Title: Hexachlorobenzene promotes angiogenesis *in vivo,* in a breast cancer model and neovasculogenesis in vitro, in the human microvascular endothelial cell line HMEC-1

Author: Carolina Pontillo Alejandro Español Florencia Chiappini Noelia Miret Claudia Cocca Laura Alvarez Diana Kleiman de Pisarev María Elena Sales Andrea Silvana Randi

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Hexachlorobenzene promotes angiogenesis *in vivo*, in a breast cancer model and neovasculogenesis *in vitro*, in the human microvascular endothelial cell line HMEC-1.

Carolina Pontillo (caroponti@hotmail.com)(*), Alejandro Español (aespan_1999@yahoo.com) (†), Florencia Chiappini (florenciachiappini@hotmail.com) (*,†), Noelia Miret (noeliamiret@hotmail.com.ar) (*), Claudia Cocca (cm_cocca@hotmail.com) (‡), Laura Alvarez (laura6alvarez@yahoo.com.ar) (*), Diana Kleiman de Pisarev (dianakleiman@yahoo.com.ar) (*), María Elena Sales (malegazpio@yahoo.com.ar) (†), Andrea Silvana Randi (andybiol@yahoo.com.ar) (*).

(*) Laboratorio de Efectos Biológicos de Contaminantes Ambientales, Departamento de Bioquímica Humana, Facultad de Medicina, Paraguay 2155, 5° piso, (CP1121), Universidad de Buenos Aires, Buenos Aires, Argentina.

(†) Centro de Estudios Farmacológicos y Botánicos (CEFYBO), Facultad de Medicina, Paraguay 2155, 16° piso, (CP1121), Universidad de Buenos Aires, Buenos Aires, Argentina.

(‡) Laboratorio de Radioisótopos, Departamento de Físico-Matemática, Facultad de Farmacia y Bioquímica, Junín 954, subsuelo (CP1113), Universidad de Buenos Aires, Buenos Aires, Argentina.

-Corresponding Author:

Andrea Silvana Randi (andybiol@yahoo.com.ar)

Laboratorio de Efectos Biológicos de Contaminantes Ambientales, Departamento de Bioquímica Humana, Facultad de Medicina, Paraguay 2155, 5° piso (CP1121), Universidad de Buenos Aires, Buenos Aires, Argentina. (TEL/FAX: 005411-4508-3672 #33).

Highlights ►

► -HCB stimulates the angiogenic switch, increasing VEGF expression in a xenograft model. ► -HCB enhances VEGFR2 expression, and activates its downstream pathways in HMEC-1. ► -HCB induces COX-2 and VEGF expression protein levels in AhR-dependent manner in HMEC-1. ► -HCB increases HMEC-1 migration and neovasculogenesis involving AhR, COX-2 and VEGFR2. ►

Abstract

Exposure to environmental pollutants may alter proangiogenic ability and promotes tumor growth. Hexachlorobenzene (HCB) is an organochlorine pesticide found in maternal milk and in lipid foods, and a weak ligand of the aryl hydrocarbon receptor (AhR). HCB induces migration and invasion in human breast cancer cells, as well as tumor growth and metastasis *in vivo*. In this study, we examined HCB action on angiogenesis in mammary carcinogenesis. HCB stimulates angiogenesis and increases vascular endothelial growth factor (VEGF) expression in a xenograft model with the human breast cancer cell line MDA-MB-231. Human microvascular endothelial cells HMEC-1 exposed to HCB (0.005, 0.05, 0.5 and 5 μM) showed an increase in cyclooxygenase-2 (COX-2) and VEGF protein expression involving AhR. In addition, we found that HCB enhances VEGF-Receptor 2 (VEGFR2) expression, and activates its downstream pathways p38 and ERK1/2. HCB induces cell migration and neovasculogenesis in a dose-dependent manner. Cells pretreatment with AhR, COX-2 and VEGFR2 selective inhibitors, suppressed these effects. In conclusion, our results show that HCB promotes angiogenesis *in vivo* and *in vitro*. HCB-induced cell migration and tubulogenesis are mediated by AhR, COX-2 and VEGFR2 in HMEC-1. These findings may help to understand the association among HCB exposure, angiogenesis and mammary carcinogenesis.

Keywords: Hexachlorobenzene, breast cancer, angiogenesis, HMEC-1 cells, vascular endothelial growth factor, aryl hydrocarbon receptor.

Abbreviations:

HCB: Hexachlorobenzene

AhR: Aryl hydrocarbon receptor

VEGF: Vascular endothelial growth factor

COX-2: Cyclooxygenase-2

VEGFR2: Vascular endothelial growth factor Receptor 2

HMEC-1: human microvascular endothelial cells -1
HER-1: Epidermal growth factor receptor

1.Introduction

Epidemiological data show increases in incidence and prevalence of diseases associated with endocrine-disrupting chemicals, such as breast and prostate cancer, diabetes, obesity, and decreased fertility over the last 50 years (De Coster et al., 2012). Hexachlorobenzene (HCB) is an organochlorine pesticide well known as a widespread environmental pollutant. Despite a long-term ban on its use as a fungicide, HCB is a by-product in several processes, such as production of chlorinated solvents (Polder et al., 2009). Regional studies demonstrated the presence of this pollutant in human breast milk (Der Parsehian, 2008). Animal exposure to HCB elicits a number of effects such as thyroid disruption (Chiappini et al., 2009), immunological disorders (ATSDR, 2002) and co-carcinogenesis in rat mammary tumors (Peña et al., 2012). We have also reported that HCB induces cell migration and activates c-Src/HER1/STAT5b and HER1/ERK1/2 signaling pathways in the human breast cancer cell line MDA-MB-231 (Pontillo et al., 2011). In addition, we have also observed that the pesticide increased MDA-MB-231 cell invasion and metastasis in different breast cancer experimental models in mice (Pontillo et al., 2013).

HCB is a dioxin-like compound and a weak ligand of the aryl hydrocarbon receptor (AhR) (Hahn et al., 1989), which is a ligand-dependent transcription factor that modulates processes such as angiogenesis (Roman et al., 2009), proliferation and migration (Dietrich and Kaina, 2010). Upon dioxin binding, AhR can translocate to the nucleus where it regulates transcription of several genes including cyclooxygenase-2 (COX-2) (Degner et al., 2007). On the other hand, AhR-dioxin may release c-Src from its cytosolic complex, which can stimulate growth factor receptors, like the vascular endothelial growth factor receptor 2 (VEGFR2) (García-Martín et al., 2013).

Angiogenesis, the formation of new blood vessels from preexisting vasculatures, is necessary for many physiologic functions, while abnormal angiogenesis is usually considered as a sign of several diseases including cancer. Healthy vessels are arranged in a

hierarchical manner, whereas the tumor vasculature is disorganized and morphologically abnormal. Tumors vessels are leaky, in part due to the deficient perivascular support, but also due to abundant expression of the vascular endothelial growth factor A (VEGFA) (Fukumura and Jain, 2007). VEGFA acts on tumor endothelial cells to increase their proliferation, survival, migration and permeability, and by inhibiting vessel maturation (Greenberg et al., 2008). VEGF which is secreted by epithelial and endothelial cells, exerts its effects through binding to the receptor tyrosine kinases vascular endothelial growth factor –receptor 1 (VEGFR1) and VEGFR2, of which VEGFR2 is believed to be the main signal transducer in endothelial cells (Koch et al., 2011). VEGFR2 mediates the full range of VEGF responses in endothelial cells, like proliferation, migration and formation of the vascular tube. These physiological responses activate downstream mediators, including ERK1/2, c-Src, Akt, endothelial nitric oxide and p38 MAPK (Koch et al., 2011). Enhanced COX-2-induced synthesis of prostaglandin E2 (PGE2) stimulates cancer cell proliferation, promotes angiogenesis (Gately, 2000) and increases metastatic potential (Kakiuchi et al., 2002). Increase in COX-2 mRNA and protein levels are known to be associated with esophageal, head and neck, breast, lung, prostate, and other cancers, indicating a close involvement of COX-2 in tumor progression and other pathological phenotypes in various malignant tumors (Tsuji et al., 2001).

