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Activation of c-Src/HER1/STAT5b and HER1/ERK1/2 Signaling Pathways and Cell Migration by Hexachlorobenzene in MDA-MB-231 Human Breast Cancer Cell Line

Carolina A. Pontillo,* María A. García,* Delfina Peña,* Claudia Cocca,† Florencia Chiappini,* Laura Alvarez,* Diana Kleiman de Pisarev,* and Andrea S. Randi*,[1]

*Laboratorio de Efectos Biológicos de Contaminantes Ambientales, Departamento de Bioquímica Humana, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina; and †Laboratorio de Radioisótopos, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina

[1]To whom correspondence should be addressed at Laboratorio de Efectos Biológicos de Contaminantes Ambientales, Departamento de Bioquímica Humana, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, 5to piso, (1121) Buenos Aires, Argentina. Fax: +0054-11-4508-3672. E-mail: andybiol@yahoo.com.ar.

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Hexachlorobenzene (HCB) is a widespread environmental pollutant. It is a dioxin-like compound and a weak ligand of the aryl hydrocarbon receptor (AhR) protein. HCB is a tumor cocarcinogen in rat mammary gland and an inducer of cell proliferation and c-Src kinase activity in MCF-7 breast cancer cells. This study was carried out to investigate HCB action on c-Src and the human epidermal growth factor receptor (HER1) activities and their downstream signaling pathways, Akt, extracellular-signal-regulated kinase (ERK1/2), and signal transducers and activators of transcription (STAT) 5b, as well as on cell migration in a human breast cancer cell line, MDA-MB-231. We also investigated whether the AhR is involved in HCB-induced effects. We have demonstrated that HCB (0.05μM) produces an early increase of Y416-c-Src, Y845-HER1, Y699-STAT5b, and ERK1/2 phosphorylation. Moreover, our results have shown that the pesticide (15 min) activates these pathways in a dose-dependent manner (0.005, 0.05, 0.5, and 5μM). In contrast, HCB does not alter T308-Akt activation. Pretreatment with a specific inhibitor for c-Src (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo[3,4-d]pyrimidine [PP2]) prevents Y845-HER1 and Y699-STAT5b phosphorylation. AG1478, a specific HER1 inhibitor, abrogates HCB-induced STAT5b and ERK1/2 activation, whereas 4,7-orthophenanthroline and α-naphthoflavone, two AhR antagonists, prevent HCB-induced STAT5b and ERK1/2 phosphorylation. HCB enhances cell migration evaluated by scratch motility and transwell assays. Pretreatment with PP2, AG1478, and 4,7-orthophenanthroline suppresses HCB-induced cell migration. These results demonstrate that HCB stimulates c-Src/HER1/STAT5b and HER1/ERK1/2 signaling pathways in MDA-MB-231. c-Src, HER1, and AhR are involved in HCB-induced increase in cell migration. The present study makes a significant contribution to the molecular mechanism of action of HCB in mammary carcinogenesis.

Key Words: **hexachlorobenzene; MDA-MB-231; cell migration; AhR; c-Src; HER1.**

Exposure to ubiquitous persistent organic pollutants (POPs) such as polychlorinated biphenyls, dichlorodiphenyldichloroethylene (DDE), and hexachlorobenzene (HCB) has attracted attention in breast cancer etiology. Although the mechanistic actions of these chemicals in carcinogenesis remain unclear, studies showed that some POPs have the potential to promote cancer development in various experimental models such as rodents and human cell lines (Verner *et al*., 2008).

HCB is a chlorinated hydrocarbon with a high "lipophilicity" and a strong tendency to accumulate in food chains and lipid-rich tissues. It was used as a fungicide until the 1970's when such use was prohibited. However, considerable amounts are still generated, as waste by-products of industrial processes are emitted into the environment. Exposure of laboratory animals to HCB can elicit a number of effects, including hepatic porphyria (Mylchreest and Charbonneau, 1997), hypothyroxinemia (Kleiman de Pisarev *et al*., 1990), reproductive dysfunctions (Alvarez *et al*., 2000), and immunomodulation (Ezendam *et al*., 2003). HCB is also known to be a promoter of liver foci growth (Ou *et al*., 2001) and rat mammary tumors (Randi *et al*., 2006). The International Agency for Research on Cancer and the U.S. Environmental Protection Agency have classified HCB as a probable human carcinogen (group 2B) (ATSDR, 2002).

HCB is a weak agonist of the aryl hydrocarbon receptor (AhR) (Hahn *et al*., 1989), which is a ligand-dependent transcription factor present in cytosol. It has been proposed that upon ligand binding, the AhR dissociates from the original cytosolic multiprotein complex and two cellular signaling events are initiated. The first one is the translocation of the AhR to the nucleus where it dimerizes with the AhR nuclear translocator and binds to xenobiotic-responsive elements (XREs) in the enhancer region of target genes, like cytochrome P4501A1 (CYP1A1), to stimulate their transcription (Matsumura, 1994). The second one is the stimulation of c-Src release from its cytosolic multiprotein complex

upon which c-Src translocates to the cell membrane where it can interact with a variety of growth factor receptors, including the human epidermal growth factor receptor (HER1) family (Biscardi *et al*., 1999; Park *et al*., 2007). Studies from our laboratory in WB-F344 rat hepatic cells demonstrated that HCB stimulates HER1 transactivation in a c-Src–dependent manner (Randi *et al*., 2008).

Coexpression of c-Src and HER1 in breast cancer cell lines results in epidermal growth factor (EGF)-induced physical association of the HER1 with c-Src and in the phosphorylation on tyrosine 845 (Y845) within the catalytic domain of HER1. This modification is not required for catalytic activity but highly increases proliferation, transformation, and tumor formation *in vivo* (Biscardi *et al*., 1999). It has also been suggested that phosphorylation of Y845-HER1 by c-Src may be critical for signal transducers and activators of transcription (STAT) 5b phosphorylation in its activating Y699 (Kloth *et al*., 2003). STAT proteins regulate processes involved in cancer development and progression, like proliferation, survival, and angiogenesis (Bernaciak *et al*., 2009). Together, these findings indicate that c-Src cooperates with the HER1 in the processes of both mitogenesis and transformation in breast cancer. Moreover, activated HER1 binds Grb2 and Sos, resulting in the activation of the Ras/Raf pathway and phosphorylation of mitogen-activated protein kinases (Parsons and Parsons, 2004). Furthermore, HER1 can stimulate phosphatidylinositol 3-kinase, which in turn activates Akt. These two pathways are involved in cell growth, apoptosis, invasion, and migration (Wang *et al*., 2007).

In previous studies of our laboratory, we found that HCB has a cocarcinogenic effect in N-Nitroso N-methyl urea–induced mammary tumors in rats, enhancing their development and malignancy (Randi *et al*., 2006). We have recently demonstrated that HCB induces cell proliferation and insulin-like growth factor I (IGF-I) signaling pathway in an estrogen receptor (ERα)–dependent manner in MCF-7 breast cancer cell line (García *et al*., 2010). On the other hand, we observed that HCB has no effect in cell proliferation but induces IGF-I signaling pathway in the ERα (−) MDA-MB-231 (García et al., unpublished results). Other studies have reported that in ERα (−) breast cancer cells, activation of IGF signaling pathway promotes growth-unrelated processes, such as migration and invasion (Bartucci *et al*., 2001).

In view of the important role of c-Src and HER1 in breast tumor progression, in this work, we examined the ability of HCB to alter c-Src and HER1 activities and its effect on their downstream signaling pathways STAT5b, extracellular-signal-regulated kinase ERK1/2, and Akt in the ERα (−) MDA-MB-231 cells, which overexpress both c-Src and HER1. Furthermore, we also investigated the HCB action on cell migration and whether the AhR is involved in HCB-induced effects.

