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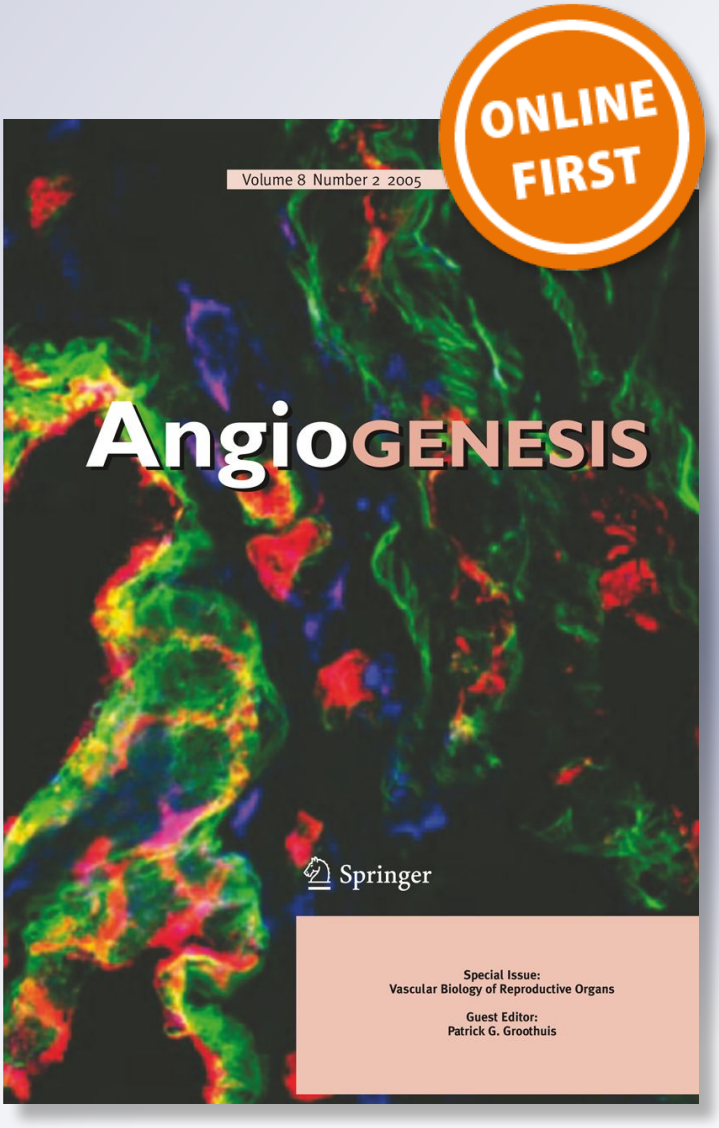

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ORIGINAL PAPER

Estrogen receptor alpha as a key target of organochlorines to promote angiogenesis

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Abstract Epidemiological studies report that exposure to pesticides like chlordecone and lindane increases risk of cancer. They may act as endocrine disruptors via the activation of estrogen receptor α (ERα). Carcinogenesis involved angiogenesis and no available data regarding these organochlorines have been reported. The present study aimed at investigating the effects of lindane and chlordecone on cellular processes leading to angiogenesis through an involvement of ERα. Angiogenesis has been analyzed both in vitro, on human endothelial cells, and in vivo by quantifying neovascularization with the use of ECMgel® plug in mice. Both pesticides increased endothelial cell proliferation, migration and MMP2 activity. These toxics potentiated cell adhesion by enhancing FAK phosphorylation and stress fibers. The two organochlorines increased nitric oxide production via an enhancement of eNOS activity without modification of oxidative stress. Evidence has been provided that the two toxins increased in vivo neovascularization. Most interestingly, all the above processes were either partially or completely prevented after silencing of ERα. Altogether, these data highlight that organochlorines modulate cellular angiogenic processes through activation of ERα. This study further reinforces the harmful effects of these pesticides in carcinogenesis, particularly in the modulation of angiogenesis, a critical step in tumor promotion, through ERα.

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Keywords Endothelial cell · Lindane · Chlordecone · Pro-angiogenic effects · Estrogen receptor alpha

Introduction

Organochlorine pesticides are widespread persistent organic pollutants that bioaccumulate in wildlife and humans. They persist in the environment and contaminate animals and plants, leading to instances of food contamination [1] and eventually dietary exposure in humans [2]. Among these molecules, lindane and chlordecone are organochlorine chemicals that have been commonly used in agriculture and pharmaceutical applications for many years. Despite the fact that the use and production of lindane and chlordecone are banned in most developed countries, the general population is still exposed to these pollutants [3, 4]. Indeed, both lindane and chlordecone have been detected in serum from patients with breast cancer [5] and with prostate tumor [6, 7].

Angiogenesis, defined as the formation of new blood vessels from nearby pre-existing capillaries, represents an essential step in tumor development and metastasis [8]. Then, during the vascular stage, tumor nutrition through diffusion is no longer sufficient and formation of new vasculature is necessary for tumor growth [9, 10]. The formation of functional blood vessels involves a series of coordinated biological processes such as cell proliferation, guided migration, differentiation and cell–cell communication [11]. Moreover, the production, by endothelial cells, of angiogenic factors such as vascular endothelial growth factor (VEGF), metalloproteinases 2 (MMP2), nitric oxide (NO) and superoxide anion (O_2^-) plays a key role in angiogenesis [12].

Estrogens represent an important class of hormones that can promote angiogenesis through the activation of

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estrogen receptors (ERs) α and β [13, 14]. These receptors are distributed between the nuclear compartment, where they act as ligand-activated transcription factors of target genes, and at the plasma membrane, where they mediate rapid, non genomic effects of signal transduction [15, 16]. Estrogens, when bound to their receptors, activate both genomic and non-genomic mechanisms that may require from minutes to days to occur. For example, in endothelial cells, it has been reported that 17β-estradiol binding to ERα is rapidly followed by a potentiation of re-endothelialization and a decrease in smooth muscle cell migration and proliferation [17]. On the vascular system, in addition to these rapid, non-genomic actions, estrogens exert long-term effects, which involve transcriptional events.

It has been reported that lindane and chlordecone may act as endocrine disrupting chemicals because of their affinity to ERα [18, 19]. Although lindane and chlordecone involvement has been described in many hormone-dependent cancers [20, 21], no study focused on the role of these chemicals in the regulation of angiogenesis.

Then, the present study was designed to test the potential effect of lindane and chlordecone on different processes leading to angiogenesis through ERα activation. For this purpose, low and high concentrations corresponding to that found in plasma of exposed individuals or present in drinking water, respectively, were tested on in vitro cell migration, proliferation, adhesion and formation of capillary-like structures. Then, the involvement of lindane and chlordecone in the regulation of angiogenesis was confirmed in a model of ECMgel® plug in vivo.

Materials and methods

Cell culture

Freshly delivered umbilical cords were obtained from a nearby hospital. HUVECs, from male newborns, were obtained as previously described [22] and grown on plastic flasks in MCDB-131 medium (Invitrogen, Carlsbad, CA) containing 1 % L-glutamine, 1 % streptomycin/penicillin, 500 ng/L epidermal growth factor, 1 µg/L basic fibroblast growth factor (bFGF), supplemented with 10 % of heat-inactivated FBS. HUVECs were used at the second to fourth passage. Cells were grown for 24 h in the absence or presence of organochlorines, VEGF (20 ng/mL), fulvestrant (30 nM) or Rho-associated coiled-coil-containing protein kinase (ROCK) inhibitor (Y-27632, 10 µM).

Also, primary endothelial cells (ECs) were isolated from Swiss mice aortas. The extraction method was adapted from Kobayashi's protocol [23]. ECs were cultured in growth medium EBM-2 (Lonza, Basel, Switzerland) supplemented with 5 % FBS. ECs were used until their third–fourth passage.

RNA interference and transient transfection

In order to silencing ERα, siRNA duplexes specific for human ERα and control, non-silencing siRNA, were obtained from Santa Cruz Biotechnology. Transient transfection of HUVECs was done according to the manufacturer's protocol. Briefly, cells were seeded in six-well plates, grown for 24 h (60 % confluence) and then transiently transfected with 100 nM of ERα-specific or control siRNA using the transfection reagent provided, which also served as control without siRNA. Medium was replaced 24 h later by fresh medium and cells were grown for an additional 24 h, prior to either western blot analysis of ERα expression (showing a significant inhibition of ERα—*data not shown*) as previously described by our team [24].

In vitro capillary network formation on ECM gel®

After 24 h of incubation with lindane, chlordecone or VEGF (20 ng/mL), HUVECs were detached with trypsin ethylenediaminetetraacetic acid. Cells were seeded with a density of 150×10^3 cells per well precoated with ECM gel® (Sigma–Aldrich). Briefly, 150 µL of ECM gel® was added into a four-well plate and allowed to solidify for 1 h at 37 °C. Then, cells were incubated with medium containing 10 % of FBS and allowed to adhere for 1 h after which the different *stimuli* were added. Tube formation was examined by phase-contrast microscopy (MOTIC AE21) after 24 h and the average length of capillaries was quantified using ImageJ software.

