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Enhanced cyclooxygenase-2 expression levels and metalloproteinase 2 and 9 activation by Hexachlorobenzene in human endometrial stromal cells

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ABSTRACT

Hexachlorobenzene (HCB) is an organochlorine pesticide that induces toxic reproductive effects in laboratory animals. It is a dioxin-like compound and a weak ligand of the aryl hydrocarbon receptor (AhR). Endometriosis is characterized by the presence of functional endometrial tissues outside the uterine cavity. Experimental studies indicate that exposure to organochlorines can interfere with both hormonal regulation and immune function to promote endometriosis. Altered expression of metalloproteinases (MMPs) in patients with endometriosis, suggests that MMPs may play a critical role. In the endometriotic lesions, prostaglandin E_2 (PGE_2) produced by cyclooxygenase-2 (COX-2), binds to its EP4 receptor (EP4), and via c-Src kinase induces MMPs activation, promoting endometriosis. We examined the HCB action on MMP-2 and MMP-9 activities and expression, COX-2 levels, PGE_2 signaling, and the AhR involvement in HCB-induced effects. We have used different *in vitro* models: (1) human endometrial stromal cell line T-HESC, (2) primary cultures of Human Uterine Fibroblast (HUF), and (3) primary cultures of endometrial stromal cells from eutopic endometrium of control (CESC) and subjects with endometriosis (EESC). Our results show that HCB enhances MMP-2 and MMP-9 activities in T-HESC, HUF and ESC cells. The MMP-9 levels were elevated in all models, while the MMP-2 expression only increased in ESC cells. HCB enhanced COX-2 and EP4 expression, PGE_2 secretion and the c-Src kinase activation in T-HESC. Besides, we observed that AhR is implicated in these HCB-induced effects. In conclusion, our results show that HCB exposure could contribute to endometriosis development, affecting inflammation and invasion parameters of human endometrial cells.

1. Introduction

Endometriosis is an estrogen-dependent inflammatory disease of reproductive-age women characterized by the presence and growth of endometrial tissue in ectopic sites [1]. The prevalence of the disease is 10–20% in childbearing-age women, increased to 20–30% in women with subfertility, and to 40–60% in women with dysmenorrhea [1,2]. Organochlorine pollutants may play a role in the development of this disease, however, the results of clinical trials are discordant, and it is not clear how the effect of exposure to these compounds is linked to endometriosis. Dioxins and dioxin-like compounds have in particular been associated with the disease, mainly on the basis of their effects on cytokines, immune system, hormones, and growth factors which are thought to increase the risk of endometriosis [3]. Given their capacity to persist in the environment, these compounds tend to accumulate

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in the food chain and the body, being found in adipose tissue, maternal milk and blood, and umbilical blood [4]. Several studies that focused on dioxins or dioxin-like compounds and endometriosis have observed significantly higher concentrations in serum of women with endometriosis than in those without the condition [5].

Hexachlorobenzene (HCB) is a widely distributed organochlorine pesticide and has been classified as a probable human carcinogen [6]. It has been reported that HCB induces toxic reproductive effects [7] and is an endocrine disruptor in laboratory animals [8]. Regional studies demonstrate the presence of this pollutant in mother's milk [9] as well as in samples of bovine milk for human consumption [10]. HCB is a "dioxin-like" compound that binds to the aromatic hydrocarbon receptor (AhR), accumulates in lipid tissue and induces the expression of xenobiotic metabolic enzymes [11]. Some of the biological effects of these compounds are mediated by activation of cytosolic AhR complex (AhR-dioxin-c-Src), triggering: (a) membrane actions, where c-Src activates growth factor receptors, and (b) nuclear actions, where AhR regulates gene transcription including cyclooxygenase-2 (COX-2) [12,13].

COX is the rate-limiting enzyme in the metabolic conversion of arachidonic acid to prostaglandins. Two isoforms of COX have been described: COX-1 is constitutively expressed in many tissues, while COX-2 is inducible by mitogen, growth factors [14] and cytokines [15]. A positive feedback cycle indicates that aromatase, an enzyme involved in estrogen biosynthesis, and COX-2, are responsible for the continuous local formation of estrogen and prostaglandin E_2 (PGE_2) in endometriotic stromal cells [2]. PGE_2 is a major mediator of the pain resulting from the pathophysiology of endometriosis [16]. Multifaceted actions of PGE_2 are mediated through G-protein coupled receptors designated EP1, EP2, EP3 and EP4 [17]. Selective inhibition of PGE_2 receptors EP2 and EP4 suppresses expression and/or activity of matrix metalloproteinases (MMPs) and thereby decreases migration and invasion of human immortalized endometriotic epithelial and stromal cells [17]. In this investigation, the authors found that interactions between EP2/EP4 and MMPs are mediated through c-Src kinase [17]. Takenaka et al. [18] ascertained that EP4 was higher than other EP1–3 gene expression in endometriotic stromal cells, suggesting that EP4 would be the crucial factor for PGE_2 signaling.

Involvement of MMPs in the development of endometriosis is being increasingly confirmed by several research groups, with studies indicating that the pattern of MMPs expression in the endometrium of these women significantly differs from that of healthy women [19]. Studies have shown that the expression of MMPs is increased in the ectopic endometrium, indicating that MMPs may participate in the displacement of the endometrium. Among the MMP family, MMP-2 and MMP-9 are closely associated with the formation of endometriosis [20,21]. In previous studies, we have observed that HCB induces MMP-2 expression and MMP-9 activation in human breast cancer cell line MDA-MB-231, and stimulates tumor growth and metastasis in breast cancer animal models [22].

The objective of the present study was to investigate if HCB exposition may induce alterations that promote endometriosis. We have used three *in vitro* models: (1) human endometrial stromal cell line T-HESC, (2) primary culture of Human Uterine Fibroblast (HUF), and (3) primary cultures of endometrial stromal cells from eutopic endometrium of control patients (CESC) and patients with endometriosis (EESC). We examined the HCB mechanism of action on cell viability, MMP-2 and MMP-9 expressions and activities, COX-2 expression levels and PGE_2 signaling. In addition, we evaluated the AhR role in HCB-induced MMPs activities, COX-2 expression and c-Src activation.

2. Materials and methods

2.1. Chemicals

HCB (>99% purity, commercial grade) was obtained from Aldrich-Chemie GmbH & Co. (Steinheim, Germany). Anti-c-Src and anti-phospho-Y416-c-Src antibodies were purchased from Cell Signalling Technology Inc. (MA). Anti-MMP-2 antibody was obtained from Santa Cruz Biotechnology (CA, USA); and anti-MMP-9 antibody was purchased from Millipore (Temecula, California, USA). Anti-COX-2, anti-AhR and anti-cytokeratin antibodies were obtained from Abcam Ltd. (Cambridge, UK). Monoclonal antibody anti-β-Actin, antibiotic–antimycotic, trypsin, glutamine, α-naphthoflavone (ANF) and 4,7-orthophenanthroline (4,7 PHE) specific inhibitors for AhR, as well as gelatin of zymography assay were obtained from Sigma Chemical Co. (St. Louis, MO). Polyclonal anti-EP4 antibody was purchased from Santa Cruz Biotechnology, INC (Santa Cruz, CA). For PGE_2 determination, [5,6,8,9,11,12,14,15 (n)-3H]-PGE_2 (130 Ci/mmol) was obtained from Amersham Corporation (Arlington Heights, IL, USA). PGE_2 antiserum was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-vimentin antibody was obtained from DAKO Cytomation, Glostrup, Denmark. The secondary antibodies peroxidase-accomplished goat, rabbit and mouse anti-immunoglobulin, and polyvinylidene difluoride (PVDF) membranes were purchased from Bio-Rad Laboratories (CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Promega (Southampton, UK). The enhanced chemiluminescence kit (ECL) and protein electrophoresis molecular markers were purchased from GE Healthcare Life Sciences (Buckinghamshire, UK). Phenol red-free DMEM: F-12 and RPMI culture media were obtained from Beckton and Dikinson. Fetal bovine serum (FBS) was purchased from Invitrogen Life Technology (Carlsbad, CA). All other reagents used were of analytical grade.

2.2. Collection of human biopsies

A total of 19 women in reproductive age who underwent diagnostic laparoscopy for infertility participated in this study were: 12 with untreated endometriosis (Stages I, II, III and IV according to the Revised American Society for Reproductive Medicine Classification 1997) [23] and 7 controls. Endometriosis was confirmed by laparoscopy and histological documentation. Control subjects were infertile women with tubal factor or unexplained infertility undergoing diagnostic laparoscopy. To avoid false negatives, only patients who did not complain of pelvic pain were considered for the control group. All patients were infertile, showed regular menstrual cycles and had not received any hormonal medical treatment for the last 6 months. All subjects signed informed consent prior to evaluation. Biopsies of eutopic endometrium were obtained from all subjects as described previously [24]. Biopsies were taken with a metal Novak curette from the posterior uterine wall. This study was approved by the Ethics and Research Committee from the Biology and Experimental Medicine Institute (IBYME-CONICET) of Buenos Aires, Argentina.

2.3. Isolation and culture of human endometrial stromal cells (ESCs)

The eutopic tissue biopsies were collected under sterile conditions, placed in 1:1 formula of DMEM:F-12 culture medium and transported to the laboratory. Endometrial stromal cells (ESCs) were isolated from endometrial biopsies as was previously described [25] with minor modifications. The eutopic endometrium was minced with scissors and digested with collagenase type IA in culture medium for 2 h at 37 °C. Then, the samples were

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centrifuged for 5 min at 400×g and the supernatant was discarded, the pellet was resuspended in DMEM:F-12 and filtered through a 40 μm sieve to separate the epithelial cell fraction. The filtered cellular suspension containing the stromal cell fraction was centrifuged again for 5 min at 400×g. The pellet was resuspended in 1 ml of DMEM:F-12 with 10% fetal bovine serum (FBS) and the cell number was determined employing a Neubauer chamber. The primary ESCs were seeded in a 25 mm^2 culture flask and were cultured with phenol red-free DMEM:F-12 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin and 250 ng/ml amphotericin B (complete medium). When the culture reached a confluence of 90% the cells were detached with 0.25% trypsin and 0.05 mM EDTA and split 1:3 in a 25 mm^2 culture flask. Afterward, the cells were detached again with trypsin–EDTA and cultured in a 75 mm^2 flask up to fourth passage, when the ESCs were used in the respective experiments included in this study.

