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A physiological concentration of anandamide promotes the migration of human endometrial fibroblast and the interaction with endothelial cells *in vitro*

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ABSTRACT

Introduction: The mechanisms that govern fibroblast behavior during the vascular adaptations of the uterus at early pregnancy remain unknown. Anandamide, an endocannabinoid, binds to cannabinoid receptors (CBs), and regulates gestation and angiogenesis. Its tone is regulated by fatty acid amide hydrolase (FAAH) within the uterus. We investigated the role of anandamide in endometrial fibroblasts migration and whether anandamide modulates fibroblasts-endothelial crosstalk.

Methods: T-hESC and EA.hy926 cell lines were used as models of endometrial stromal and endothelial cells, respectively. T-hESC were incubated with anandamide plus different agents. Migration was tested (wound healing assay and phalloidin staining). Protein expression and localization were studied by Western blot and immunofluorescence. To test fibroblast-endothelial crosstalk, EA.hy926 cells were incubated with fibroblast conditioned media obtained after T-hESC migration.

Results: Anandamide 1 nM increased T-hESC migration via CB1 and CB2. Cyclooxygenase-2 participated in anandamide-stimulated fibroblast migration. Prostaglandin F2alpha, and not prostaglandin E2, increased fibroblast wound closure. CB1, CB2, cyclooxygenase-2 and FAAH were expressed in T-hESC. Anandamide did not alter cyclooxygenase-2 localization but induced its cytoplasmic and nuclear expression through CB1 and CB2. URB-597, a FAAH selective inhibitor, also increased T-hESC migration via both CBs, and augmented cyclooxygenase-2 expression. Conditioned media from anandamide-induced T-hESC wound healing closure stimulated endothelial migration and did not alter their proliferation. Soluble factors from cyclooxygenase-2 were secreted by T-hESC and participated in T-hESC-induced EA.hy926 migration. Although anandamide-conditioned media augmented in EA.hy926 the expression of γH2AX, a marker of DNA damage, cyclooxygenase-2 was not involved in this effect.

Discussion: Our results provide novel evidence about an active role of anandamide on endometrial fibroblast behavior as a mechanism regulating uterine vascular adaptations in early gestation.

Abbreviations: AEA, Anandamide; CB1, cannabinoid receptor type 1; CB2, cannabinoid receptor type 2; FAAH, fatty acid amide hydrolase; COX-2, cyclooxygenase-2

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1. Introduction

The migratory behavior of human endometrial stromal fibroblasts is proposed to contribute to the deep tissue remodeling related to embryo implantation, trophoblast invasion and endometrial regeneration. In the middle of the menstrual cycle, the stroma of the endometrium is composed mainly of fibroblasts. Stromal fibroblasts begin to transform into decidual cells after ovulation, and if pregnancy occurs, the decidualization reaction is exacerbated. Nonetheless, non-differentiated endometrial stromal cells stay in the decidua and placenta [1,2]. Yet, their contribution in early gestation remains poorly understood.

It has been described that anandamide (AEA, N-arachidonoylethanolamine), the best studied endocannabinoid, plays a critical role during pregnancy and regulates fibroblast behavior in different biological systems [3,4]. Once released, AEA binds to the cannabinoid receptors type 1 and type 2 (CB1 and CB2). Also, AEA could exert its action by receptor-independent pathways through the production of metabolites that result from the activity of fatty acid amide hydrolase (FAAH) and cyclooxygenase-2 (COX-2). Degradation of AEA by FAAH is essential to ensure an adequate AEA tone throughout pregnancy [5–8]. Indeed, dysregulations in AEA level correlate with implantation failure and recurrent abortion [9,10].

The coordination of vascular processes at the maternal-fetal interface is crucial for the maintenance of gestation and requires a profound reorganization of uterine and fetal tissues. Endometrial fibroblasts might be part of this dialogue that ensures an adequate blood flow to the growing embryo. Besides its role in reproduction, AEA displays vasoactive properties and regulates angiogenesis and vascular growth [11,12]. Also, COX-2-derived prostaglandins increase vascular permeability and angiogenesis in mice implantation sites [13]. Women with repeated implantation failure have dysregulated COX-2 expression in the endometrium [14]. Previously, we showed that AEA regulates COX-2 expression and prostaglandin synthesis in the rat uterus during the window of implantation and pseudopregnancy [15,16].

