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Paradoxical effects of ethoxidine, a topoisomerase I inhibitor, in the cellular processes leading to angiogenesis on endothelial cells

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Angiogenesis, a critical step in tumorigenesis, is defined by different processes leading to neovascularization. Topoisomerase I (Top I) is the target for some of the most successful anticancer drugs that decrease tumor cell proliferation. Ethoxidine, a benzo[*c*]phenanthridines derivative, camptothecin analogue, has been identified as a potent inhibitor of Top I in various cancer cell lines. This study was aimed to investigate the impact of ethoxidine on angiogenesis and cellular processes including migration, proliferation and adhesion since these processes play an important role in tumor progression. Ethoxidine was incubated for 24 h at low (10^{-9} M) and high (10^{-5} M) concentrations on two types of human endothelial cells: EaHy.926 and human umbilical endothelial cells. Vascular endothelial growth factor (VEGF, 20 ng/ml) was used as a positive control. Ethoxidine at low concentration increased cell proliferation and migration that was associated with enhanced metalloproteinase 2 expression and activity, whereas high concentration of ethoxidine inhibited all of these effects. The two concentrations of ethoxidine did not affect endothelial cell adhesion. Low concentration of ethoxidine increased VEGF expression and endothelial nitric oxide (NO) synthase expression, NO and superoxide anion productions, whereas high concentration of ethoxidine did not induce any effect. Taken together, the present results highlight paradoxical effects of ethoxidine on angiogenesis depending on the concentration used. This study underscores that in addition to its anti-proliferative properties, ethoxidine may affect the generation of vascular network in tumorigenesis.

Introduction

Angiogenesis represents an essential step in tumor proliferation, expansion and metastasis (1). It is generally accepted that there are two stages of tumor progression regarding its vasculature (2,3). During the initial avascular stage of tumor growth (tumor mass $<$ 0.5 mm), nutrition and oxygen delivery can be achieved by diffusion. When tumor mass grows $>$0.5 mm, nutrition through diffusion is no longer sufficient and formation of new vasculature is necessary for further growth (vascular stage) (4,5). The tumor remains in a dormant state until it can stimulate blood vessel growth from nearby pre-existing capillaries, a process known as angiogenesis. First, the formation of functional blood vessels involves a series of coordinated biological processes such as cell proliferation, guided migration, differentiation and cell–cell communication (6). 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 (7). Anti-angiogenic agents are now considered as an important cancer therapy option.

Eukaryotic DNA topoisomerase I (Top I) efficiently relaxes DNA supercoils during basic cellular processes such as DNA replication, transcription, recombination, repair and chromatin remodeling (8). Consistent with the role of Top I in promoting cell growth, Top I enzyme activity and/or levels are elevated in several types of cancers (9). Top I is recognized as the specific target of camptothecin, a commonly used inhibitor of Top I in cancer therapy (10). Other Top I inhibitors (e.g. indolocarbazoles, phenanthridines and indenoisoquinolines) have also been described (11). Among these molecules, fagaronine derivatives such as ethoxidine, a camptothecin analogue, and its metabolites (ethoxy-fagaronine) have been synthesized by our group (12); these latter agents have been shown to inhibit the cell growth of different cell lines including the human leukemia, L1210, and a number of those from solid tumors (HT-29, A-549, MCF-7 and CaOv3 cells) (11,13). Although ethoxidine inhibits the proliferation of various tumor cell lines (11), no study has, to date, focused on the influence of this molecule in the regulation of angiogenesis.

Top I is highly expressed in endothelial cells (14,15) and several studies have focused on the role of Top I inhibitors in the regulation of angiogenic steps. For example, previous studies documented that the Top I inhibitor topotecan inhibits angiogenic growth in the *in vivo* rat disc angiogenesis model (16). Similar results have also been reported in a mouse cornea angiogenesis model *in vivo* (17). Furthermore, Top I inhibition decreases VEGF expression in a model of neuroblastoma (18). Interestingly, inhibition of Top I regulates differential endothelial NO synthase (eNOS) expression in human umbilical vein endothelial cells (HUVECs) (19) suggesting its involvement in the regulation of endothelial function.

The anti-proliferative properties of several Top I inhibitors (camphothericin, irinotecan) including ethoxidine have been tested using a wide range of concentration from 10^{-9} to 10^{-5} M (11,13,20,21). Beside, ethoxidine has been reported to be 10-fold greater more potent in inhibiting Top I on K562 and A549 cancer cell lines, compared with other inhibitors. However, the effects of these concentrations of ethoxidine on angiogenesis are not known. Since the bioavailability of this compound is not completely known, we have chosen to test its effects on *in vitro* angiogenesis in two types of human endothelial cells (i.e. EaHy.926 cell line and HUVECs), using two concentrations of ethoxidine. For this purpose, low (10^{-9} M) and high (10^{-5} M) concentrations of ethoxidine were tested on cell migration, proliferation, adhesion and formation of capillary-like structures.

Materials and methods

Material

Ethoxidine was synthesized as described previously (11) and was dissolved in sterile water. Trypsin ethylenediaminetetraacetic acid and culture 'media' were obtained from Lonza (Basel, Switzerland). VEGF was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

The EaHy.926 (American Type Culture Collection, Manassas, VA,) endothelial cell line was maintained at 37°C in a humidified incubator gassed with 5% CO_2 in air and was cultured in growth medium (Dulbecco's modified Eagle's medium:-Ham's F-12, 1:1; Lonza) supplemented with 1% L-glutamine, 1% non-essential amino acids, 1% Na-pyruvate, 1% streptomycin/penicillin (Lonza), 1% hypoxanthine, aminopterin, thymidine (Sigma–Aldrich, St Louis, MO) and 10% of heat-inactivated fetal bovine serum (FBS) (Invitrogen, Cergy Pontoise, France).

Also, freshly delivered umbilical cords were obtained from a nearby hospital. HUVECs were obtained as described previously (22) and grown on plastic flasks in MCDB 131 medium (Invitrogen) containing 1% L-glutamine, 1% streptomycin/penicillin, 500 ng/l epidermal growth factor, 1 μg/l basic

Abbreviations: DETC, diethyldithiocarbamate; eNOS, endothelial NO synthase; EPR, electron paramagnetic resonance; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; HUVECs, human umbilical vein endothelial cells; MMP2, metalloproteinases 2; mRNA, messenger RNA; NO, nitric oxide; PCR, polymerase chain reaction; Top I, topoisomerase I; VEGF, vascular endothelial growth factor.

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fibroblast growth factor, 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 10^{-9} or 10^{-5} M ethoxidine or VEGF (20 ng/ml).