In previous studies, we found that HCB increases MDA-MB-231 cell invasion, and enhances mammary tumor growth and metastasis in different breast cancer models in mice (Pontillo et al., 2013). The aim of the present study was to investigate the effect of HCB on angiogenesis in mammary carcinogenesis. We sought to evaluate HCB capability to induce *in vivo* angiogenesis, using a xenograft model of breast cancer with MDA-MB-231 cell line. Furthermore, we examined the effect of HCB on cell proliferation, migration and neovasculogenesis in HMEC-1. We also investigated whether the AhR, COX-2 and VEGFR2 are involved in HCB-induced effects.

2. Materials and Methods

2.1.Chemicals

HCB (>99% purity, commercial grade) was obtained from Aldrich-Chemie GmbH & Co. (Steinheim, Germany). Anti-COX-2, anti-AhR and anti-VEGF antibodies were purchased from Abcam Ltd. (Cambridge, UK). Anti-VEGFR2, anti-phospho-p38 and anti-phospho-ERK1/2 antibodies were obtained from Cell Signaling Technology Inc., (Beverly, MA). Anti-ERK1/2 was purchased from Upstate (Lake Placid, NY), and anti-p38 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-β-Actin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 4,7-orthophenanthroline (PHE) were purchased from Sigma Chemical Co. (St Louis, MO). The specific inhibitors for COX-2, N-[2-(Cyclohexyloxy)-4-nitrophenyl] methanesulfonamide (NS-398) and for AhR, α-naphthoflavone (ANF), as well as antibiotic–antimycotic, trypsine and glutamine were obtained from Sigma Chemical Co. (St. Louis, MO). The inhibitor for VEGFR2, 4-Hydroxy-3-benzimidazol-2-ylhydroquinolin-2-one (ABYO) was purchased from Calbiochem (CAS 144335-37-5, Merck Millipore). The enhanced chemiluminescence kit (ECL) was from GE Healthcare Life Sciences (Buckinghamshire, UK). DMEM high glucose culture medium was purchased from HyClone Laboratories, Inc. (Logan, UT). Matrigel was purchased from Becton Dickinson Biosciences (San José, CA). Fetal bovine serum was obtained from Invitrogen Life Technology (Carlsbad, CA). All other reagents used were of analytical grade.

2.2. Mammary tumor- induced angiogenesis

Six-week-old female nude Swiss mice (La Plata Laboratory Animal Facility, Buenos Aires, Argentina) were maintained in a controlled environment: 24 ± 2 °C, 50 ± 10% relative humidity, and a 12–12 hour light/dark cycle. Female nude mice were housed into germ free environmental conditions. Animals were given free access to a powdered certified rodent diet obtained from a commercial source (ACA- Nutrición Animal, 16-014007 Rata-Ratón) and given tap water ad libitum. Tumor cells induced angiogenesis was quantified using an *in vivo* bioassay previously described (Monte et al., 1997). HCB (0.3, 3 and 30 mg/kg body weight) was dissolved in corn oil and then administered to female mice by intraperitoneal injection (i.p.) (0.1 ml) three times a week during 4 weeks (n=5 mice per group). Then, MDA-MB-231 cells ($5x10^4$) in 0.1 ml DMEM were inoculated intradermically (i.d.) in both flanks of female nude mice. On day 5, animals were euthanized, the skin from the

angiogenic site was carefully separated from the underlying tissues and the vascular response was observed in that place with a dissecting microscope (Konus USA Corporation, Miami, FL) at a 7.5 X magnification and photographed with an incorporated digital camera (Canon Power Shot A45, Canon USA, Inc. Lake Success, NY). Photos were projected on a reticular screen to count the number of vessels per mm^2 of skin. Angiogenesis was quantified as vessel density, calculated as the total number of vessels divided by the total number of squares. To detect VEGF protein levels in skin mice, skin from the angiogenic sites was dissected and homogenates were prepared. All the procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, National Research Council, USA. This research project was evaluated and accepted by the Institutional Animal Care and Use Committee (CICUAL) of School of Medicine, University of Buenos Aires, Argentina (Res.1146/2011). The HCB doses used in this model were the same we assayed previously in our laboratory. We have observed that these doses increased mammary tumor volume and weight, and significantly enhanced metastatic focus in mice lungs and liver (Pontillo et al., 2013). These concentrations are similar to those found in the environment. The HCB exposition to human populations varies among different countries. It has been detected levels of HCB in serum of umbilical cord (1 mg/kg) and in maternal serum (0.6 mg/kg) in China (Guo et al., 2014). Other authors have analyzed HCB levels in human serum samples in France (0.02 mg/kg) (Saoudi et al., 2014), and in Italy (0.043 mg/kg) (Mrema et al., 2013).

2.3. Cell culture and treatment

HMEC-1 is an immortalized cell line of human microvascular endothelial cells. It retains the characteristic of normal human microvascular endothelial cells in morphology, phenotype and function. HMEC-1 cells (Centers for Disease Control, Atlanta, GA, USA) were cultured in high glucose DMEM supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic mixture (10, 000 Units/ml penicillin, 10 mg/ml streptomycin sulphate, and 25 μg/ml amphotericin B). After 24 hours of starvation with DMEM 2% FBS, cells at 70–80% confluence were treated with HCB (0.005, 0.05, 0.5 and 5 μM) dissolved in absolute ethanol. Final ethanol concentration in each treatment was 0.5% and had no influence on the analyzed parameters as shown previously (Pontillo et al., 2011). In the

present study, the highest HCB dose (5 μM) used is in the same range of order as that found in human serum from a highly contaminated population (To-Figueras et al., 1997). In addition, the HCB dose of 0.5 μM is similar that observed in human serum samples from general population in France (Saoudi et al., 2014). For inhibitors treatment, cells were pretreated for 3 hours with the specific inhibitors dissolved in dimethyl sulphoxide (DMSO), ABYO for VEGFR2, NS-398 for COX-2, and α-naphthoflavone (ANF) or 4,7-orthophenanthroline (PHE) for AhR, in different concentrations according to the assay. Then, HCB or vehicle was added to the media in the presence or absence of inhibitors.

2.4. Western blotting

For VEGF immunodetection, skin from the angiogenic sites of mice was dissected and homogenates were prepared. Total cell protein lysates and skin homogenates (30–100 μg) were used by Western Blot analysis as described Pontillo et al., (2011). Polyclonal anti-VEGF (1:500), anti-VEGFR2 (1:500), anti-COX-2 (1:500) or monoclonal AhR (1:500) antibodies were used. Polyclonal anti-phosho-ERK1/2 (1:500), anti-phospho-p38 (1:250), anti-p38 (1:500) and monoclonal anti- ERK1/2 (1:500) were used. After incubation, membranes were washed 5 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 enhanced chemiluminiscence kit (ECL, Amersham Biosciences Inc., UK) and quantified by scanning laser densitometry in a Fotodyne (Foto/Analyst), Gel-Pro Analyzer 3.1. Monoclonal Anti-β Actin (1:2000) antibody was used as control blottings.

2.5. Proliferation assay

The measurement of cell proliferation was evaluated by MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay. This process requires active mitochondria, and even freshly dead cells do not cleave significant amounts of MTT. The cell viability and cell number are proportional to the value of absorbance measured by spectrophotometry at 570 nm. Briefly, $4x10^3$ HMEC-1 cells were seeded in 96-well plates, and maintained in high glucose DMEM 10% FBS for 24 h. The next day, medium was removed and high glucose DMEM 2% FBS was added. After 24 hours of starvation,

medium was removed and HCB (0.005, 0.05, 0.5 and 5 μM) or ETOH, in high glucose DMEM 3% FBS was added for 24 or 48 h. Finally, MTT (0.5 mg/ml) solution dissolved in high glucose DMEM was added to each well and incubated for 4 h at 37°C. Formazan crystals were dissolved in 100 μl DMSO, and the absorbance of the solution was measured at 570 nm using the microplate reader Synergy HT (Biotek Instruments, Inc., USA).

