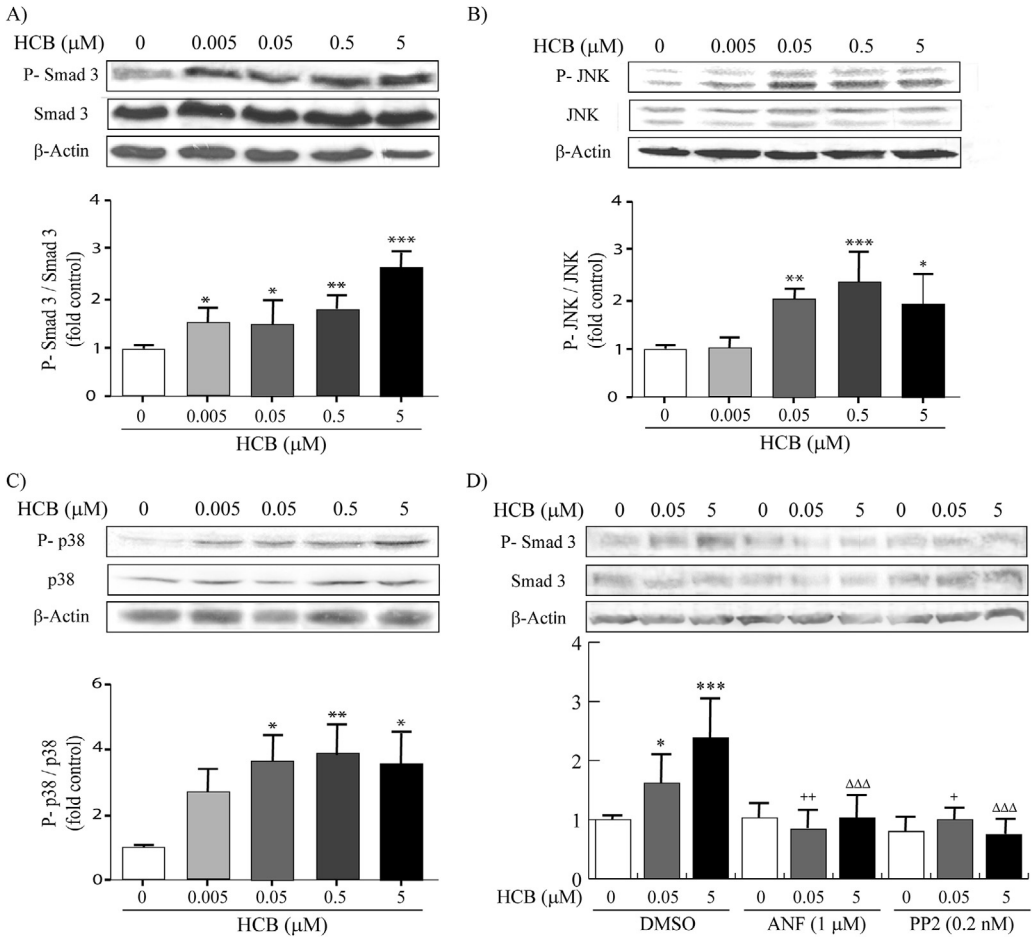

Fig. 5. HCB-induced Smad3, JNK, and p38 activation. Role of AhR and c-Src in HCB-mediated Smad3 phosphorylation. (A, D) Total and phospho-Smad3; (B) total and phospho-JNK; (C) total and phospho-p38 levels. The cells were exposed to HCB (0.005, 0.05, 0.5, and 5 μM) or vehicle for (A) 5 or (B, C) 15 min. (D) The cells were pre-incubated with ANF (1 μM), PP2 (0.2 nM), or vehicle (DMSO) for 2 h, and then, the cells were exposed to HCB (0.05 and 5 μM) or vehicle for 5 min in the presence or absence of the inhibitors. Whole cell lysates were prepared, and protein was resolved by 12% SDS-PAGE and blotted for p-Smad3, p-p38, and p-JNK, and reblotted for total Smad3, p38, and JNK protein levels. β-Actin protein expression was used as loading control. Western blots from one representative experiment are shown in the upper panels. Quantification of phosphorylated protein/total ratio by densitometry scanning of the immunoblots were normalized to arbitrary units, and the results are shown in the lower panels. The data are expressed as means ± SD of three independent experiments. Asterisks indicate significant differences vs. control ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Crosses and triangles indicate significant differences versus HCB (0.05 and 5 μM, respectively) ($^{*}p < 0.05$, $^{++}p < 0.01$, and $^{\Delta\Delta\Delta}p < 0.001$). ANOVA and Tukey post hoc test.