Migration assay

HUVECs were detached, washed twice in phosphate-buffered saline (PBS) and re-suspended in MCDB-131 medium with 10 % FBS. Fifty thousand cells were seeded in the upper chamber of a Transwell® insert (PTFE membrane with 8 µm diameter pores, Corning, NY). The lower chamber was filled with 1 mL of MCDB-131 with 10 % FBS with or without lindane or chlordecone. After 24 h, migrated cells were fixed with 4 % of paraformaldehyde for 15 min at room temperature. Cells were rinsed two times with washing buffer, stained with crystal violet (Sigma–Aldrich) (1 mg/mL in 2 % of ethanol) for 10 min at room temperature and extensively washed with distilled water. Then, sodium dodecyl sulfate 2 % was added and incubated for 30 min at room temperature. Absorbance was then evaluated using a microplate reader at 550 nm (Snergy HT®, Biotek).

Gelatin zymography

Equal amounts of conditioned media (10 μg) were analyzed by gelatin zymography under non-reducing conditions using a 10 % (v/v) polyacrylamide gel containing 1 mg/mL gelatin. After electrophoresis, gels were washed twice in 50 mM Tris–HCl pH 7.5, 5 mM $CaCl_2$ and 2.5 % (v/v) Triton X-100 for 30 min each wash, and then incubated in 50 mM Tris–HCl pH 7.5, 5 mM $CaCl_2$ at 37 °C overnight. Gels were stained with 0.25 % (w/v) Coomassie Brilliant Blue (G-250) dye in 10 % (v/v) acetic acid and 10 % (v/v) ethanol, then destained in 10 % (v/v) acetic acid and 10 % (v/v) ethanol until clear bands of MMP2 were visualized.

Proliferation assay on HUVECs

Effects of lindane and chlordecone on HUVECs proliferation were analyzed by using CyQUANT® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR). Briefly, 5×10^3 cells per well were seeded into 96-well plates and allowed to attach overnight and then cells were treated with lindane or chlordecone for 24 h. After growth medium removal, dye-binding solution was added into each microplate well and cells were incubated at 37 °C for 30 min. The fluorescence levels were read on a fluorescent microplate reader (Synergy HT®, Biotek) with filters for 485 nm excitation and 530 nm emission.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromure (MTT) cell proliferation assay

HUVECs were seeded at 10^4 cells/well on 96-well plates. Cells were treated with lindane or chlordecone for 24 h. Viability was assessed by colorimetric analysis of MTT (Sigma-Aldrich). Absorbance values were obtained at a wavelength of 570 nm on a microplate reader (Synergy HT®, Biotek, Winooski, VT).

Apoptosis measurement by flow cytometry

HUVECs were exposed to organochlorines or actinomycin D (10^{-6} M as positive control) for 24 h and then fixed in 70 % ethanol at 4 °C for at least 4 h. After a centrifugation at 15,000*g* for 5 min, cells were re-suspended in PBS containing 0.05 mg/mL RNase (Sigma-Aldrich, St. Louis, MO) and 10 μg/mL propidium iodide (Sigma-Aldrich). Cellular DNA content was analyzed on a Cytomics FC 500 MPL flow cytometer (Beckman Coulter, Villepinte, France). In all cases at least 10,000 events were collected for analysis.

Adhesion assay on HUVECs

Evaluation of adherent cells was performed using crystal violet staining. Then, 5×10^3 cells per well were seeded into 96-well plates and were treated for 24 h. After incubation, the plate was shacked for 15 s. The supernatant with non-adherent cells was removed by three washes with washing buffer (0.1 % BSA in medium without serum). Attached cells were fixed with 4 % of paraformaldehyde for 15 min at room temperature. Cells were rinsed two times with washing buffer, stained with crystal violet (Sigma–Aldrich) (1 mg/mL in 2 % of ethanol) for 10 min at room temperature and extensively washed with distilled water. Then, sodium dodecyl sulfate 2 % was added and incubated for 30 min at room temperature. Absorbance was then evaluated using a microplate reader at 550 nm (Synergy HT®, Biotek).

Confocal microscopy

After treatment, HUVECs were fixed with 4 % paraformaldehyde for 15 min at room temperature, permeabilized with 0.1 % Triton X-100 in PBS and then blocked with 5 % bovine serum albumin (BSA) in PBS for 1 h at room temperature. Cells were treated with a rabbit polyclonal p-FAK (Y925, Cell Signaling Technology, Danvers, MA) antibody in 5 % BSA in PBS overnight at 4 °C. After washing with PBS, cells were treated with Alexa 488-conjugated goat anti-rabbit antibody (Interchim, Montluçon, France) in 5 % BSA in PBS for 1 h at room temperature.

In another set of experiment, tetramethylrhodamine isothiocyanate–phalloidin (Sigma–Aldrich) was used in order to label actin fibers. Briefly after treatments, cells were fixed with 4 % paraformaldehyde and then stained with phalloidin (50 μg/mL) for 30 min at room temperature. After washing with PBS, cells were mounted and visualized with a confocal microscopy (CLMS 700, Zeiss, ZEN software). All images were acquired using a 40× or 63× objective.

NO and superoxide anion (O_2^-) determinations by electron paramagnetic resonance (EPR)

Detection of NO production was performed using Fe^{2+} diethyldithiocarbamate (DETC, Sigma-Aldrich) as spin trap. Briefly, cells were treated with lindane, chlordecone or VEGF (20 ng/mL) for 24 h; medium was replaced with 250 μL of Krebs solution, then treated with 250 μL of colloid $Fe(DETC)_2$ and incubated for 45 min at 37 °C. Cells were then scrapped and frozen in plastic tubes. NO detection was measured in situ by EPR. Values are

expressed as amplitude of signal per protein concentration (units/μg/μL of endothelial cell proteins).

For O_2^- quantification, cells were allowed to equilibrate in deferoxamine-chelated Krebs-Hepes solution containing 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidin (CMH; Noxygen, Mainz, Germany) (500 μmol/L), deferoxamine (25 μmol/L), and DETC (5 μmol/L) under constant temperature (37 °C) for 20 min. Cells were then scrapped and frozen in plastic tubes and analyzed by EPR spectroscopy. Values are expressed as units/μg/μL of proteins.

Western blot

After treatment, cells were homogenized and lysed. Proteins (30 μg) were separated on 10 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Blots were probed with peNOS-Ser, peNOS-Thr (Cell Signaling), eNOS (BD Biosciences, San Jose, CA), Rho-A and VEGF antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Monoclonal anti-β-actin antibody (Sigma–Aldrich) was used to visualize protein gel loading. The membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ). The protein–antibody complexes were detected by Enhanced chemiluminescence plus (Amersham Biosciences).

In vivo ECMgel® plug assay

Six-week-old male Swiss mice (Service Commun Animalerie Hospitalo-Universitaire, Université d'Angers) were used. All studies involving mice were in accordance with European guidelines (A49065). Primary mouse ECs were cultured in a 25 cm^2 flask and were treated with DMSO, 2×10^{-11} or 5×10^{-8} M chlordecone, 7×10^{-12} or 5×10^{-8} M lindane or VEGF for 24 h. After treatment, cells were detached and were mixed with 500 μL of ECMgel® (Sigma-Aldrich) with recombinant bFGF (300 ng/mL, Peprotech, Rocky Hill, NJ). This mixture was injected subcutaneously on the back of male Swiss mice treated or not with 10 mg/week fulvestrant, a dose known to inhibit significantly ERα in tumor cells [25]. At day 14, ECMgel® plugs were removed and homogenized in lysis buffer and incubated for 24 h at 4 °C and then, disrupted with a Polytron (PRO250, Monroe, CT). Hemoglobin concentration was measured in the supernatants with Drabkin's reagent (Sigma-Aldrich) according to the manufacturer's instructions.

Statistical analysis

Data are presented as mean ± SEM, n represents the number of experiments repeated at least in triplicate. Statistical analyses were performed by ANOVA followed by a Bonferroni test. $p < 0.05$ was considered to be statistically significant.

Results

Lindane and chlordecone promoted in vitro angiogenesis

After 24 h, HUVECs formed capillaries in ECMgel®. Compared to control, VEGF (20 ng/mL) increased the formation of capillaries (Fig. 1a). Similarly, treatment with lindane (7×10^{-12} or 5×10^{-8} M) or chlordecone (2×10^{-11} or 5×10^{-8} M) significantly increased capillary length. Although no concentration/effect relationship was observed with lindane, high concentration chlordecone (5×10^{-8} M) induced a greater increase of the capillary length than observed with low concentration (2×10^{-11} M) (Fig. 1b). These data suggest that lindane and chlordecone possess pro-angiogenic properties.