2.6. Migration assay

The HMEC-1 capability to migrate was evaluated by scratch motility assay. 4 $x10^6$ HMEC-1 cells were plated in a 6-well plate and grown overnight to confluency in DMEM, with 10% FBS. Next, the cells were serum starved for 24 hours in DMEM 2% FBS. The monolayer was scratched with a pipette tip, washed with PBS to remove floating cells, and then were exposed to different concentrations of HCB (0.005, 0.05, 0.5 and 5 μM) in 3% FBS, or vehicle for 18 hours. Then, the scratched area was photographed at 0 and 18 hours, and the distance of wound healing in each well was evaluated (D t0= distance at 0 hours; D t18= distance at 18 hours). Then, the migration rate was calculated by D t0 –D t18 /(D t0 x 100). For assays performed in the presence of specific VEGFR, COX-2 and AhR inhibitors, cells were pretreated for 3 hours with different inhibitors dissolved in DMSO: 0.1 or 1 μM ABYO for VEGFR2, or 10 μM NS-398 for COX-2, or 1 or 2 μM ANF for AhR. HCB 0.5 μM or vehicle was added to the media during 18 hours in the presence or absence of inhibitors, and then were washed with PBS.

2.7. Neovasculogenesis assay

Matrigel (50 μl aliquots) was gelled at 37˚C for 1 h. After starving with high glucose DMEM 2% FBS, HMEC-1 ($1x10^5$ cells) were plated in 96 well in DMEM 3% FBS containing ETOH or HCB (0.005, 0.05, 0.5 and 5 μM), and the formation of capillary-like structures was photographed 24 hours later. Quantification of the total tube length and the number of branch points were performed using Image J program. For assays performed in the presence of specific VEGFR, COX-2 and AhR inhibitors, cells were pretreated for 3 hours with 0.1 μM ABYO for VEGFR2, 1 μM NS-398 for COX-2, or 0.1 μM ANF for AhR. HCB 0.5 μM or vehicle was added to the media during 24 hours in the presence or absence of inhibitors, and then were washed with PBS.

2.8. Statistical analysis

For *in vivo* studies, we used n=5 mice per group. 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.

3. Results

3.1. In vivo study of angiogenesis

To evaluate the potential role of HCB to promote angiogenesis *in vivo*, MDA-MB-231 cells were injected into the mammary fat pad of female nude mice previously HCB (0.3, 3 and 30 mg/kg b.w.)-treated three times a week during 4 weeks. After 5 days, the animals were euthanized and the inoculated sites were photographed and the number of vessels/mm2 was counted in the skin. Our results showed that HCB treatment (3 and 30 mg/kg b.w.) significantly enhanced (20 and 40%) the angiogenic switch, inducing the formation of blood vessels (Fig. 1A and B). To detect VEGF protein levels, skin from the angiogenic sites was dissected, homogenates were prepared, and VEGF was evaluated by Western Blot. Our findings indicate that HCB (3 and 30 mg/kg b.w.) exposure increases VEGF protein expression (90 and 110%) in mice skin (Fig. 1C).

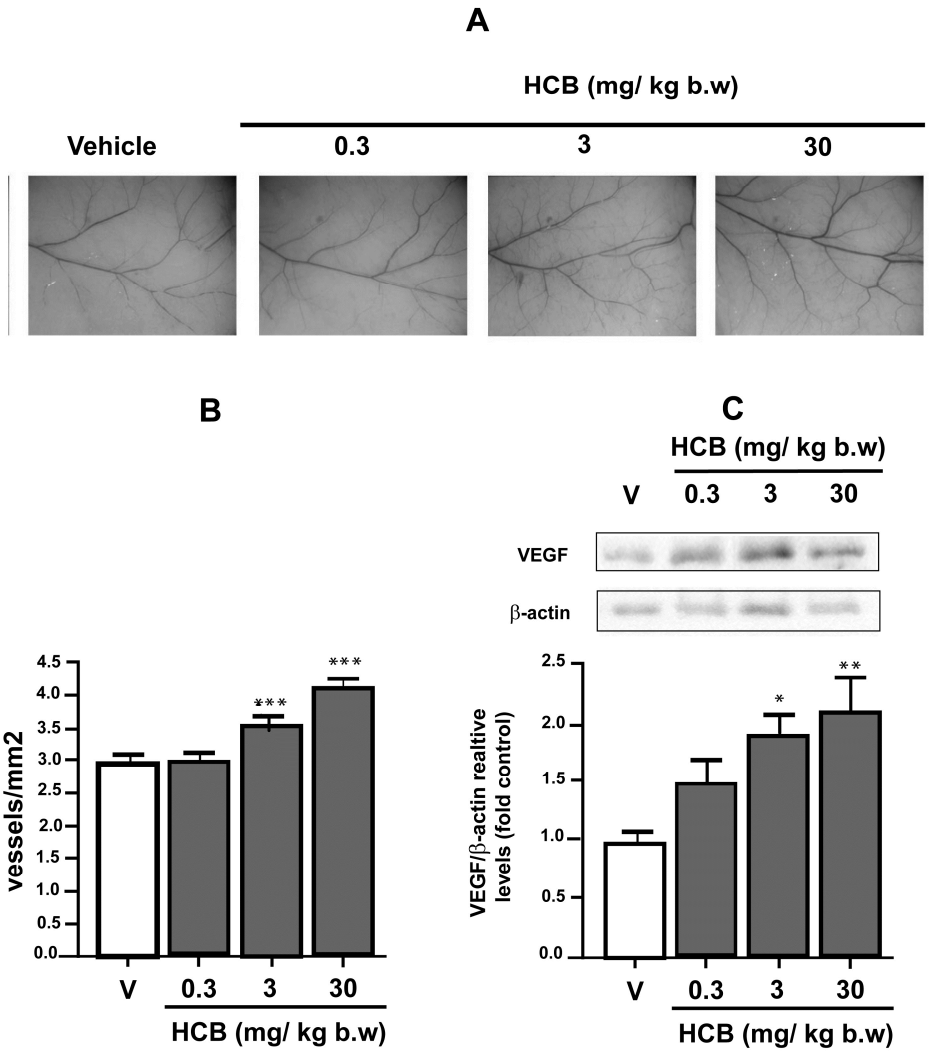


Fig. **1. HCB promotes angiogenesis in a mouse MDA-MB-231 xenograft model**. MDA-MB-231 cells were injected into the mammary fat pad of female nude mice previously HCB (0.3, 3 and 30 mg/kg b.w.)-treated three times a week during 4 weeks. After 5 days, animals were euthanized and the inoculated sites were photographed and were projected onto a reticular screen to count the number of vessels/mm^2 in skin. (A) Skin photographs of HCB or vehicle–treated mice. (B) Number of vessels/mm^2 in HCB or vehicle-treated mice inoculated with MDA-MB-231 cells. (C) VEGF protein levels in mice skin evaluated by immunoblot. Western Blot from one representative experiment is shown in the upper panel. Quantification by densitometry scanning of the immunoblots is shown in the lower panel. Data are expressed as means ± SD of three independent experiments with n=5 mice per group. Asterisks indicate significant differences vs. vehicle (*$p<0.05$, **$p<0.01$, ***$p<0.001$), ANOVA and Tukey post hoc test.

3.2. HCB alters VEGF secretion and VEGFR2 protein levels in HMEC-1

VEGF is secreted by epithelial and endothelial cells. Therefore, we sought to evaluate if HCB has a direct action on endothelial cells, *in vitro*. For this purpose we explore whether HCB alters VEGF secretion in HMEC-1. Cells were treated with HCB (0.005, 0.05, 0.5 and 5 µM) during 24 hours. The secretion of VEGF was evaluated in the conditioned medium of the cells, whereas the intracellular VEGF levels were analyzed in whole-cell lysates. As shown in Figure 2A, HCB had no effects on VEGF protein levels in cell lysates; in contrast, VEGF secretion was significantly enhanced by all assayed doses (76, 106, 91 and 62%). We also evaluated VEGFR2 protein expression in this cell line. Our results show that HCB-treatment resulted in significantly increased VEGFR2 protein levels, only at 0.5 µM (39%) (Fig. 2B).