2.4. Isolation and culture of Human Uterine Fibroblasts (HUFs)

Human term placentae of cesarean section were granted by the Hospital Italiano, Buenos Aires, Argentina. Human Uterine Fibroblasts (HUFs) were isolated from decidua parietalis of normal term placentae as described [26] but slightly modified. Briefly, cells were isolated as described previously [27] within 1–2 h after delivery. HUF cells were used for experiments between passage 3 and 5 and their morphology was studied by hematoxylin and eosin staining. To evaluate the number of purified cells and its viability we used a Neubauer chamber and dye Trypan blue. We assessed the expression of cytoskeletal proteins, vimentin and cytokeratin by immunofluorescence staining. The purity of the stromal cells used in these studies was greater than 90%. We determined by immunofluorescence positive staining of vimentin (fibroblast cell) and negative expression of cytokeratin (epithelial cell). The cells were cultured at 37 °C in a 5% CO_2 atmosphere with phenol red-free RPMI culture medium supplemented 10% FBS, 1% antibiotic–antimycotic mixture (10,000 U/ml penicillin, 10 mg/ml streptomycin sulfate, and 25 μg/ml amphotericin B), 1% glutamine and 10 mM sodium pyruvate (complete medium).

2.5. Cell culture

Telomerase-immortalized Human Endometrial Stromal Cells line (T-HESC) was purchased from American Type Culture Collection (ATCC, CRL-4003). T-HESCs were cultured at 37 °C in a 5% CO_2 atmosphere with phenol red-free DMEM:F-12 (1:1) culture medium supplemented with 10% FBS, 1% antibiotic–antimycotic mixture (10,000 U/ml penicillin, 10 mg/ml streptomycin sulfate, and 25 μg/ml amphotericin B), and 1% glutamine (complete medium).

2.6. Cell treatment for time-course and dose–response studies

T-HESC, HUF and ESC cells were seeded in 6-well plates (2.5×10^5 cells) and cultured with complete medium followed by overnight incubation to allow cells to attach. Afterward, the medium was withdrawn and replaced with fresh culture medium supplemented with 2% FBS (HUF and ESC) or without FBS (T-HESC) for 24 h and finally cells were treated with HCB (0.005, 0.05, 0.5, and 5 μM) dissolved in absolute ethanol, according to the assay. Final ethanol concentration in each treatment was 0.5% and had no influence on the analyzed parameters as shown previously [28]. In dose–response studies, cells were exposed for 1, 3, 6 and 24 h, depending on the assay, to HCB (0.005, 0.05, 0.5, and 5 μM) in complete medium. The selected higher dose of HCB (5 μM) was in the same range as that found in human serum from a highly contaminated population [29]. In addition, the HCB dose of 0.5 μM is similar to that observed in human serum samples from general population in France [30]. After HCB exposure, cells were washed twice with ice-cold phosphate-buffered saline and processed according to the experiment. For assays performed in the presence of specific AhR inhibitors, cells were pretreated for 1 h with 6, 10, 20 and 30 μM 4,7-orthophenanthroline (4,7 PHE) or with 0.1, 0.2, 0.5 and 1 μM α-naphthoflavone (ANF). HCB (0.5 μM) or vehicle was added to the media for 3 and 24 h in the presence or absence of inhibitors. The HCB dose and time of exposure in this assay were selected because they were effective to induce MMPs activities, COX-2 expression and c-Src activation.

2.7. Viability assay

The measurement of cell viability 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 spectrophotometer at 570 nm. Briefly, 6×10^3 cells were seeded in 96-well plates and maintained in DMEM:F-12 (T-HESC) or RPMI (HUF) complete media for 24 h. The next day media were removed, and serum-free media were added. Finally, cells were treated with HCB (0.005, 0.05, 0.5, and 5 μM) or ETOH in complete DMEM:F-12 medium for 24 h. After that, MTT (0.5 mg/ml) solution dissolved in DMEM:F-12 or RPMI without phenol-red was added to each well and incubated for 1 h at 37 °C. Formazan crystals were dissolved in 100 μl dimethyl sulfoxide, and the absorbance of the solution was measured at 570 nm using the microplate reader Synergy HT (Biotek Instruments, Inc.). The results were expressed as percentage of ETOH treated cells. For each experiment, at least three independent assays were performed.

2.8. Gelatin zymography

Aliquots (10–50 μl) of cell-conditioned media were resuspended in Laemmli modified buffer [10 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 0.03% bromophenol blue, 10% glycerol], and denatured at 95 °C for 5 min. Samples were loaded on 6 or 7.5% SDS–polyacrylamide gel electrophoresis gels containing 1% gelatin and electrophoresed. After electrophoresis, gels were washed twice with Rinsing I buffer [50 mM Tris–HCl (pH 7.4), 2.5% Triton X-100], for 30 min, and then three times with Rinsing II buffer [50 mM Tris–HCl (pH 7.4)], for 5 min, to remove SDS, and subsequently incubated for 24, 48 or 72 h at 37 °C in developing buffer containing [50 mM Tris–HCl, 0.15 M NaCl and 10 mM $CaCl_2$ (pH 7.4)]. MMP-2 and MMP-9 activities were visualized by 0.5% Coomassie brilliant blue R-250 staining. Gels were scanned and clear bands were quantified by scanning laser densitometry in a Fotodyne (Foto/Analyst), Gel-Pro Analyzer 3.1. Values were normalized by immunoblotting using anti-β-Actin antibody. Each experiment was repeated at least three times, and the results were normalized to arbitrary units, designating a value of 100 to control assays.

2.9. Western blotting

Total cellular protein lysates were electrophoresed in 10–12% SDS–PAGE, prior to transfer to polyvinylidene difluoride membranes in a semidry transfer cell at 18 V for 1.5 h. Membranes were blocked overnight at 4 °C with 5% nonfat dry milk-2.5% BSA in Tris saline solution with Tween 20 (TBST) buffer (10 mM Tris–HCl, pH 8.0, 0.5% Tween 20, 150 mM NaCl). Membranes were incubated with rabbit polyclonal antibodies, anti-c-Src (1:500), anti-phospho-Y416-c-Src (1:500), anti-MMP-2 (1:250), anti-MMP-9

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(1:500), anti-EP4 (1:500) or anti-COX-2 (1:500). On the other hand, membranes were incubated with mouse monoclonal antibody anti-AhR (1:500) and anti-β-actin (1:2000) overnight at 4 °C as loading control. After incubation, membranes were washed five times with TBST, one time with TBS (without Tween 20), and the suitable peroxidase-conjugated anti species-specific antibodies were used for protein detection. After washing, blots were reacted using ECL detection kit (Amersham Biosciences, Inc., UK) and quantified by scanning laser densitometry in a Fotodyne (Foto/Analyst), Gel-Pro Analyzer 3.1.

2.10. PGE_2 determination

The amount of PGE_2 was assayed in the collected media by specific radioimmunoassay according to the manufacturer's protocol (Sigma). Specific antiserum for PGE_2 was used. Labeled [3H]-PGE_2 was added to each tube. The incubation was performed for 90 min at 4 °C. Bound and free radioligands were separated by dextran-coated charcoal and the tubes were centrifuged for 15 min at 2000×*g*. The sensitivity of the assay was 5–10 pg/ml and the cross-reactivity was less than 0.1% with other PGs. Intra- and inter-assay variations were each <8.0%. Results were expressed as pg PGs/ml/24 h.

2.11. Statistical analysis

Data were evaluated by a 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. Viability assay

It has been shown that organochlorine pesticides or their metabolites could induce cell proliferation in estrogen-sensitive cells [28], or cytotoxicity causing a significant loss of cell viability [31]. The viability of T-HESC and HUF cells was determined by an MTT assay. Cells were treated with HCB (0.005, 0.05, 0.5 and 5 μM) for 24 h. Our results show that the pesticide didn't produce changes in cell viability in T-HESC or HUF cells ($p > 0.05$) (Fig. 1A and B).

3.2. Metalloproteinase activities

Secretion and activities of MMPs are critical for tissue remodeling throughout the menstrual cycle and has been postulated to be a major player in the pathogenesis of the endometriosis [32]. We have previously reported that HCB significantly increases MMP-9 secretion and activity in human breast cancer cells MDA-MB-231 [22]. T-HESC, HUF, and ESCs were treated with HCB (0.005, 0.05, 0.5, and 5 μM) or ETOH for 24 h, and then culture medium was used to analyze MMP-2 and MMP-9 activities by gelatin zymography. Secretion of active forms of MMP-2 and MMP-9 proteins by cells into the culture medium was clearly evident by gelatinolytic activity seen at 66 kDa and 82 kDa, respectively.

Results indicate that HCB increased MMP-2 (50 and 54%) ($p < 0.05$) and MMP-9 (75 and 51%) activities at 0.5 ($p < 0.01$) and 5 μM ($p < 0.05$), respectively in T-HESC cells (Fig. 2A). Besides, the pesticide significantly enhanced the activities of MMP-2 (50%) and MMP-9 (53%) at 0.5 μM in HUF cells ($p < 0.05$) (Fig. 2B). When we evaluated human ESC, our results show that HCB treatment enhanced MMP-9 (87%) ($p < 0.05$) and MMP-2 (190%) ($p < 0.01$) activities at 0.05 μM in cell culture from CESC (Fig. 2C). In EESC cultures, HCB increased MMP-9 activity (48%) at 0.005 μM ($p < 0.05$) and MMP-2 activity at 0.5 μM (65%) ($p < 0.001$) (Fig. 2D).