MATERIALS AND METHODS

Chemicals

HCB (> 99% purity, commercial grade) was obtained from Aldrich-Chemie GmbH & Co. (Steinheim, Germany). Anti-HER1, anti-c-Src, anti-phospho-Y416-c-Src, anti-phospho-T308-Akt, anti-Akt, and anti-phospho-ERK1/2 anti-STAT5 b antibodies were purchased from Cell Signaling Technology, Inc. (MA). Anti-ERK1/2, anti-STAT5 b and anti-phospho-Y699-STAT5b were obtained from Upstate (Lake Placid, NY) and anti-phospho-Y845-HER1 was obtained from Abcam Ltd (Cambridge, UK). Anti-phosphotyrosine antibody was purchased from BD Biosciences/Clontech (CA). Anti-β-actin, EGF, 4,7-orthophenanthroline, and α-naphthoflavone were obtained from Sigma Chemical Co. (St Louis, MO). The enhanced chemiluminescence kit (ECL) was from GE Healthcare Life Sciences (Buckinghamshire, UK). 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo[3,4-d]pyrimidine (PP2) inhibitor was obtained from Calbiochem (La Jolla, CA) and Tyrphostin AG1478 inhibitor was purchased from A.G. Scientific, Inc. (San Diego, CA). RPMI-1640 culture medium was purchased from HyClone Laboratories, Inc. (Logan, UT). Antibiotic-antimicotic, trypsine, and glutamine were obtained from PAA Laboratories GmbH (Pasching, Austria). All reagents used were of analytical grade.

Cell Culture

MDA-MB-231 cell line was obtained from American Type Culture Collection. MDA-MB-231 cells derive from a metastatic site (pleural effusion) of a human breast adenocarcinoma. Cells were cultured at 37°C in a 5% CO_2 incubator with RPMI-1640 complete growth medium that consisted of RPMI supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimicotic mixture (10,000 Units/ml penicillin, 10 mg/ml streptomycin sulfate, and 25 μg/ml amphotericin B), and 1% glutamine. Cells at 70–80% confluence were treated with HCB dissolved in absolute ethanol. Final ethanol concentration in each treatment was 0.5% and had no influence on the analyzed parameters as shown previously (García *et al*., 2010). All experiments were repeated at least three times, and the results shown are representative of similar findings.

Cell Treatment for Time Course and Dose-Response Studies

MDA-MB-231 cells were seeded in 100-mm dishes (1.5×10^6 cells) in RPMI complete growth medium followed by overnight incubation to allow cells to attach. The next day, the medium was changed to RPMI without FBS, and 24 h later, cells were exposed to HCB according to the assay. For time-course studies, cells were treated with HCB (0.05μM) in RPMI 0.5% FBS or vehicle for 5, 15, and 30 min, and 2 and 24 h. For dose-response studies, cells were exposed for 15 min to HCB (0.005, 0.05, 0.5, and 5μM) in RPMI 0.5% FBS or vehicle. After HCB exposure, cells were washed with PBS, and total cellular protein lysates were obtained by scraping cells with lysis buffer (0.1M Tris-HCl, 1% Triton X-100, 1mM ethylene glycol tetraacetic acid, 0.1mM sodium fluoride (NaF), 0.02 mg/ml leupeptin, 1mM Na_3VO_4, and 1mM phenylmethanesulfonylfluoride). Samples were centrifuged at 16,000 × g for 10 min, and supernatants were kept at −80°C. Doses were selected according to previous studies from our laboratory (García *et al*., 2010) and considering that a dose of 5μM is in the same range order as that found in serum from humans from a highly contaminated population (To-Figueras *et al*., 1997).

Cell Treatment for Inhibitor Assays

For assays performed in the presence of specific c-Src and HER1 inhibitors, cells were pretreated for 3 h with 0.2nM of PP2 or 0.5μM AG1478, respectively. HCB (0.5μM) or vehicle was added to the media during 15 min in the presence or absence of inhibitors and then were washed with PBS. HCB dose (0.5μM) and time of exposure were selected because they were effective to stimulate c-Src, HER1, ERK1/2, and STAT5b phosphorylation.

To determine whether HCB activation of signaling pathways could involve AhR, two structurally unrelated antagonists, 4,7-orthophenanthroline and α-naphthoflavone, were used. Cells were pretreated for 3 h with 5 or 10μM of 4,7-orthophenanthroline and 1μM of α-naphthoflavone, and then HCB or vehicle was added to the media during 15 min. Total cellular protein lysates were obtained as described above. In view of the fact that HCB is a weak agonist of AhR, the lowest dose (0.05μM) and the highest dose (5μM), which activated the signaling pathways, were used in this assay to analyze if HCB effects are mediated by this receptor.

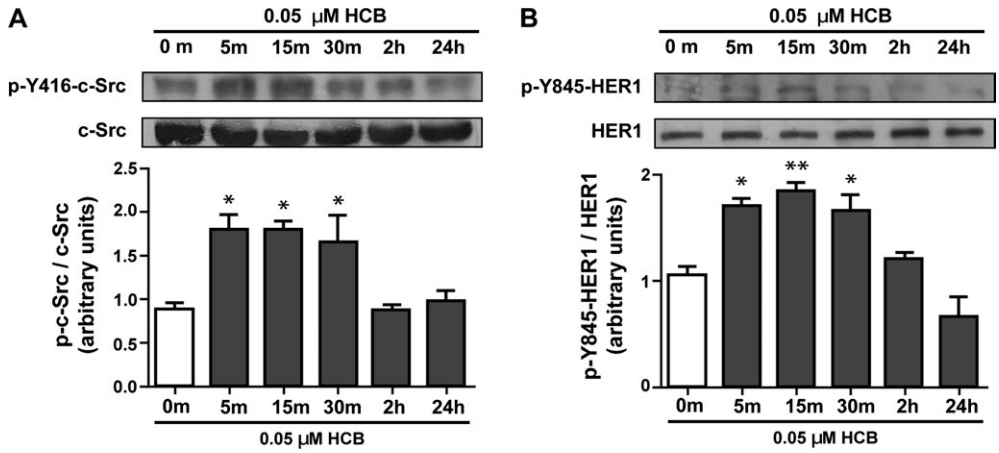

FIG. 1. Time courses of HCB effect on c-Src and HER1 activation. (A) Total and phospho-Y416-c-Src levels and (B) total and phospho-Y845-HER1 levels. Cells were exposed to HCB (0.05μM) or vehicle during 5, 15, and 30 min, and 2 and 24 h. Whole-cell lysates were resolved by SDS-PAGE and immunoblotted for p-Y416-c-Src and p-Y845-HER1 and reblotted for total c-Src and HER1 as described in the ''Materials and Methods'' section. A Western blot from one representative experiment is shown in the upper panels. Quantification of (A) p-Y416-c-Src/total c-Src ratio and (B) p-Y845-HER1/total HER1 ratio by densitometry scanning of the immunoblots are shown in the lower panels. Data are expressed as means ± SDs of three independent experiments. Asterisks indicate significant differences versus control (*$p < 0.05$ and **$p < 0.01$; ANOVA and Tukey *post hoc* test).

HER1 Immunoprecipitation

Cell lysates (300–500 μg) suspended in immunoprecipitation buffer (200mM Tris, pH 7.4, 1M NaCl, 100mM EDTA, 1% [octylphenoxy] polyethoxyethanol, and 2% Triton X-100) containing 1mM leupeptin, 200mM PMSF, 1 mg/ml aprotinine, 2mM Na_3VO_4, and 1M NaF were incubated with 3 μg of anti-HER1 antibody and 25 μl of A/G plus agarose in a final volume of 0.5 ml for 16–24 h at 4°C under constant shaking. Precipitates were washed four times with immunoprecipitation buffer, centrifuged at 12,000 × g, and denatured in Laemmli buffer at 95°C for 5 min.

Western Blotting

The protein concentration of cell lysates was determined according to Bradford (1976). Total cellular protein lysates (30–100 μg) were electrophoresed in 6 or 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), prior to transfer to polyvinylidene difluoride membrane (PVDF) in a semidry transfer cell at 15 V for 2 h in transferring buffer (25mM Tris, 200mM glycine, 0.1% SDS, 20% methanol), and blocked overnight in tris-buffered saline (TBS) (10mM Tris, 150mM NaCl, pH 8) with 5% bovine serum albumin for anti-phosphotyrosine or 5% milk for the remaining antibodies. Polyclonal anti-phospho-Y845-HER1 (1:250), anti-phospho-Y416-c-Src (1:500) or anti-phospho-ERK1/2 (1:1000), anti-phospho-T308-Akt (1:500), and anti-phospho-Y699-STAT5b (1:500) antibodies were used. For immunoprecipitates, PVDF membranes were incubated overnight with monoclonal anti-phosphotyrosine antibody (1:1000) and reprobed using polyclonal anti-HER1 (1:500). After incubation, membranes were washed five times with TBS-T (TBS 0.1% Tween 20) and the suitable peroxidase-conjugated anti–species-specific antibodies (1:1000) were used for protein detection. The immune complexes were visualized by enzyme-linked ECL kit (Amersham Biosciences Inc., UK) and quantified by scanning laser densitometry in a Fotodyne (Foto/Analyst), Gel-Pro Analyzer 3.1. Polyclonal anti-HER1 (1:500), anti-c-Src (1:500), anti-ERK1/2 (1:1000), anti-STAT5b (1:500), and anti-Akt2 (1:500), as well as monoclonal anti-β-actin (1:2000), antibodies were used for total protein receptor blottings.