Lindane and chlordecone potentiated endothelial cell migration and activation of MMP-2

As migration of endothelial cells contributes to angiogenesis by dissemination from the pre-existing vessel to form new vessels, the effects of lindane and chlordecone were studied on HUVECs migration. A significant increase of endothelial cell migration was shown in cells treated with lindane or chlordecone. Interestingly, organochlorines enhanced cell migration to a similar extent to that observed with VEGF (Table 1). Since metalloproteinases, including MMP2, control cell migration, its activity was evaluated in HUVEC cell line. As shown in Fig. 2, chlordecone significantly enhanced MMP2 activity independently of the concentrations used. In contrast, only high lindane (5×10^{-8} M) concentration increased MMP2 activity. VEGF, used as a positive control, significantly increased cell migration and MMP2 activity (Fig. 2).

Lindane and chlordecone promoted endothelial cell proliferation

Because endothelial cell proliferation represents a critical step in angiogenesis, the effect of lindane and chlordecone was investigated on this cellular process. As shown in Table 1, both lindane and chlordecone enhanced endothelial cell proliferation at any concentration tested when compared to cells treated with DMSO. As expected, VEGF treatment induced an increase in endothelial cell proliferation (Table 1). To ensure the lack of cytotoxicity and apoptotic effects of lindane and chlordecone on HUVECs, viability

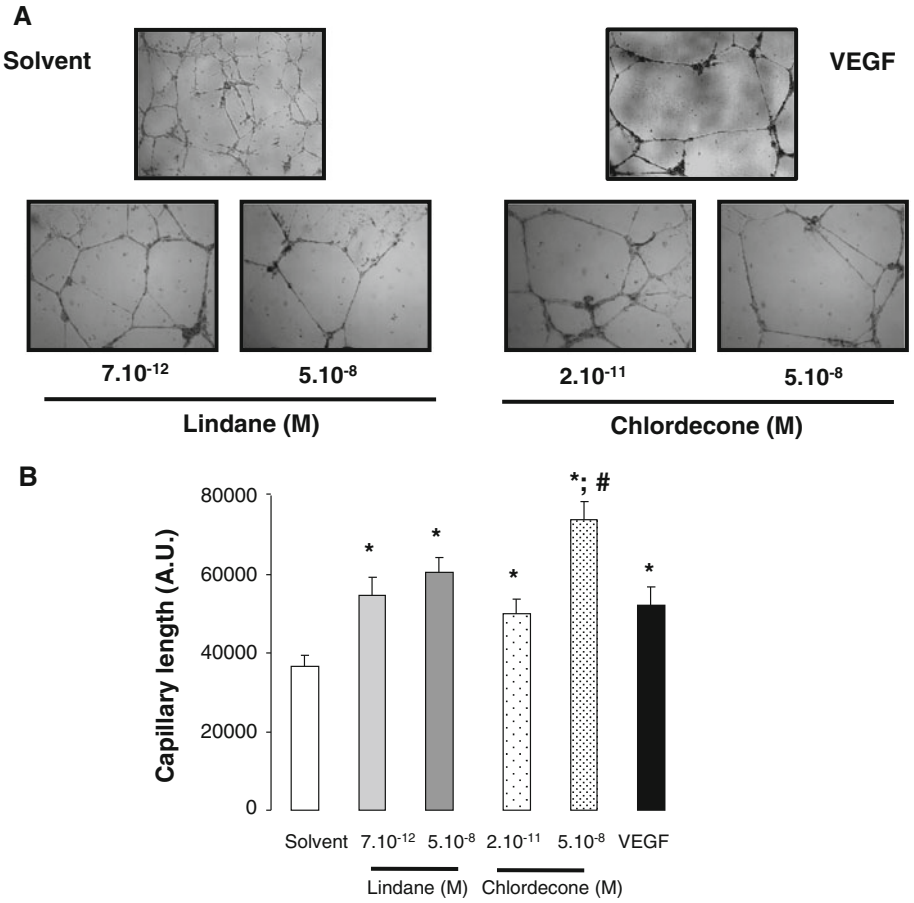


Fig. 1 Pro-angiogenic properties of lindane and chlordecone. **a** HUVECs were cultured in MCDB-131 supplemented with 10 % FBS and treated with 7×10^{-12} or 5×10^{-8} M lindane, 2×10^{-11} or 5×10^{-8} M chlordecone for 24 h. VEGF (20 ng/mL) was used as positive control. **b** Capillary length was used to quantify angiogenesis. Results are means ± SEMs from three independent experiments. $^{*}p < 0.05$ versus solvent; $^{\#}p < 0.05$ versus 2×10^{-11} M chlordecone

Table 1 Role of lindane and chlordecone in different cellular processes leading to in vitro angiogenesis

Assays	Solvent	Lindane 7×10^{-12} M	Lindane 5×10^{-8} M	Chlordecone 2×10^{-11} M	Chlordecone 5×10^{-8} M	VEGF
Proliferation (Fluorescence; A.U.)	5,662.8 ± 752.2	8,891.2 ± 1,023.3*	8,309.7 ± 409.4*	10,360.2 ± 522.0*	9,262.2 ± 481.34*	7,971.0 ± 414.1*
Viability ($OD_{570\ nm}$)	0.625 ± 0.018	0.588 ± 0.009	0.581 ± 0.011	0.590 ± 0.008	0.602 ± 0.010	
Migration ($OD_{550\ nm}$)	0.581 ± 0.029	0.742 ± 0.047*	0.732 ± 0.042*	0.749 ± 0.074*	0.718 ± 0.067*	0.733 ± 0.047*
Adhesion (% of solvent)	100.00	119.99 ± 6.50*	131.32 ± 9.55*	142.36 ± 13.38*	125.54 ± 9.57*	128.56 ± 8.67*
Adhesion (+Y27632) (% of solvent)	106.99 ± 4.96	100.28 ± 3.60	101.72 ± 6.39	109.62 ± 6.50	101.80 ± 3.53	98.81 ± 4.86

Results are mean ± SEMs from four independent experiments; * $p < 0.05$ versus solvent

and apoptosis measurements were performed for each concentration. No cytotoxic effect was observed in cells treated with lindane or chlordecone (Table 1). Analysis of apoptosis by flow cytometry showed no effect of lindane or chlordecone whereas, as expected, actinomycin D treatment (10^{-6} M) increased HUVEC apoptosis (Fig. 3).

Lindane and chlordecone induced endothelial cell adhesion through Rho A pathway

Adhesion assay using crystal violet staining showed that lindane and chlordecone significantly enhanced endothelial cell adhesion to a similar extent to that observed with

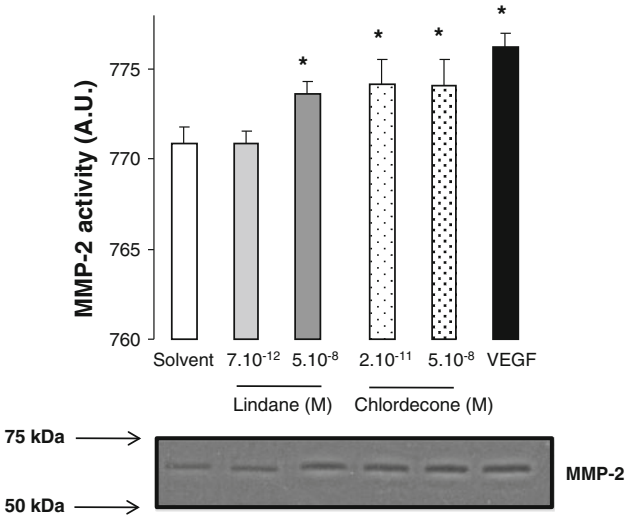


Fig. 2 Role of lindane and chlordecone in MMP2 activity. MMP2 activity was analyzed by gelatin zymography. Treatment with 5×10^{-8} M lindane enhances significantly MMP2 activity while 7×10^{-12} M lindane does not modify it. 2×10^{-11} and 5×10^{-8} M chlordecone increase significantly MMP2 activity compared with cells treated with solvent. VEGF (20 ng/mL) was used as positive control. Results are means ± SEMs from four independent experiments; $^{*}p < 0.05$ versus solvent

VEGF (Table 1). In cells treated with lindane and chlordecone, Rho-A expression measured by Western blot was significantly increased (Fig. 4a). Thereby, the inhibition of ROCK by Y27632 (10 μM) did not alter the adhesion properties of non-treated cells whereas it prevented the increased toxic-induced cell adhesion (Table 1). Then, the expression and activation of proteins, that depend on Rho-A activation, linked to cell adhesion were investigated. Western blot and immunohistochemistry data showed that both lindane and chlordecone induced an increase in FAK phosphorylation (Fig. 4b, c). No modification in FAK expression was found in both cells treated by lindane and chlordecone (Fig. 4c). These effects were inhibited by Y27632 (*data not shown*). In addition, labeling of actin fibers with phalloidin showed that both lindane and chlordecone induced the formation of stress fibers (Fig. 4d), that was prevented by ROCK inhibitor treatment (*data not shown*). These results suggest that lindane and chlordecone upregulate HUVEC adhesion through a Rho-A-dependent pathway.