In vitro capillary network formation on ECM gel®

After 24 h of incubation with ethoxidine or VEGF (20 ng/ml), HUVECs and EaHy.926 cells 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® substrate diluted with FBS-free medium (1:1 dilution) 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. To determine pro- or anti-angiogenic properties of ethoxidine, cells were cultured either with 10^{-9} or 10^{-5} M ethoxidine for 24 h in media completed or not with 10% of heat-inactivated FBS. Tube formation was examined by phase-contrast microscopy (MOTIC AE21) after 4 and 24 h and was quantified using ImageJ software.

Adhesion assay on EaHy.926 cells or HUVECs

Evaluation of adherent cells was performed using crystal violet staining. For adhesion experiments, 5×10^3 cells per well were seeded into 96-well plates for 24 h before addition of ethoxidine (10^{-9} and 10^{-5} M). After 24 h of incubation, the plate was shacked for 15 s. The supernatant with non-adherent cells was removed by three washes with washing buffer (0.1% bovine serum albumin 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

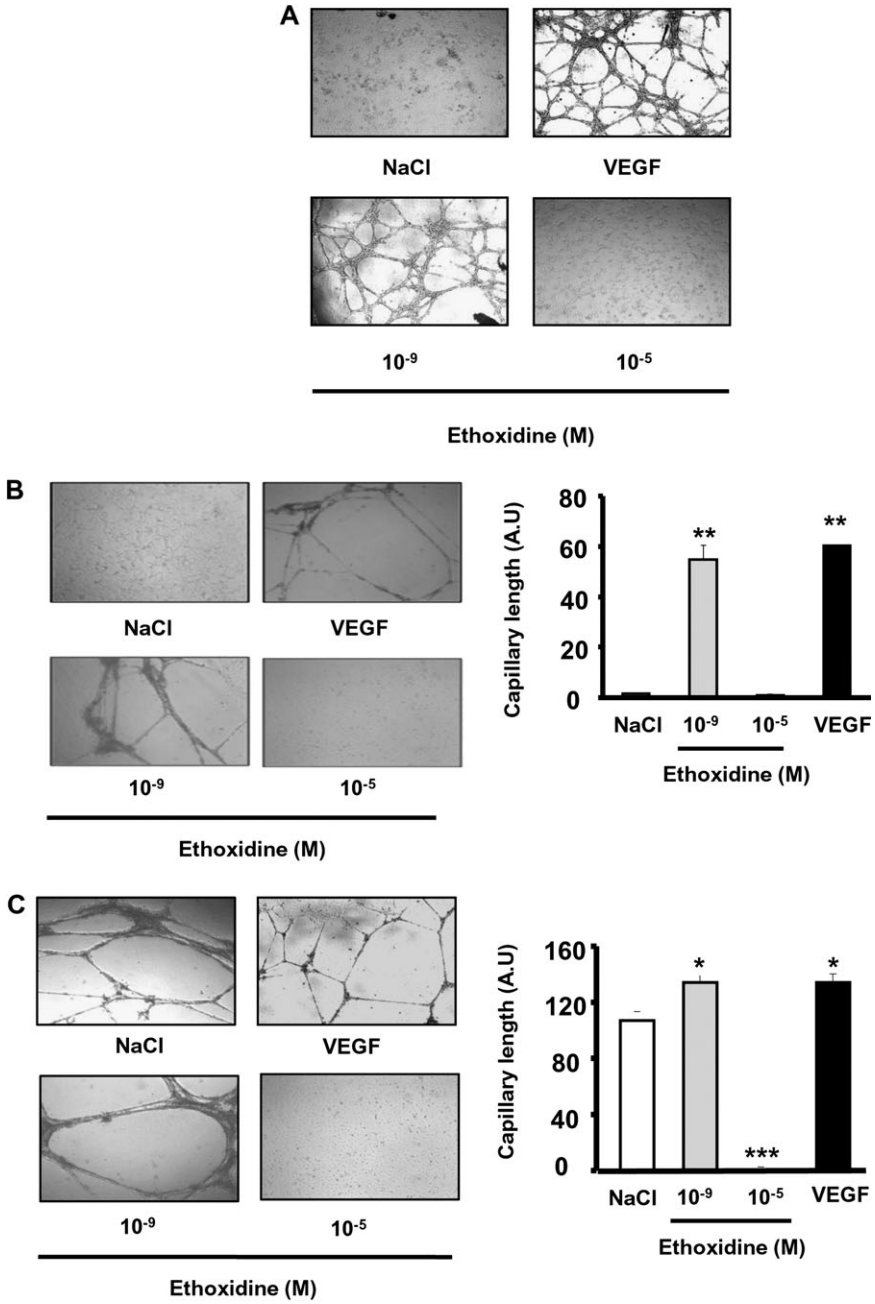

Fig. 1. Pro-angiogenic properties of 10^{-9} M ethoxidine and anti-angiogenic properties of 10^{-5} M ethoxidine. (**A**) EaHy.926 cells were cultured in medium supplemented with 10% FBS and treated with 10^{-9} or 10^{-5} M ethoxidine for 24 h. VEGF (20 ng/ml) was used as positive control. (**B**) HUVECs were cultured in medium without FBS and treated with 10^{-9} or 10^{-5} M ethoxidine for 24 h. VEGF (20 ng/ml) was used as positive control. Capillary length was used to quantify angiogenesis. Reproducible data were obtained from three independent experiments. $^{**}P < 0.01$ versus NaCl. (**C**) HUVECs were cultured in medium supplemented with 10% FBS. VEGF (20 ng/ml) was used as positive control. Capillary length was used to quantify angiogenesis. Reproducible data were obtained from three independent experiments; $^{*}P < 0.05$ versus NaCl; $^{***}P < 0.001$ versus NaCl.

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, Winooski, VT).

Proliferation assay on EaHy.926 cells or HUVECs

Effects of ethoxidine on proliferation on EaHy.926 cells or HUVECs 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 ethoxidine (10^{-9} and 10^{-5} M) 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.

Since similar effects of ethoxidine were obtained in the two endothelial cell types, we have chosen to use EaHy.926 in the following experiments.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide cell viability assay

EaHy.926 were seeded at 10^4 cells per well on 96-well plates. Cells were treated with 10^{-5} M ethoxidine for 24 h. Viability was assessed by colorimetric analysis of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma–Aldrich). Absorbance values were obtained at a wavelength of 570 nm on a microplate reader (Synergy HT Biotek).

Apoptosis measurement by flow cytometry

EaHy.926 were exposed to 10^{-5} M ethoxidine for 24 h and then fixed in 70% ethanol at 4°C for 4 h. After a centrifugation at 15 000*g* for 5 min, cells were resuspended in PBS containing 0.05 mg/ml RNase (Sigma–Aldrich) and 10 µg/ml propidium iodide (Sigma–Aldrich). Cellular DNA content was analyzed on a Cytomics FC500 MPL flow cytometer (Beckman Coulter, Villepinte, France). In all cases, at least 10 000 events were collected for analysis.