Based on previous observations that AhR can regulate VEGF levels in HMEC-1 cell line (Roman et al., 2009), we evaluated if HCB-induced VEGF secretion is mediated through the bind of this pollutant to their receptor. The pretreatment with two structurally different AhR antagonists (0.1 µM ANF or 5 µM PHE), blocked HCB-induced VEGF secretion (Fig. 2C). These results indicate clearly that HCB-enhanced VEGF secretion is mediated by AhR in HMEC-1 cells.

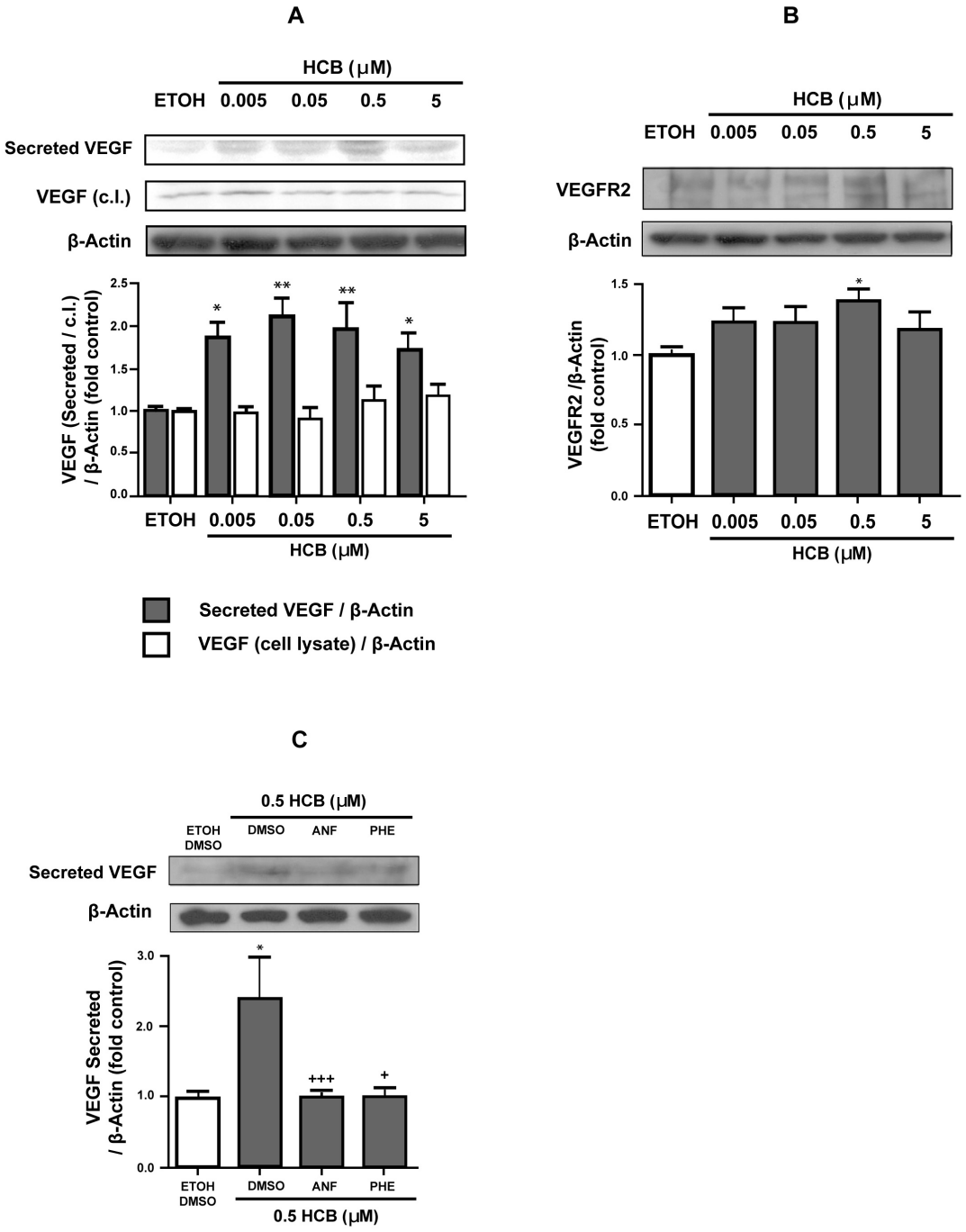


Fig. **2. HCB alters VEGF secretion and VEGFR2 protein levels.** HMEC-1 human microvascular endothelial cells were treated with HCB (0.005, 0.05, 0.5, and 5 μM) or vehicle (ETOH) during 24 hours. HMEC-1–conditioned medium was used to measure secreted VEGF protein levels and whole-cell lysates were used to analyze intracellular VEGF (A) or VEGFR2 (B) protein levels by Western blot. (C) Role of AhR in HCB-mediated VEGF secretion. Cells were pretreated for 3 hours with 0.1 μM ANF or 5 μM PHE and then exposed to HCB (0.5 μM) or vehicle during 24 hours, in the presence or

absence of inhibitors. Values were normalized by immunoblotting using anti-β-Actin antibody. Western blots from one representative experiment are shown in the upper panels. Quantification by densitometry scanning of the immunoblots is shown in the lower panels. Data are expressed as means ± SD of three independent experiments. Asterisks indicate significant differences vs. vehicle (*p< 0.05, **p<0.01), ANOVA and Tukey post hoc test. Crosses indicate significant differences vs. HCB (0.5 µM), (+p< 0.05 and +++p<0.001), ANOVA and Tukey post hoc test.

3.3. HCB action on AhR and COX-2 protein expression in HMEC-1

We examined next the effect of HCB on AhR expression at 24 hours of treatment. Our results show that HCB (0.05, 0.5 and 5 µM) significantly increased AhR protein levels in a dose-dependent manner (100, 139 and 160% respectively) (Fig. 3A).

The inhibition of COX-2 activity in animal models has been associated with a decrease of new blood vessel production in tumors, and an increase in tumor cell apoptosis (Kobayashi et al., 2004). Because VEGF up-regulates COX-2 mRNA, protein and enzymatic activity levels in HMEC-1 (Tamura et al., 2002), we next analyzed whether HCB could alter COX-2 protein expression in this cell line. Cells were treated with different doses of HCB (0.005, 0.05, 0.5 and 5 µM) during 24 hours, and cell lysates were analyzed by Western Blot. Fig. 3A shows that HCB significantly increased COX-2 levels at all assayed doses (50, 45, 48 and 50% respectively).

In order to evaluate if AhR could be mediating HCB-enhanced COX-2 levels, cells were pretreated for 3 hours with ANF (0.1 µM) or PHE (5 µM) and then exposed to HCB (0.5 µM) or vehicle during 24 hours. Fig. 3B clearly demonstrated that HCB enhances COX-2 protein levels through an AhR- dependent mechanism.