Lindane and chlordecone promoted NO production

Treatment with lindane or chlordecone significantly increased NO production, compared to cells treated with DMSO. As positive control, VEGF enhanced NO release (Fig. 5a). To determine the molecular changes governing the enhancement of NO release induced by lindane and chlordecone, the expression and activation of eNOS were analyzed by Western blot. Although treatment with lindane or chlordecone did not modify eNOS expression, these toxics were able to increase eNOS phosphorylation on its activator site (Ser-1177) and to decrease its phosphorylation at the inhibitor site (Thr-495) in comparison with solvent-treated cells (Fig. 5b). In addition, after normalization of the amount of phosphorylated eNOS to total amount of the enzyme, the ratio of phosphorylated eNOS at the activator and inhibitor sites was calculated. Interestingly, this ratio was significantly increased in HUVECs treated with lindane or chlordecone compared with non-treated cells. As positive control, VEGF increased p-eNOS-Ser-1177 and decreased p-eNOS-Thr-495 without modifying eNOS expression (Fig. 5c).

The quantification of anion superoxide (O_2^-) production by EPR revealed that neither 7×10^{-12} or 5×10^{-8} M lindane (77.8 ± 12.0 and 93.1 ± 19.4 %, respectively) nor 2×10^{-11} or 5×10^{-8} M chlordecone (82.8 ± 10.9 and 84.0 ± 8.9 %, respectively) did not modify O_2^- expression compared with solvent-treated cells (100 %). As positive control, VEGF (160.33 ± 44.38 %) enhanced significantly ($p < 0.05$) O_2^- production in HUVECs.

Lindane and chlordecone promoted in vitro angiogenesis through ERα activation

Because lindane and chlordecone are described as endocrine disruptors and since the involvement of estrogens in the regulation of angiogenesis has convincingly been demonstrated by a large amount of experimental studies, the hypothesis that these toxics mediate their actions through the activation of ER to promote angiogenesis was investigated. Fulvestrant, a specific antagonist of ER, alone had no effect on endothelial capillary formation, but it significantly prevented the ability of both lindane and chlordecone to increase capillary length. Interestingly, fulvestrant failed to inhibit VEGF-induced increase in capillary length. To specify the isoform of ER implicated in the effect of the organochlorines, we have silenced ERα with specific siRNA. Silencing ERα significantly prevented the ability of lindane and chlordecone to increase capillary length (Fig. 6a). Since similar effects in cells treated with fulvestrant or siRNA against ERα were obtained in the regulation of in vitro angiogenesis. Thus, we have chosen to use only fulvestrant in the following experiments.

The influence of ERα in HUVEC migration was investigated in cells treated either with lindane or chlordecone. In the absence of toxics, fulvestrant did not modify HUVEC migration but it prevented the increase of HUVEC migration induced by lindane or chlordecone, as determined by Transwell® migration assay (Fig. 6b).

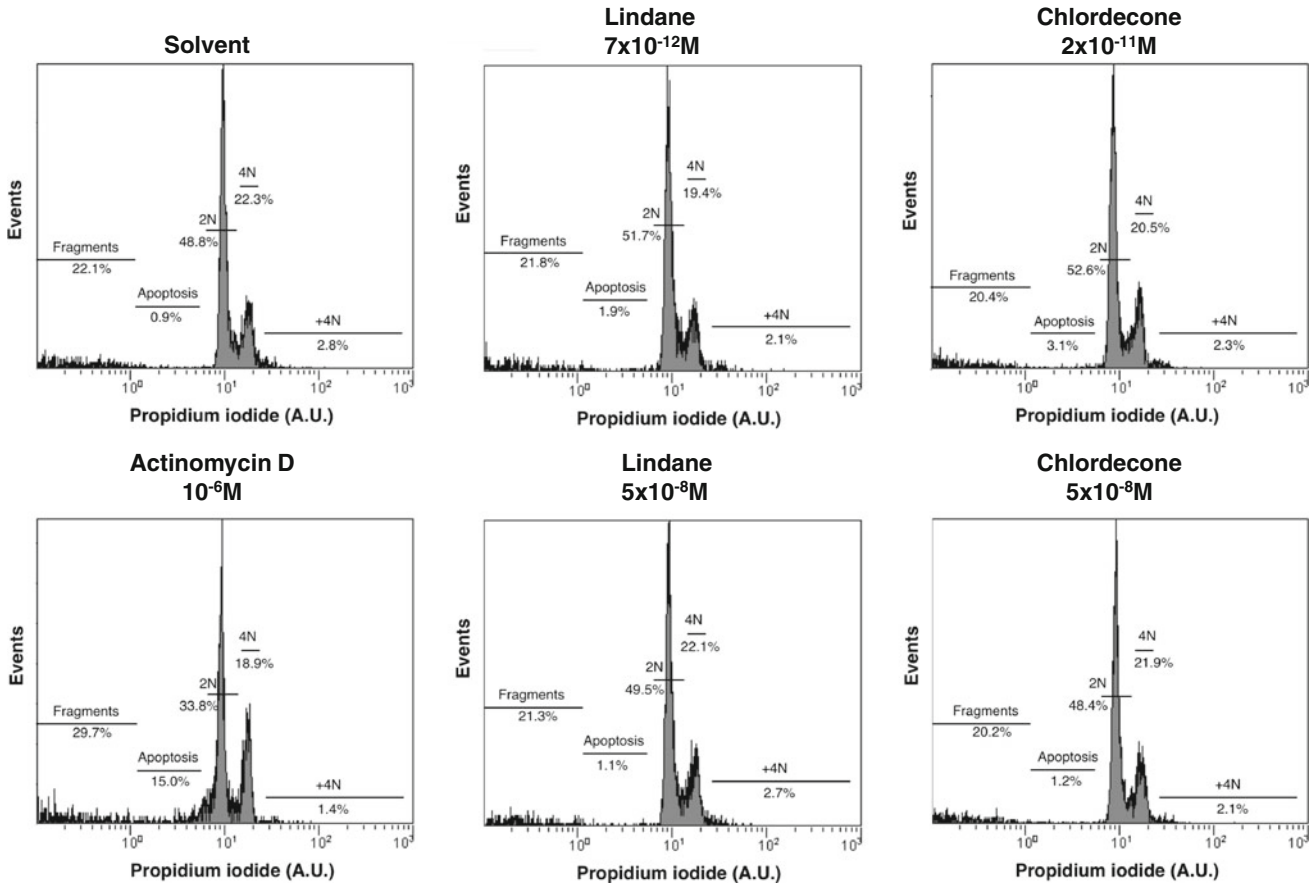


Fig. 3 Lindane and chlordecone do not induce apoptosis. Flow cytometric DNA content histograms (fluorescence in arbitrary units, AU) of HUVECs exposed to lindane or chlordecone for 24 h, showing that at these concentrations, organochlorines fail to induce apoptosis. Actinomycin D (10^{-6} M) was used as positive control

Also, the influence of ERα on toxic-treated-cell proliferation was investigated. No change in HUVECs proliferation was found in cells treated with fulvestrant in comparison with solvent-treated cells (Fig. 6c). Fulvestrant partially prevented the increase of HUVEC proliferation induced by organochlorines.

Moreover, the influence of ERα in HUVECs adhesion was studied in cells treated either with lindane or chlordecone. In the absence of toxics, fulvestrant did not modify HUVECs adhesion, but it prevented the increase in HUVEC adhesion induced by lindane or chlordecone (Fig. 6d). These observations were confirmed by a decrease in FAK phosphorylation (Fig. 6e) and in the formation of stress fibers in the presence of fulvestrant (Fig. 6f) both in cells treated with lindane or chlordecone.

The expression of one of the major pro-angiogenic factors, VEGF, was analyzed. The treatment of HUVECs with lindane and chlordecone significantly enhanced VEGF expression at any concentration tested. Interestingly, fulvestrant prevented the VEGF expression induced by lindane and chlordecone (Fig. 6g).

Lindane and chlordecone promoted in vivo angiogenesis by a mechanism sensitive to ER blockade

Finally, to investigate the effects of lindane and chlordecone on the formation of the new vessels, in vivo angiogenesis was studied by using ECMgel® plug assay. At day 14, a blush of vessel proliferation was observed in plugs from mice injected with mouse-derived endothelial cells treated with lindane or chlordecone. Fulvestrant (10 mg/week, s.c.) seemed to prevent the increase of vessel formation induced by organochlorines (Fig. 7a). These effects were further quantified by measuring the hemoglobin content of the plug. Hemoglobin content was significantly increased in mice injected with mouse-derived endothelial cells treated with lindane or chlordecone. Fulvestrant partially prevented the increase of hemoglobin content induced by organochlorines. As expected, vascularization of the plugs was greater in mice injected with cells treated with VEGF (Fig. 7b).