Migration Assays

Scratch motility assay. 0.5×10^6 MDA-MB-231 cells were plated in a 6-well plate and grown overnight to confluency in RPMI with 10% FBS. The cells were serum starved for 24 h and were exposed to HCB (0.005, 0.05, 0.5, and 5μM) in 5% FBS or vehicle for 24 h. The monolayer was scratched with a pipette tip, washed with PBS to remove floating cells, and then were further exposed to different concentrations of HCB. Then, the scratched area was photographed at 0, 10, and 20 h, and cells in five randomly selected fields were counted. Mean value per field was expressed.

Transwell migration assay. MDA-MB-231 cells were exposed to HCB (0.005, 0.05, 0.5, and 5μM) in 5% FBS for 24 h. Cells (3×10^4 cells) suspended in serum-free media were added to the top chambers of 24-well transwell plates. RPMI with 2% serum was added to the bottom chamber as chemoattractant. After incubation for 20 h at 37°C, cells on the top of each filter were removed by wiping with cotton swabs. The cells on the lower surface of the filters were fixed with methanol and stained with toluidine blue. The number of migrating cells was counted. For inhibitor treatment, cells were pretreated for 3 h with the specific inhibitors, 2nM PP2 for c-Src, 10μM AG1478 for HER1, and 50μM 4,7-orthophenanthroline for AhR. Each experimental condition was repeated at least thrice.

Statistical Analysis

Data were evaluated by one-way ANOVA, followed by Tukey *post hoc* test to identify significant differences between controls and treatments. Differences were considered significant when p values were < 0.05. Results represent the mean ± SD of at least three independent experiments.

RESULTS

Time Courses of HCB-Induced c-Src and HER1 Phosphorylation

Because of the importance of c-Src and HER1 in breast tumor progression (Biscardi *et al.*, 1999), we investigated whether HCB stimulates their phosphorylation in the ERα (−) MDA-MB-231 breast cancer cell line. Cells were treated with HCB (0.05μM) during 0, 5, 15, and 30 min, and 2 and 24 h, and total cell lysates were electrophoresed and immunoblotted using anti-phospho-Y416-c-Src antibody, a specific phosphorylation site for activated c-Src (Frame, 2002), and anti-phospho-Y845-HER1, a specific site of c-Src phosphorylation. As shown in Figure 1A, HCB significantly increased Y416-c-Src phosphorylation (99, 98, and 86%) at 5, 15, and 30 min of

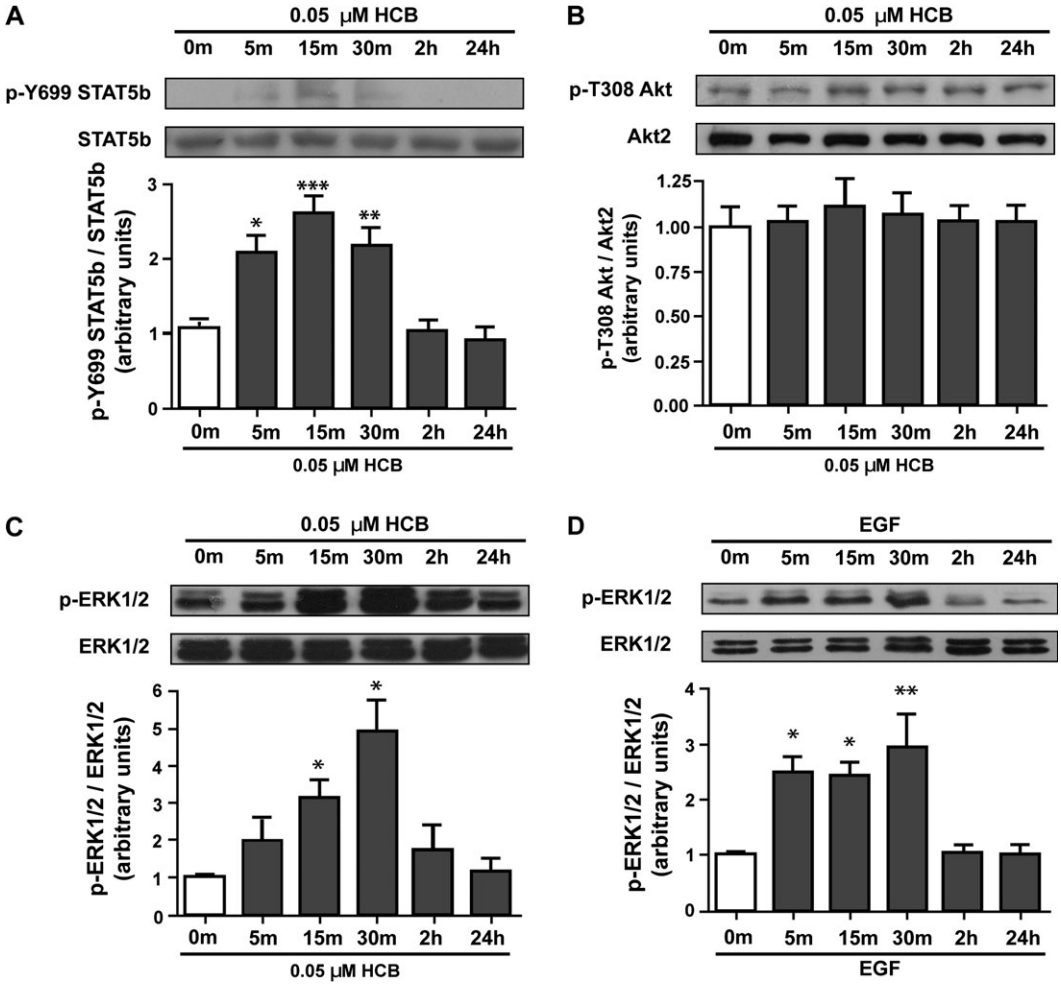

FIG. 2. Time course of HCB effects on ERK1/2, Akt, and STAT5b phosphorylation (A–C) or EGF effect on ERK1/2 phosphorylation (D). (A) Total STAT5b and phospho-Y699-STAT5b, (B) total Akt2 and phospho-T308-Akt, and (C and D) total ERK1/2 and phospho-ERK1/2 levels. Cells were exposed to HCB (0.05μM), vehicle (A, B, and C), or EGF (10 ng/ml) (D) during 5, 15, and 30 min, and 2 and 24 h. Whole-cell lysates were prepared, and protein was resolved by SDS-PAGE and blotted for p-ERK1/2, p-T308-Akt, and p-Y699-STAT5b and then reblotted for total proteins as described in the ''Materials and Methods'' section. A Western blot from one representative experiment is shown in the upper panels. Quantification of (A) p-Y699-STAT5b/total STAT5b ratio, (B) p-T308-Akt/total Akt2 ratio, and (C and D) p-ERK1/2/total ERK1/2 ratio by densitometry scanning of the immunoblots are shown in the lower panels. Data are expressed as means ± SDs of three independent experiments. Asterisks indicate significant differences versus control (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; ANOVA and Tukey *post hoc* test).

exposure, respectively. In a similar manner, HCB induced Y845-HER1 phosphorylation (60, 72, and 56%) at 5, 15, and 30 min of exposure, respectively (Fig. 1B). Total c-Src and HER1 protein levels were not modified.