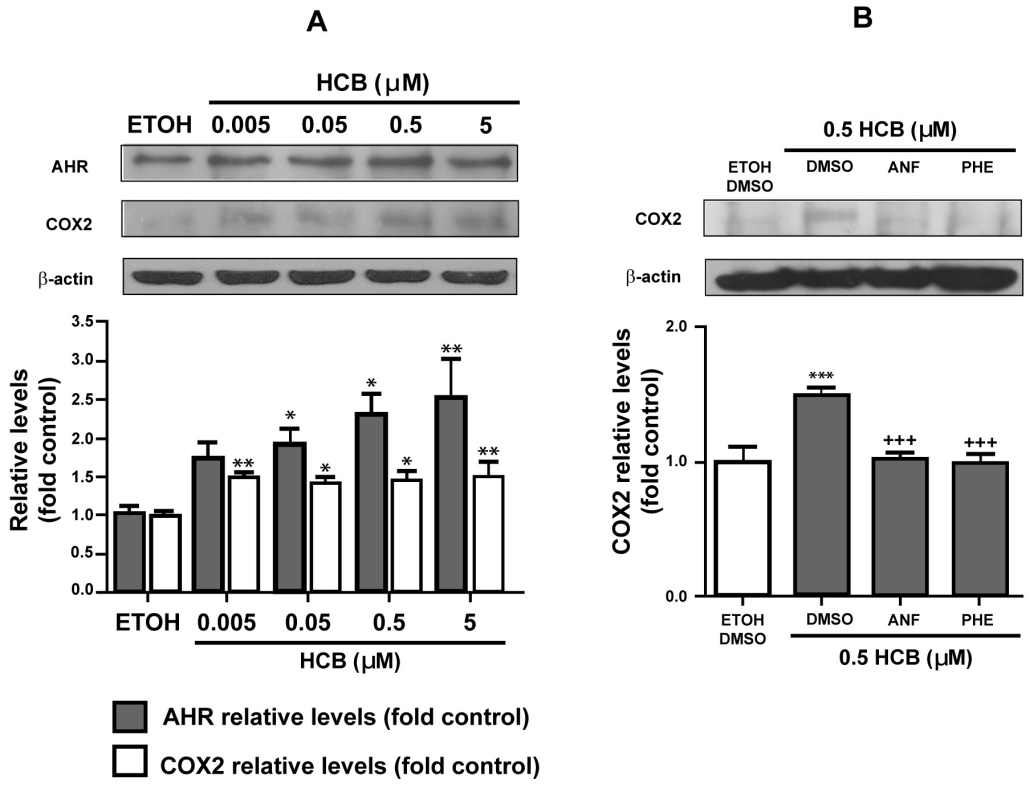


Fig. **3. HCB increases AhR and COX-2 protein expression in HMEC-1.** (A) Cells were treated with HCB (0.005, 0.05, 0.5, and 5 μM) or vehicle (ETOH) during 24 hours. Whole-cell lysate was used to analyze AhR protein levels and COX-2 protein levels by Western Blotting. (B) Role of AhR in HCB-induced COX-2 protein levels. Cells were pretreated for 3 hours with 0.1 μM ANF or 5 μM PHE and then exposed to HCB (0.5 μM) or vehicle during 24 hours, in the presence or absence of inhibitors. Whole-cell lysate was used to analyze COX-2 protein levels by Western Blotting. Data were normalized to β-Actin expression. Western blots from one representative experiment are shown in the upper panels. Quantification by densitometry scanning of the immunoblots is shown in the lower panels. Data are expressed as means ± SD of three independent experiments. Asterisks indicate significant differences vs. vehicle (*p< 0.05, **p<0.01 and ***p<0.001), ANOVA and Tukey post hoc test. Crosses indicate significant differences vs. HCB (0.5 μM), (+++p<0.001), ANOVA and Tukey post hoc test.

3.4. HCB activates VEGFR2 in HMEC-1

VEGFR2-specific ligands induce signaling pathways known to operate downstream of most tyrosine kinase receptors such as extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK), and the stress kinase p38 MAPK (Koch et al, 2011). Because HCB increases VEGF protein levels, we examined whether the

pesticide activates these pathways. HMEC-1 was treated with HCB (0.005, 0.05, 0.5 and 5 µM) during 5 minutes, and then ERK1/2 and p38 phosphorylation were evaluated by Western Blot. As shown in Figure 4A and B, the pesticide increased ERK1/2 phosphorylation (150, 147 and 165%), as well as p38 phosphorylation (72, 50 and 64%) at HCB 0.05, 0.5 and 5 µM, respectively.

Further, we examined the ability of VEGFR2 to mediate ERK1/2 and p38 activation when HMEC-1 cells were treated with HCB (0.5 µM). The pretreatment with VEGFR2 inhibitor (0.1 µM ABYO), blocked HCB-induced p38 and ERK1/2 phosphorylation (Fig. 4C). These results indicate that HCB-induced ERK1/2 and p38 phosphorylation is mediated by VEGFR2 activation in HMEC-1 cells. Consistently, VEGF treatment resulted in the phosphorylation of ERK1/2 in a similar manner as that elicited by HCB (Supplementary Figure). To determine whether HCB-induced activation of VEGFR2 could involve COX-2 and AhR, HMEC-1 cells were preincubated for 3 hours in the presence or absence of AhR inhibitor (0.1 µM ANF) or COX-2 inhibitor (1 µM NS-398), and then exposed to HCB (0.5 µM) for 5 min. As shown in Fig. 4D, ANF and NS-398 completely blocked HCB-induced ERK1/2 and p38 phosphorylation. In this respect, we have also observed that 1 nM TCDD, a strong AhR agonist, induced ERK1/2 activation in a similar manner as that elicited by 0.5 µM HCB. Further, when we incubated cells in the presence of 4,7-orthophenanthroline (PHE), a structurally ANF not related inhibitor of AhR, we found that ERK1/2 phosphorylation was blocked by two inhibitors by a similar manner (Supplementary Figure). These results suggest that AhR and COX-2 are involved in HCB-induced VEGFR2 activation.