Migration assay

Evaluation of EaHy.926 cell migration was performed using Oris® cell migration assembly kit-flex (Platypus Technologies, Madison, WI) according to manufacturer's instructions. Briefly, Oris® cell seeding stoppers were inserted into 96-well plates to create a detection zone. Then, when cells were confluent, the stoppers were removed and cells were treated with ethoxidine (10^{-9} and 10^{-5} M) or VEGF (20 ng/ml) for 48 h. Finally, cell migration was assessed using an inverted microscope (MOTIC AE21).

Gelatin zymography

Equal amounts of conditioned media (10 µg) were analyzed by gelatin zymography under non-reducing conditions using a 10% (vol/vol) 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% (vol/vol) 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% (wt/vol) Coomassie Brilliant Blue (G-250) dye in 10% (vol/vol) acetic acid and 10% (vol/vol) ethanol and then destained in 10% (vol/vol) acetic acid and 10% (vol/vol) ethanol until clear bands of MMP2 were visualized.

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

Detection of NO production was performed using Fe^{2+} diethyldithiocarbamate (DETC; Sigma–Aldrich) as spin trap. Briefly, cells were treated with ethoxidine (10^{-9} or 10^{-5} M) 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 electron paramagnetic resonance (EPR). Values are expressed as amplitude of signal per protein concentration (units per microgram per microliter of endothelial cell proteins).

For O_2^- quantification, cells were allowed to equilibrate in deferoxamine-chelated Krebs–acide 4-(2-hydroxyéthyl)-1-pipérazine éthane sulfonique solution containing 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidin (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 per microgram per microliter 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, phospho-p42/p44, p42/p44, pAkt, Akt (Cell Signaling, Beverly, MA), eNOS (BD Biosciences, San Jose, CA) and VEGF antibodies (Santa Cruz Biotechnology). 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).

Quantitative real-time reverse transcription–polymerase chain reaction analysis

EaHy.926 cells were grown for 24 h in the presence or absence of ethoxidine (10^{-9} and 10^{-5} M) or VEGF (20 ng/ml). Cells were detached using trypsin and after two subsequent steps of centrifugation at 500*g* for 10 min, the pellet containing cells were frozen in liquid N_2 and used to investigate the expression of messenger RNA (mRNA) for eNOS, VEGF and MMP2 transcripts by real-time reverse transcription–polymerase chain reaction (PCR). Reverse transcription–PCR analyses were carried out by Service Commun de Cytométrie et d'Analyses Nucléotidiques from Angers University on Chromo 4TM (Bio-Rad, Hercules, CA) using the SYBR Green PCR Master Mix (Invitrogen). The PCR consisted of 7.5 µl of SYBR Green PCR Master Mix, 10 nM of forward and reverse primers and 2.0 µl of 1:20-diluted template complementary DNA in a total volume of 20 µl. Cycling was performed using the conditions 10 min at 95°C, followed by 40 rounds of 15 s at 95°C and 1 min at 60°C. To verify that the used primer pair produced only a single product, a dissociation protocol was added after thermocycling, determining dissociation of the PCR products from 65°C to 95°C. We selected the *HPRT* gene as an endogenous control. Finally, the quantification of mRNA was performed according to the ΔCt method.

Statistical analysis

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

Results

Low concentration of ethoxidine promotes in vitro angiogenesis and high concentration decreased it

To determine the pro- and anti-angiogenic properties of ethoxidine, EaHy.926 (Figure 1A) and HUVECs (Figure 1B and C) were treated

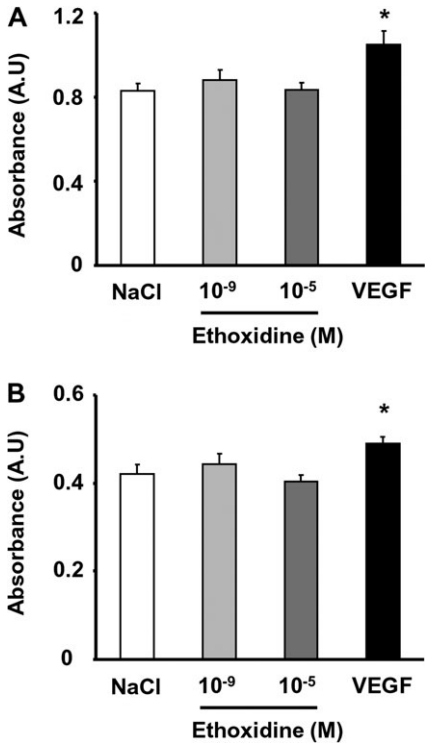

Fig. 2. Ethoxidine does not modulate endothelial cell adhesion. Whatever the concentration of ethoxidine, no difference in the number of (**A**) EaHy.926 cells and (**B**) HUVECs that resulted positive to crystal violet staining. VEGF (20 ng/ml) was used as positive control. Results are means ± SEMs from four independent experiments; $^*P < 0.05$ versus NaCl.

with 10^{-9} or 10^{-5} M ethoxidine in a medium with or without 10% FBS.

As shown in Figure 1A, EaHy.926 endothelial cells failed to organize in capillary-like structures in medium supplemented with 10% FBS. After 24 h of treatment with 10^{-9} M ethoxidine, EaHy.926 reorganized and formed capillaries on ECM gel®. In contrast, 10^{-5} M ethoxidine was not able to promote the organization and the formation of capillaries. As control, VEGF (20 ng/ml) increased the formation of capillaries. Likewise, under basal conditions in the absence of FBS, EaHy.926 cells failed to organize in capillary-like structures, whereas treatment with 10^{-9} M ethoxidine promoted the formation of capillaries and 10^{-5} M inhibited their formation (*data not shown*). HUVECs failed to organize in capillary-like structures in basal conditions, without FBS (Figure 1B). After 24 h of treatment with 10^{-9} M ethoxidine, HUVECs formed capillaries, whereas 10^{-5} M had no effect. The summary of capillary length measurements confirmed the differential effects of ethoxidine at the two concentrations used (Figure 1B). Although in the absence of treatment, HUVECs were able to form capillaries, in medium supplemented with 10% FBS, 10^{-9} M ethoxidine enhanced capillary-like structure formation as illustrated by the capillary length measurement, whereas 10^{-5} M ethoxidine completely inhibited this process (Figure 1C). As control, VEGF (20 ng/ml) favored the formation of capillaries. These data strongly suggest ethoxidine posses either pro- or anti-angiogenic properties depending on the concentrations used.