Time-Course Effects of HCB on STAT5b, Akt, and ERK1/2 Phosphorylation

Given the precedence for the function of STAT proteins in oncogenesis (Kloth *et al.*, 2003), we have investigated the potential role of STAT5b in the signaling pathways activated by HER1 and c-Src in MDA-MB-231 cell line in response to HCB. Cells were treated with HCB (0.05μM) during 5, 15, and 30 min, and 2 and 24 h, and total cell lysates were electrophoresed and immunoblotted using specific antibodies. As shown in Figure 2A, HCB-induced Y699-STAT5b phosphorylation was readily observed at 5, 15, and 30 min of treatment (91, 139, and 100%, respectively).

We also examined whether the pesticide activates ERK1/2 and Akt by phosphorylation, two corresponding signaling pathways downstream to HER1. We found that HCB (0.05μM) did not increase T308-Akt phosphorylation at any time of exposure (Fig. 2B). Conversely, the pesticide increased ERK1/2 phosphorylation levels (209 and 386%) at 15 and 30 min, respectively, returning to control values at 2 and 24 h (Fig. 2C). As shown in Figure 2D, EGF treatment resulted in the phosphorylation of ERK1/2 in a similar pattern as that elicited by HCB (Fig. 2C).

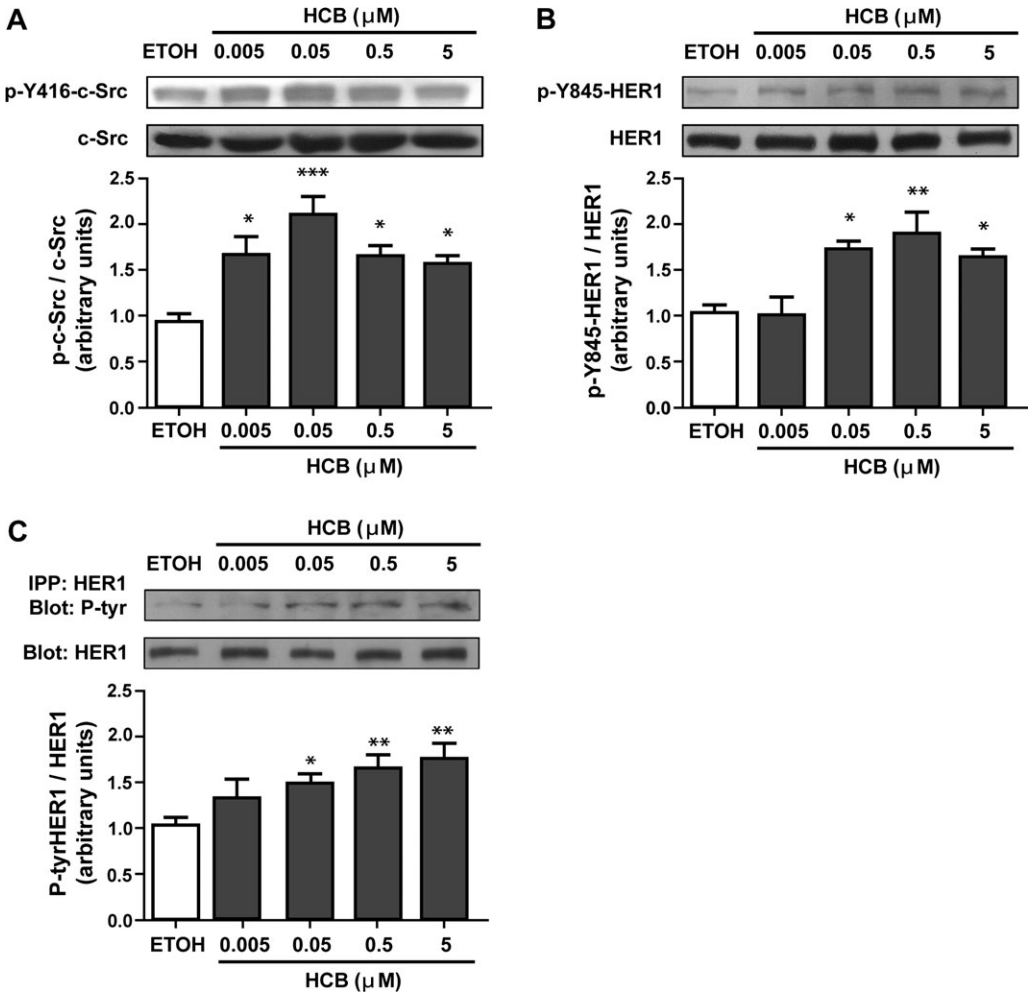

FIG. 3. HCB-induced c-Src and HER1 activation is dose dependent. (A) Total c-Src and phospho-Y416-c-Src, (B) total HER1 and phospho-Y845-HER1, and (C) total HER1 and phospho-Tyr-HER1 levels. Cells were exposed to HCB (0.005, 0.05, 0.5, and 5μM) or vehicle during 15 min. For c-Src and HER1 detection, whole-cell lysates were prepared, and protein was resolved by SDS-PAGE and blotted for p-Y416-c-Src and p-Y845-HER1 and reblotted for total c-Src and HER1 protein levels. For HER1 and p-Tyr detection, whole-cell lysates were precipitated with anti-HER1 antibody and resolved by SDS-PAGE. Membranes were blotted for total phosphotyrosine and reblotted for HER1 as described in the ''Materials and Methods'' section. Western blots from one representative experiment are shown in the upper panels. Quantification of (A) p-Y416-c-Src/total c-Src ratio, (B) p-Y845-HER1/total HER1 ratio, and (C) p-Tyr-HER1/total HER1 ratio by densitometry scanning of the immunoblots are shown in the lower panels. Data are expressed as means ± SDs of three independent experiments. Asterisks indicate significant differences versus control (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; ANOVA and Tukey *post hoc* test).

HCB Action on c-Src and HER1 Phosphorylation Is Dose Dependent

Previous 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay demonstrated that HCB concentrations used in the present study did not alter cell viability (results not shown). To evaluate dose-response effects of HCB on c-Src and HER1 activation, MDA-MB-231 cells were treated with HCB (0.005, 0.05, 0.5, and 5μM) or vehicle during 15 min. Total cell lysates were electrophoresed and immunoblotted using specific antibodies. As shown in Figure 3A, the pesticide significantly increased phospho-Y416-c-Src levels at all assayed doses, with a maximum effect at 0.05μM HCB (125%). Moreover, treatment of cells with HCB (0.05, 0.5, and 5μM) enhanced (66, 82, and 60%, respectively) Y845-HER1 phosphorylation (Fig. 3B). We also studied the action of HCB on HER1 total tyrosine phosphorylation because it has been shown that Y845-HER1 exerts a low contribution to full receptor kinase activity and its ability to activate ERK1/2 in response to EGF in breast cancer cells (Tice *et al.*, 1999). We observed dose-dependent increases (41, 51, and 66%) in total p-Tyr-HER1 phosphorylation after HCB (0.05, 0.5, and 5μM) treatment, respectively (Fig. 3C).

Dose-Response Effects of HCB on Akt, STAT5b, and ERK1/2 Phosphorylation

To evaluate dose-response effects of HCB on Akt, STAT5b, and ERK1/2 activation, MDA-MB-231 cells were treated with HCB (0.005, 0.05, 0.5, and 5μM) or vehicle during 15 min.