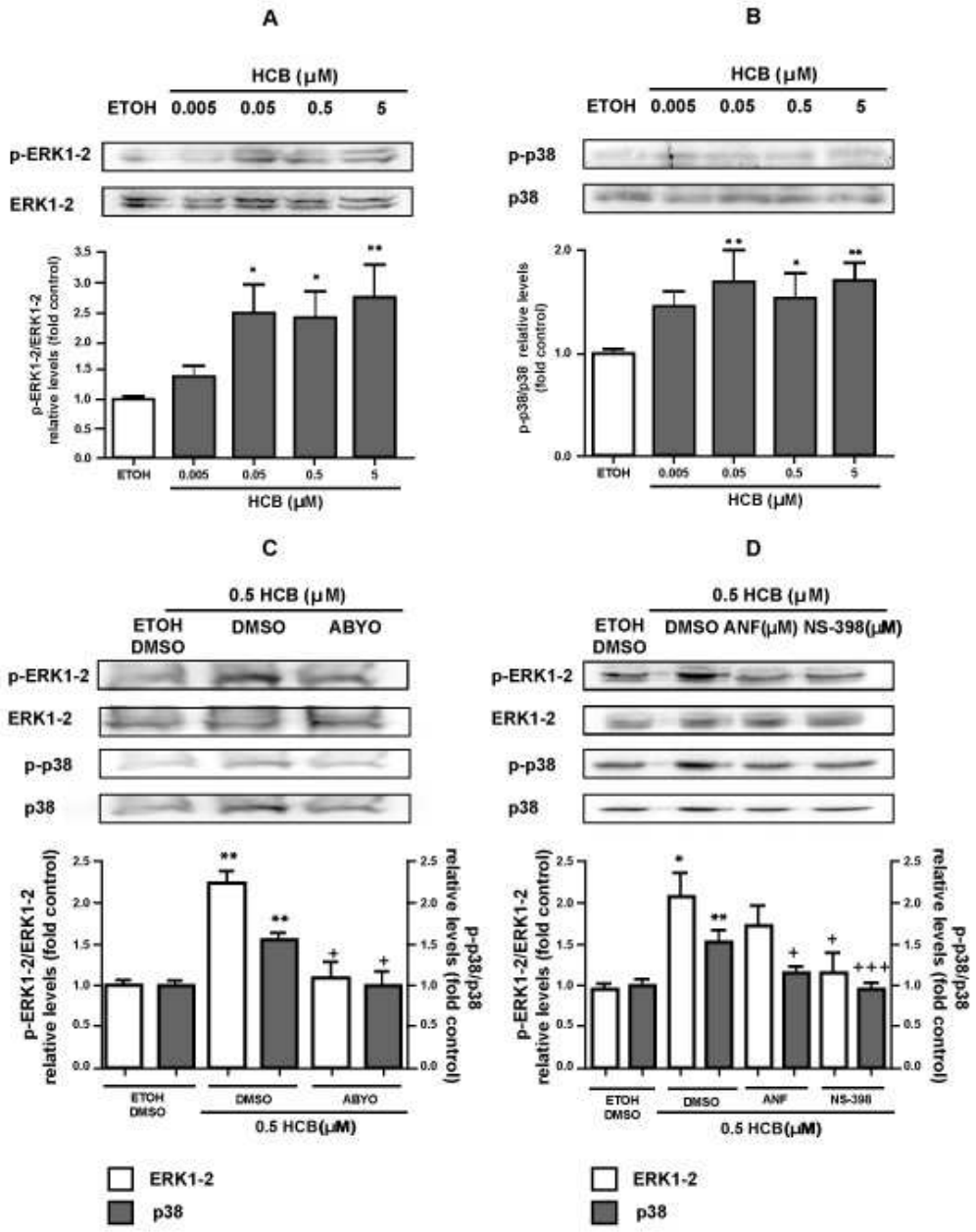


Fig. 4. HCB activates VEGFR2 in HMEC-1 (A) Phospho-ERK1/2 and total ERK1/2, (B) phospho-p38 and total p38. Cells were exposed to HCB (0.005, 0.05, 0.5 and 5 μM) or vehicle during 5 minutes. Whole-cell lysate was used to analyze phospho-ERK1/2 and phospho-p38 and total protein levels by Western Blot. (C) Role of VEGFR2, and (D) roles of AhR and COX-2 in HCB-mediated activation of ERK1/2 and p38. Cells were pretreated for 3 h with 0.1 μM ABYO (for VEGFR2) in (C), and pretreated for 3 hours with 0.1 μM ANF (for AhR) or 1 μM NS-398 (for COX-2) in (D) and then exposed to HCB (0.5 μM) or vehicle during 5 minutes, in the presence or absence of inhibitors. Whole-cell lysate was used to analyze phospho-ERK1/2 and phospho-p38 and total protein levels by Western Blot. 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. Data are expressed as means ± SD of three independent experiments. Asterisks indicate significant differences vs. vehicle (*p< 0.05, **p<0.01), ANOVA and Tukey post hoc test. Crosses indicate significant difference vs. HCB (0.5 μM), (+p< 0.05, ++p<0.01 and +++p<0.001), ANOVA and Tukey post hoc test.

3.5. HCB effect on cell proliferation and migration

VEGFA acts on endothelial cells to increase their proliferation, survival and migration (Greenberg et al., 2008). Because we have demonstrated that HCB increases VEGF secretion levels we next examined the ability of the pesticide to alter HMEC-1 cell proliferation. Cells were treated with different HCB doses (0.005, 0.05, 0.5 and 5 μM) during 24 and 48 hours and MTT assay was made. Our results show that HCB did not alter this parameter (data not shown).

We next examined whether HCB exposure affects HMEC-1 cell migration. Then, wound healing assay was made at different HCB doses (0.005, 0.05, 0.5 and 5 μM), and the scratched area was photographed at 0 and 18 hours. Finally, the distance of wound width was measure on photographed area. We observed that the pesticide induces an enhancement on cell migration at all assayed doses (Fig. 5A). The activation of VEGFR2 and COX-2 has been reported to mediate several aspects of tumor growth and progression, including migration, invasion and angiogenesis (Koch et al., 2011; Gately, 2000). It has also been shown that cell migration is regulated by AhR and its toxic ligands (Barouki et al., 2007). To determine whether VEGFR2, COX-2 and AhR are involved in HCB-induced cell migration, HMEC-1 cells were pretreated for 3 hours with the specific inhibitors, 0.1 and 1 μM ABYO for VEGFR2, 10 μM NS-398 for COX-2 and 1 and 2 μM ANF for AhR. Next, wound healing assay was made at HCB 0.5 μM, in the presence or absence of inhibitors. Our results show that the specific inhibitors prevented HMEC-1 cells migration, indicating that VEGFR2, COX-2 and AhR are implicated in HCB-induced cell migration (Fig. 5B).

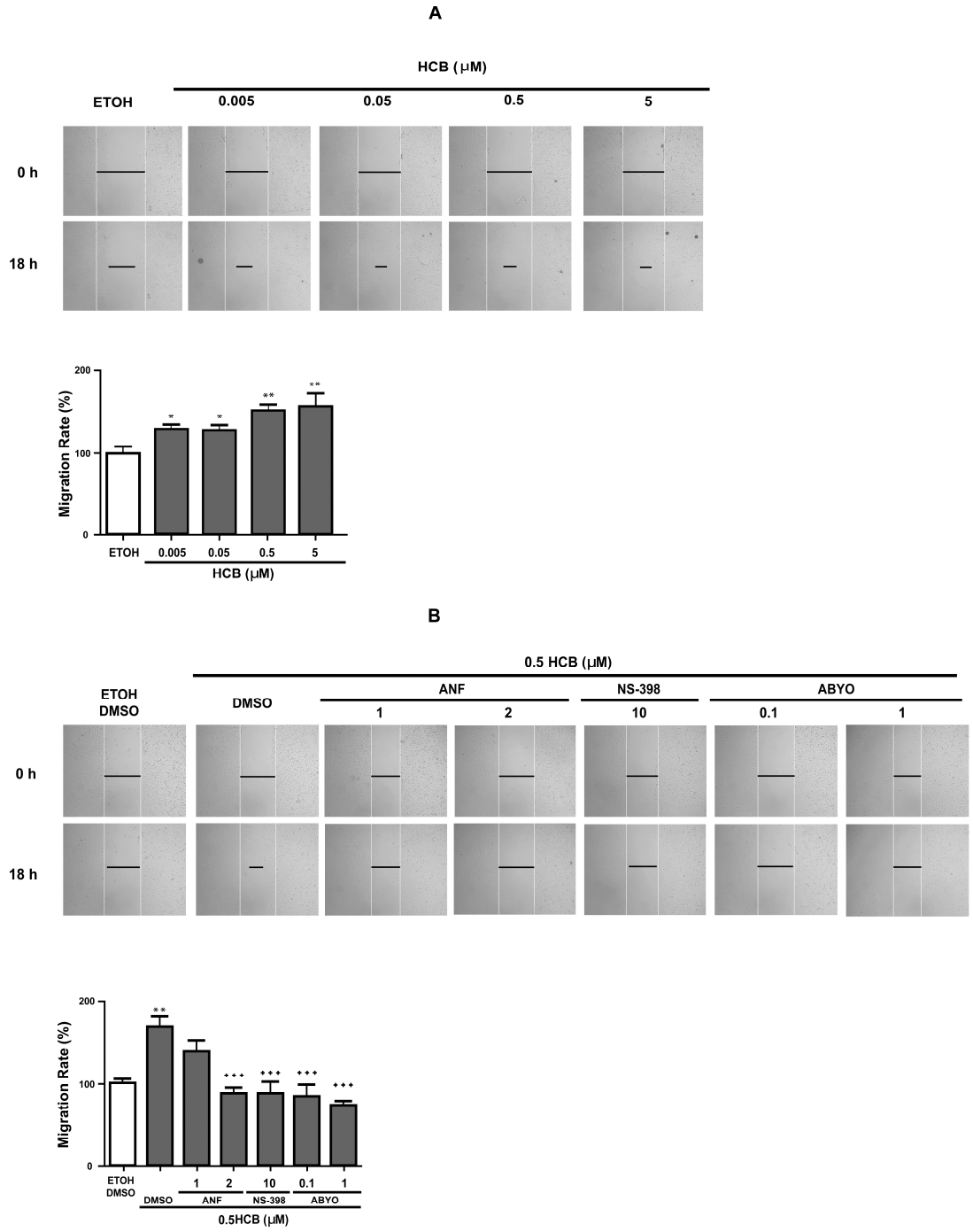
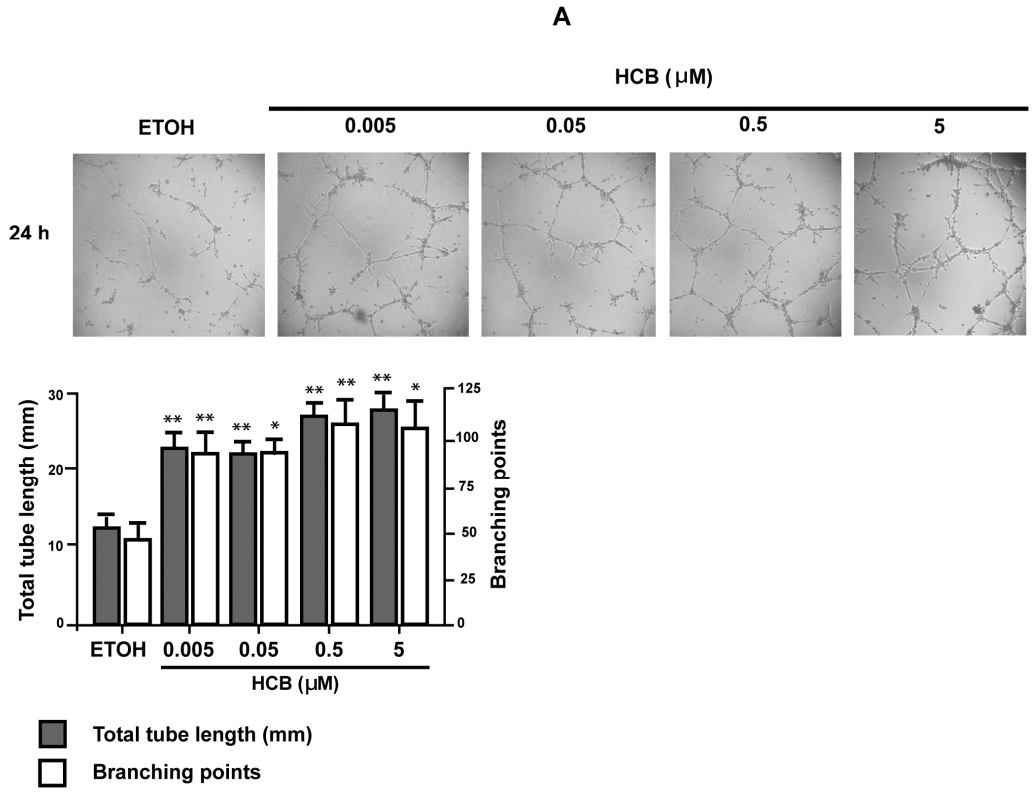