expected, HCB treatment increased the number of cells with nuclear Smad3 (94%, $p < 0.05$, 157%, $p < 0.01$, 190%, $p < 0.01$, and 140%, $p < 0.01$, respectively) in a dose-dependent manner, confirming the activation of TGF-β1 signaling pathway (Fig. 6A and B).

3.5. HCB induces cell migration involving the Smad, JNK, p38, and ERK signaling pathways

In the current study, we found that HCB can enhance Smad3, JNK, and p38 phosphorylation in MDA-MB-231 cells. In addition, we have previously demonstrated that the pesticide produces an early increase in ERK1/2 phosphorylation without changes in Akt (Pontillo et al., 2011). Given that several studies have reported these pathways contribute to cell migration into neighboring tissues (Kim et al., 2003; Mitra et al., 2011; Muraoka-Cook et al., 2005), we assessed their possible involvement in the HCB-induced cell migration observed in our laboratory (Pontillo et al., 2011). And, although previously we demonstrated that 0.05, 0.5, and 5 μM HCB enhances MDA-MB-231 cell migration, this specific study was only conducted at the lowest dose (0.05 μM) because it is the most environmentally relevant. The cells were pretreated with specific inhibitors and then exposed to HCB in the presence or absence of the inhibitors. Then, the scratch motility assay was performed. The results show that pretreatment with the inhibitor in the absence of HCB had no effect on cell migration and, as we have previously reported, HCB treatment in the absence of inhibitors produces a significant increase in wound closure (40%, $p < 0.001$) (Fig. 7). However, this increase was not observed in cells that were both pretreated with the inhibitors and exposed to HCB, which indicates that Smad, JNK, p38, and ERK1/2 are involved in HCB-induced cell migration (Fig. 7).

3.6. The role of Smad, JNK, p38, and ERK1/2 signaling pathways in HCB-induced cell invasion

Recently, we demonstrated that 5 μM of HCB increases MDA-MB-231 cell invasion (Pontillo et al., 2013). Because it is well-established that Smad, JNK, p38, and ERK1/2 enhance breast cancer cells invasion (Huang et al., 2000; Nasrazadani and Van Den Berg, 2011; Sundqvist et al., 2012), we investigated whether these proteins were also involved in HCB-induced cell invasion. MDA-MB-231 cells were treated with specific inhibitors and later exposed to HCB (5 μM), as described in the Materials and Methods section. As shown in Fig. 8A–B and G, the results confirmed the previous findings on HCB-induced cell invasion (200%, $p < 0.01$) and showed this effect to be Smad, JNK, and p38-dependent