Ethoxidine does not affect endothelial cell adhesion

Neither a low (10^{-9} M) or high (10^{-5} M) concentration of ethoxidine had an effect on the adhesion of EaHy.926 (Figure 2A) or HUVEC (Figure 2B) cells, as determined with the adhesion assay using crystal violet. However, VEGF (20 ng/ml, for 24 h) increased cell adhesion under similar experimental conditions.

Ethoxidine modulates both endothelial cell proliferation and migration

Because endothelial cell proliferation represents a critical step in angiogenesis, we investigated the effect of ethoxidine on this cellular

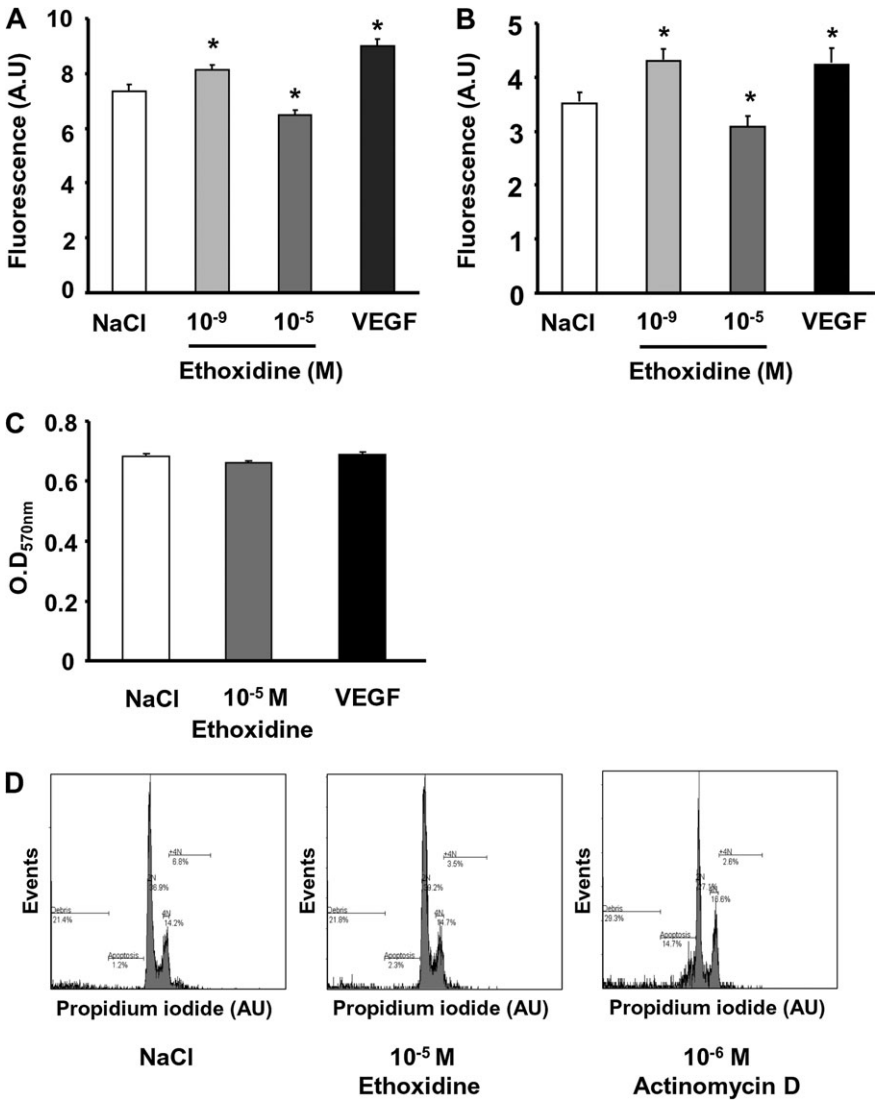

Fig. 3. Paradoxical properties of ethoxidine in endothelial cell proliferation. (**A**) In EaHy.926 cells, 10^{-9} M ethoxidine favors cell proliferation, whereas 10^{-5} M ethoxidine has anti-proliferative properties. VEGF (20 ng/ml) was used as positive control. Results are means ± SEMs from four independent experiments; $^{*}P < 0.05$ versus NaCl. (**B**) Analysis of HUVECs proliferation confirmed proliferative properties for 10^{-9} M ethoxidine and anti-proliferative properties for 10^{-5} M ethoxidine. VEGF (20 ng/ml) was used as positive control. Results are means ± SEMs from four independent experiments; $^{*}P < 0.05$ versus NaCl. (**C**) *In vitro* cell viability was assessed by colorimetric analysis of MTT reduction after a treatment with 10^{-5} M ethoxidine for 24 h. Data are expressed as mean ± SEMs. (**D**) Flow cytometric DNA content histograms (fluorescence in arbitrary units, AU) of EaHy.926 cells exposed to 10^{-5} M for 24 h, showing that at this concentration, ethoxidine fails to induce apoptosis.

process. We highlighted proliferative properties for ethoxidine at low concentration. Indeed, 10^{-9} M ethoxidine was able to increase significantly EaHy.926 (Figure 3A) and HUVECs (Figure 3B) proliferation. In contrast, high concentration of ethoxidine reduced proliferation of both EaHy.926 (Figure 3A) and HUVECs (Figure 3B). As expected, VEGF treatment induced an increase of endothelial cell proliferation in the two endothelial cell types studied.

Since similar effects of ethoxidine were obtained in the two endothelial cell types, we have chosen to use only EaHy.926 in the following experiments. Thus, to ensure the absence of cytotoxicity and apoptosis of 10^{-5}M ethoxidine on EaHy.926 cells, viability and apoptosis measurements were performed by MTT assay and DNA hypodiploid quantification by flow cytometry, respectively. No cytotoxic effect was observed in cells treated by high concentration of ethoxidine (Figure 3C). Analysis of apoptosis by flow cytometry showed no effect of high concentration ethoxidine. As expected, actinomycin D treatment increased the apoptosis of EaHy.926 cells (Figure 3D).

As migration of endothelial cells contributes to angiogenesis by dissemination from the pre-existing vessel to form new vessels, we studied the effects of ethoxidine on endothelial cell migration on EaHy.926 cell line. We observed that low concentration of ethoxidine increased endothelial cell migration to a similar extent to that observed with VEGF. Conversely, treatment with high concentration of ethoxidine decreased cell migration (Figure 4A). These results were confirmed by the migration area measurement (Figure 4B). Since metalloproteinases, including MMP2, control cell migration, its expression and activity were evaluated in EaHy.926 cell lines. Both MMP2 mRNA expression (Figure 4C) and activity (Figure 4D) were enhanced after treatment of EaHy.926 cell line with 10^{-9} M ethoxidine. In contrast, 10^{-5} M ethoxidine reduced both the expression (Figure 4C) and the activity (Figure 4D) of MMP2 under the same experimental conditions. As positive control, VEGF increased migration, MMP2 expression and activity in EaHy.926 cell line.