Fig. 5. HCB-induces HMEC-1 cell migration. (A) Wound healing assay with HMEC-1 cells exposed to HCB (0.005, 0.05, 0.5 and 5 μM) or vehicle for 18 hours. (B) Role of AhR, COX-2 and VEGFR2 in HCB- mediated cell migration. Cells were pretreated with specific inhibitors (1 and 2 μM ANF for AhR, 10 μM NS-398 for COX-2 and 0.1 and 1 μM ABYO for VEGFR2) and then were exposed to HCB 0.5 μM for 18 hours. The cell monolayer was scratched with a pipette tip, and then treated with HCB for 18 hours, or pretreated for 3

hours with inhibitors and then exposed at HCB (0.5 µM), and relative wound closure was observed under microscope and photographed. Migration rate (%) was calculated on the photography of scratched area, measuring the distance of wound width at 0 and 18 h. Asterisks indicate significant differences vs. control (*p< 0.05, **p< 0.01), ANOVA and Tukey post hoc test. Crosses indicate significant difference vs. HCB (0.5 µM), (+++p<0.001), ANOVA and Tukey post hoc test.

3.6. In Vitro neovasculogenesis assay

Because HCB promotes angiogenesis *in vivo*, and increases VEGF secretion in HMEC-1, we next examined the pesticide effect on neovasculogenesis *in vitro* in this cell line. Briefly, the formation of HMEC-1 capillary-like structures in marigel was analyzed under treatment with different HCB doses (0.005, 0.05, 0.5 and 5 µM). Our data indicate that HCB significantly induced neovasculogenesis, enhancing total tube length (82, 77, 123 and 127 %) as well as branching points (100, 97, 130 and 125 %) in a dose-dependent manner (Fig. 6A). To investigate, whether COX-2 and VEGFR2 were involved in HCB-induced neovasculogenesis, HMEC-1 cells were pretreated with specific inhibitors (1 µM NS-398 for COX-2 and 0.1 µM ABYO for VEGFR2). As shown in the Figure 6B, NS-398 and ABYO abrogated HCB-induced tubulogenesis. It has also been shown that neovasculogenesis is regulated by AhR in HMEC-1 cells (Roman et al., 2009). Therefore we have also investigated whether AhR was involved in HCB-induced neovasculogenesis. Our results show that the specific AhR-inhibitor, 0.1 µM ANF, suppresses HCB-induced tubulogenesis (Fig. 6B). Altogether, these data indicate that COX-2, VEGFR2 and AhR are implicated in HCB-induced neovasculogenesis in this cell line.



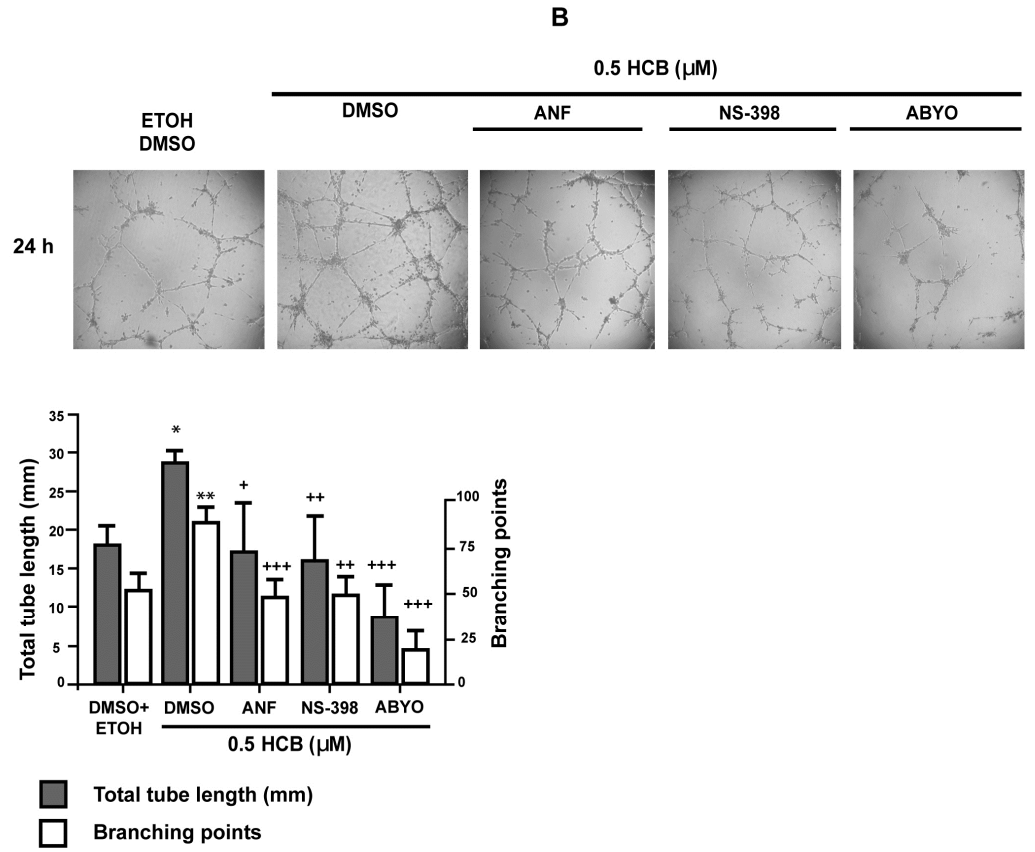


Fig. 6.Neovasculogenesis induced by HCB in HMEC-1 cells. (A) Photographs of capillary –like tube formation in Matrigel with cells exposed to HCB (0.005, 0.05, 0.5 and 5 µM). (B) Role of AhR, COX-2 and VEGFR2 in HCB-mediated neovasculogenesis. Cells were pretreated with specific inhibitors (0.1 µM ANF for AhR, 1 µM NS-398 for COX-2 and 0.1 µM ABYO for VEGFR2) for 3 hours, and then were exposed to HCB 0.5 µM for 24 hours. The total tube length and branching points were calculated and represented as mean ± SD. Asterisks indicate significant differences versus control (*p< 0.05, **p< 0.01), ANOVA and Tukey post hoc test. Crosses indicate significant difference vs. HCB (0.5 µM), (+ p<0.05, ++p<0.01, +++p<0.001), ANOVA and Tukey post hoc test.