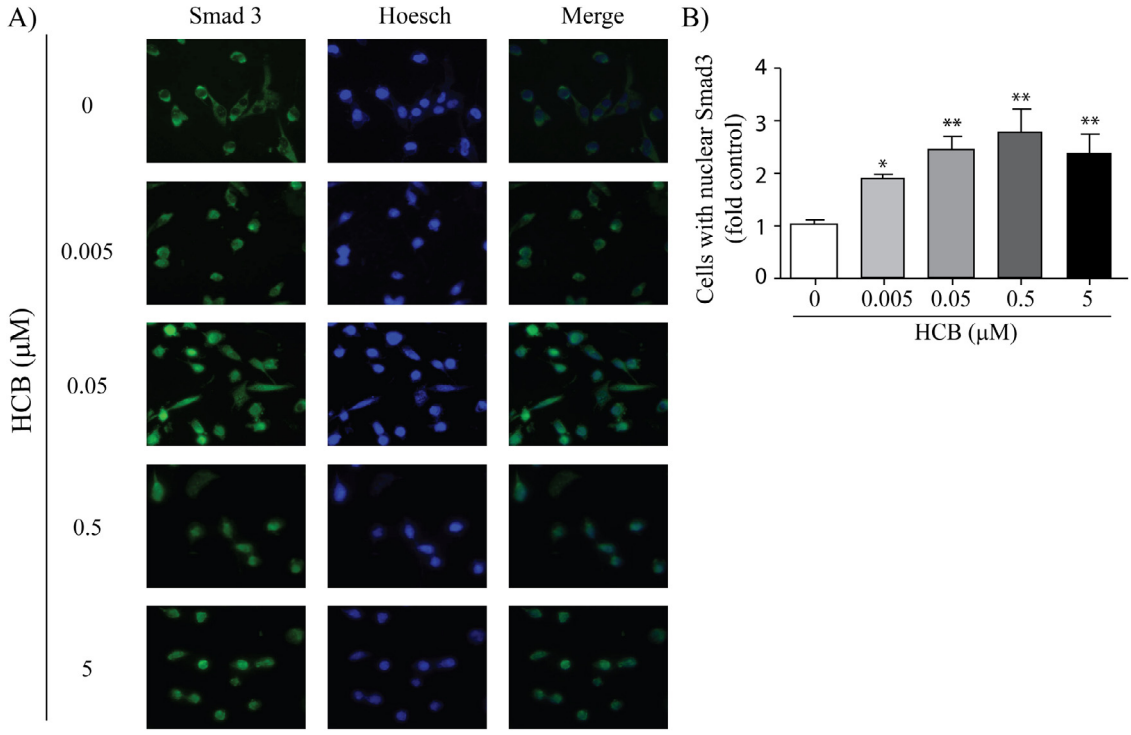

Fig. 6. HCB alters the Smad3 localization. (A) Smad3 translocation to the nucleus was evaluated by immunofluorescence. The cells were treated with HCB (0.005, 0.05, 0.5, and 5 μM) for 5 min, and then, the cells were fixed with methanol, stained with Smad3 specific antibody, and incubated with secondary antibody Alexa488. After that, Hoescht stain was performed. Magnification ×600. (B) Quantification of nuclear Smad3-positive cells was normalized to arbitrary units. We chose random fields counting at least 1000 cells/treatment. Values represent the mean ± SD of three independent experiments. Asterisks indicate significant differences vs. control (*$p < 0.05$, **$p < 0.01$). ANOVA and Tukey post hoc test.

(Fig. 8C–E and G). However, no change in cell invasion was observed, compared to the HCB treatment, when the cells were pretreated with the ERK1/2 pathway inhibitor, suggesting that ERK1/2 is not involved in HCB-induced cell invasion (Fig. 8F and G).

4. Discussion

Considering the potential adverse effects of pesticides on various human diseases, including breast cancer, the mechanism of action of these compounds remains an active area of scientific research. Therefore, we were interested in studying the potential effect of HCB, an environmental pollutant that accumulates in humans, as a breast cancer risk factor. In previous studies, we have reported that HCB enhances MDA-MB-231 cell migration and invasion involving the AhR-c-Src signaling pathway (Pontillo et al., 2011, 2013). In agreement, D'Amato et al. (2015) demonstrated that AhR activation by an endogenous ligand is critical to support anchorage independent survival and invasive capacity in response to detachment. Furthermore, *in vitro* and *in vivo* analyzes have demonstrated that AhR is necessary to maintain the levels of TGF-β1 activity (Gómez-Durán et al., 2009). In studies from our laboratory, we found that HCB enhances TGF-β1 expression in the rat thyroid gland (Chiappini et al., 2009) and activates TGF-β1/Smad signaling in rat thyroids cells (Chiappini et al., 2014). According to the experimental data of the present investigation, we propose a mechanism that integrates AhR/TGF-β1 crosstalk and the pro-migratory phenotype exerted by HCB in the MDA-MB-231 human breast cancer cell line (Fig. 9).