Ethoxidine regulates NO release in endothelial cells by modulating eNOS activity

Treatment of EaHy.926 cells with ethoxidine (10^{-9} M) significantly increased NO production (Figure 5A), whereas high concentration of ethoxidine (10^{-5} M) was without effect on release of NO as measured by the EPR method using $Fe(DETC)_2$. To determine the molecular changes governing the reduction of NO release induced by ethoxidine in endothelial cells, we analyzed expression and activation of enzymes linked to NO pathway by real-time reverse transcription–PCR and western blotting. An increase in eNOS mRNA was observed

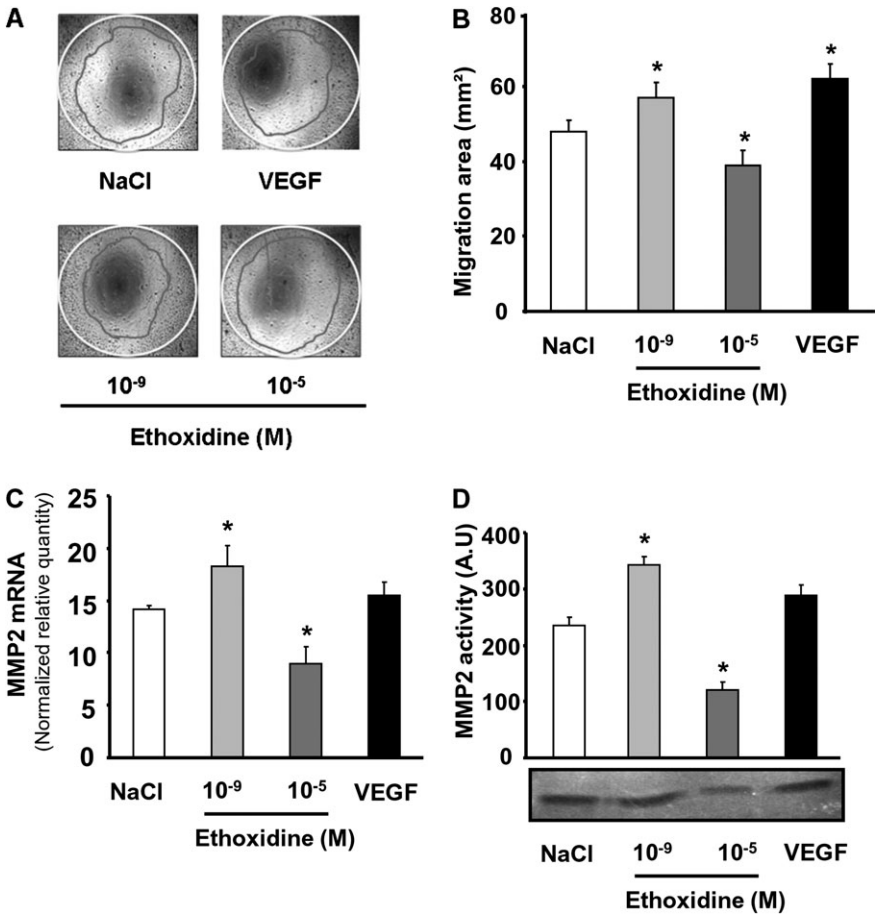

Fig. 4. Paradoxical properties of ethoxidine in endothelial cell migration. (**A**) Phase-contrast micrographs show that treatment with 10^{-9} M ethoxidine enhances cell migration compared with the control conditions and 10^{-5} M ethoxidine decreases it. The space between white circle (before the initiation of ethoxidine treatment) and grey circle (at the end of the treatment) represents the index of endothelial cell migration. Reproducible data were obtained from four independent experiments. (**B**) Evaluation of migration area shows that 10^{-9} M ethoxidine favors cell migration, whereas 10^{-5} M inhibits this process. VEGF (20 ng/ml) was used as positive control. Results are means ± SEMs from four independent experiments; $^{*}P < 0.05$ versus NaCl. (**C**) Quantification of MMP2 mRNA expression by real-time reverse transcription–PCR revealed that 10^{-9} M ethoxidine increases significantly MMP2 transcripts expression, whereas 10^{-5} M ethoxidine decreases it. VEGF (20 ng/ml) was used as positive control. Results are means ± SEMs from four independent experiments; $^{*}P < 0.05$ versus NaCl. (**D**) MMP2 activity was analyzed by gelatin zymography. Treatment with 10^{-9} M ethoxidine enhances significantly MMP2 activity and 10^{-5} M reduces it. VEGF (20 ng/ml) was used as positive control. Results are means ± SEMs from four independent experiments; $^{*}P < 0.05$ versus NaCl.