3.3. Metalloproteinase expression levels

In order to evaluate if HCB-induced increase in MMP-2 and MMP-9 activities is related to an enhancement on protein levels, cells were treated with HCB (0.005, 0.05, 0.5 and 5 μM) or ETOH for 24 h, and MMPs expression protein levels were assayed by Western blot. T-HESC exposure to HCB resulted in an increase in MMP-9 protein levels (67%, 60% and 55%) at 0.05, 0.5 and 5 μM respectively ($p < 0.05$) (Fig. 3A), while in HUF cells the pesticide raised the MMP-9 protein levels only to the dose of 0.5 μM (125%) ($p < 0.001$) (Fig. 3B). HCB did not produce changes on MMP-2 protein levels in both models (Fig. 3A and B). Severity of endometriosis is primarily associated with degree of expression or activity of MMP-2 and MMP-9 [33,34]. When we studied HCB

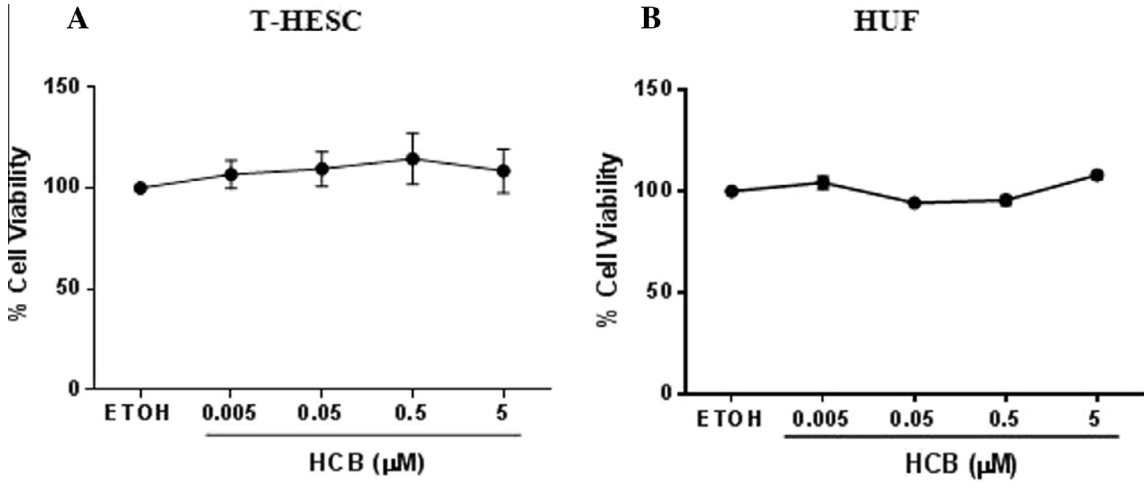

Fig. 1. Effect of HCB on cell viability of T-HESC and HUF cells. (A) Viability of T-HESC cells, and (B) HUF cells. The viability of cells was evaluated using the MTT assay. Cells were incubated with MTT (0.5 mg/ml) for 1 h at 37 °C after HCB (0.005, 0.05, 0.5, and 5 μM) treatment for 24 h. Then the absorbance was measured at 570 nm and the results were expressed as percentage of ETOH treated cells. Data are expressed as mean ± SDs of three independent experiments. Statistical comparisons were made by an analysis of variance (one-way ANOVA), with a 95% confidence interval followed by Tukey post hoc test to identify significant differences between mean values and indicated controls.

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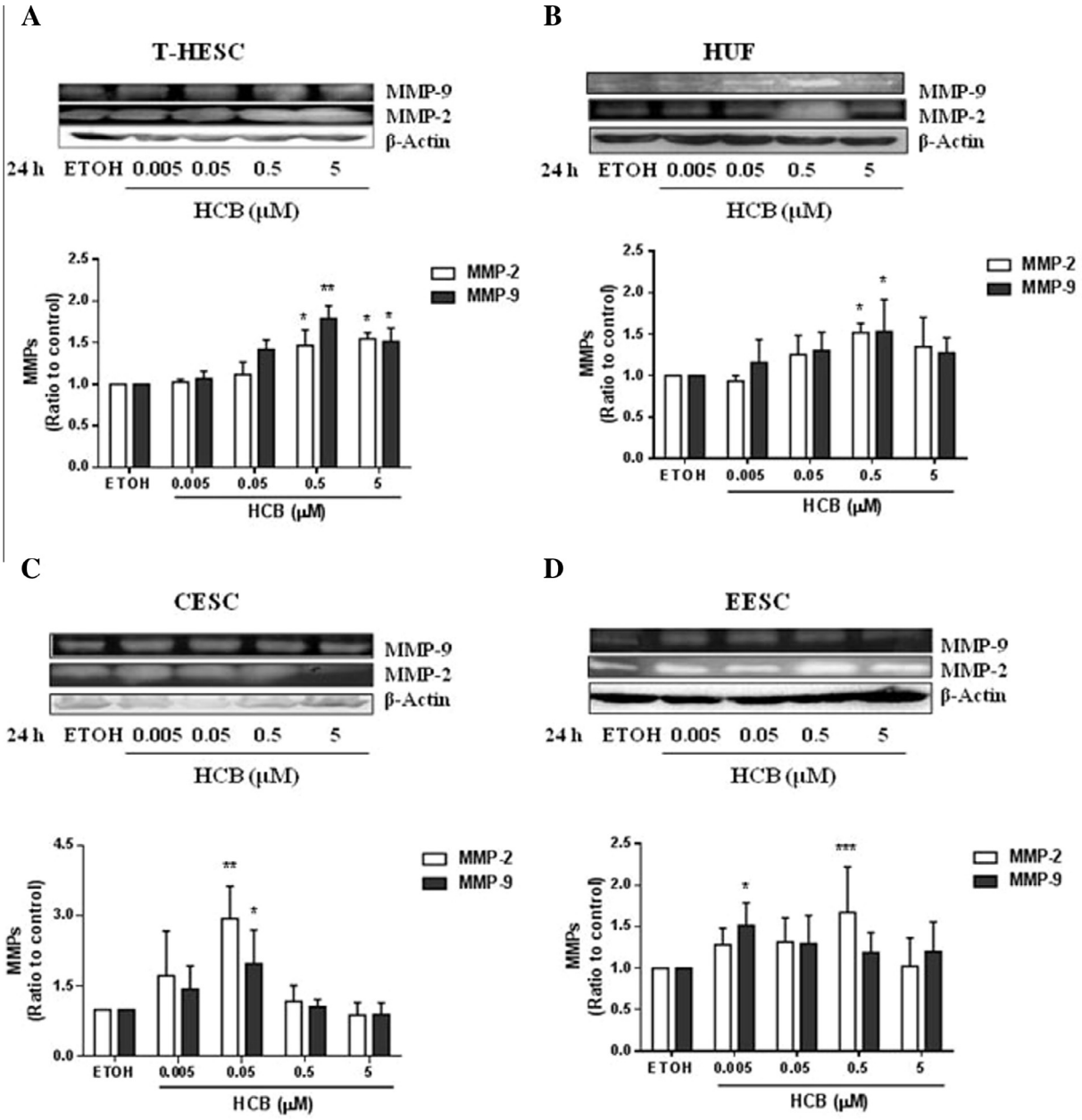

Fig. 2. HCB effects on MMP-2 and MMP-9 activities. (A) MMP-2 and MMP-9 activities in T-HESCs, (B) in HUF cells; (C) in CESC, and (D) EESC cultures. Cells were treated with HCB (0.005, 0.05, 0.5, and 5 μM) or vehicle (ETOH) for 24 h. Cell culture-conditioned media were collected and MMP-2 and MMP-9 activities quantified by gelatin zymography. Secreted MMPs activities were relativized to β-Actin expression. One representative experiment is shown in the upper panels. Quantification by densitometric scanning of the MMPs activities is shown in the lower panels. Data are expressed as mean ± SDs of three independent experiments. Asterisks indicate significant differences vs. control ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$), ANOVA and Tukey posthoc test.

effects on MMPs protein levels in ESC cultures, we found that the pesticide increased MMP-9 (65%) and MMP-2 (52%) protein expression at 0.05 μM in CESC ($p < 0.05$) (Fig. 3C). In EESC, HCB enhanced MMP-9 protein expression at 0.005 (100%) ($p < 0.05$) and 0.5 μM (97%) ($p < 0.01$), as well as MMP-2 protein levels at 0.05 (100%) and 0.5 μM (185%) ($p < 0.05$) (Fig. 3D).

3.4. HCB action on COX-2 protein levels

In vitro studies indicated that COX-2 inhibitor prevented growth of primary cultured eutopic endometrial epithelial cells from endometriosis patients [35], as well as decreased establishment of endometriotic implants in rats [36]. To examine if HCB exposure produces COX-2 protein expression alterations, we treated T-HESC, HUF cells, and the ESC for 24 h with HCB (0.005, 0.05, 0.5 and 5 μM). Our results show that HCB did not change COX-2 protein expression levels at 24 h in T-HESC and HUF cells ($p > 0.05$) (Fig. 4A and B). Conversely, in CESC cultures, we observed that HCB heavily increased the COX-2 protein expression at 0.05 μM (385%) ($p < 0.05$), finding a non-significant increase at 0.005 μM ($p > 0.05$) (Fig. 4C). On the other hand, in EESC, COX-2 protein expression was induced at 0.5 μM (120%) ($p < 0.05$), while we have not observed any significant increase at 0.005 and 0.05 μM ($p > 0.05$) (Fig. 4D).