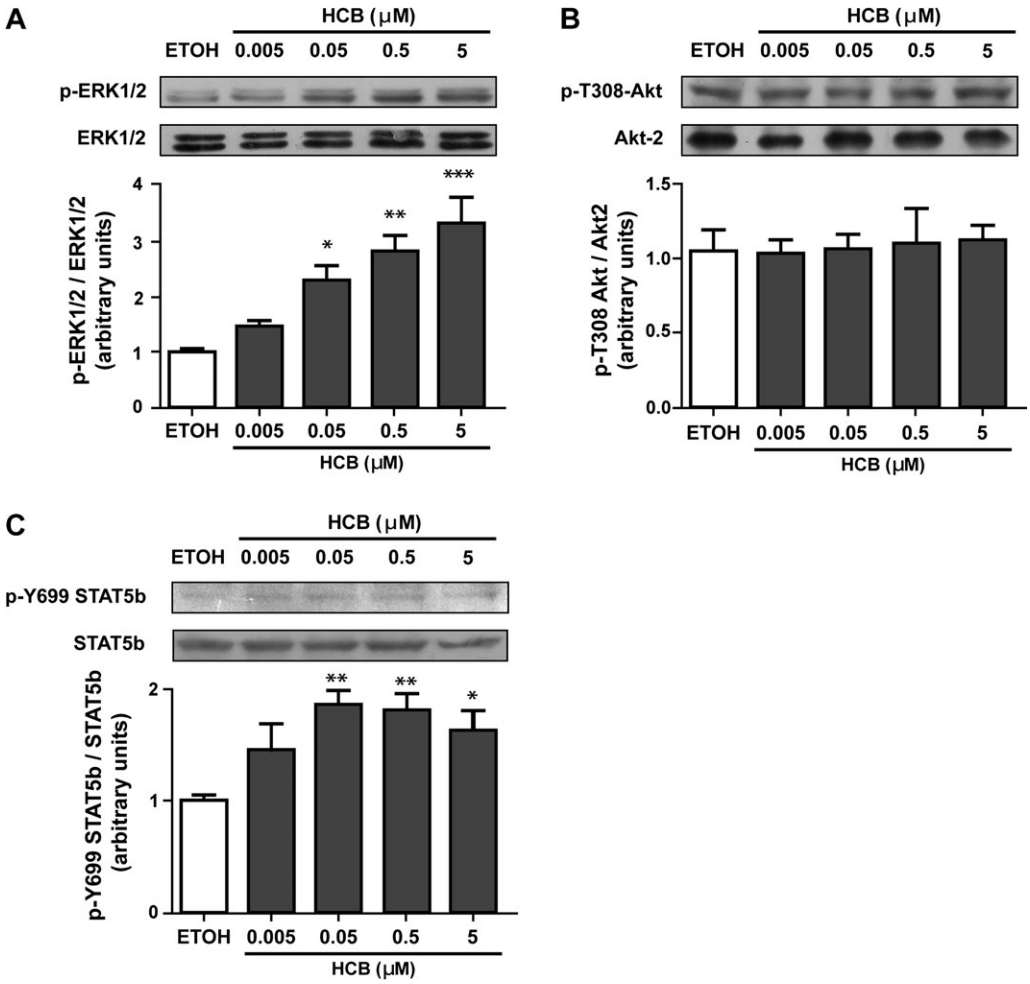

FIG. 4. Dose-response of HCB effects on ERK1/2, Akt, and STAT5b phosphorylation. (A) Total ERK1/2 and phospho-ERK1/2, (B) total Akt2 and phospho-T308-Akt, and (C) total STAT5b and phospho-Y699-STAT5b levels. Cells were exposed to HCB (0.005, 0.05, 0.5, and 5μM) or vehicle during 15 min. Whole-cell lysates were prepared, and proteins were resolved by SDS-PAGE and blotted for p-ERK1/2, p-T308-Akt, and p-Y699-STAT5b and then reblotted for total proteins as described in the ''Materials and Methods'' section. A Western blot from one representative experiment is shown in the upper panels. Quantification of (A) p-ERK1/2/total ERK1/2 ratio, (B) p-T308-Akt/total Akt2 ratio, and (C) p-Y699-STAT5b/total STAT5b ratio by densitometry scanning of the immunoblots are shown in the lower panels. Data are expressed as means ± SDs of three independent experiments. Asterisks indicate significant differences versus control (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; ANOVA and Tukey *post hoc* test).

Total cell lysates were electrophoresed and immunoblotted using specific antibodies. Similar to the results observed with total p-Tyr-HER1, HCB induced a dose-dependent increase of p-ERK1/2 levels (114, 176, and 221%), except at the lowest dose (Fig. 4A). Conversely, treatment of cells with HCB did not alter T308-Akt phosphorylation (Fig. 4B). As shown in Figure 4C, HCB (0.05, 0.5, and 5μM) significantly increased Y699-STAT5b phosphorylation (89, 82, and 65%), respectively.

Role of c-Src and HER1 Kinases in HCB-Mediated Activation of ERK1/2 and STAT5b

In order to evaluate the possible involvement of c-Src and HER1 on HCB-mediated activation of ERK1/2 and STAT5b, MDA-MB-231 cells were pretreated with specific inhibitors (0.2nM PP2 for c-Src and 0.5μM AG1478 for HER1) during 3 h. Then, cells were exposed to 0.5μM HCB or vehicle for 15 min in the presence or absence of inhibitors. We showed that PP2 prevented HCB stimulation of Y845-HER1 phosphorylation, a specific site of c-Src kinase (Fig. 5A). Conversely, PP2 did not alter HCB-induced ERK1/2 activation, indicating that c-Src activity is not involved in this effect, whereas AG1478 completely inhibited HCB-induced ERK1/2 phosphorylation. These results indicate that only HER1 is involved in ERK1/2 activation (Fig. 5B). Furthermore, we examined the ability of c-Src and HER1 kinase to mediate STAT5b activation with HCB treatment. As shown in Figure 5C, pretreatment with PP2 partially decreased HCB-induced STAT5b phosphorylation (20%), whereas pretreatement with AG1478 completely

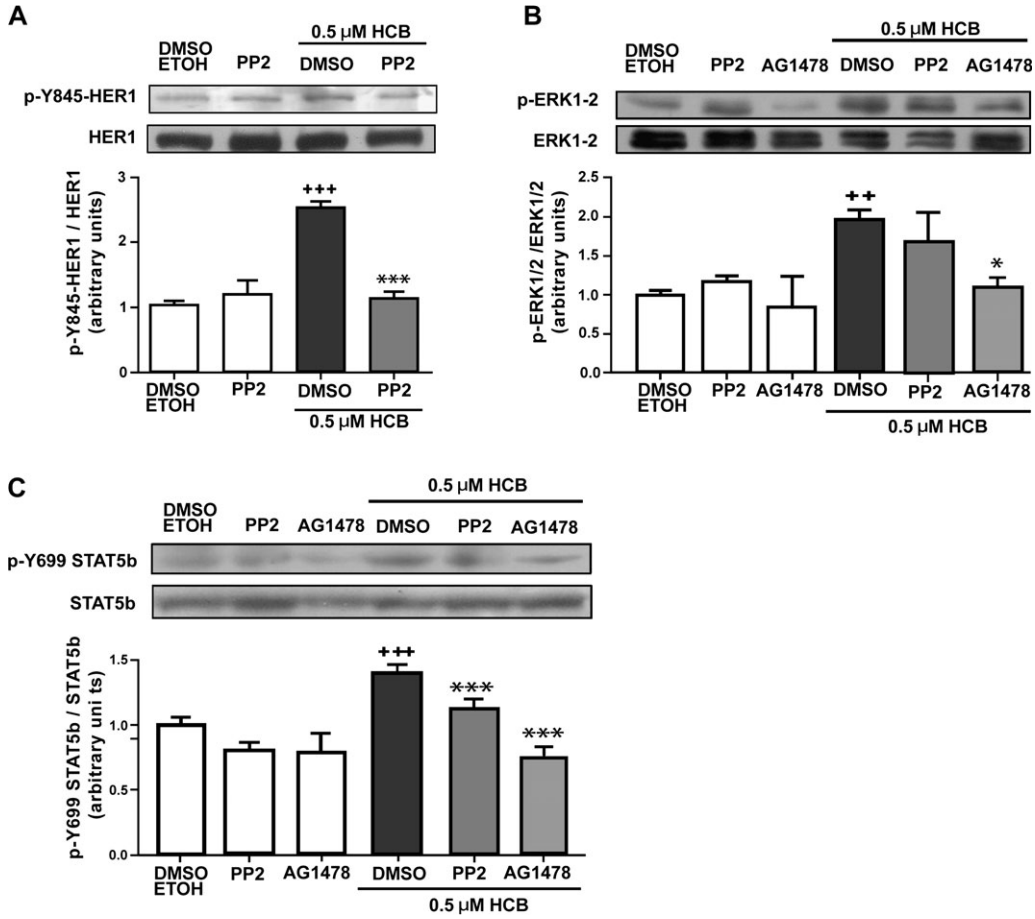

FIG. 5. Role of c-Src and HER1 kinases in HCB-mediated activation of ERK1/2 and STAT5b. (A) Total HER1 and phospho-Y845-HER1, (B) total ERK1/2 and phospho-ERK1/2, and (C) total STAT5b and phospho-Y699-STAT5b levels. Cells were pretreated for 3 h with 0.2nM PP2 or 0.5μM AG1478 and exposed to HCB (0.5μM) or vehicle during 15 min in the presence or absence of inhibitors. Whole-cell lysates were prepared, and proteins were resolved by SDS-PAGE and blotted for p-Y845-HER1, p-ERK1/2, and p-Y699-STAT5b and then reblotted for total proteins as described in the ''Materials and Methods'' section. A Western blot from one representative experiment is shown in the upper panels. Quantification of (A) p-Y845-HER1/total HER1 ratio, (B) p-ERK1/2/total ERK1/2 ratio, and (C) p-Y699-STAT5b/total STAT5b ratio by densitometry scanning of the immunoblots are shown in the lower panels. Data are expressed as means ± SDs of three independent experiments. Asterisks indicate significant differences versus HCB (*$p < 0.05$ and ***$p < 0.001$). $^{++}p < 0.01$ and $^{+++}p < 0.001$ indicate significant difference versus vehicle, ANOVA and Tukey *post hoc* test.

returned Y699-STAT5b phosphorylation to control values. These results indicate that both c-Src and HER1 are involved in STAT5b activation by the pesticide.