In the current study, we found that HCB activates downstream pathways of TGF-β1 following short times of exposure (Fig. 9A). We demonstrated that early Smad3 phosphorylation is mediated by c-Src activation via the AhR signaling pathway. These data support the previous observation that c-Src can phosphorylate TβRII and thus activate TGF-β1 signaling pathways (Galliher and Schiemann, 2006). However, we cannot exclude that small active TGF-β1 levels present in the extracellular medium at early times of exposure could contribute to the activation of these signaling pathways. In addition, the phosphorylation of JNK, p38, and ERK1/2 after HCB exposure could be mediated by the activation of non-canonical TGF-β1 signaling, as described Parvani et al. (2011).

Several investigations have reported a connection between the AhR and the TGF-β1 signaling pathways (Gómez-Durán et al., 2009; Haarmann-Stemmann et al., 2009). Given this functional crosstalk, it is reasonable to assume that a reciprocal regulation exists between the AhR and TGF-β1. At longer times of exposure, our results showed that HCB enhances TGF-β1 expression but decreases AhR mRNA content and exacerbates the pro-migratory phenotype (Fig. 9B). Accordingly, Rico-Leo et al. (2013) have observed that TGF-β1 promotes EMT in both AhR-expressing and AhR-depleted mammary epithelial cells, although the effects on the latter were more pronounced. The molecular mechanisms that explain the motile and invasive phenotype in response to TGF-β1 are diverse and include both Smad-dependent gene regulation and MAPK signaling activation in carcinoma (Shen et al., 2001). Herein, we have demonstrated that HCB modulates cell migration and invasion through Smad-dependent and -independent signaling pathways. We found that Smad, JNK, and p38 activation are essential for HCB-induced cell migration and invasion, whereas ERK1/2 is only implicated in HCB-induced cell migration. Although it is known that ERK1/2 is involved in cell invasion in breast cancer, our results show that the increase in HCB-induced MDA-MB-231 invasion is not mediated by ERK1/2.

In the present study, we have demonstrated that HCB increases TGF-β1 expression (mRNA and protein levels) and active isoform levels after 2 h of exposure. In addition, we observed that the HCB-induced increase in TGF-β1 mRNA levels is AhR-dependent, which