3.5. Time and dose–response study of HCB effects on COX-2 protein levels in T-HESC

In order to determine HCB effects on COX-2 protein levels and PGE_2 signaling pathway, we have decided to evaluate these

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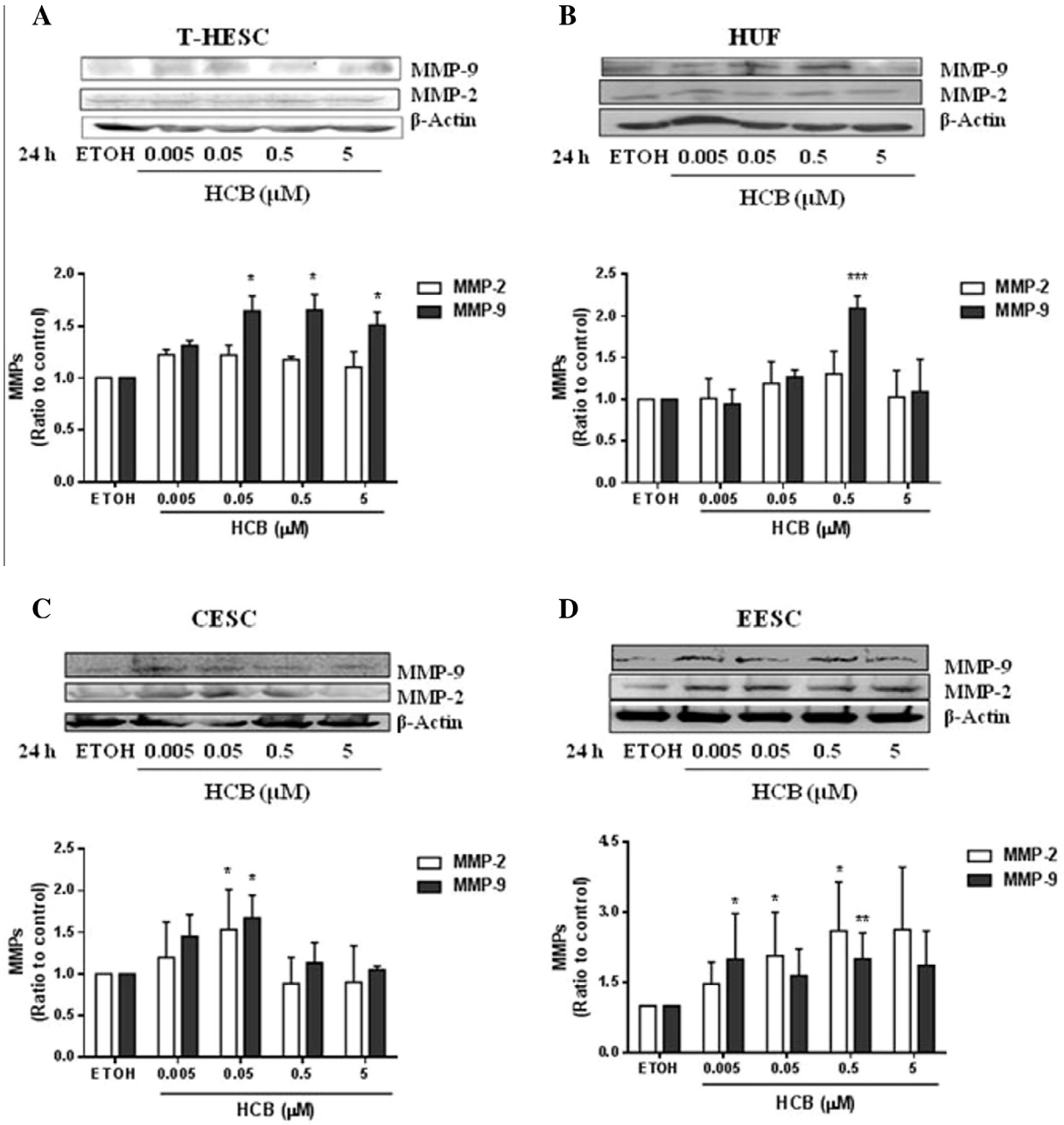

Fig. 3. HCB action on MMP-2 and MMP-9 protein expression levels. (A) MMP-2 and MMP-9 protein expression levels in T-HESC, and (B) in HUF cells; (C) in CESC, and (D) EESC cultures. Cells were treated with HCB (0.005, 0.05, 0.5, and 5 μM) or vehicle for 24 h. Whole-cell lysates were prepared and protein levels were analyzed by Western blot. Intracellular protein MMPs levels were relativized to β-Actin expression. One representative experiment is shown in the upper panels. Quantification by densitometric scanning of the immunoblots is shown in the lower panels. Data are expressed as mean ± SDs of three independent experiments. Asterisks indicate significant differences vs. control ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$), ANOVA and Tukey posthoc test.

parameters only in T-HESC. The decision was supported by our similar results obtained in the three different models T-HESC, HUF and ESC. As COX-2 is an inducible enzyme, and herein we observed that HCB did not alter COX-2 expression after 24 h of treatment in T-HESC, we examined COX-2 levels in dose–response curves at 1, 3 and 6 h with HCB (0.005, 0.05, 0.5 and 5 μM) or vehicle. Our results show that HCB exposure at 1 h did not produce changes in COX-2 protein levels ($p > 0.05$) (Fig. 5A). Conversely, at 3 h of treatment the pesticide significantly enhanced COX-2 protein levels at 0.05 ($p < 0.05$) and 0.5 μM ($p < 0.01$) (95 and 105%) (Fig. 5B). In addition, HCB exposure for 6 h increased COX-2 expression protein at 0.5 ($p < 0.01$) and 5 μM ($p < 0.001$) (85 and 84%) (Fig. 5C).

3.6. Effect of HCB exposure on PGE_2 secretion in T-HESC

In endometriosis, stromal and epithelial cells from the endometrium form extrauterine lesions and persist in response to estrogen and PGE_2. Stromal cells produce excessive quantities of estrogen and PGE_2 in a feed-forward manner [37]. In this study, we have observed that HCB increased COX-2 expression levels in T-HESC and in ESCs cultures. In order to evaluate if HCB induce changes in PGE_2 secretion, we have examined PGE_2 levels in conditioned medium from T-HESC exposed to HCB (0.005, 0.05, 0.5 and 5 μM) for 24 h. As shown in Table 1, the pesticide significantly enhanced PGE_2 secretion at 0.05 μM in T-HESC (52%) ($p < 0.05$). No changes were observed at higher HCB doses ($p > 0.05$).

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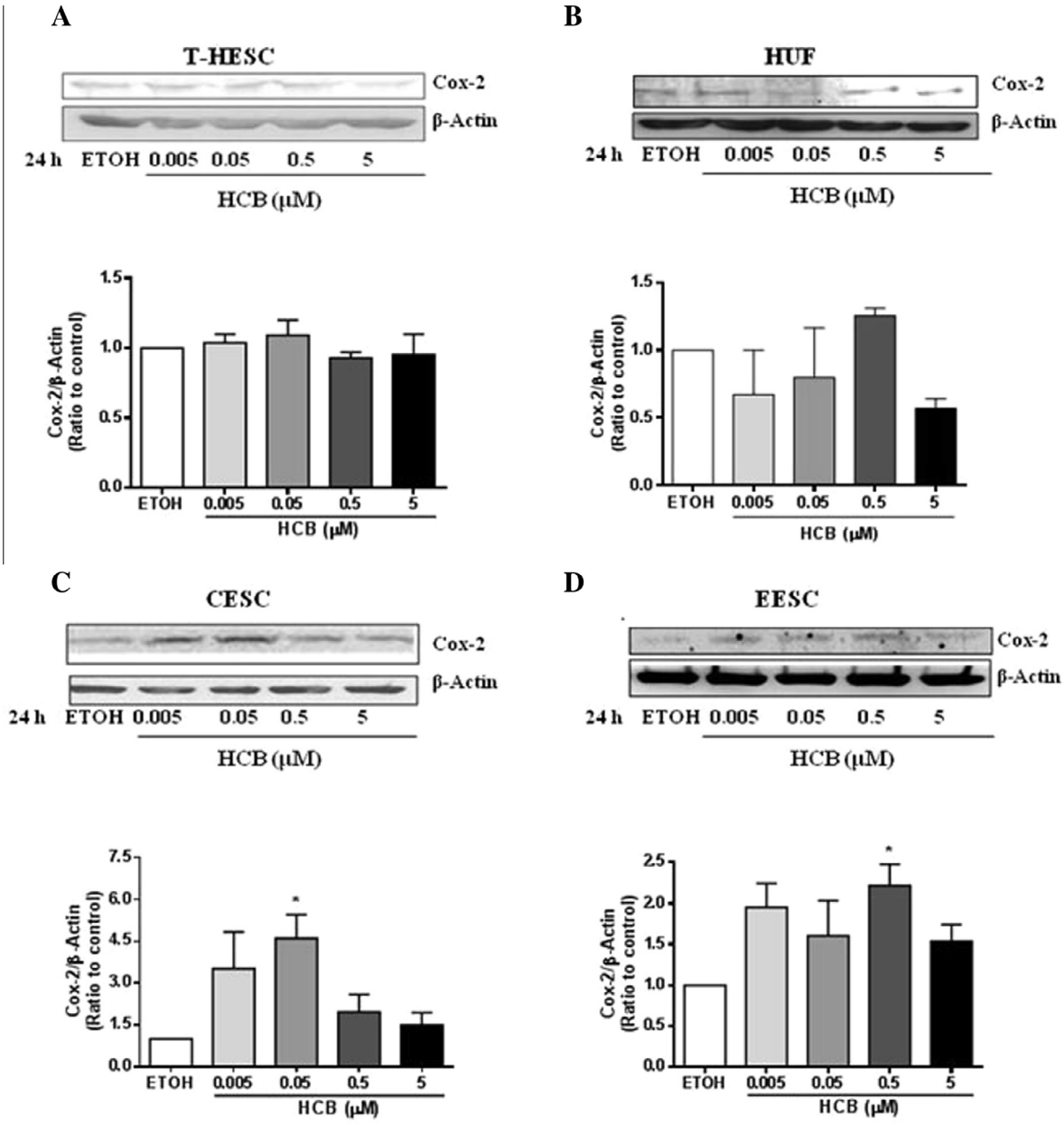

Fig. 4. COX-2 expression levels in HCB-exposed human endometrial cells. (A) COX-2 expression levels in T-HESC, (B) HUF cells; (C) CESC, and (D) EESC cultures. Cells were treated with HCB (0.005, 0.05, 0.5, and 5 μM) or vehicle for 24 h. Whole-cell lysates were prepared and protein levels were analyzed by Western blot. Intracellular protein COX-2 levels were relativized to β-Actin expression. One representative experiment is shown in the upper panels. Quantification by densitometric scanning of the immunoblots is shown in the lower panels. Data are expressed as mean ± SDs of three independent experiments. Asterisks indicate significant differences vs. control ($^{*}p < 0.05$), ANOVA and Tukey posthoc test.