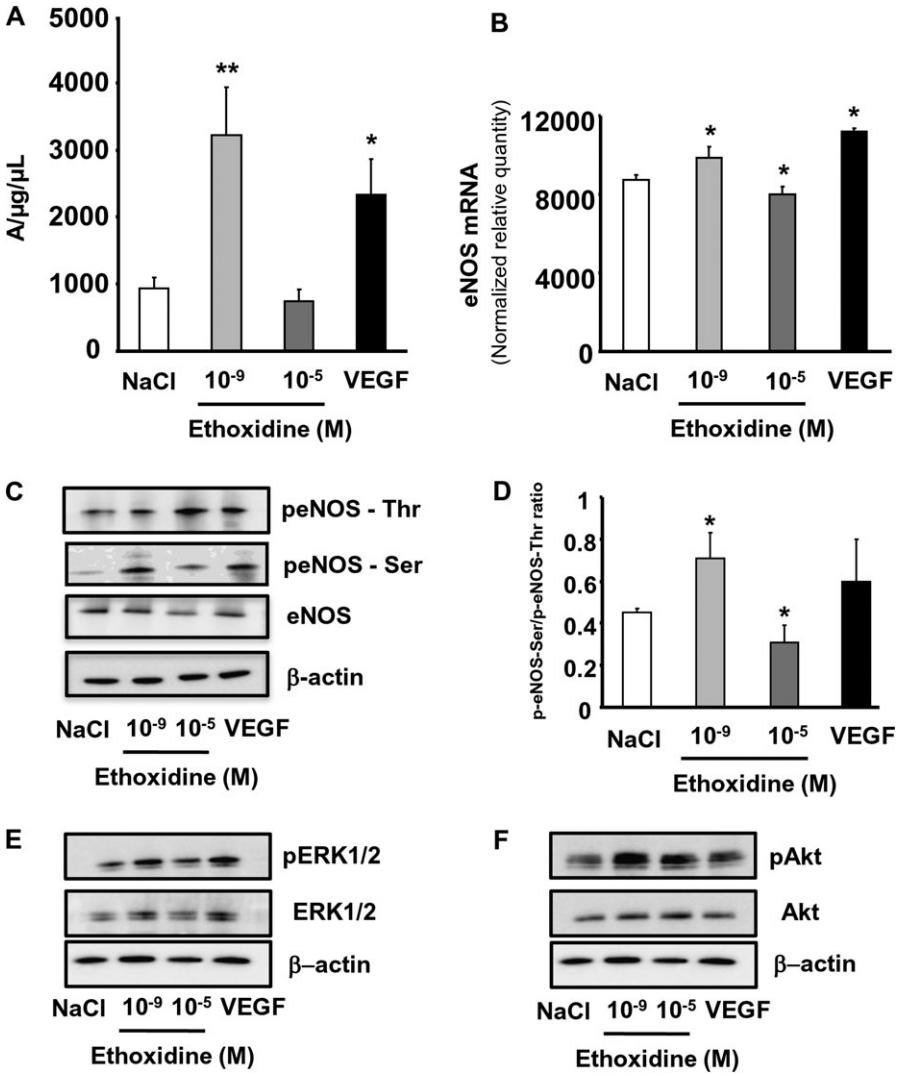

Fig. 5. (**A**) Quantification of the amplitude of the NO-Fe(DETC)$_2$ complex signal in EaHy.926 endothelial cells revealed a significant increase of NO production in cells treated with 10^{-9} M ethoxidine. No difference was found in cells treated with 10^{-5} M ethoxidine compared with control cells. 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. $^{**}P < 0.01$ versus NaCl; $^{*}P < 0.05$ versus NaCl. (**B**) Quantification of eNOS mRNA expression by real-time reverse transcription–PCR showed a significant increase in cells treated with 10^{-9} M ethoxidine and a significant decrease in endothelial cells treated with 10^{-5} M ethoxidine. VEGF (20 ng/ml) was used as positive control. Results are means ± SEMs from four independent experiments; $^{*}P < 0.05$ versus NaCl. (**C**) 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. (**D**) The 10^{-9} M ethoxidine increases the ratio between p-eNOS-Ser and p-eNOS-Thr, whereas 10^{-5} M ethoxidine decreases it. VEGF (20 ng/ml) was used as positive control. Results are means ± SEMs from four independent experiments; $^{*}P < 0.05$ versus NaCl. (**E**) Western blot revealed ERK1/2 expression and phosphorylation. β-Actin control was included. Data are representative of four separate blots. (**F**) Western blots showed Akt expression and phosphorylation. β-Actin control was included. Data are representative of four separate blots.

in cells treated with 10^{-9} M ethoxidine, whereas 10^{-5} M ethoxidine decreased its expression (Figure 5B). Western blot analysis revealed that 10^{-9} M ethoxidine did not modify eNOS expression, whereas 10^{-5} M ethoxidine decreased it. Furthermore, 10^{-9} M ethoxidine enhanced eNOS phosphorylation on its activator site (Ser-1177) but did not modify its phosphorylation at the inhibitor (Thr-495) site. Conversely, 10^{-5} M ethoxidine decreased eNOS phosphorylation on its activator site and enhanced its phosphorylation at the inhibitor site (Figure 5C). 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 greater in cells treated with 10^{-9} M ethoxidine and was lower in cells treated with 10^{-5} M ethoxidine compared with control cells (Figure 5D). As positive control, VEGF increased eNOS and p-eNOS-Ser-1177 and decreased p-eNOS-Thr-495 expressions in EaHy.926 endothelial cells although the ratio of phosphorylation was not statistically different.

Because the activation of extracellular signal-regulated kinase (ERK) 1/2 is an important signaling event for endothelial angiogenic processes, we determined whether ethoxidine modulates the phosphorylation of ERK1/2 in EaHy.926 endothelial cells. Treatment of EaHy.926 endothelial cells with 10^{-9} M ethoxidine increased both ERK1/2 expression and its phosphorylation like VEGF, whereas 10^{-5} M ethoxidine was without effect compared with untreated cells (Figure 5E).

It has been well known that the activation of Akt directly phosphorylates eNOS at its activator site Ser-1177 and subsequently increases NO production. Interestingly, low concentration of ethoxidine did not alter Akt expression but stimulated its phosphorylation. High concentration of ethoxidine did not affect both Akt expression and phosphorylation (Figure 5F).

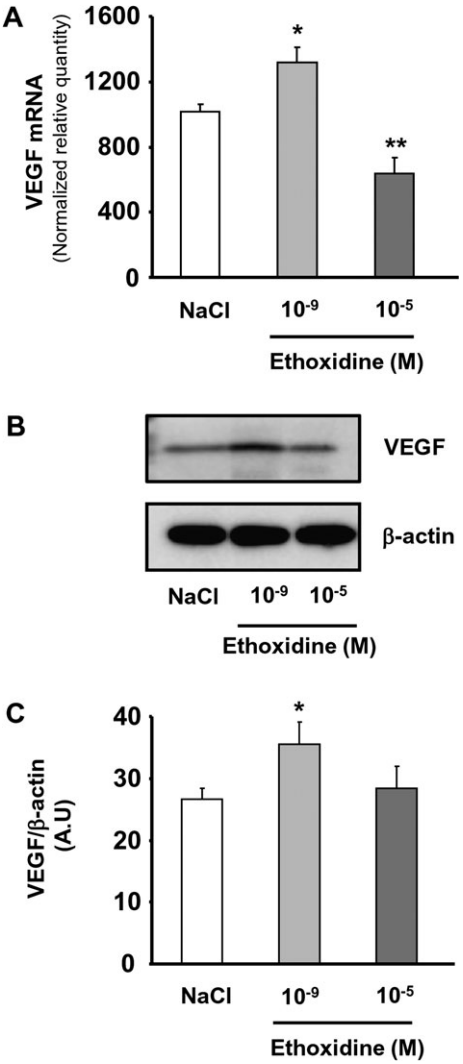

Fig. 6. (**A**) Quantitative reverse transcription–PCR analysis was conducted on total RNA from four independent EaHy.926 cultures. VEGF mRNA expression level was enhanced by 10^{-9} M and decreased by 10^{-5} M ethoxidine. $^{*}P < 0.05$ versus NaCl; $^{**}P < 0.01$ versus NaCl. (**B**) Western blot shows VEGF protein expression after treatment with ethoxidine for 36 h. (**C**) Ratio between VEGF expression and β-actin expression. The 10^{-9} M ethoxidine increased VEGF expression and 10^{-5} M decreased it. Data are representative of four separate blots. Results are means ± SEMs from four independent experiments; $^{*}P < 0.05$ versus NaCl.