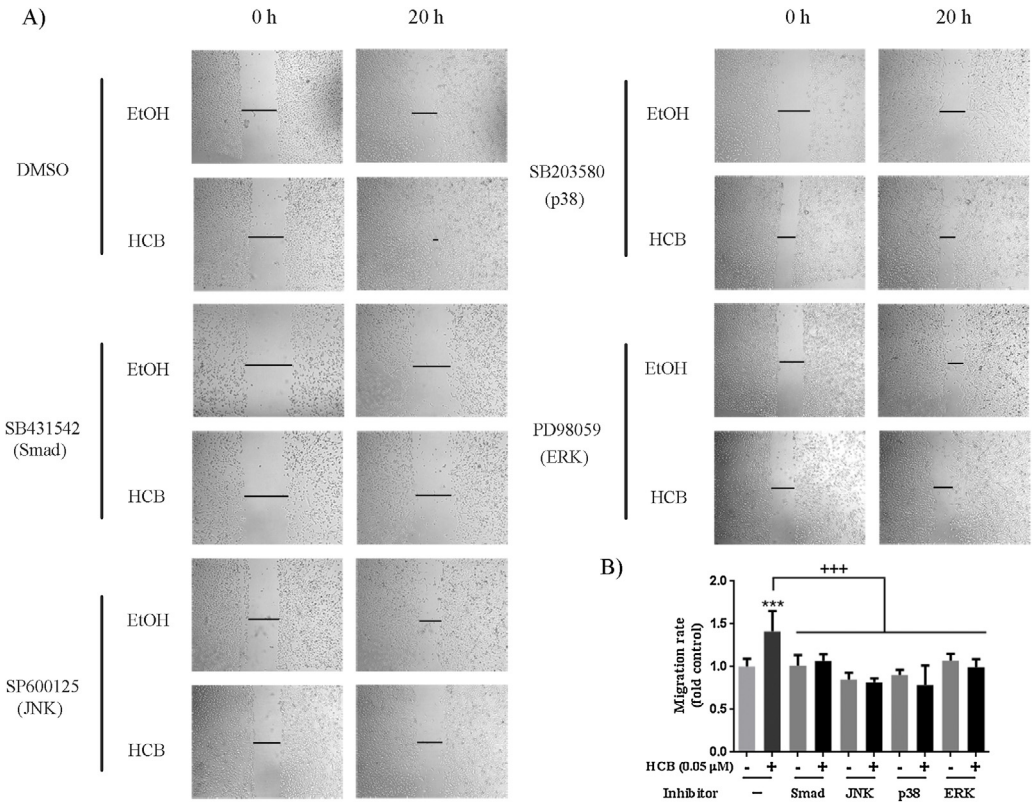

Fig. 7. Role of Smad, JNK, p38, and ERK1/2 signaling pathways in HCB-induced cell migration. (A) Wound healing assay. The serum-starved MDA-MB-231 cells were pretreated with specific inhibitors (2 μM SB431542 for Smad, 12.5 μM SP600125 for JNK, 10 μM SB203580 for p38, and 10 μM PD98059 for ERK1/2 pathways). The monolayer was scratched with a pipette tip, and the cells were treated with HCB 0.05 μM in the presence or absence of the inhibitors. Relative wound closure was observed at 20 h under microscope and photographed. (B) The migration rate was calculated on the photography of scratched area, measuring the distance of wound width at 0 and 20 h. Asterisks indicate significant differences vs. control without inhibitors (***$p < 0.001$). Crosses indicate significant differences vs. HCB without inhibitors (+++$p < 0.001$). ANOVA and Tukey post hoc test.

may involve a nuclear or membrane AhR pathway. Our findings are in agreement with those of other authors, who reported that both intracellular and extracellular depositions of TGF-β1 are enhanced in mammary tumors (Dalal et al., 1993). Because there is a correlation between TGF-β1 and disease stage (Tan et al., 2009), our results suggest that HCB exposure could be activate signaling pathways involved in the promotion of breast tumors. In this study, we observed that TGF-β1 mRNA levels were up-regulated after stimulation with 0.05 μM of HCB, whereas its protein levels were increased at all doses evaluated. These data suggest that different mechanisms of regulation are involved in TGF-β1 expression, depending on the dose of exposure. Post-transcriptional regulation of TGF-β1 may be implicated in the whole range of HCB doses. Conversely, the up-regulation of the active isoform of TGF-β1 in the extracellular medium from MDA-MB-231 cells could be the result of matrix metalloproteinase activity releasing this active isoform, as suggested by Curran and Keely (2013). In this respect, we have previously reported that HCB increases the activity of metalloproteinase 9 in MDA-MB-231 cells (Pontillo et al., 2013). Despite the increase in the active isoform of TGF-β1, Smad3 and MAPK phosphorylation returns to control values at longer times of exposure (2–24 h). This phenomenon could be explained by different mechanisms involved in the decrease in TGF-β1 signaling, which are a) TGF-β receptors are rapidly internalized and then downregulated in an ubiquitin-dependent manner (Di Guglielmo et al., 2003) or b) the Smad3/Smad4 complex upregulates Smad7, a potent inhibitor of TGF-β1 signaling (Nagarajan et al., 1999).

In this study, we found that the activation of Smad-dependent signaling is necessary for the HCB-induced reduction in AhR levels. Supporting our results, Wolff et al. (2001) demonstrated that TGF-β1/Smad regulates AhR transcription through an interaction with a TGF-β1-responsive element located in the AhR-promoter region. These authors found that Smad recruits factors that result in transcriptional repression of AhR. Although HCB reduces AhR mRNA levels, we observed that the pesticide (0.05 μM) increases AhR protein levels, suggesting that some mechanism of protein degradation could be reduced. Given that TGF-β1 did not alter AhR protein levels, this pesticide action could be independent of TGF-β1.