3.7. EP4 levels in T-HESC

PGE_2 binds to EP4 and triggers c-Src kinase phosphorylation and MMPs activation, cell migration and invasion as described [17]. EP4 gene expression was higher than other EP1–3 expressions in endometriotic stromal cells, being a crucial factor for PGE_2 signaling [18]. To assess whether HCB exposure alters EP4 protein levels, we evaluated those in dose–response assays at 1, 3 and 6 h. The results obtained demonstrated that HCB 0.005, 0.5 and 5 μM (55, 35, 40%) significantly increased EP4 expression at 1 h of treatment ($p < 0.001$) (Fig. 6A). On the other hand, we found that at 3 h of exposure, HCB 0.005 ($p < 0.01$), 0.05 ($p < 0.001$) and 0.5 μM ($p < 0.01$) (96, 167, 99%) enhanced EP4 levels, showing a greater effect than the one observed at 1 h of exposure (Fig. 6B). Finally, no changes in EP4 protein levels were observed after HCB treatment for 6 h ($p > 0.05$) (Fig. 6C).

3.8. c-Src kinase activation in T-HESC

Previous data of our laboratory have demonstrated that HCB activates c-Src kinase in human breast cancer cell line MDA-MB-231 [38]. As c-Src kinase transduces EP4 signaling in endometriotic stromal and epithelial cells, and we reported in this study that HCB increases EP4 levels, we evaluated the HCB effect on Tyr416 phosphorylation of c-Src in T-HESC. As shown in Fig. 7A and B, HCB significantly increased c-Src activation at 1 h of exposure at 0.5 μM (54%) ($p < 0.05$), as well as at 3 h of treatment at 0.005, 0.05 and 0.5 μM (40, 45, 47% respectively) ($p < 0.05$). Finally, when the cells

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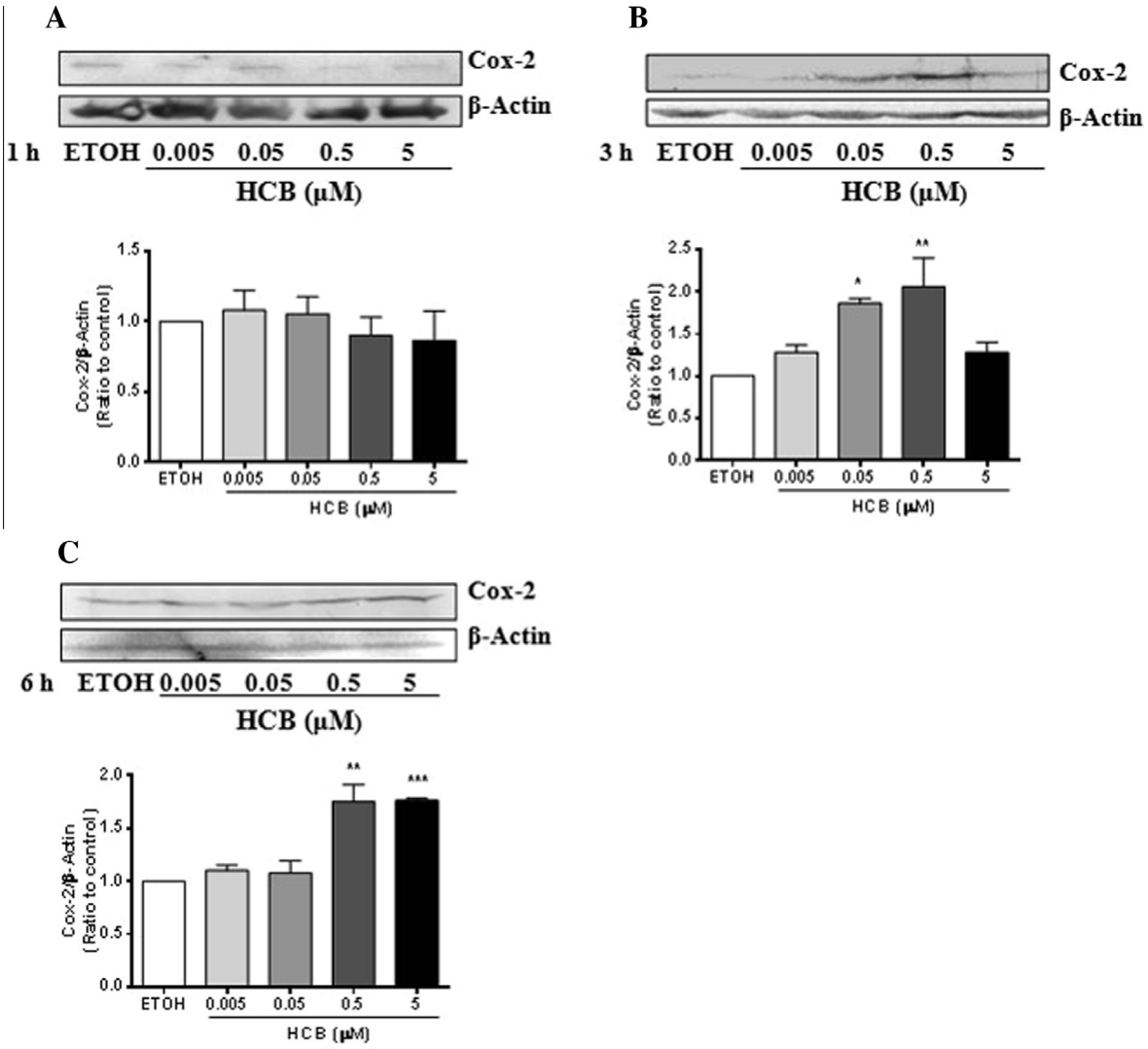

Fig. 5. Time and dose–response study of HCB effects on COX-2 expression levels in T-HESC. (A) COX-2 expression levels for 1 h, (B) 3 h and (C) 6 h after HCB (0.005, 0.05, 0.5, and 5 μM) exposure. Whole-cell lysates were prepared and protein levels were analyzed by Western blot. Intracellular protein COX-2 levels were relativized to β-Actin expression. One representative experiment is shown in the upper panels. Quantification by densitometric scanning of the immunoblots is shown in the lower panels. Data are expressed as mean ± SDs of three independent experiments. Asterisks indicate significant differences vs. control ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$), ANOVA and Tukey posthoc test.

Table 1
Effect of HCB exposure on PGE_2 secretion. T-HESC cells were treated with HCB (0.005, 0.05, 0.5 and 5 μM) or vehicle for 24 h. The amount of PGE_2 was assayed in the collected media by specific radioimmunoassay according to the manufacturer's protocol (Sigma). Specific antiserum for PGE_2 was used. Labeled [3H]-PGE_2 was added to each tube. The incubation was performed for 90 min at 4 °C. Intra- and inter-assay variations were each <8.0%. Results were expressed as pg PGs/ml/24 h. Data are expressed as mean ± SDs of three independent experiments. Asterisks indicate significant differences vs. control ($^{*}p < 0.05$), ANOVA and Tukey posthoc test.

PGE_2 (pg/ml)	ETOH	HCB 0.005 μM	HCB 0.05 μM	HCB 0.5 μM	HCB 5 μM
T-HESCs	1.54 ± 0.177	1.48 ± 0.32	2.34 ± 015(*)	1.73 ± 0.20	155 ± 0.15

were exposed for 6 h, we observed an increase in c-Src phosphorylation in a dose-dependent manner at 0.005 ($p < 0.05$), 0.05 ($p < 0.05$) and 0.5 μM ($p < 0.01$) (390%, 470% and 540%), without changes at 5 μM ($p > 0.05$) (Fig. 7C).

3.9. Role of AhR in HCB-mediated activation of c-Src and MMPs, and COX-2 protein expression in T-HESC

In order to examine if AhR could be mediating the pesticide effects, first we evaluated the AhR protein levels in this cell line. Besides, we examined the HCB action on the AhR protein expression. Our results showed that AhR is expressed in T-HESC, and the pesticide exposure increases these levels (110% and 115%; $p < 0.05$) in the dose-dependent manner (0.5 and 5 μM HCB) (Fig. 8A). To evaluate the role of AhR in HCB-induced MMPs activation, T-HESC was pretreated for 1 h with two structurally different AhR antagonist 4,7 PHE (6, 10, 20 and 30 μM) or ANF (0.1, 0.2, 0.5 and 1 μM). Then, cells were exposed to HCB (0.5 μM) or vehicle for 24 h. In Fig. 8B and C we show that HCB enhanced MMPs activities through an AhR-dependent mechanism ($p < 0.05$ and $p < 0.01$).