Based on these antecedents, here we explored the contribution of AEA and endometrial stromal fibroblasts to the vascular remodeling of the uterus, a crucial event leading to a successful pregnancy. We investigated which factors contribute to AEA specific action on endometrial fibroblast migration and the crosstalk between endometrial fibroblasts and endothelial cells. Understanding the processes underlying the role of AEA on endometrial fibroblasts would further shed light on the cellular and molecular basis that governs early pregnancy.

2. Materials and methods

2.1. Cell culture

T-hESC human endometrial stromal cell line and EA.hy926 endothelial cell line were used. Telomerase-immortalized Human Endometrial Stromal Cells line (T-hESC) was purchased to American Type Culture Collection (ATCC, CRL-4003). EA.hy926 cell line was donated by Dr. Gareth Owen (Pontifical Catholic University of Chile, Santiago, Chile). Both cell lines were incubated at 37 °C with 5% CO2 in DMEM/F12 (Gibco) supplemented with 10% fetal bovine serum (Natocor), 100 U/ml penicillin (Gibco), 100 μg/mL streptomycin (Gibco) and 1% glutamine (Microvet SRL).

Final concentrations of the vehicles used to dilute the agents in which both cell lines were incubated are specified in Supplementary Table 1.

2.2. Wound healing assay and evaluation of actin filaments in T-hESC and EA.hy926

T-hESC and EA.hy926 were plated at 10^4/well until confluence. A wound was made with a sterile tip. Unattached cells were washed. T-hESC cells were treated for 15 h with different agents (AEA, URB-597, AM630, AM251, methanandamide, meloxicam). EA.hy926 were incubated for 18 h with conditioned media (CM). CM consists of a dilution of 1:7 of T-hESC migration supernatants with media. Media without fetal bovine serum was taken as basal (negative control). Both cell lines were incubated at 37 °C in DMEM/F12. Photographs were taken at initial wounding (t = 0 h) and 15 h or 18 h (the optimal time to quantify T-hESCand EA.hy926 migration respectively). Images were analyzed using the Image J software (open source). Results were expressed as the percentage of cell migration.

Actin filaments were evaluated as described [17,18]. T-hESC (40×10^3/well) and EA.hy926 (30×10^3/well) were seeded, grown to confluence and incubated with AEA 1 nM for 24 h or with the CM for 18 h respectively. Actin was stained with TRITC-phalloidin (Sigma Aldrich), mounted (DABCO mounting medium, Sigma Aldrich), and observed by fluorescence microscopy at 40× (Nikon Eclipse 200, USA).

2.3. Western blot of FAAH and CB1 in T-hESC and γH2AX in EA.hy926

T-hESC (400×10^3/well) and EA.hy926 (50×10^4/well) were plated and grown to confluence. T-hESC cells were treated as described [17]. EA.hy926 were incubated with CM (1:7) for 24 h at 37 °C and treated as described [19]. Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with anti-FAAH (1:250, #ab54615, Abcam), anti-CB1 (1:250, #ab3558, Abcam), anti-γH2AX (1:1000, #ab26350, Abcam) or anti-GAPDH (1:8000, #2118, Cell Signaling Technology) [19]. The signal was detected by chemiluminescence. Each membrane was exposed to CL-XPosure films (Kodak) and photographed. A homogenate from rat uterus on day 5 of gestation was used as positive control for CB1 and FAAH [15,20]. T-hESC Western blots are representative of three independent experiments. In the case of EA-hy926 Western blots, protein levels were analyzed by densitometry using Scion Image for Windows (Scion Corporation, Worman's Mill, CT, USA). Optical density is expressed as arbitrary units ± SEM. Blots shown are representative of four independent experiments.