It is important to highlight that AhR and TGF-β1 expression are differently modulated, depending on HCB doses. The highest HCB dose (5 μM) used in these studies is similar to that found in human serum from a highly contaminated population (To-Figueras et al., 1997), whereas 0.05 μM of HCB is ten times less than the observed concentration in human serum from the general population (Saoudi et al., 2014). At a low dose (0.05 μM), we found a clear regulation of AhR/TGF-β1 crosstalk because the pesticide reduces AhR mRNA expression through the TGF-β1 pathway, while it enhances TGF-β1 mRNA expression involving AhR signaling. However, at a high dose (5 μM), we show that more than one mechanism could be involved. First, the HCB-induced decrease in AhR mRNA levels was not only blocked in the presence of the TGF-β1 inhibitor but also increased above their control values. These observations require further studies to clarify the mechanism. Additionally, given that HCB is a weak ligand of AhR, we expected the highest dose of the pesticide to increase TGF-β1 mRNA content in a similar way as TCDD. Nevertheless, only a non-significant enhancement was observed in TGF-β1 mRNA levels after HCB-exposure. We hypothesize that AhR protein content could be

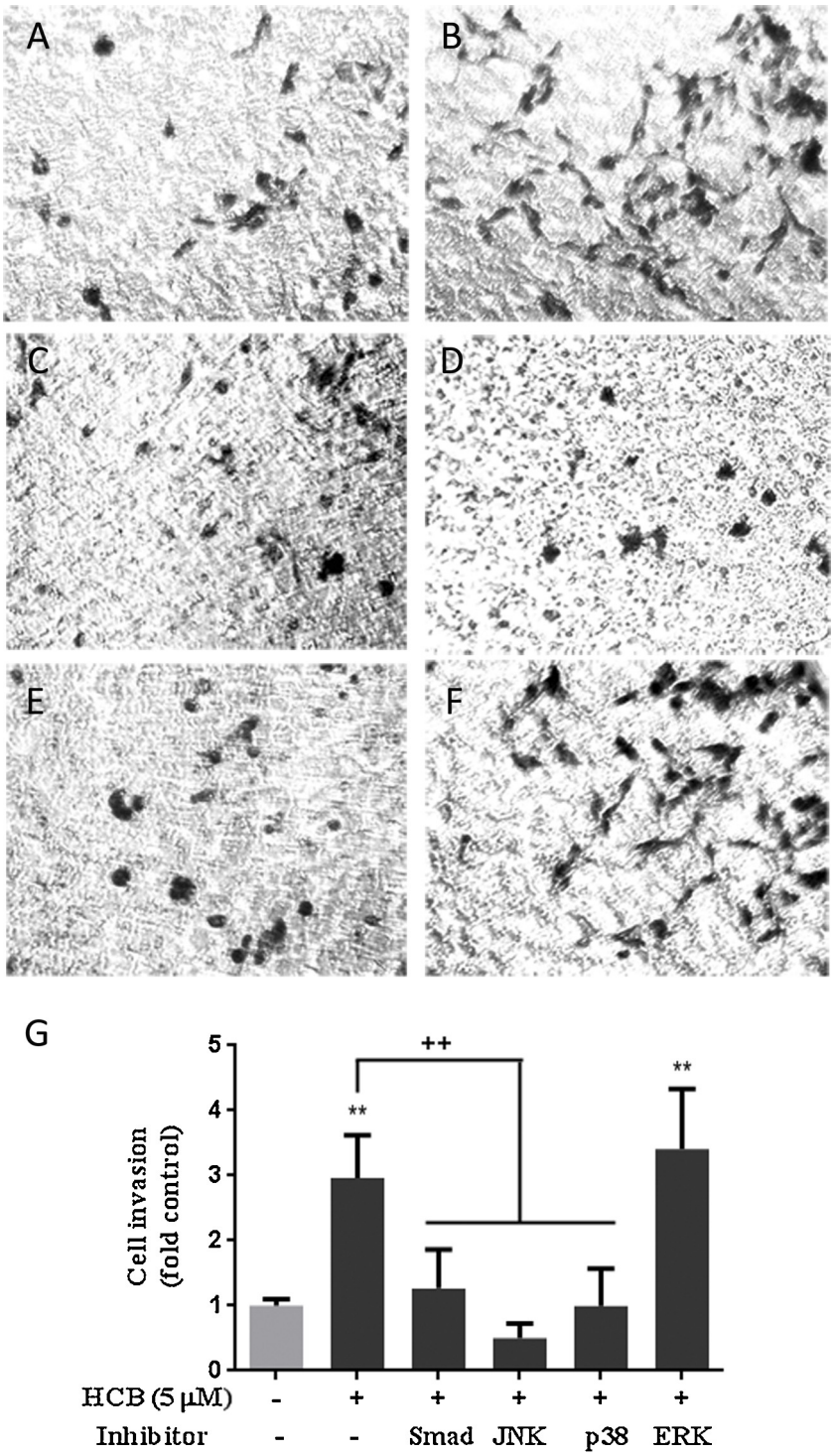

Fig. 8. HCB action on MDA-MB-231 cell invasion: role of Smad, JNK, p38, and ERK1/2. MDA-MB-231 cells were exposed to (A) EtOH or (B-F) HCB (5 μM) in the presence of (A-B) DMSO or (C-F) specific inhibitors: (C) 2 μM SB431542 for Smad, (D) 12.5 μM SP600125 for JNK, (E) 10 μM SB203580 for p38, and (F) 10 μM PD98059 for ERK1/2 pathways. Then, the cells were placed on the top of transwell chambers covered with matrigel. RPMI with 10% FBS was placed in the lower chamber as chemoattractant. After incubation for 72 h, the cells on the lower surface of the filters were fixed, stained, and counted under an optic microscope. (G) The number of cells that have invaded through the filter was normalized to arbitrary units. Values represent the mean ± SD of three independent experiments. Asterisks indicate significant differences versus control without inhibitors (**$p < 0.01$). Crosses indicate significant differences versus HCB without inhibitors (++$p < 0.01$). ANOVA and Tukey post hoc test.