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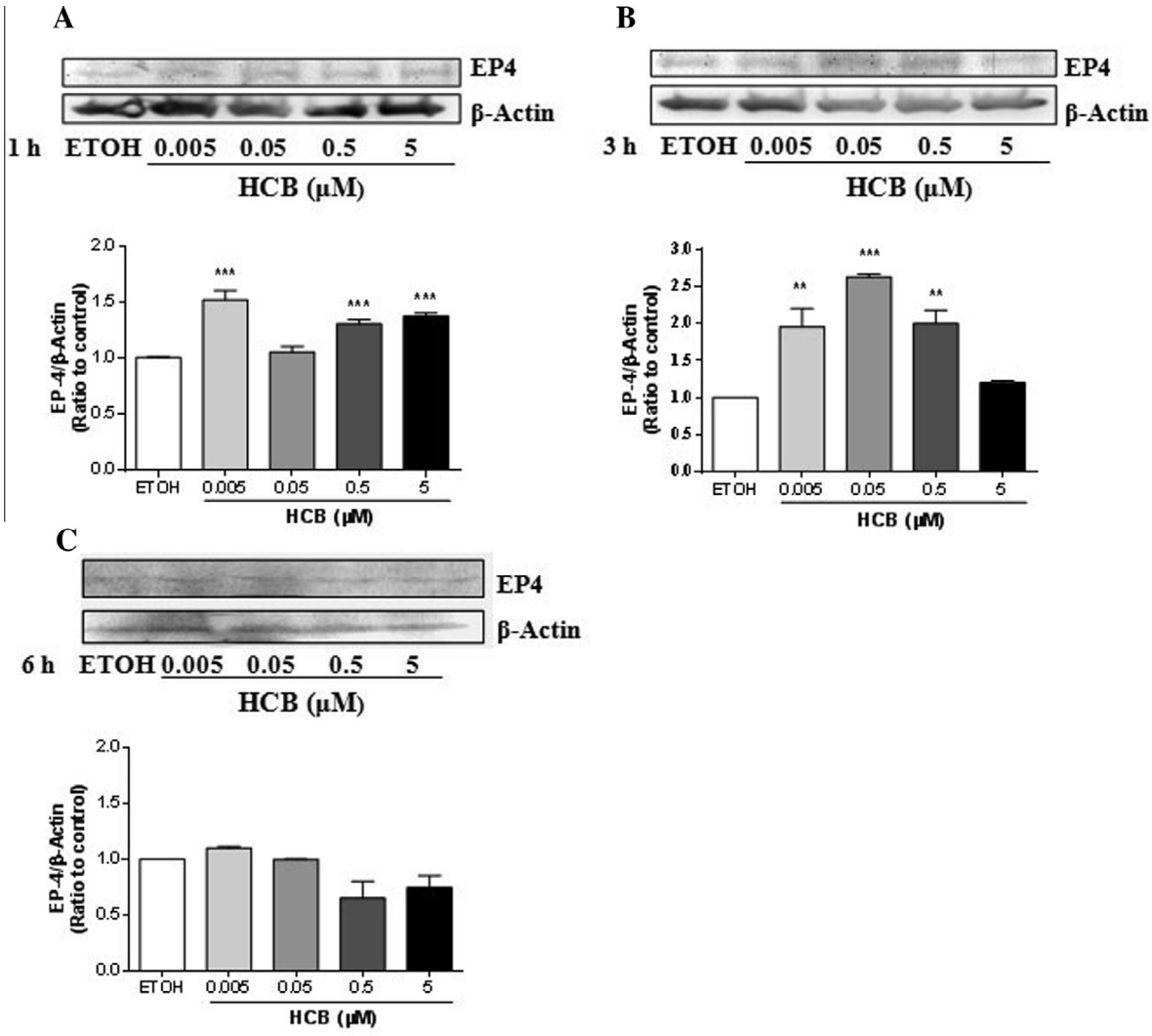

Fig. 6. HCB exposure alters EP4 protein levels in T-HESC. (A) EP4 expression levels in T-HESC for 1 h, (B) 3 h and (C) 6 h after HCB (0.005, 0.05, 0.5, and 5 μM) exposure. Whole-cell lysates were prepared and protein levels were analyzed by Western blot. Intracellular protein EP4 levels were relativized to β-Actin expression. One representative experiment is shown in the upper panels. Quantification by densitometric scanning of the immunoblots is shown in the lower panels. Data are expressed as mean ± SDs of three independent experiments. Asterisks indicate significant differences vs. control ($^{**}p < 0.01$, and $^{***}p < 0.001$), ANOVA and Tukey posthoc test.

The role of AhR in promoting inflammatory responses through the induction of COX-2 protein is well known [39]. In order to determine the contribution of AhR in HCB effect on increase in c-Src activation and COX-2 protein levels, we pretreated T-HESC with 4,7 PHE or ANF and then cells were exposed to HCB (0.5 μM) or vehicle for 3 h. Our results showed that the enhancement in c-Src phosphorylation ($p < 0.05$, $p < 0.01$) and COX-2 expression levels ($p < 0.05$) by the pesticide was AhR-dependent (Fig. 9A and B).

4. Discussion

Experimental studies on rodents and primates indicate that adult exposure to endocrine disruptors as organochlorines can promote endometriosis [40]. However, data linking organochlorine exposure and endometriosis in humans are equivocal [41]. Investigations into the pathophysiology of endometriosis suggest that disease onset and progression involve steroid-related alterations of the endometrium and peritoneal cavity, excess estrogen production by ectopic endometriotic lesions, and changes in ovarian steroidogenesis [1,2]. Thus, environmental chemicals that are endocrine disruptive, or that mimic or alter endogenous hormonal activity, may plausibly affect endometriosis risk. In the present study, we have observed that HCB treatment affects inflammation and invasion parameters of human endometrial cells that could contribute to endometriosis development. Degradation of the extracellular matrix is a basic step in the invasiveness and angiogenesis [42]. Severity and extent of endometriosis is primarily associated with degree of expression or activity of MMP-2 and MMP-9 at endometrial–peritoneal interface [33,34]. In this study, we found that HCB induces an increase in MMP-2 and MMP-9 activities in T-HESC and HUF as well as in ESC cultures derived from endometriosis and control subjects. The detection for active forms of MMPs proteins and their gelatinolytic activities in these experiments were in agreement with previous reports [17]. Our results show a correlation between the increase produced by HCB in protein levels of MMPs and their activities. However, in some cases the pesticide increases the MMPs activities, without changes in the protein levels, as previously observed [43] on MMP-9 in human samples. Yu et al. [44] have reported that the combination of 17β-estradiol with 2,3,7,8-tetrachloro dibenzo-p-dioxin (TCDD) enhanced the expression and activity of MMP-9 and MMP-2 in endometrial stromal cells from patients with

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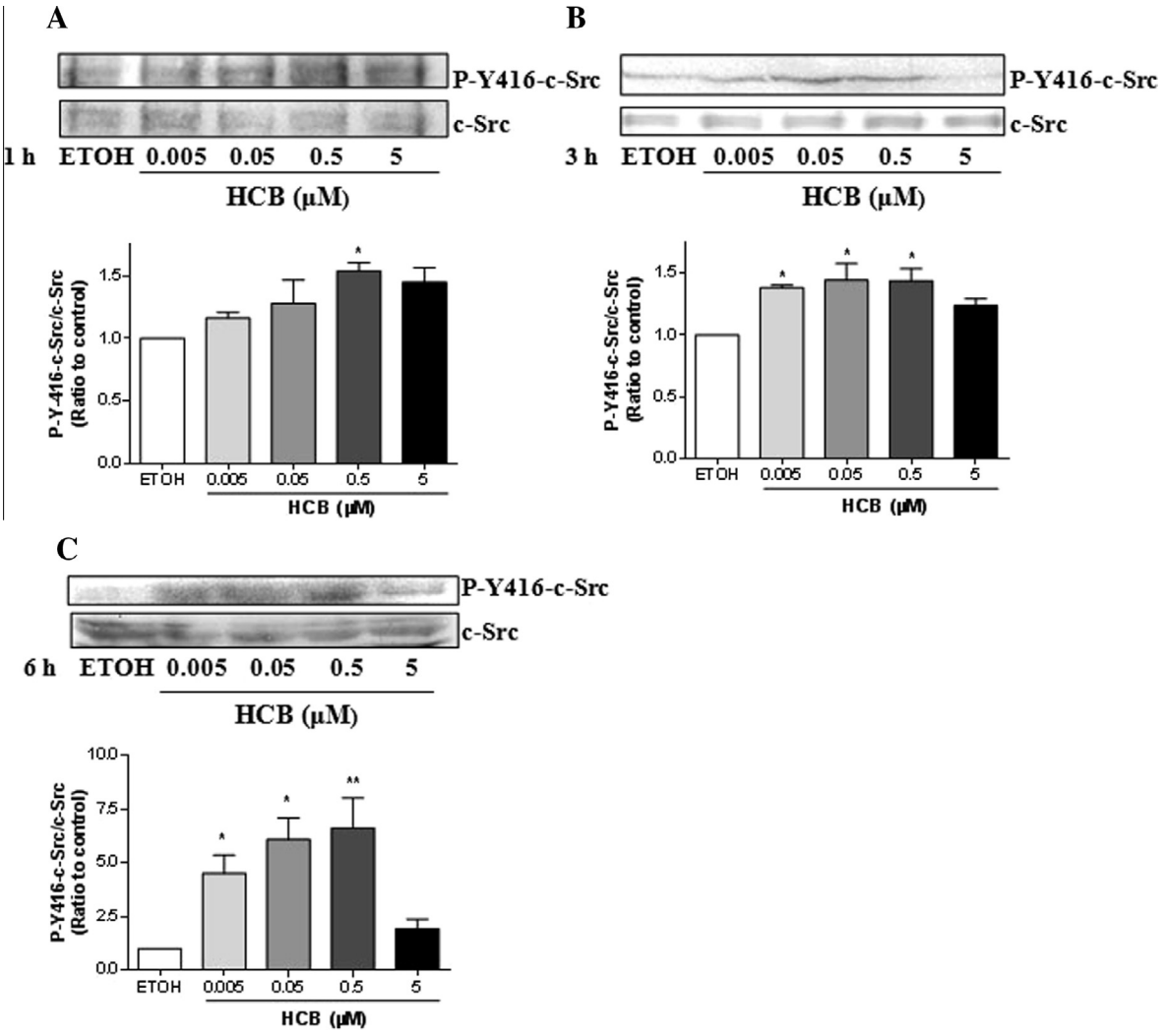

Fig. 7. Time-courses of HCB effect on c-Src activation. (A) c-Src activation in T-HESC for 1 h, (B) 3 h and (C) 6 h after HCB exposure. Cells were treated with HCB (0.005, 0.05, 0.5, and 5 μM) or vehicle for 1, 3 or 6 h. Whole cell lysates were resolved by SDS–PAGE and immunoblotted for p-Y416-c-Src, and reblotted for total c-Src as described in Section 2. Intracellular protein p-Y416-c-Src levels were relativized to total c-Src levels. A Western blot from one representative experiment is shown in the upper panels. Quantification of p-Y416-c-Src/total c-Src ratio by densitometry scanning of the immunoblots is shown in the lower panels. Data are expressed as mean ± SDs of three independent experiments. Asterisks indicate significant differences vs. control ($^{*}p < 0.05$, $^{**}p < 0.01$), ANOVA and Tukey posthoc test.

endometriosis. Therefore, the altered regulation of endometrial MMPs expression in response to steroids may represent a mechanism linking the invasive potential of refluxed endometrium to the establishment of this disease, as occurs in certain women [45]. We have previously found that HCB increases MMP-2 and MMP-9 levels, MMP-9 activation and cell invasion in MDA-MB-231 breast cancer cell line [22].