2.4. FAAH, CB1, CB2 and COX-2 immunocytochemistry in T-hESC

T-hESC were treated as described previously [17]. Briefly, cells (40×10^3/well) were grown to confluence on coverslips. In the case of COX-2 immunostaining, cells were incubated for 24 h with medium (control), AEA 1 nM, AEA + AM 630, AEA + AM251 or URB-597. Then, the monolayer was fixed (10 min, room temperature, 3.7% w/v paraformaldehyde) and permeabilized with 0.1% TRITON X-100 in PBS for 5 min. For CB receptors, samples were also incubated without TRITON X-100. Non-specific binding sites were blocked (60 min, 40 mg/mL BSA in PBS). Slices were incubated overnight at 4 °C with anti-COX-2 (1:50, #60126, Cayman Chemical), anti-FAAH (1:200, #ab54615, Abcam), anti-CB1 (1:250, #ab23703, Abcam) or anti-CB2 (1:50, #101550, Cayman Chemical). Afterward, cells were incubated for 60 min at room temperature with Alexa-Fluor 555 goat anti-Mouse IgG (1:500, Abcam) for FAAH or Alexa-Fluor 555 goat anti-rabbit IgG (1:500, Abcam) for CB1, CB2 and COX-2. Nuclei were stained with Hoechst (#33352, Sigma Aldrich). Immunoreactive specificity was assessed by omitting the first antibody. Cells were mounted using DABCO mounting medium (Sigma Aldrich) and observed by fluorescence microscopy at 40× (Nikon Eclipse 200, USA). COX-2 immunofluorescence signal in the cytoplasm and the nucleus was analyzed quantitatively using the Image J (open source) software package and expressed as mean gray value.

2.5. Proliferation assay in EA.hy926

EA.hy926 cells (10 × 103/well) were grown in 96 well plates at 37 ˚C with 5% CO2. Proliferation was determined using WST-1 reagent (4-[3-(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene sulfonate; Roche Diagnostics, Mannheim, Germany), following the manufacturer's instructions. Briefly, after stimulation with CM for 18 h, 12 μL of WST-1 was added to each well and cells were incubated for 30 min. Absorbance was measured using a microplate reader at 440 nm and 620 nm.

2.6. Statistical analysis

All values represent mean ± standard error of the mean (SEM). Data was normally distributed according to the Shapiro-Wilk normality test. Homoscedasticity was tested by Brown-Forsythe test. Comparisons between values of different groups were performed using analysis of variance (ANOVA) and significance was determined using post hoc tests Bonferroni or Tukey's multiple comparisons. Differences between means were considered significant when $p < 0.05$. Statistical analysis was performed using GraphPad Prism software version 8.0.1 (244).

3. Results

3.1. AEA promoted endometrial fibroblast migration

It is postulated that the migratory nature of endometrial fibroblasts would participate in the remodeling of the maternal-fetal interface during early gestation. Therefore, we first investigated whether AEA regulated the migration of endometrial stromal cells. For this, T-hESC cells were incubated with different concentrations of AEA and tested for wound healing closure. AEA showed a concentration-response curve on T-hESC migration. AEA 1 nM stimulated the wound closure, whereas 10 and 100 nM did not modify it (Fig. 1a). Interestingly, AEA 1000 nM