Activation of the STAT5b and ERK1/2 by HCB Is Dependent on AhR

HCB is a weak agonist of AhR; therefore, it could trigger AhR-dependent or AhR-independent effects (Hahn *et al.*, 1989). To determine whether HCB activation of STAT5b and ERK1/2 pathways could involve AhR, MDA-MB-231 cells were preincubated for 3 h in the presence or absence of 4,7-orthophenanthroline (5 or 10μM) and α-naphthoflavone (1μM) and then exposed to HCB for 15 min. 4,7-Orthophenanthroline was found to competitively inhibit [1,6-3H]2,3,7,8-tetrachlorodibenzo-p-dioxin ([^{3}H]TCDD)-specific binding to the AhR under conditions *in vitro* (Mahon and Gasiewicz, 1992). For this assay, we used the lowest (0.05μM) and the highest (5μM) doses of HCB that induced an activation of ERK1/2 and STAT5b (Figs. 6A, 6B, and 6C). As shown in Figure 6A, 4,7-orthophenanthroline (5μM) inhibited HCB-induced increase of Y699-STAT5b phosphorylation at both HCB concentrations. However, 4,7-orthophenanthroline (10μM) decreased ERK1/2 phosphorylation only when cells were exposed to 5μM HCB (Fig. 6C). In order to corroborate that the effects of HCB on STAT5b phosphorylation are mediated by AhR, we used α-naphthoflavone, a structurally different AhR antagonist (Merchant *et al.*, 1990). Our results show that α-naphthoflavone inhibited HCB-induced increase of Y699-STAT5b phosphorylation at both HCB concentrations. Altogether, these results suggest that HCB activates c-Src/HER1/STAT5b and HER1/ERK1/2 signaling pathways

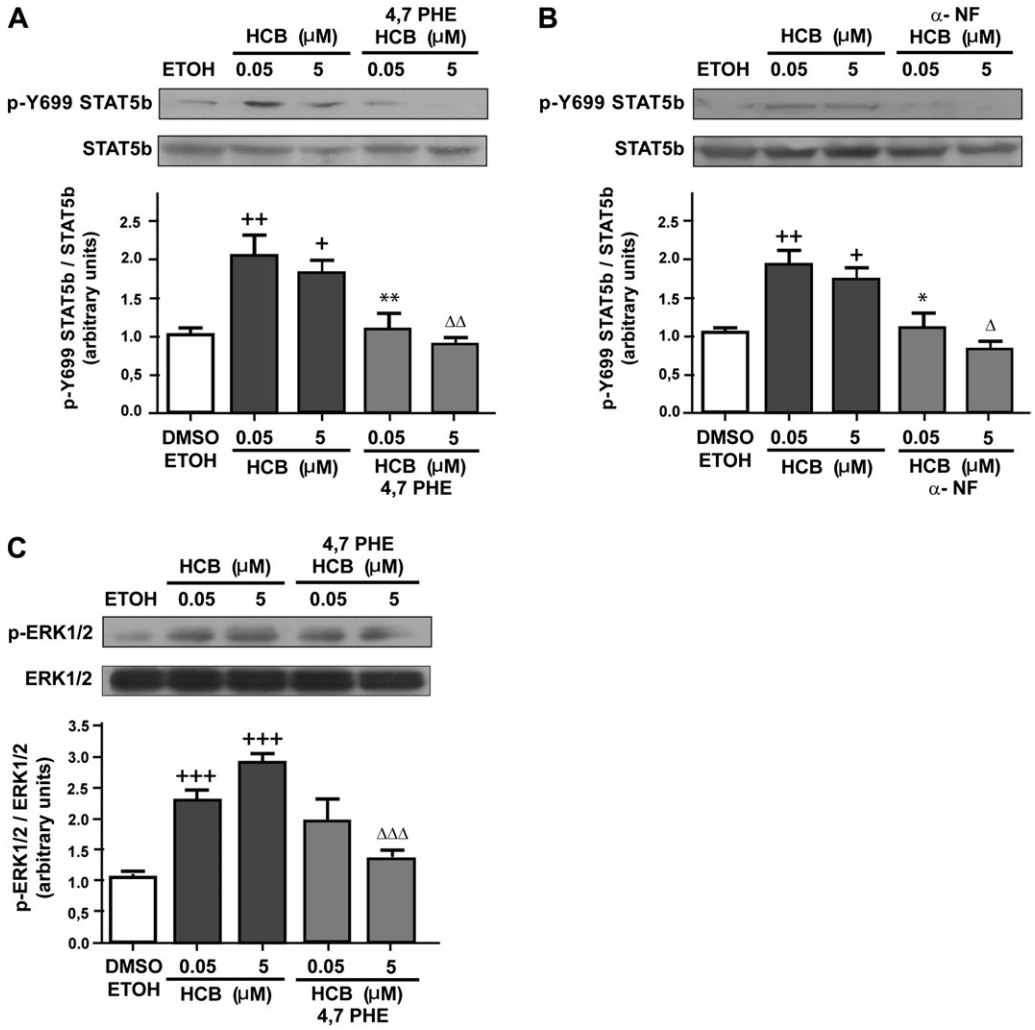

FIG. 6. HCB-induced activation of STAT5b and ERK1/2 is AhR dependent. (A and B) phospho-Y699-STAT5b and total STAT5b and (C) phospho-ERK1/2 and total ERK1/2. Cells were pretreated for 3 h with 4,7-orthophenanthroline (4,7 PHE; 5μM) or α-naphthoflavone (α-NF; 1μM) for STAT5b and with 4,7 PHE (10μM) for ERK1/2 and exposed to HCB (0.05 and 5μM) or vehicle during 15 min. Whole-cell lysates were prepared, and protein was resolved by SDS-PAGE and blotted for p-ERK1/2 and p-Y699-STAT5b and then reblotted for total proteins as described in the ''Materials and Methods'' section. A Western blot from one representative experiment is shown in the upper panels. Quantification of (A and B) p-Y699-STAT5b/total STAT5b ratio and (C) p-ERK1/2/total ERK1/2 ratio by densitometry scanning of the immunoblots are shown in the lower panels. Data are expressed as means ± SDs of three independent experiments. Crosses indicate significant differences versus vehicle ($^{+}p < 0.05$, $^{++}p < 0.01$, and $^{+++}p < 0.001$). Asterisks and triangles indicate significant differences versus HCB (0.05 and 5μM, respectively) ($*p < 0.05$, $**p < 0.01$, $^{\Delta}p < 0.1$, $^{\Delta\Delta}p < 0.01$, and $^{\Delta\Delta\Delta}p < 0.001$), ANOVA and Tukey *post hoc* test.

through AhR-dependent and independent mechanisms, according to HCB concentration.

HCB Effect on Cell Migration

To investigate the effect of HCB on cell migration, we assayed two different methods, the wound-healing and the transwell migration assays. For the first one, MDA-MB-231 cells were cultured to confluence and treated with HCB (0.005, 0.05, 0.5, and 5μM) or vehicle for 24 h, the monolayer cell was scratched, and wound closure was monitored after 10 and 20 h. A significantly enhanced wound closure was observed with HCB (0.05 and 0.5μM) at 10 h and with HCB (0.05, 0.5, and 5μM) at 20 h (Figs. 7A and 7B).