COX-2 is increased in ectopic and eutopic endometrial tissues of endometriosis patients compared to healthy patients [46]. In this work, we note that HCB-treatment at 24 h induced the expression of COX-2 in ESC cultures. Conversely, in T-HESC cells the pesticide increased COX-2 at 3 and 6 h. The modulation of COX-2 expression at 3 h was observed by other authors in endometriotic cells after lipopolysaccharide exposure [18]. Hirata et al. [47] observed that interleukin-17F increased COX-2 gene expression in ESC cultures with a maximal increases at 4 h, followed by a decrease with time up to 24 h. It was found that dioxin type compounds increase the COX-2 mRNA by binding to the AhR, translocation to the nucleus, and binding to xenobiotic response element (XRE) sites on COX-2 promoter [48]. Herein, our results show that AhR is expressed in T-HESC and HCB exposure increases AhR protein levels. In addition, we observed that this receptor is involved in HCB-induced COX-2 expression protein. Recent data of our laboratory clearly demonstrated that HCB enhances COX-2 levels through an AhR-dependent mechanism in HMEC-1 human microvascular endothelial cell line [49]. AhR is also known to be involved in COX-2 mRNA degradation by capturing HUR (a COX-2 mRNA stabilizer) in the nucleus [50]. Our data in T-HESC showed that COX-2 expression after HCB treatment is transient (3–6 h), therefore the COX-2 decay could be occurring mediated by HUR.

Role for PGE_2 is well accepted in the pathogenesis of endometriosis [2,17]. Other authors show that PGE_2 via EP2 and EP4 enhances aromatase activity [51]. Kershaw-Young et al. [52] postulate that estrogen regulates the expression of COX-2 and EP4 mRNA. It has been reported that EP4 gene expression was higher than other EP1–3 in endometriotic stromal cells, therefore, EP4 would be the crucial factor for PGE_2 signaling [18]. In addition, Lee et al. [17] observed that EP2/EP4 signaling pathway promotes activation of c-Src kinase, which triggers the subsequent activation of MMPs. In the present study, we found that HCB induces an increment in PGE_2 secretion, EP4 expression levels and c-Src activation in T-HESC. These HCB effects could be correlated to the

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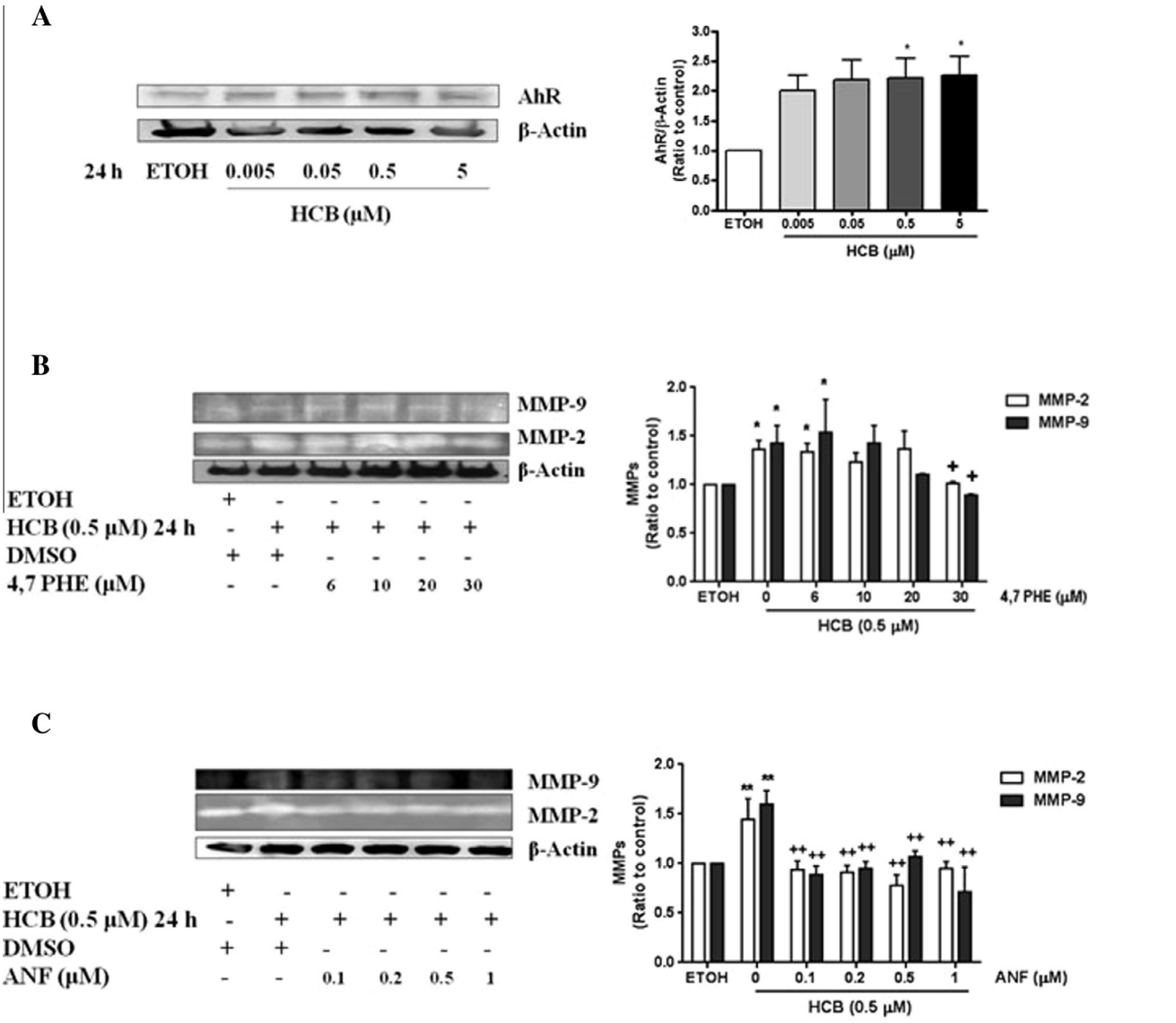

Fig. 8. Role of AhR in HCB-mediated activation of MMPs in T-HESC. (A) AhR protein expression levels. Cells were treated with HCB (0.005, 0.05, 0.5, and 5 μM) or vehicle for 24 h. Whole-cell lysates were prepared and protein levels were analyzed by Western blot. Intracellular protein AhR levels were relativized to β-Actin expression. A Western blot from one representative experiment is shown in the left panel. Quantification of AhR by densitometry scanning of the immunoblots is shown in the right panel. (B) AhR is involved in HCB-induced MMP-2 and MMP-9 activation. T-HESC was pretreated for 1 h with specific AhR antagonist 4,7 PHE (6, 10, 20 and 30 μM) and then were incubated with HCB (0.5 μM) for 24 h in the presence or absence of 4,7 PHE. (C) Cells were pretreated for 1 h with specific AhR antagonist ANF (0.1, 0.2, 0.5 and 1 μM) and then were incubated with HCB (0.5 μM) for 24 h in the presence or absence of ANF. Cell-conditioned media were collected and MMP-2 and MMP-9 activities quantified by gelatin zymography. Secreted MMPs activities were relativized to β-Actin expression. Quantification by densitometric scanning of the MMPs activities is shown in the right panel. Data are expressed as mean ± SDs of three independent experiments. Asterisks indicate significant differences vs. control ($^{*}p < 0.05$, $^{**}p < 0.01$), ANOVA and Tukey posthoc test. Crosses indicate significant differences versus HCB 0.5 μM ($^{+}p < 0.05$, $^{++}p < 0.01$).

enhancement in MMP-2 and MMP-9 activities. Other authors found that TCDD increases PGE_2 levels secreted from explants of human placenta [53]. In a previous work of our laboratory, we observed that HCB induces cell proliferation, c-Src and Estrogen Receptor α activation in human breast cancer cell line MCF-7, without estradiol presence [28]. Taking account our previous investigation and data from this study, we hypothesize that HCB could act as xenoestrogen compound, stimulating COX-2 expression, PGE_2 release, EP4 expression and c-Src phosphorylation. Ohtake et al. [54] reported that gene expression by estrogen receptors could also be regulated by the dioxin-activated AhR because they share their signaling pathways. Herein, we observed that the enhancement in MMPs activities, COX-2 expression protein and c-Src activation induced by HCB exposure are AhR-dependent.