Ethoxidine modulates O_2^- release in endothelial cells

Because reactive oxygen species promote cell proliferation and migration (23) in endothelial cells, we evaluated O_2^- in EaHy.926. EPR measurement of O_2^- production showed that treatment with 10^{-9} M ethoxidine significantly ($P < 0.05$) enhanced O_2^- production (172.70 ± 20.30%), whereas 10^{-5} M ethoxidine did not modify O_2^- production (132.01 ± 20.06%) compared with non-treated (100%) endothelial cells. As positive control, VEGF (158.33 ± 44.38%) enhanced significantly ($P < 0.05$) O_2^- production in EaHy.926 endothelial cells.

Influence of ethoxidine in the regulation of VEGF expression in endothelial cells

Treatment of EaHy.926 endothelial cells with 10^{-9} M ethoxidine enhanced both mRNA (Figure 6A) and protein expressions (Figure 6B and C) of VEGF. Furthermore, 10^{-5} M ethoxidine decreased VEGF mRNA (Figure 6A) but had no effect on its protein expression (Figure 6B and C) under the same experimental condition.

Discussion

The present study provides evidence that depending on the concentration, ethoxidine regulates the steps involved in angiogenesis. Thus, low concentration ethoxidine (10^{-9} M) increased capillary-like formation through the increase of cell proliferation and migration. These effects were associated with enhanced O_2^- and NO productions and upregulation of VEGF expression. Conversely, high concentration ethoxidine (10^{-5} M) inhibited *in vitro* angiogenesis decreasing endothelial cell proliferation and migration but it modified neither VEGF expression nor NO or O_2^- productions. Taken together, these data highlight the paradoxical effects of ethoxidine on key cellular processes involved on angiogenesis. Although the link between inhibition of Top I and angiogenic properties of ethoxidine has not been tested, the present study highlights the paradoxical effects of this inhibitor on key cellular processes involved on angiogenesis. A schematic of mechanisms of ethoxidine on angiogenesis process is illustrated on Figure 7.

The anti-proliferative properties of several Top I inhibitors (camptothecin, irinotecan) has been first studied on different tumor cell model (20,21) using concentrations ranged between 10^{-9} to 10^{-5} M. Among these drugs, it was shown that ethoxidine has a 10-fold greater potency on Top I inhibition in K562 and A549 cancer cell lines, as compared with other inhibitors (24). These effects on tumor cell proliferation were observed also with concentrations ranged between 10^{-9} to 10^{-5} M (11). Recently, similar concentrations were tested with ethoxy-fagaronine, one of the metabolites of ethoxidine, and an inhibition of L1210 leukemia cell proliferation was reported with maximal effective concentration 10^{-7} M (13). Since the bioavailability of this compound is not completely known, we have chosen to test its effects on *in vitro* angiogenesis in two types of human endothelial cells (i.e. EaHy.926 cell line and HUVECs), using ethoxidine at low and high concentrations. Although we reported paradoxical effects of ethoxidine on *in vitro* angiogenesis, it might be useful to clarify the bioavailability of this compound to determine the doses necessary to inhibit tumor growth, first in mice and then in human.

Angiogenesis is critical for tumor development (25) and neovascularization is now known as a pre-requisite to the rapid expansion of tumor cells associated with formation of macroscopic tumors (26). The ability of endothelial cells to form capillary tubes is a specialized function of this cell type resulting from a finely tuned balance between cell migration, proliferation and adhesion (27). The two concentrations of ethoxidine do not induce either cytotoxicity or apoptosis. Interestingly, 10^{-9} M ethoxidine had pro-angiogenic properties as shown by its ability to induce formation of capillary tubes both in EaHy.926 and HUVECs grown in both serum-deprived and complete media. Conversely, 10^{-5} M ethoxidine possessed anti-angiogenic properties in as much it strongly reduced capillary-like structures formation in EaHy.926 and HUVECs grown under the same experimental conditions. These results highlighted different properties of ethoxidine on *in vitro* angiogenesis depending on the concentrations used. Unlike the present work, previous *in vitro* and *in vivo* studies have reported that high concentrations of topotecan (5×10^{-8} to 2×10^{-7} M), another Top I inhibitor, had anti-angiogenic effects in Matrigel® migration assay and in morphogenesis assay (16,28). These two studies have been conducted with high concentrations known for their antitumor properties. The present study highlights paradoxical effects of ethoxidine on angiogenesis using two different concentrations.

The analysis of cellular processes involved on *in vitro* angiogenesis revealed that both cell proliferation and migration were enhanced by 10^{-9} M ethoxidine, whereas these cellular processes were inhibited by 10^{-5} M ethoxidine. In addition, ethoxidine was able to modulate MMP2 expression and activity according to the concentrations used. These results suggest that ethoxidine is able to differentially regulate events leading to angiogenesis. Similar results have been reported with topotecan, which mediates activation of transcription factors that regulate MMP2 expression both in tumor cells (29) and in endothelial cells (30) suggesting that it affects both tumor cells and endothelial cells for its therapeutic property when it is used at high concentration (10^{-7} to 10^{-6} M). Also, it is clear from the present study that