Role of c-Src, HER1, and AhR in HCB-Induced Cell Migration

In addition, the capacity of MDA-MB-231 cells to migrate toward the chemoattractant (2% FBS) in response to HCB (0.005, 0.05, 0.5, and 5μM) exposure for 24 h was measured in transwell chamber assays. As seen in Figure 8A, the migratory ability of MDA-MB-231 cells was increased 52% after 5μM HCB treatment. Lower doses of HCB have no effect.

The activation of c-Src and HER1 has been reported to mediate several aspects of tumor growth and progression, including proliferation, migration, invasion, survival, and angiogenesis (Koppikar *et al.*, 2008). It has also been shown that cellular migration is regulated by AhR and its toxic ligands (Barouki *et al.*,

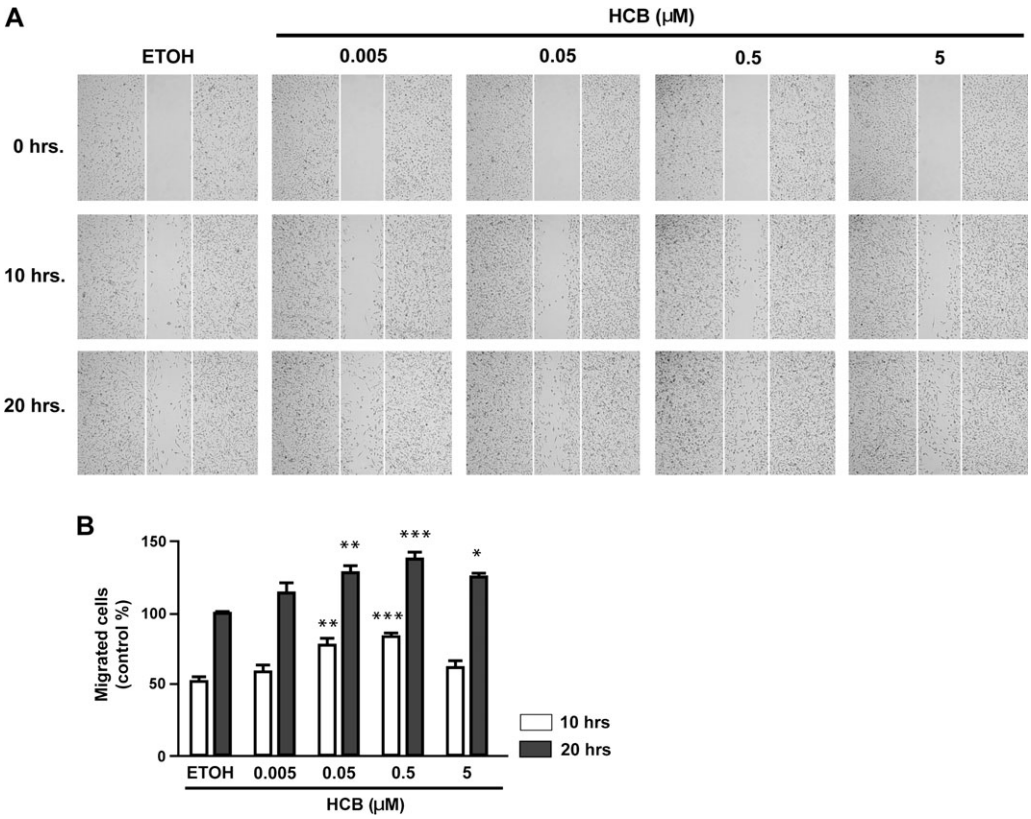

FIG. 7. HCB-induced cell migration. (A) Wound-healing assay. The serum-starved monolayer cell was scratched with a pipette tip and then treated with HCB (0.005, 0.05, 0.5, and 5μM) or vehicle for 24 h, and relative wound closure was observed at 10 and 20 h under microscope and photographed. (B) The number of cells migrated into the scratched area was counted at five randomly selected field and represented as mean ± SD. Asterisks indicate significant differences versus control (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; ANOVA and Tukey *post hoc* test).

2007). To determine whether c-Src, HER1, and AhR are involved in HCB-induced cell migration, cells were pretreated for 3 h with the specific inhibitors, 2nM PP2 for c-Src, 10μM AG1478 for HER1, and 50μM 4,7-orthophenanthroline for AhR, and then were exposed to 5μM HCB during 24 h. Our results showed that the migratory ability of MDA-MB-231 cells was prevented by the specific inhibitors (Fig. 8B), indicating that c-Src, HER1, and AhR are involved in HCB-induced cell migration.

DISCUSSION

Tumorigenesis frequently occurs as a result of the overexpression of proteins that are otherwise involved in normal cellular processes, as HER1 family and c-Src tyrosine kinase. These two proteins are overexpressed in a high percentage of certain late-stage human breast cancers, and they cooperate in tumor formation and progression (Biscardi *et al.*, 1999). Environmental chemical toxicants and ultraviolet B radiation irradiation cause enhanced and prolonged activation of growth factor receptor signaling and downstream pathways that contribute to epithelial cancer development (Rho *et al.*, 2010). HER1, and specifically phosphorylation of its Y845 by c-Src, appears to play an important, perhaps widespread, role in mediating cell responses to an array of external signals (Biscardi *et al.*, 1999). In the present study, we investigated HCB effect on c-Src and HER1 activation and their downstream signaling pathways STAT5b, ERK1/2, and Akt in MDA-MB-231 breast cancer cell line. The highest HCB dose used in the present study is in the same range of order as that found in serum from humans of a highly contaminated population (To-Figueras *et al.*, 1997). Previous reports have shown that when c-Src and HER1 are overexpressed, c-Src can bind physically to HER1 and induce Y845 phosphorylation (Tice *et al.*, 1999). The current study showed that Y416-c-Src and Y845-HER1 phosphorylation are increased by HCB at early times of exposure and that these effects are dose dependent. Our results of inhibition studies indicated that the kinase activity of c-Src is absolutely required for HCB-induced Y845-HER1 phosphorylation. In a similar manner, we have shown that HCB stimulated cell proliferation and c-Src phosphorylation in the ERα (+) MCF-7 cell line (García *et al.*, 2010). We have also demonstrated that HCB enhances the association of HER1 with c-Src and the phosphorylation of Y845-HER1 in WB-F344 rat liver epithelial cells (Randi *et al.*, 2008). An increasing number of studies support a role for the c-Src in the phosphorylation of STAT5b (Kloth *et al.*, 2003). c-Src activity is required for maximal transcriptional activation of

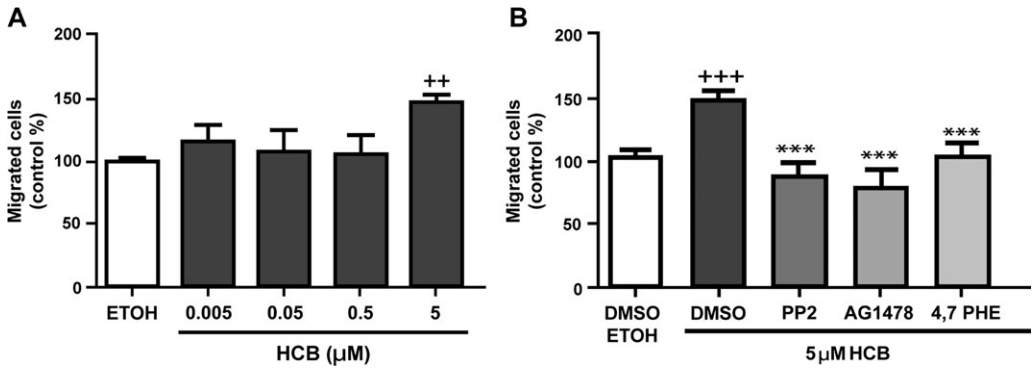

FIG. 8. Role of c-Src, HER1, and AhR in HCB-induced cell migration. (A) Cell migration by transwell assay with different HCB doses. Cells were exposed to HCB (0.005, 0.05, 0.5, and 5μM) or vehicle for 24 h. (B) Effect of different inhibitors on cell migration induced by 5μM HCB. Cells were pretreated with specific inhibitors (2nM PP2, 10μM AG1478, and 50μM 4,7-orthophenanthroline) and then were exposed to 5μM HCB for 24 h. The cells (3×10^4 cells) were suspended in serum-free media and placed in the top of transwell chamber. RPMI with 2% FBS was placed in the lower chamber as chemoattractant. After incubation for 20 h, the cells on the lower surface of the filters were fixed, stained, and counted under a microscope. The number of migrated cells was counted and represented as mean ±SD. Crosses indicate significant differences versus vehicle ($^{++}p < 0.01$ and $^{+++}p < 0.001$). Asterisks indicate significant differences versus 5μM HCB ($^{***}p < 0.001$), ANOVA and Tukey *post hoc* test.