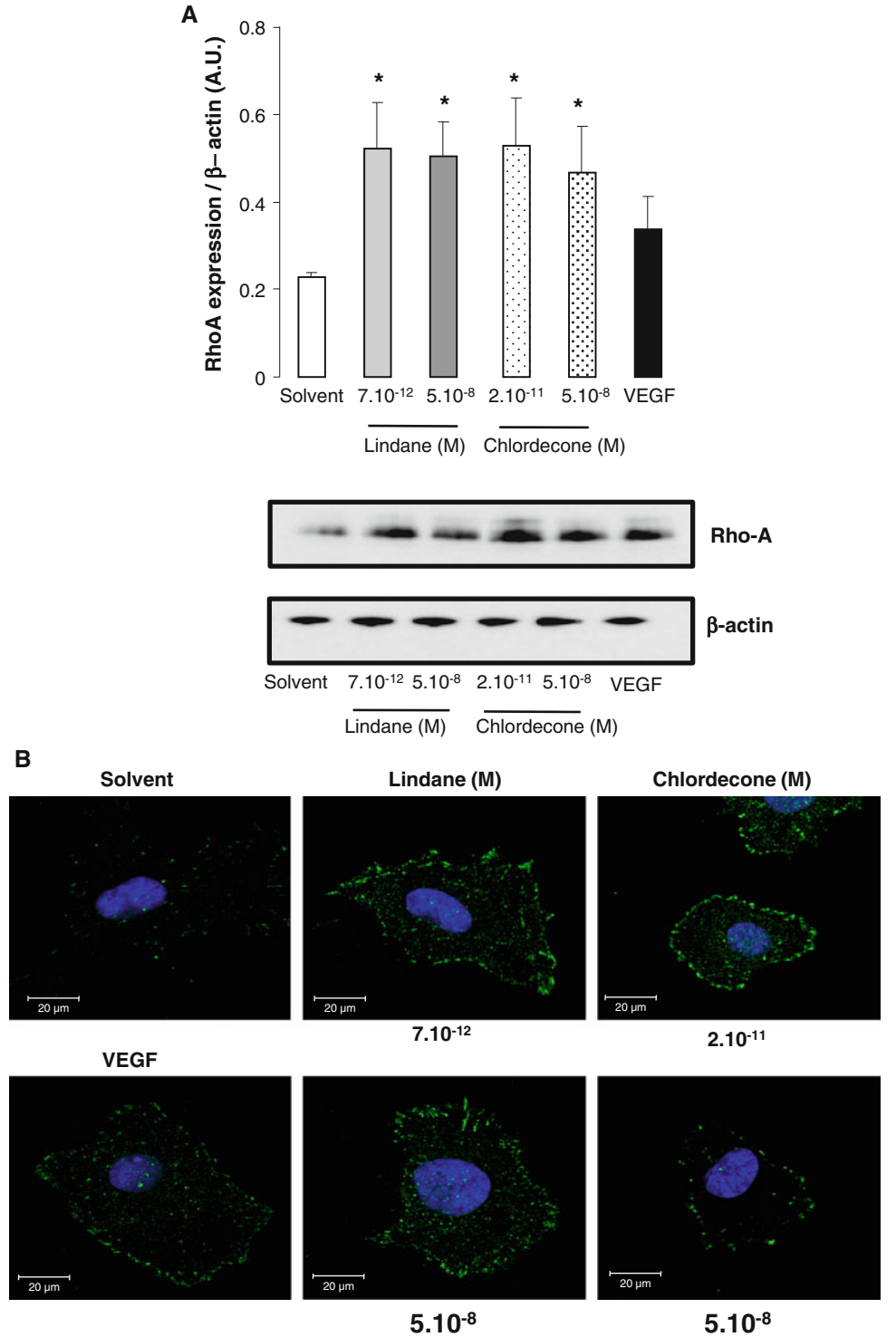
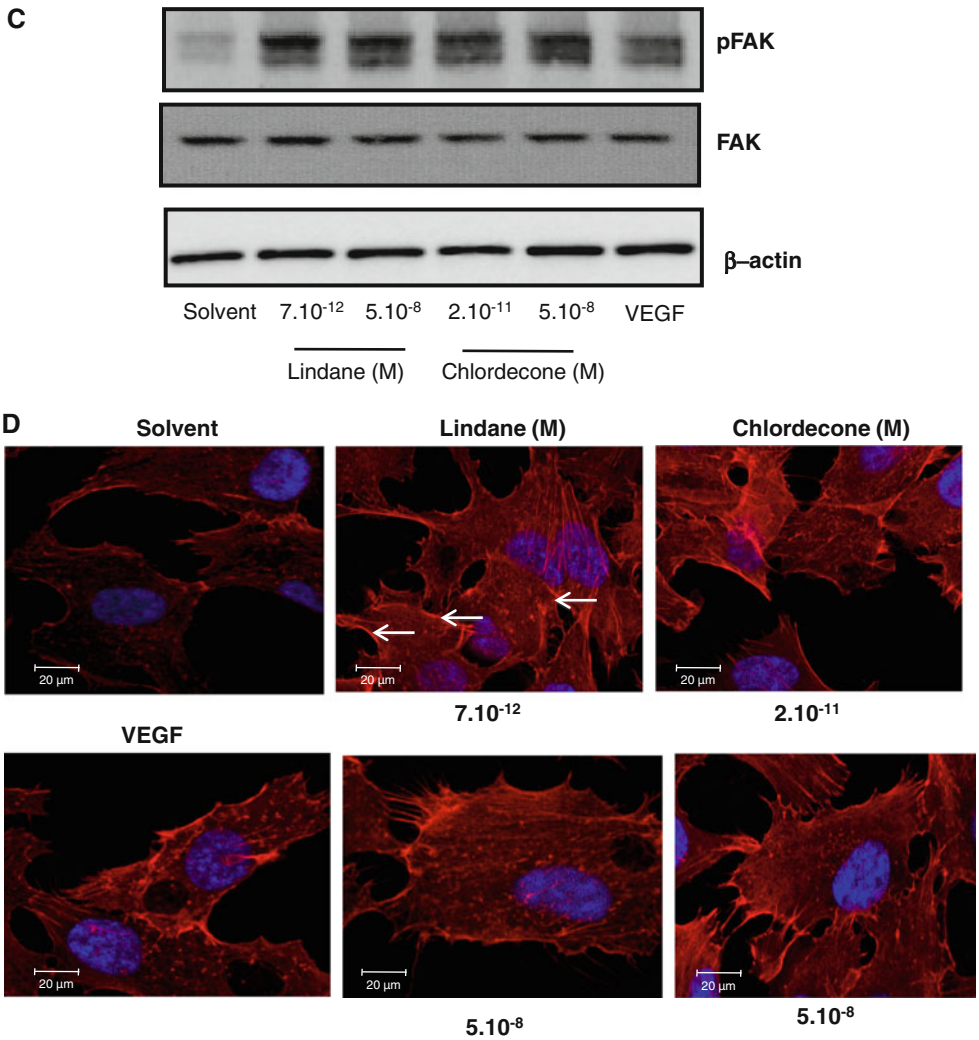


Fig. 4 Role of Rho-A pathway in toxics-induced adhesion of HUVECs. **a** Western blot shows that lindane and chlordecone increase significantly the expression of Rho-A. VEGF (20 ng/mL) was used as positive control. Results are means ± SEM from four independent experiments. Densitometry values are expressed in arbitrary units (A.U.) as mean ± SEM. $*p < 0.05$ versus solvent. **b** Immunofluorescence staining of endothelial cells for p-FAK shows an activation of FAK pathway after treatment with lindane or chlordecone. VEGF (20 ng/mL) was used as positive control. Images were taken at a magnification of ×63. *Horizontal bar* = 20 mm. **c** This was confirmed by western-blot. No modification in FAK expression was found. *β*-actin control is included. Data are representative of four separate blots. **d** To highlight actin stress fibers, HUVECs were stained with rhodamine-labelled phalloidin and visualized by confocal microscopy. Lindane and chlordecone stimulate the formation of stress fibers (white arrow). VEGF (20 ng/mL) was used as positive control. Images were taken at a magnification of ×40. *Horizontal bar* = 20 µm