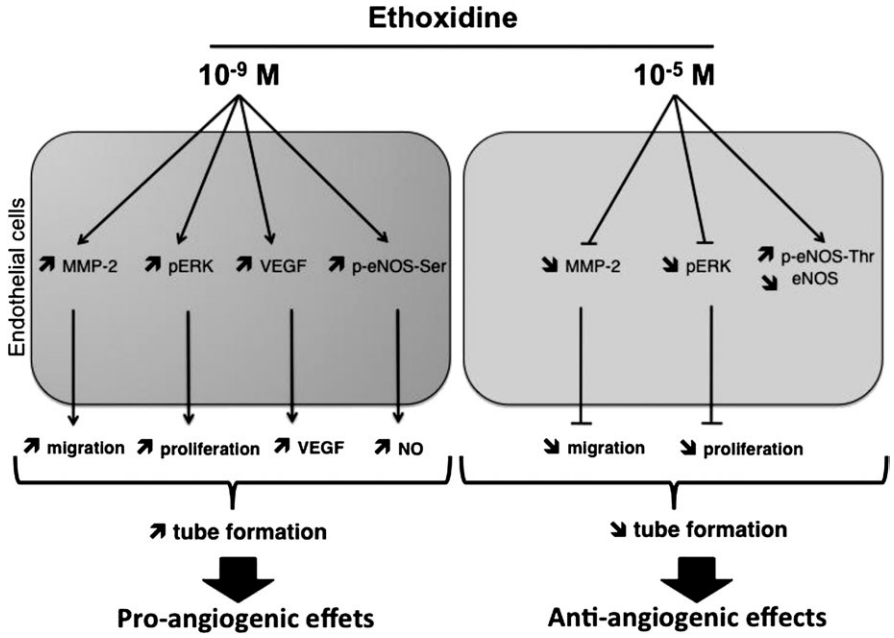

Fig. 7. Proposed model of ethoxidine implication in the regulation of *in vitro* angiogenesis. In one hand, treatment of endothelial cells with low concentration (10^{-9} M) ethoxidine increases cell migration and proliferation by enhancing MMP2 expression and activity and the phosphorylation of ERK1/2, respectively. Moreover, low concentration of ethoxidine induces an overexpression of VEGF and NO through an increase of eNOS phosphorylation on its activator site (Ser-1177). These observations confirm the pro-angiogenic properties of low concentration ethoxidine. On another hand, high concentration (10^{-5} M) ethoxidine exerts anti-angiogenic properties by decreasing endothelial cell migration and proliferation through a reduction of MMP2 expression and activity and a decrease of the phosphorylation of ERK1/2, respectively. Finally, treatment with 10^{-5} M ethoxidine decreases eNOS expression and favors its phosphorylation on its inhibitor site (Thr-495). pERK, phosphorylated extracellular signal-regulated kinases; p-eNOS-Ser, endothelial nitric oxide synthase phosphorylated on serine residue; p-eNOS-Thr, endothelial nitric oxide synthase phosphorylated on threonine residue.

ethoxidine at a concentration at which it had no reported effect on tumor cells (i.e. 10^{-9} M), it stimulates the processes leading to angiogenesis and therefore might be harmful when it is intended to treat cancers.

Beside its capacity to regulate permeability and blood flow, NO has been reported to be essential in cellular processes leading to angiogenesis (31) and to exert angiogenic properties in various tumor models (for review, see ref. 32). Low concentration (10^{-9} M) ethoxidine significantly enhanced NO production, whereas no variation was found in cells treated with high concentration (10^{-5} M) ethoxidine, in the present study. Moreover, low concentration ethoxidine increased eNOS mRNA expression and potentiated eNOS phosphorylation on its activator site, whereas high concentration decreased eNOS mRNA expression and favored eNOS phosphorylation on its inhibitor site. In HUVECs model, it was reported that NO production was mediated by phosphorylation of eNOS on its activator site (Ser-1177) and then activation of Akt (33). Interestingly, phosphorylation of Akt was greater in EaHy.926 cells treated with low concentration ethoxidine than in cells treated with high concentration. The serine/threonine protein kinase Akt is a downstream effector of Phosphoinositide-3-kinase that is activated by a variety of growth factors including those known to induce angiogenesis, such as VEGF and basic fibroblast growth factor in endothelial cells (34,35), suggesting a possible involvement of the PI3K-Akt pathway in the angiogenic process (36). If the link between DNA Top I and PI3K/Akt is not clear, the present study showed that inhibition of Top I activity with ethoxidine was associated with a decreased activity of Akt as reported by Nakashio *et al.* (30) with topotecan. In this last study, the authors claim that 10^{-7} M topotecan inhibits VEGF expression via a downregulation of the PI3K/Akt pathway.

In tumor-associated angiogenesis, angiogenic factors secreted by endothelial and tumor cells stimulate the growth of solid tumors (37). Among these factors, VEGF represents the major player in angiogenesis initiation by inducing endothelial NO production (38). Here, the analysis of VEGF expression revealed that 10^{-9} M ethoxidine enhanced mRNA expression, whereas 10^{-5} M ethoxidine inhibited it. Because mRNA transcription and protein synthesis are not simultaneous, we studied VEGF protein expression after a treatment with ethoxidine for 36 h. In these conditions, the results observed by western blot confirmed those obtained by quantitative reverse transcription–PCR. As VEGF regulates endothelial cell proliferation and migration using mitogen-activated protein kinase-dependent pathways (39), the phosphorylation of ERK1/2 has been evaluated. Low concentration of ethoxidine led to an increase of ERK1/2 phosphorylation greater than that observed for high concentration. The findings of this study suggest that paradoxical properties of ethoxidine on *in vitro* angiogenesis could be mediated by VEGF, which regulates the activity of ERK1/2 and whose expression is controlled by ethoxidine. As such, as for other molecules such as sorafenib (40), high concentration ethoxidine might be of interest in the treatment of tumor angiogenesis.

With regard to ROS production, 10^{-9} M ethoxidine was able to increase O_2^- production, whereas no change in O_2^- production was observed with 10^{-5} M. The modulation of O_2^- production by ethoxidine could explain the paradoxical properties of this molecule in the regulation of angiogenesis, particularly for low concentration ethoxidine. It was reported that ROS production stimulates induction of VEGF in various cell types (41) and promotes cell proliferation and migration (23), cytoskeletal reorganization (42) and tubular morphogenesis (43) in endothelial cells. Thus, the present study showed that low concentration ethoxidine induced an increase of O_2^- production associated with overexpression of VEGF and MMP2, which could explain its pro-angiogenic property. However, it is important to note that overproduction of ROS could be deleterious for Top I structure in endothelial cells. Indeed, recently, it was shown that ROS overproduction by endothelial cells could induce an oxidation of DNA Top I leading to the formation of oxidized DNA Top I that plays a direct pathogenic role in systemic sclerosis (44).

The main finding of the present study is the demonstration of paradoxical properties of a potent Top I inhibitor, ethoxidine in the regulation of *in vitro* angiogenesis. Although components of angiogenesis such as endothelial cell proliferation, migration and tube formation *in vitro* do not always correlate with angiogenesis *in vivo*, preliminary studies indicated that low dose of ethoxidine enhanced post-ischemic revascularization (SF, unpublished results). We can advance the hypothesis of a dual therapeutic benefit or deleterious depending on the concentrations used. First, ethoxidine at high concentrations could be used as anticancer drug targeting both tumor growth and metastasis by inhibiting angiogenesis. These properties confirmed those highlighted with other Top I inhibitors such as topotecan showing simultaneously anti-proliferative and anti-angiogenic properties on different cell models (45,46). A second finding of the present study provides a rational explanation for the probable beneficial effects of low dose of ethoxidine against ischemic diseases, although no study had yet clearly demonstrated their pro-angiogenic effect in ischemic conditions, especially *in vivo*. These data suggest a new therapeutic approach involving ethoxidine, a Top I inhibitor, to promote or inhibit angiogenesis depending on the concentrations used.

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