STAT5b by EGF, and Y845-HER1 phosphorylation is essential for both STAT5b-HER1 association, as well as tyrosine phosphorylation of STAT5b (Biscardi *et al.*, 1999). Herein, we reported that Y699-STAT5b phosphorylation was increased very fast after HCB exposure, similar to the time courses of Y416-c-Src and Y845-HER1 phosphorylation. In the dose-response assays, the toxic enhanced STAT5b activity at the same doses that Y416-c-Src and Y845-HER1 phosphorylation were increased. Furthermore, we have shown that c-Src and HER1 are involved in the process of STAT5b activation in experiments with specific inhibitors. These results showed that HCB induces the activation of c-Src/HER1/STAT5b signaling pathway.

Our results clearly indicate that HCB induced ERK1/2 activation at early times of exposure. Moreover, it is interesting to remark that both p-ERK1/2 levels and total tyrosine HER1 phosphorylation showed a dose-dependent increase. Bulayeva and Watson (2004) have also found that organochlorines, DDE, and dieldrin activated ERK1/2 phosphorylation in a dose-dependent manner in GH3/B6/F10 prolactinoma cell line. Our results showing that HCB- and EGF-induced ERK1/2 activation have similar time-course patterns suggest that HER1 is involved in the pesticide mechanism of action. In this respect, we have shown that ERK1/2 activation is not mediated by c-Src but is dependent on HER1 activity, as demonstrated in studies in the presence of the corresponding specific inhibitors. Tice *et al.* (1999) found that Y845-HER1 exerts a low contribution to full HER1 kinase activity and its ability to activate ERK1/2 in breast cancer cells. In summary, our results clearly demonstrate that HCB stimulates HER1/ERK1/2 signaling pathway. In the case of Akt phosphorylation, we found that it was not affected by HCB exposure. Other authors demonstrated that HCB increased Akt levels in rat liver (Plante *et al.*, 2005). It has been reported that activated Akt efficiently blocks migration as well as invasion in breast cancer cell lines expressing a constitutively active Akt (Yoeli-Lerner *et al.*, 2005). Therefore, it is not unexpected that Akt activity is not altered in our study, in which HCB increased MDA-MB-231 cell migration.

HCB is a weak agonist of the AhR (Hahn *et al.*, 1989); thus, this compound could elicit actions through AhR-dependent and independent pathways. It has been proposed that liganded AhR can affect cellular functions by activating cellular kinases, such as c-Src, which upon ligand binding dissociates from the original cytosolic complex and functions as a cotransducer of transmembrane signals emanating from a variety of growth factor receptors, including HER1 (Kohle *et al.*, 1999). Thus, AhR, c-Src, and HER1 interactions may represent one of the most important upstream signaling regarding dioxin toxicity (Haarmann-Stemmann *et al.*, 2009). In the present study, we demonstrated that HCB (0.05 and 5μM)-induced Y699-STAT5b phosphorylation was AhR dependent because cotreatment with 4,7-orthophenanthroline or α-naphthoflavone prevented the stimulatory effect. We propose that HCB, after its binding to AhR, could stimulate c-Src activation and translocation to the cell membrane, inducing Y845-HER1 and Y699-STAT5b phosphorylation. On the other hand, our results showed that only 5μM HCB enhanced ERK1/2 activation through an AhR-dependent pathway. One possibility is that high doses of HCB could activate AhR, increasing intracellular cyclic AMP and stimulating protein kinase A activity, which directly phosphorylates HER1, as suggested by Haarmann-Stemmann *et al.* (2009). In a different way, the stimulatory effect of 0.05μM HCB on ERK1/2 phosphorylation was AhR independent, suggesting that a different mechanism of HER1 activation may be operating. It has been reported that HCB, TCDD, and other aromatic polyhalogenated compounds induced oxidative stress in breast cancer cell lines (Lin *et al.*, 2007). Thus, a possibility in our investigation is that HER1 activation induced by 0.05μM HCB was mediated by increased cellular

reactive oxygen species content as reported by other authors (Chen *et al*., 2007). The very low effective doses reported in the present study for HCB alterations in signaling pathways demonstrate that many environmental contamination levels previously thought to be subtoxic may very well exert significant signaling disruptive effects.

We have determined by reverse transcription PCR (RT-PCR) analysis that no induction of CYP1A1 messenger RNA was observed in HCB (0.05–5μM)-treated cells (results not shown). In the present study, we have also demonstrated that the early response (15 min) of HCB induction of STAT5b and ERK1/2 phosphorylation is AhR dependent. Altogether, these results suggest that the AhR is mediating these effects by a nongenomic mechanism. Similarly, Park *et al*. (2004) found in MCF10A human breast cells that TCDD causes functional activation of Src, which is accompanied with activation of ERK1/2 that takes place within 15 min of action of TCDD when there is no sign of induction of CYP1A1 in that cell line.

It has been demonstrated that HER1 and c-Src co-overexpression unleashes an oncogenic signaling program that leads to hyperproliferation and loss of polarity, marked enhancement of migratory and invasive behavior, and anchorage-independent growth (Dimri *et al*., 2007). Similarly, previous reports have indicated that high levels of c-Src in HER1-expressing metastatic breast cells promoted cell migration (Toker and Yoeli-Lerner, 2006). Src's catalytic activity initiates intracellular signal transduction pathways that influence cell growth and adhesion strength, the latter contributing to control of cell migration. Herein, we observed that HCB induced cell migration in a dose-dependent manner in the wound-healing assay. However, only 5μM HCB enhanced cell migration in the transwell assay. The reason for the difference in dose responsiveness between both assays is still not understood. Interestingly, we have found that HCB (5μM)-induced cell migration was c-Src, HER1, and AhR dependent. Plante *et al*. (2002) reported that in the rat liver, HCB decreases the expression of gap junctional intercellular communication (GJIC) proteins. This observation is interesting because gap junctions are involved in cell migration and are targets of c-Src. Normal human mammary epithelial cells express connexins (Cx43 and Cx26), which form functional gap junction channels. However, MDA-MB-231 cells express no Cx26 and only modest amounts of Cx43 and exhibit low levels of GJIC compared with normal breast epithelial cells (Qin *et al*., 2002). Further research would be necessary to clarify the role of HCB on Cx43 and cell migration. Miller *et al*. (2005) also demonstrated that benzopyrene-induced cell invasion in MDA-MB-231 cells is mediated through an AhR pathway. An AhR-dependent increase in cell migration after MCF-7 treatment with TCDD had previously been observed by Diry *et al*. (2006). Biscardi *et al*. (1999) support the hypothesis that c-Src-HER1 interactions contribute to tumor progression in certain late-stage breast tumor cells. Although here we have only directly assessed c-Src and HER1 activation in HCB-induced cell migration, the participation of downstream signaling pathways may also be implicated. In this respect, we have also found that cell migration depends on ERK1/2 activation (Pontillo *et al.*, unpublished results); thus, we could hypothesize that STAT5b may also be involved in migratory signaling. Bernaciak *et al*. (2009) found that STAT5b knockdown inhibited serum and fibronectin-stimulated migration at both BT-549 and MDA-MB-231 human breast cancer cell lines.

In conclusion, our results demonstrate for the first time that HCB induces the activation of c-Src/HER1/STAT5b and HER1/ERK1/2 signaling pathways, as well as cell migration in the breast cancer cell line MDA-MB-231. We have also shown that c-Src, HER1, and AhR are involved in HCB-enhanced cell migration. Taken together, our results provide a clue to the molecular events involved in the mechanism of action of HCB in mammary cancer development.

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