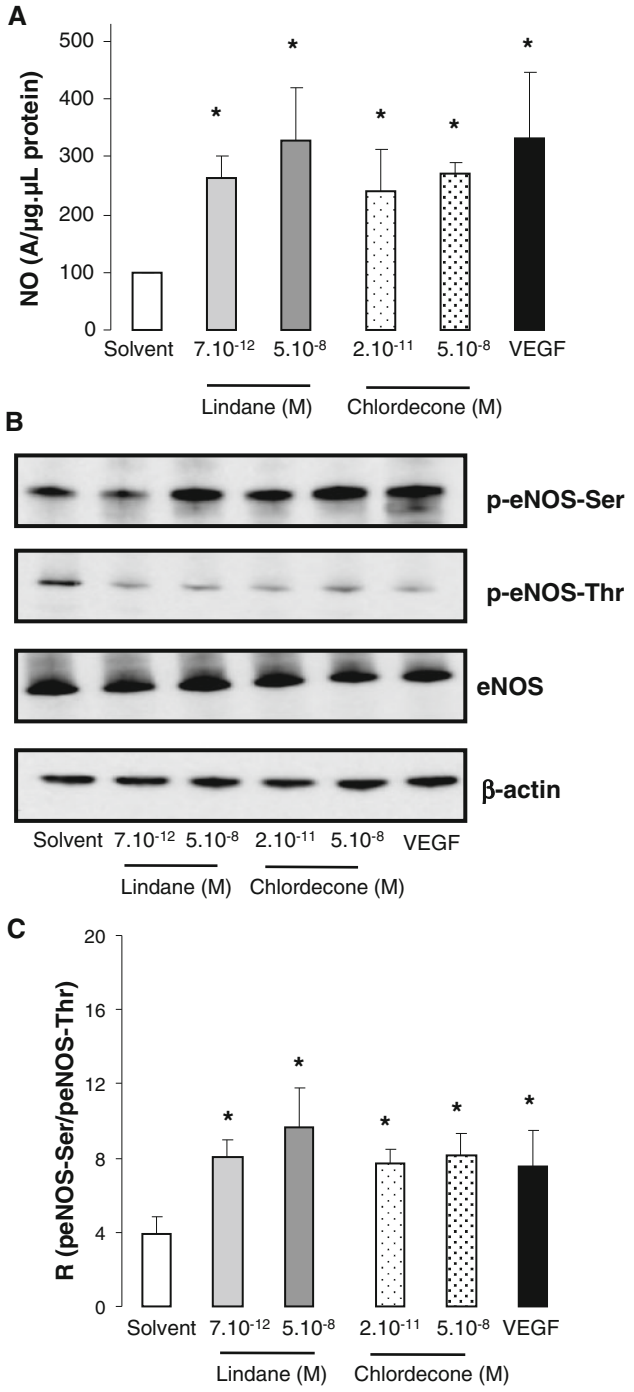
Fig. 4 continued



Discussion

According to in vitro and in vivo experiments, the present study identifies ERα as the, or at least one of, key receptor transducing angiogenic effects exerted by lindane and chlordecone. Indeed, in in vitro HUVEC model, lindane and chlordecone, at environmental and circulating concentrations, enhanced capillary-like formation through an increase in cell proliferation, migration and adhesion. These effects were associated with an increase in VEGF expression and NO production. Interestingly, these cellular effects are prevented by fulvestrant, a selective ERα antagonist. Most importantly, evidence is provided that ERα trigger the in vivo pro-angiogenic effects of lindane and chlordecone inasmuch their effets are prevented after silencing of ERα.

Several epidemiological studies have been conducted until the early eighty's on the toxicity of lindane [26, 27] and chlordecone [28, 29] in humans. Indeed, it was reported that lindane, chlordecone and their metabolites are stored in the blood [30], adipose tissue, prostate [7], breast [31] explaining the influence of these chemicals on tumor development and obesity, two pathophysiological situations in which angiogenesis plays an important role [32]. According to these epidemiological studies, the World Health Organization established a correlation between maximum plasmatic values for lindane and chlordecone (5×10^{-8} M) and the risk of developing cancer or metabolic diseases. Moreover, although lindane and chlordecone are currently prohibited in many parts of the world, traces of these toxics can be found in the air but also in the rain and in the rivers. Therefore, studies have determined the amount of lindane [33] and chlordecone [34] found in water and reported the maximum amount of these compounds in drinking water being 7×10^{-12} and 2×10^{-11} M, respectively. Once introduced in the blood circulation, lindane, chlordecone and theirs metabolites can remain for extended time periods making that endothelial cells are one of the primary targets of the toxicity of these compounds. Thus, to assess the impact of these

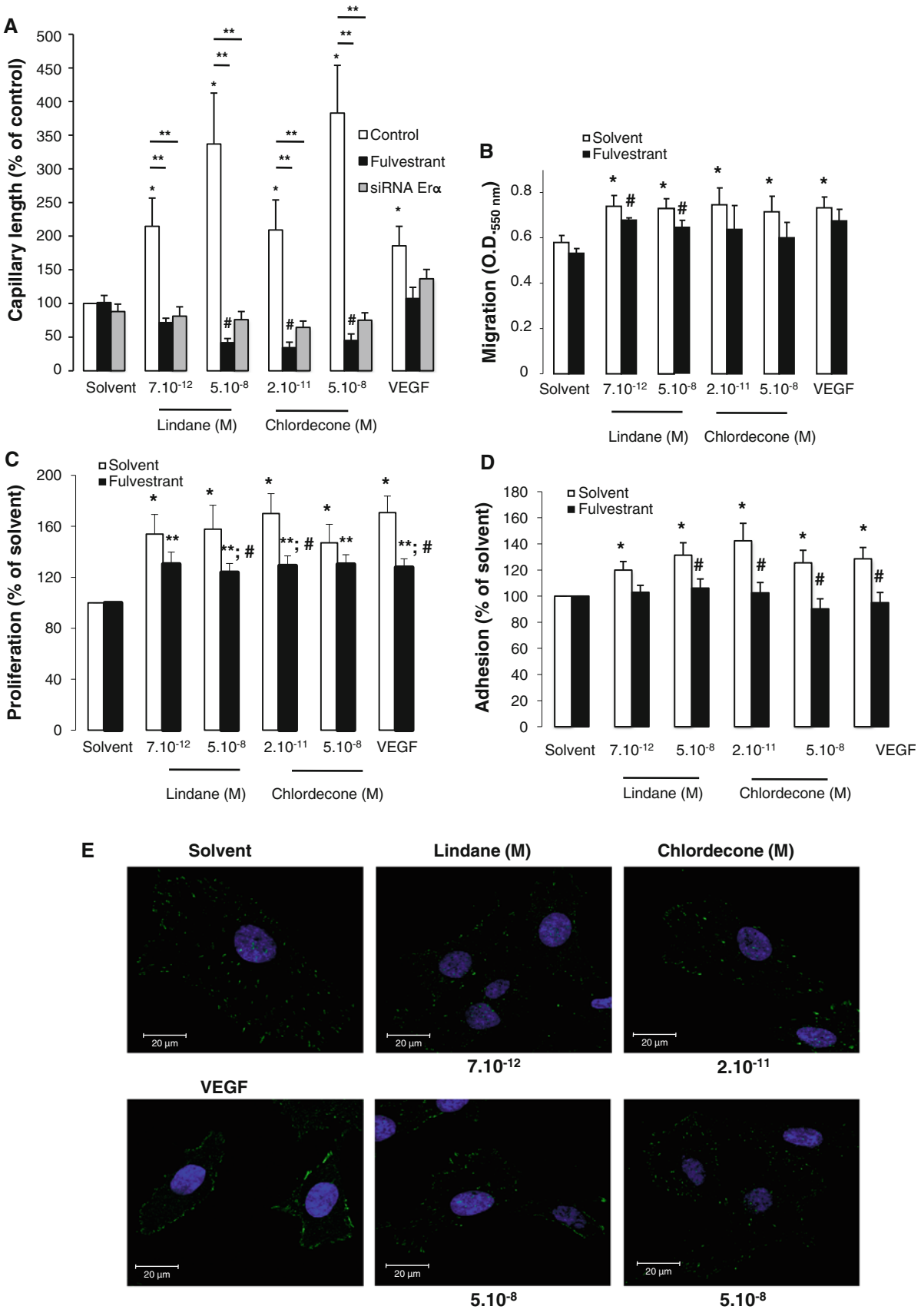


◀ **Fig. 5** Lindane and chlordecone enhance NO production. **a** Quantification of the amplitude of the NO-Fe(DETC)$_2$ complex signal in HUVECs reveals a significant increase of NO production in cells treated with lindane (7×10^{-12} or 5×10^{-8} M) or chlordecone (2×10^{-11} or 5×10^{-8} M) compared with control cells. Values are expressed as units per microgram per microliter of protein in the samples. VEGF (20 ng/mL) was used as positive control. Results are means ± SEMs from four independent experiments. $^{*}p < 0.05$ versus solvent. **b** Western blot revealed eNOS expression and phosphorylation of Ser-1177 (activator site) and of Thr-495 (inhibitory site). Data are representative of four separate blots. β-actin control is included. **c** Lindane (7×10^{-12} and 5×10^{-8} M) or chlordecone (2×10^{-11} and 5×10^{-8} M) increase the ratio between p-eNOS-Ser and p-eNOS-Thr. VEGF (20 ng/mL) was used as positive control. Results are means ± SEMs from four independent experiments; $^{*}p < 0.05$ versus solvent