With regard to the HCB effective doses in this study, the alterations observed in ESC cultures occurred at low doses (0.005 and 0.05 μM) in samples from control subjects. This is interesting, since it would mean that low doses (environmental concentrations) are sufficient to induce changes in endometrial cells. These doses acting in a chronic way, could contribute into the development of endometriosis. Since organochlorine pesticides are considered metabolically stable, it seems reasonable to speculate that even low concentrations continuously activate AhR sufficient to maintain the long-term biological effects of these compounds. In contrast, in cultures from samples of women with endometriosis (EESC), the HCB actions have a variable pattern with effects at all assayed doses. Perhaps this behavior is due to multiple pre-existing alterations, and that the exposure to the pesticide would

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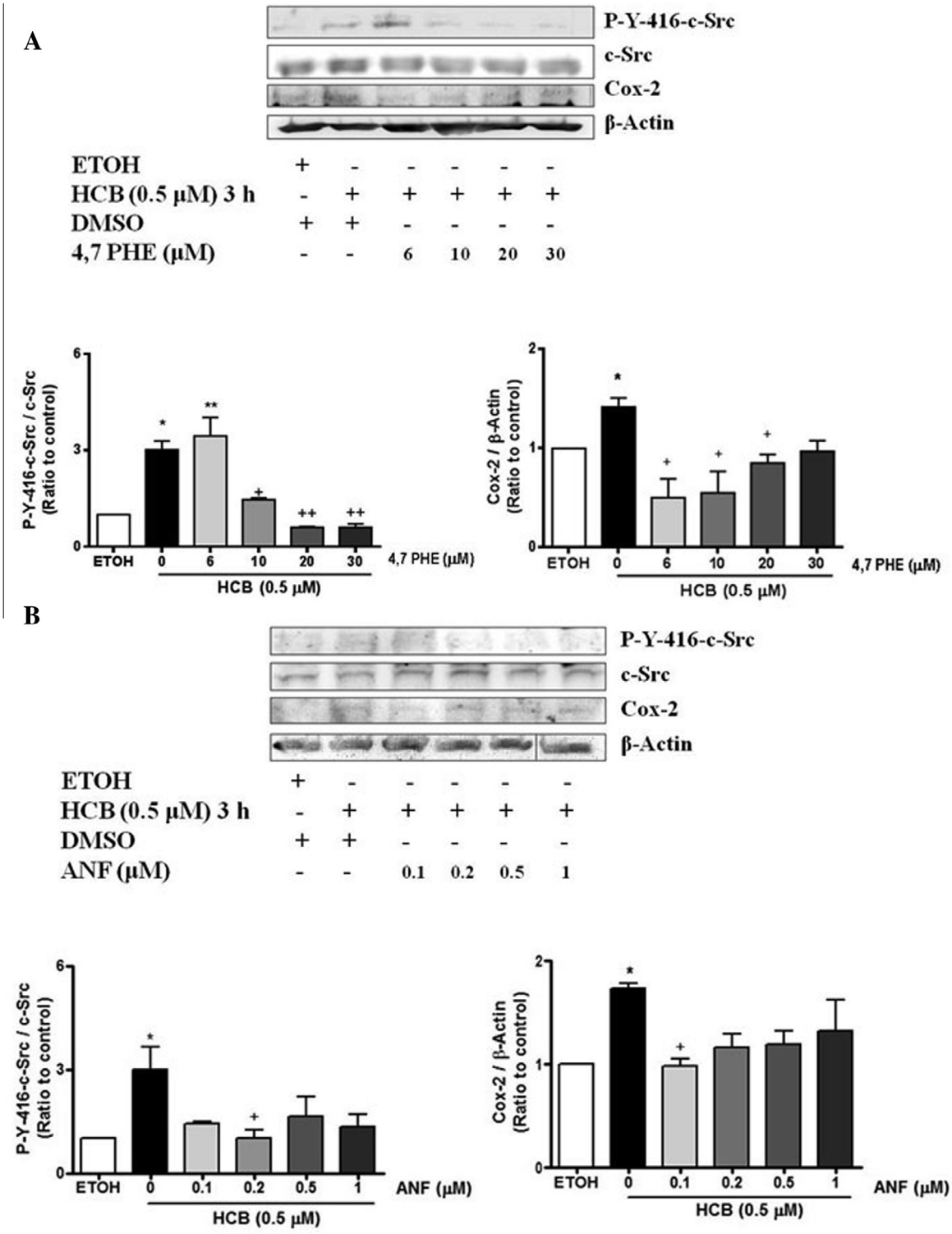

Fig. 9. Role of AhR in HCB-mediated activation of c-Src and COX-2 protein expression in T-HESC. (A) AhR is involved in HCB-induced c-Src activation and COX-2 expression in T-HESC. Cells were pretreated for 1 h with specific AhR antagonist 4,7 PHE (6, 10, 20 and 30 μM) and then were incubated with HCB (0.5 μM) for 3 h in the presence or absence of 4,7 PHE. (B) Cells were pretreated for 1 h with specific AhR antagonist ANF (0.1, 0.2, 0.5 and 1 μM) and then were incubated with HCB (0.5 μM) for 3 h in the presence or absence of ANF. Whole cell lysates were resolved by SDS–PAGE and immunoblotted for COX-2, p-Y416-c-Src, and reblotted for total c-Src as described in Section 2. Intracellular protein COX-2 levels were relativized to β-Actin expression, while p-Y416-c-Src levels were relativized to c-Src levels. Quantification of p-Y416-c-Src/total c-Src ratio and COX-2 by densitometry scanning of the immunoblots is shown in the lower panel. Data are expressed as mean ± SDs of three independent experiments. Asterisks indicate significant differences vs. control ($^{*}p < 0.05$, $^{**}p < 0.01$), ANOVA and Tukey posthoc test. Crosses indicate significant differences versus HCB 0.5 μM ($^{+}p < 0.05$, $^{++}p < 0.01$).

participate in the severity of the disease. Several properties of endocrine disruptors have caused controversy, such as that low levels of exposure may cause endocrine or reproductive abnormalities (low doses may even exert more potent effects than higher doses) [55]. In respect to the effective HCB doses in T-HESC, HCB enhances COX-2 expression, PGE_2 secretion levels, as well as EP4

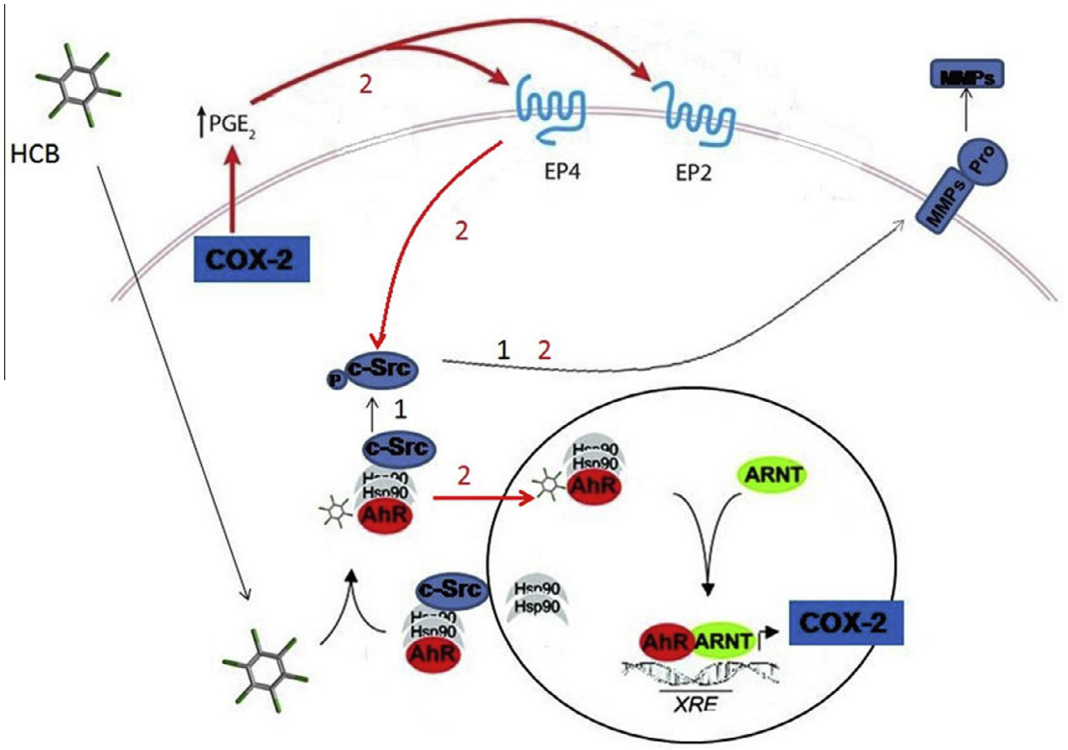

Fig. 10. HCB mechanism of action in endometrial stromal cells. (1) Signaling pathway at early time (1 h), where HCB across the cell membrane and binds to the cytosolic aryl hydrocarbon receptor (AhR), which is attached kinase c-Src. Then, it triggers c-Src phosphorylation and MMPs activation. (2) Signaling pathway at late time (3–6 h), where HCB-AhR complex enters to the nucleus and forms heterodimer with ARNT, binds to XRE in COX-2 promoter and increases COX-2 expression levels. The COX-2 enhances the secretion of PGE_2 levels, out of cell and binds to EP2-EP4 receptors, and activates c-Src and then c-Src triggers MMPs activation.

levels and c-Src activation at 0.05 and 0.5 μM. Besides, we found that the pesticide increases MMPs activation and expression to the high doses (0.5 and 5 μM). Herein, the highest HCB dose (5 μM) is in the same range of order as that found in human serum from a highly contaminated population [29]. In addition, the 0.5 μM HCB effective dose in our data is similar to that observed in human serum samples from general population in France [30].

Lee et al. [17] postulate a molecular mechanism of action in human epithelial and stromal endometriotic cells, where EP2/EP4 signaling promotes phosphorylation of c-Src kinase, which triggers MMPs activation, and finally cell migration and invasion. Similarly, in the present study, we found that HCB increases PGE_2 secretion, EP4 expression, c-Src phosphorylation and MMPs activation. Given that c-Src phosphorylation (1 h) occurs before the increased COX-2 levels (3 h), we postulate a model of HCB mechanism of action in two pathways. One pathway at early time (1 h), and other at late time (3–6 h). At the first pathway (Path 1, early time, Fig. 10), HCB binds to the AhR-c-Src complex, triggering c-Src phosphorylation and MMPs activation. At the second pathway (Path 2, late time, Fig. 10), HCB-AhR complex translocates to the nucleus, binds to the AhR nuclear translocator protein (ARNT) and leads COX-2 gene expression (XRE in COX-2 promoter). This increase in COX-2 expression produces an enhancement in the PGE_2 synthesis and secretion. Finally, this pathway induces stimulation of EP4 signaling, activation of c-Src and the consequent activation of MMPs.

In conclusion, we have demonstrated for the first time that HCB induces MMPs activities, COX-2 expression and PGE_2 signaling pathway in human endometrial stromal cells through AhR mechanism. Our results suggest that the exposure of human endometrial stromal cells to HCB, could contribute to the pathophysiology of endometriosis, increasing parameters of inflammation and invasion that allow acquiring a phenotype to form appropriate endometriotic lesions.

Conflict of interest

No potential conflicts of interest relevant to this article were reported.

Acknowledgements

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