4. Discussion

Accumulating literature suggests that breast cancer may have an environmental origin and of particular concern are hormonally active environmental agents such as organochlorine compounds that bio-accumulate within the food chain. However, there are few available data regarding organochlorine pesticides involvement in the modulation of angiogenesis, a critical step in tumor promotion. In this study, we observed that HCB-treatment induces neovascularization and VEGF expression, surrounding MDA-MB-231 inoculation site, thus stimulating the "angiogenic switch". This is a prevascular phase during early tumor development where few or no tumor cells are angiogenic. As virtually all solid tumors are neovascularized by the time they are detected, is more interesting to evaluate the effect of the pesticide in this step. These data confirm our previous findings, demonstrating that HCB increased tumor growth in a xenograft model with MDA-MB-231 in nude mice (Pontillo et al., 2013). Other authors also found that prenatal exposure to bisphenol A increased the relative vascular area and VEGF expression in mammary glands in rats (Durando et al., 2011).

VEGFR2 transduces VEGF responses in endothelial cells, via ERK1/2 and p38 pathways, regulating endothelial survival, proliferation, migration and formation of the vascular tube (Koch et al., 2011). Our data revealed that HCB activates VEGFR2 through an AhR and COX-2-dependent mechanism in HMEC-1. Besides, we observed that HCB increases VEGF secretion levels in this cell line. In this respect, other authors observed that the polychlorinated biphenyl PCB104, increases VEGF expression in HMEC-1 cells (Eum et al., 2004). Furthermore, our results demonstrated that HCB increases COX-2 protein levels in HMEC-1. Similarly Anderson et al, (2011), demonstrated that the polychlorinated

biphenyl PCB126, increased COX-2 mRNA expression in human umbilical vascular endothelial cells (HUVEC). Moreover, in this study we observed that HCB increases AhR protein levels in HMEC-1. We also demonstrated that HCB-induced VEGF secreted levels and COX-2 protein levels are mediated by AhR. It is well known the role of AhR in angiogenesis. In this respect, Roman et al., *(*2009), reported that the lack of AhR expression in mice severely impairs their capacity to support the growth and angiogenesis of tumors, suggesting that an AhR-competent microenvironment is needed for such an effect. On the other hand, we have shown that HCB enhances VEGFR2 protein levels, which has been correlated with breast cancer and poor survival (Ryden et al., 2010). Other authors found that VEGFR2 expression was up-regulated by VEGF in HMEC-1 cells (Hervé et al., 2006). Hence, our results suggest that increased VEGF secreted levels, might be associated with HCB-induced VEGFR2 protein levels.

Angiogenesis is a complex process including endothelial cell proliferation, migration, basement membrane degeneration, and new tube formation, involved in a variety of physiologic processes, as well as pathological mechanisms as cancer (Carmeliet and Jain, 2000). Herein, we observed that HCB doesn't modify cell proliferation or viability in HMEC-1 cells. Recent studies found that organochlorine pesticides lindane and chlordecone increased endothelial cell proliferation (Clere et al., 2012). Conversely, other authors have demonstrated that 3-methylcholantrene is a potent anti-proliferative molecule in HUVEC, acting mainly through the AhR (Juan et al., 2006). In the present study, we show that HCB increases HMEC-1 cell migration and neovasculogenesis involving AhR, COX-2 and VEGFR2 proteins.

In respect to the effective HCB doses *in vitro*, we observed that the pesticide enhances the AhR protein expression, as well as VEGFR2 activation levels at 0.05 to 5 µM. Similarly, we found that the pesticide increases cell migration and tubulogenesis at all doses, but has greater effect to the higher doses (0.5 and 5 µM). Therefore, we observed that there is a relationship between the pesticide effect on the activation of the important molecules for angiogenesis, and the biological actions such as migration and tubulogenesis.

Our hypothesis is that HCB would be enhancing angiogenesis in breast cancer, in part, through the direct activation of the endothelial cells. Particularly in HMEC-1, HCB might be binding to its receptor AhR and inducing VEGF expression as reported previously by Roman et al., (2009), in this cell line. Other authors have found that AhR mediates TCDD induced VEGF expression and angiogenesis in mice (Takeuchi et al., 2009). Then, VEGF could be stimulating COX-2 levels. Similarly, Tamura et al., (2002), demonstrated that VEGF up-regulates COX-2 mRNA levels in HMEC-1. Furthermore, we thought that COX-2 might be increasing VEGF expression in HCB treated HMEC-1. In this respect, it has been reported that VEGF expression is regulated by COX-2/ PGE2/ PGE2 receptor EP2-EP4 pathway in gastric cancer cells (Liu et al., 2014). Then, HCB could be stimulating HMEC-1 migration and neovasculogenesis through an AhR, COX-2 and VEGFR2 dependent mechanism (Fig.7).

In conclusion, our results demonstrate for the first time, that HCB increases angiogenesis and VEGF expression *in vivo* in a breast cancer model in mice. Furthermore, HCB-induced enhancement of HMEC-1 cell migration and neovasculogenesis are mediated by AhR, COX-2 and VEGFR2 proteins in this cell line. These findings may help understanding the association between HCB exposure, angiogenesis and mammary carcinogenesis.

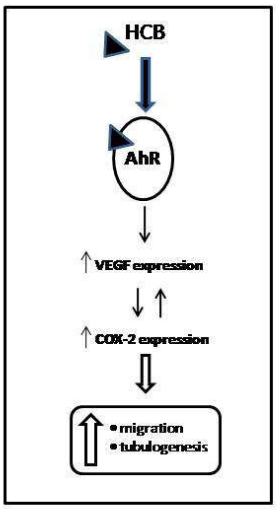
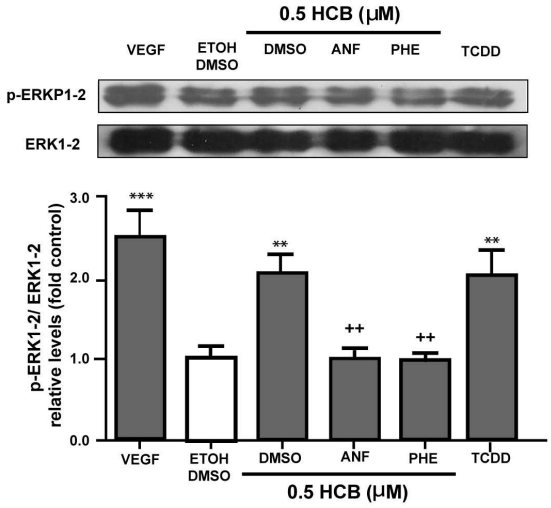


Fig 7. HCB induces cell migration and tubulogenesis through an AhR, COX-2 and VEGFR2 dependent mechanism in HMEC-1. The overall scheme for HCB-induced effects on neovasculogenesis and cell migration in HMEC-1. HCB might be binding to its receptor AhR and inducing VEGF expression. Then, VEGF could be stimulating COX-2 levels. Moreover, COX-2 might be increasing VEGF expression in HCB treated HMEC-1 cells.

Supplementary Figure



New Figure: Effect of HCB on ERK1/2 phosphorylation in HMEC-1, and comparative effects of VEGF and TCDD. Phospho-ERK1/2 and total ERK1/2 protein. Cells were exposed to HCB (0.5 μM), VEGF (10 ng/ml), TCDD (1 nM) or vehicle during 5 minutes. Cells were pretreated for 3 hours with 0.1 μM ANF or 5 μM PHE and then exposed to HCB (0.5 μM) or vehicle, in the presence or absence of inhibitors. Whole-cell lysate was used to analyze phospho-ERK1/2 and total protein levels by Western Blot. A western blot from one representative experiment is shown in the upper panel. Quantification by densitometry scanning of the immunoblots is shown in the lower panel. Data are expressed as means ± SD of three independent experiments. Asterisks indicate significant differences vs. vehicle (**p< 0.01, ***p<0.001), ANOVA and Tukey post hoc test. Crosses indicate significant difference vs. HCB (0.5 μM), (++p<0.01), ANOVA and Tukey post hoc test.

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