Fig. 6 Influence of ERα blockade on cellular processes leading to angiogenesis and on VEGF expression. **a** Capillary length was used to quantify angiogenesis from HUVECs treated with lindane or chlordecone. Pharmacological blockade with fulvestrant or silencing with specific siRNA against ERα have been used. Reproducible data were obtained from three independent experiments. $^{*}p < 0.05$ versus solvent; $^{**}p < 0.05$ versus without fulvestrant; $^{\#}p < 0.05$ versus HUVECs treated only with fulvestrant. **b** Transwell® migration assay shows that treatment with 7×10^{-12} or 5×10^{-8} M lindane and 2×10^{-11} or 5×10^{-8} M chlordecone enhances cell migration compared with the control conditions. Treatment with fulvestrant (30 nM) increases significantly cell migration in HUVECs treated with lindane (7×10^{-12} and 5×10^{-8} M). VEGF (20 ng/mL) was used as positive control. Results are means ± SEMs from four independent experiments; $^{*}p < 0.05$ versus solvent; $^{\#}p < 0.05$ versus HUVECs treated only with fulvestrant. **c** Analysis of HUVECs, treated or not with fulvestrant, shows proliferative properties for each concentration lindane and chlordecone. Cell proliferation is significantly decreased in HUVECs treated simultaneously with fulvestrant and 5×10^{-8} M lindane or 2×10^{-11} M chlordecone. VEGF (20 ng/mL) was used as positive control. Results are means ± SEMs from four independent experiments; $^{*}p < 0.05$ versus solvent; $^{**}p < 0.05$ versus without fulvestrant; $^{\#}p < 0.05$ versus HUVECs treated only with fulvestrant. **d** Lindane and chlordecone enhance the number cells that resulted positive to crystal violet staining. Fulvestrant (30 nM) significantly decreases the adhesion of HUVECs treated with 5×10^{-8} M lindane or chlordecone (2×10^{-11} or 5×10^{-8} M). Results are means ± SEMs from four independent experiments. $^{*}p < 0.05$ versus solvent; $^{\#}p < 0.05$ versus HUVECs treated only with fulvestrant. **e** Immunofluorescence staining of endothelial cells for p-FAK shows no activation of FAK pathway after simultaneous treatment with fulvestrant and lindane or chlordecone. VEGF (20 ng/mL) was used as positive control. Images were taken at a magnification of ×63. *Horizontal bar* = 20 µm. **f** With fulvestrant, nor lindane no chlordecone do not stimulate the formation of stress fibers. VEGF (20 ng/mL) was used as positive control. Images were taken at a magnification of ×40. *Horizontal bar* = 20 µm. **g** Western blot shows VEGF protein expression after treatment with lindane (7×10^{-12} and 5×10^{-8} M) or chlordecone (2×10^{-11} and 5×10^{-8} M) with or without fulvestrant. β-actin control is included. In absence of fulvestrant, ratio between VEGF expression and β-actin expression shows that lindane (7×10^{-12} and 5×10^{-8} M) or chlordecone (2×10^{-11} and 5×10^{-8} M) increased VEGF expression. When cells were treated simultaneously with fulvestrant and organochlorines, ratio between VEGF expression and β-actin expression was significantly decreased. Data are representative of four separate blots. Results are means ± SEMs from four independent experiments; $^{*}p < 0.05$ versus solvent

concentrations, known to induce metabolic diseases or cancers, on the cellular processes leading to angiogenesis, the present study was performed using the maximum concentrations in drinking water and plasma doses permitted.

Angiogenesis results from a finely tuned balance between cell migration, proliferation and adhesion [35]. Then, although lindane and chlordecone, under the experimental conditions used, do not induce either cytotoxicity



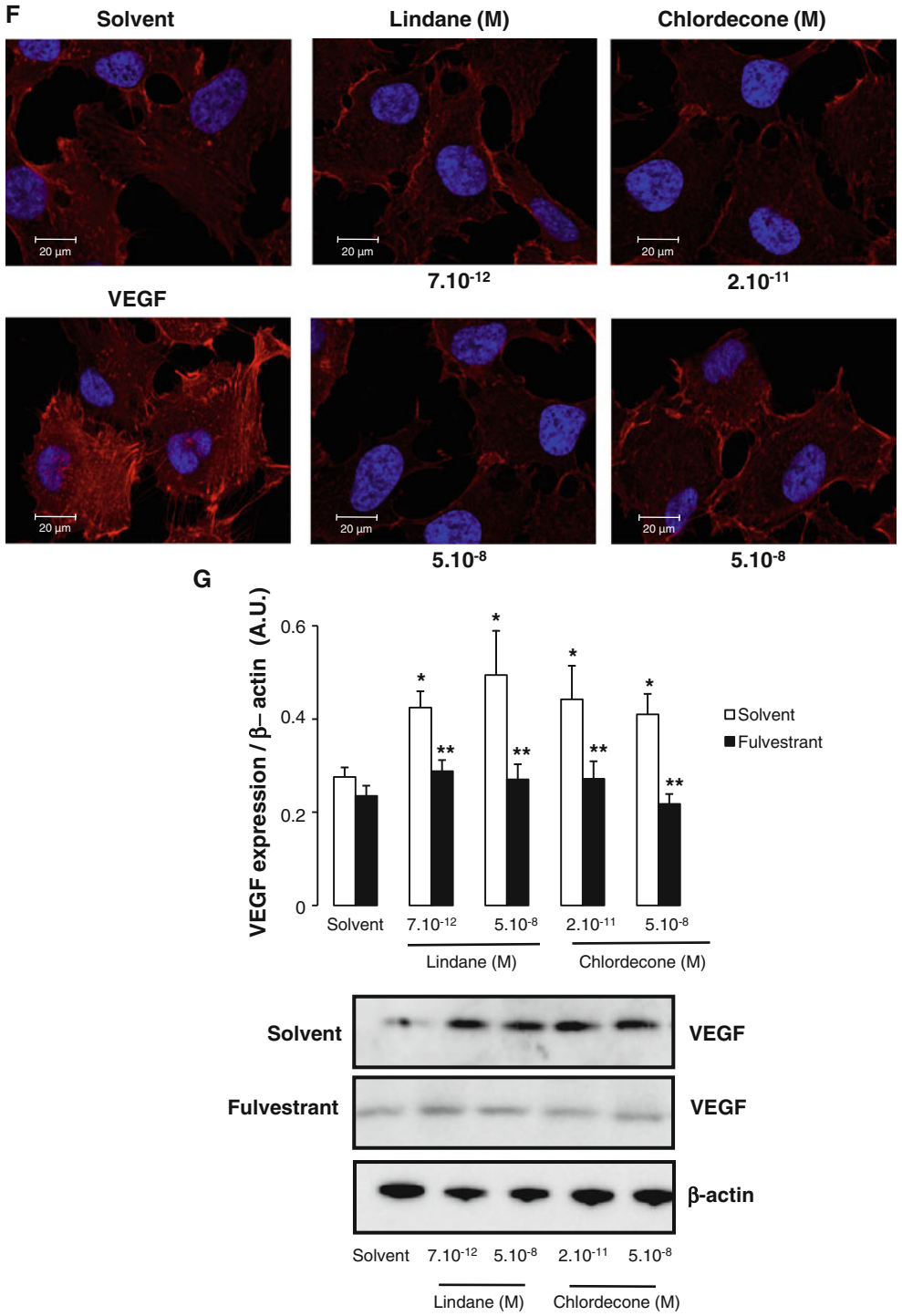


Fig. 6 continued

or apoptosis, this study provides evidence that both lindane and chlordecone have pro-angiogenic properties because of their ability to increase the formation of capillary tubes.

The analysis of cellular processes involved in in vitro angiogenesis reveals that both cell proliferation and migration are enhanced by lindane and chlordecone, independently of the concentration used. These effects are associated with potentiation of MMP2 activity. These findings suggest that lindane and chlordecone are able to control events leading to angiogenesis confirming data with various PCBs (PCB-126, -188 and -153), which regulate MMP2 expression both in melanoma and in endothelial