related to its intracellular localization. Specifically, 0.05 μM of HCB increases AhR protein levels retained in the cytosol, whereas 5 μM of HCB does not alter this parameter but increases its nuclear translocation. Because AhR nuclear signaling is activated at 5 μM of HCB, our results suggest that proteosomal degradation is triggered at the highest HCB concentration. In this respect it has been demonstrated that AhR is rapidly degraded via the proteasome pathway following exposure to ligands (Feng et al., 2013).

In conclusion, we demonstrated that HCB modulates the interaction between AhR and TGF-β1 signaling and, as a result, exacerbates a motile and invasive phenotype in MDA-MB-231

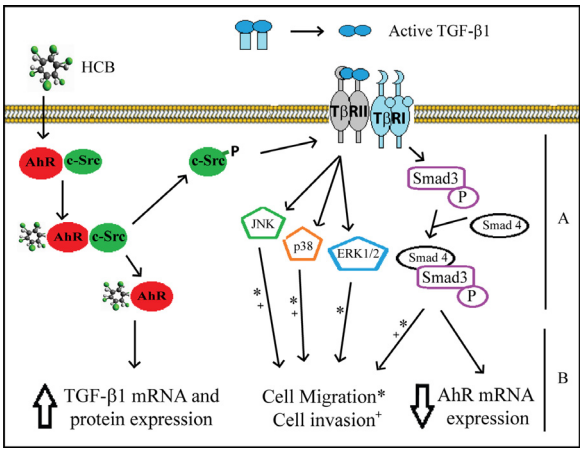

Fig. 9. Model depicting molecular mechanisms of HCB action in MDA-MB-231 breast cancer cells. (A) At short times (5–30 min), HCB binds to AhR-c-Src complex and triggers c-Src activation, enhancing the phosphorylation of TGF-β1 downstream pathways Smad3, JNK, p38, and ERK1/2. (B) At longer times, these signaling pathways induce cell migration and invasion. In addition, HCB-AhR complex and/or others transcription factors could translocate to the nucleus, and may lead TGF-β1 gene expression, which is secreted and accumulated into de culture medium. On the other hand, the pesticide activates TGF-β1/Smad signaling and thus decreases the AhR mRNA content. Asterisks indicate pathways involved in cell migration. Crosses represent signaling involved in cell invasion.

cells. Our results suggest that alteration in this crosstalk could have implications in breast cancer progression.

Conflict of interest

None.

Acknowledgements

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