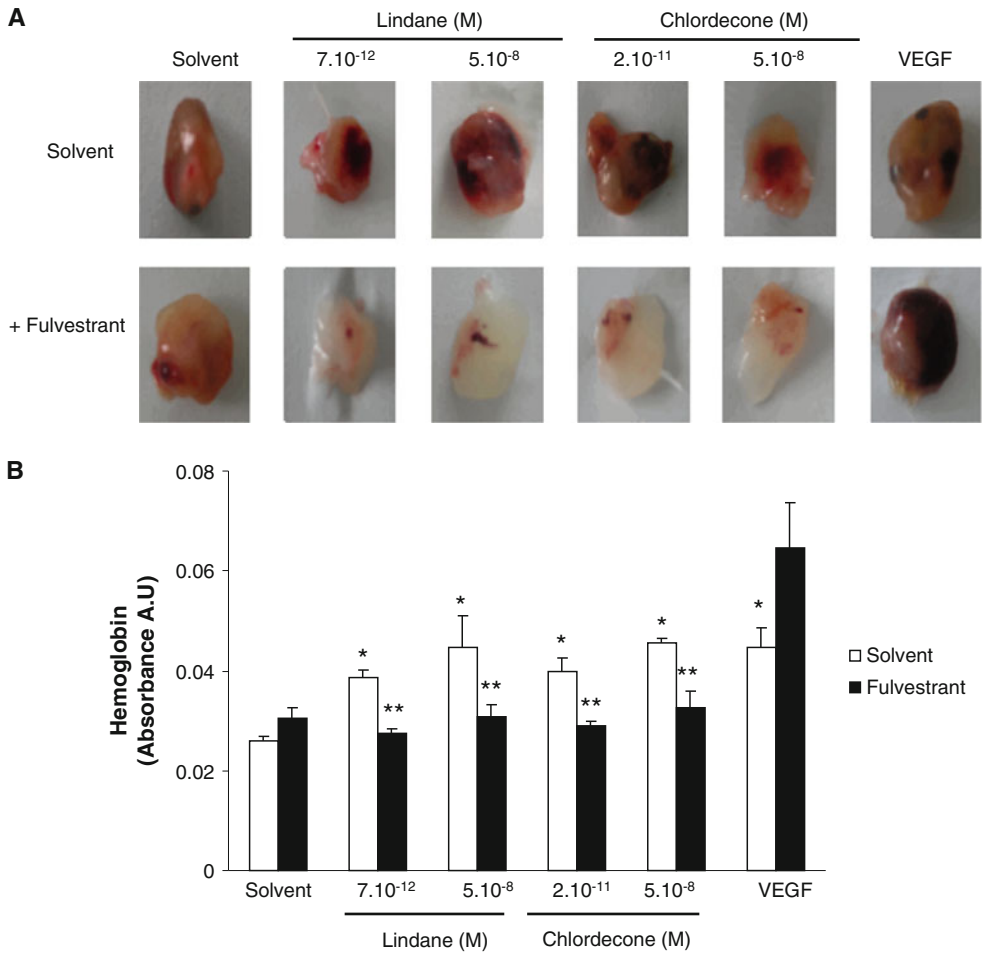


Fig. 7 Lindane and chlordecone promote in vivo angiogenesis. **a** At day 14, lindane (7×10^{-12} and 5×10^{-8} M) or chlordecone (2×10^{-11} and 5×10^{-8} M), enhance neovascularization in plugs containing mouse aortic-derived endothelial cells pretreated with lindane or chlordecone. In mice treated with fulvestrant (10 mg/week, s.c), no vascularization is observed in plugs containing mouse aortic-derived endothelial cells pretreated with toxics. **b** Quantitative measurement of hemoglobin was reported as absorbance (arbitray units)/weight of plugs. Hemoglobin content is increased in plugs containing mouse aortic-derived endothelial cells pretreated with lindane or chlordecone, in absence of fulvestrant. This content significantly decreases in plugs from mice treated with fulvestrant (10 mg/week, s.c) containing mouse aortic-derived endothelial cells pretreated with lindane or chlordecone, in comparison with plugs from control mice. *$p < 0.05$ versus plug from control mice, in the absence of fulvestrant; **$p < 0.05$ versus plug from mice treated with fulvestrant. Figures are representative of at least four independent experiments for each condition

cells [36]. However, although MMP2 plays an important role in controlling endothelial cell migration, this enzyme is not the main mediator involved in the regulation of this cellular process. Indeed, an increased migration has been observed with low concentration lindane (7×10^{-12} M) with no change in MMP2 activity. Therefore, FAK phosphorylation and stress fibers, that play a significant role in migrating cells, have been studied. As shown by Western blot and confocal microscopy, lindane and chlordecone (1) induce FAK phosphorylation without modifying its expression and (2) favor the formation of stress fibers. These effects are reversed by the ROCK inhibitor Y-27632 suggesting an implication of RhoA/Rho kinase pathway in the effect of both lindane and chlordecone on HUVEC adhesion. The involvement of Rho A and ROCK has been shown only in breast cancer cells in which PCBs enhance the metastatic propensity by activating the ROCK signaling [37]. Moreover, PCBs has been reported to increase both aortic [38] and brain [39] endothelial cell adhesion through an enhancement of VCAM-1 expression. Data from the present study reinforce and bring molecular explanation that lindane and chlordecone are able to regulate both HUVEC migration and adhesion.

Beside its capacity to regulate permeability and blood flow, NO has been reported to be essential in cellular processes leading to angiogenesis [40]. Lindane and chlordecone significantly enhance NO production through an increase in eNOS phosphorylation on its activator site and a decrease phosphorylation on its inhibitor site. Few studies have described the influence of organochlorines in

the regulation of NO production. However, it has been reported that PCB-77 activated human-derived vascular endothelial cells through an enhancement of eNOS phosphorylation [41]. Moreover, in another study performed on HUVECs, authors claimed that PCB-77 and -126 increased eNOS mRNA influencing both fetal circulation and angiogenesis [42]. In an in vivo study conducted on rats, lindane (40 mg/kg) also increased NO production without affecting O_2^- production [43]. The lack of effects of lindane and chlordecone on the O_2^- production was also found in the present study. Indeed no changes in O_2^- production were noted independently of the treatment used. Unlike the present work, previous in vivo studies have reported that lindane, but not chlordecone, is able to induce cytotoxicity through a significant increase in oxidative stress. Indeed, in rats treated with lindane (8 mg/kg, *i.p.*), the hepatotoxicity induced by this chemical is mainly due to a potentiation of oxidative stress [44]. Another study performed on C57/BL6 mice confirmed that the toxicity of lindane in thymocytes is explained by an enhancement of O_2^- and hydrogen peroxyde [45]. These paradoxical effects could be explained by greater doses of lindane and chlordecone used by these authors. Therefore, the present work suggests that pro-angiogenic properties of lindane and chlordecone might be explained by a potentiation of NO production independently of increased O_2^- production.

The major finding of this work is the identification of ERα as one of the targets involved in the pro-angiogenic activity of lindane or chlordecone. Although ERα is known to be a target of organochlorines, studies on the effects of these toxics on endothelial cells are very limited. Indeed, some studies report that both lindane and chlordecone might act as an agonist of ERα [19, 46], and activation of this type receptor has been shown to up-regulate angiogenesis by other authors [47, 48]. Interestingly, in the present study, up-regulation of angiogenesis by lindane or chlordecone is prevented by fulvestrant, a specific ERα antagonist or after silencing of ERα by a specific siRNA. Indeed, they strongly reduced capillary-like structures formation in HUVECs. Moreover, fulvestrant inhibits both proliferation and adhesion of HUVECs treated with lindane or chlordecone. Conversely, no change in HUVECs migration is observed in cells treated with toxics and fulvestrant compared with HUVECs treated with lindane or chlordecone only. According to these findings, it is difficult to conclude whether the effects of lindane and chlordecone are non-genomic or genomic process. Indeed, on one hand, some authors have reported that the stimulation of ERα can induce changes both in mRNA and protein expressions, suggesting that a genomic mechanism was involved after 24 h treatment of endothelial cells [14]. Identical treatment time has been used in the present study. On the other hand ER-α activation has been shown to stimulate $G\alpha_{13}$ and triggers actin remodelling and migration either of endothelial or breast cancer cells through a RhoA/ROCK/Moesin pathway [49, 50] through a non-genomic process. Nevertheless, the present study provide evidence of the implication of ERα on the effects of the two compounds on endothelial cells but the nature of its actions whether non-genomic or genomic remains to be established.

The estrogenic activities of chlordecone in vivo have already been demonstrated in rodents. In ovariectomized mice, the effects of chlordecone injection (7.5 mg/kg) have been determined on uterine accumulation of two estrogen-responsive genes, and it increases lactoferrin and progesterone receptor mRNA levels [51]. To further validate our obtained data concerning the effects of lindane and chlordecone on cellular processes leading to in vitro angiogenesis, the in vivo effects of lindane- or chlordecone-treated endothelial- cells have been investigated on neovascularization. Thus, lindane or chlordecone enhance the formation of new vessels as assessed by increased hemoglobin content in ECM gel® plugs; interestingly, these effects are prevented by 10 mg/week fulvestrant (s.c), a dose known to inhibit significantly ERα in tumor cells [25]. These findings demonstrate that isolated endothelial cells, expanded by in vitro treatment with lindane or chlordecone, are able to generate in vivo functional vessels and confirm the ERα-dependent pro-angiogenic activities of these toxics.

VEGF plays an essential role in the regulation of angiogenesis. As previously described with PCB-104 in human microvascular endothelial cell 1 (HMEC-1), this organochlorine induces VEGF overexpression that potentiates endothelial permeability and angiogenesis [52]. In the present study, we found that treatment with lindane or chlordecone enhanced VEGF expression in HUVECs. Moreover, we reported that the VEGF overexpression induced by organochlorines has been prevented by fulvestrant, suggesting that the modulation of VEGF expression is dependent of an activation of ERα. These findings corroborate the conclusions of previous studies performed on various tumor cells [53, 54] but also, in HUVEC [55]. In absence of fulvestrant, all the effects of organochlorines on cellular processes leading to angiogenesis was equal that the effect of VEGF whereas in the cases that fulvestant was used these effects were fully prevented. Altogether these findings suggest that cellular processes involved in organochlorines-induced angiogenesis are consequences of VEGF induction rather than additionnal effects.

There is considerable evidence in the role of lindane and chlordecone in the etiology of hormono-dependent cancers such as breast or prostate cancer [5, 56]. The agonistic effects on ERα of organochlorines may increase proliferation of estrogen-sensitive tissues, increasing the risk of cancer. Since no studies have shown the involvement of

these environmental disruptors in the regulation of angiogenesis, the main finding of this present study is the demonstration of the role of ERα in the regulation of in vitro and in vivo angiogenesis. In conclusion, the present study provides strong evidence that, in addition to their interaction with ERα, lindane and chlordecone may activate estrogen-signaling pathways to promote both tumor growth and tumor development through a potentiation of angiogenesis.

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