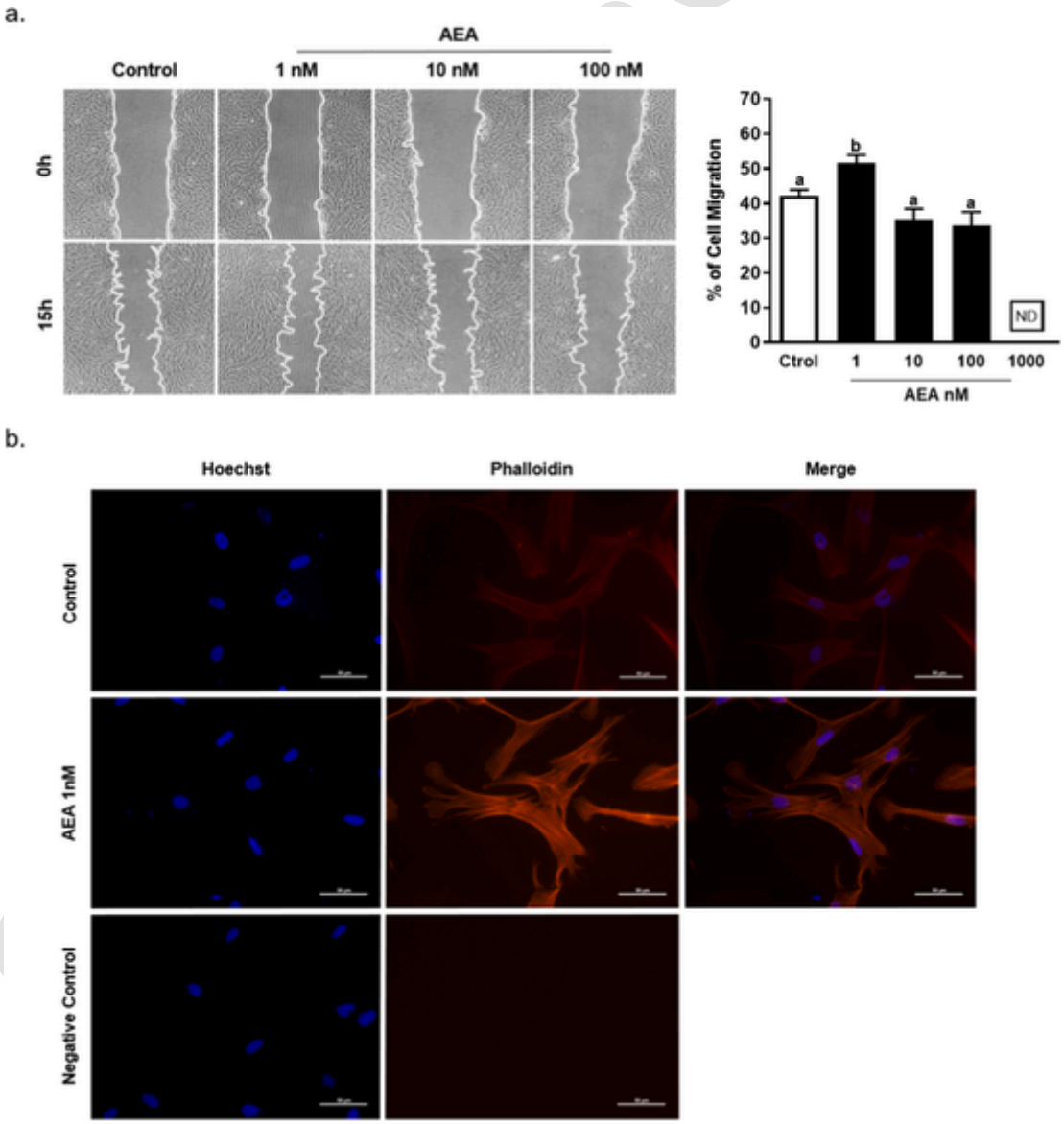

Fig. 1. Anandamide (AEA) stimulated human endometrial stromal cells (T-hESC) migration. (a) T-hESC were incubated with medium (control) or AEA (1, 10, 100 and 1000 nM) and assayed for migration (wound healing closure). Representative photographs at 0 and 15 h are shown (4×). Data is expressed as mean ± S.E.M. Three replicates per treatment, each experiment repeated 3–5 times. Different letters indicate statistical differences ($p < 0.05$). ND: not detectable. (b) Effect of AEA 1 nM on T-hESC cytoskeleton arrangement. Actin filaments were stained with TRITC-phalloidin after 24 h of incubation with AEA. Representative photographs under a fluorescent microscope are shown (40×). Photographs represent three independent experiments. Negative control: phalloidin was omitted.

affected T-hESC viability. Remarkably, the physiological concentration of AEA detected in different reproductive fluids (1 nM) [21] stimulated T-hESC migration, while AEA in micromolar concentration (1000 nM) showed deleterious effects. Afterward, actin filament polymerization was used to confirm the migration of T-hESC cells incubated with AEA. AEA 1 nM induced a rearrangement of the actin filaments compared to the control characterized by the elongation of stress fibers (Fig. 1b). Therefore, AEA stimulated endometrial fibroblast migration. The concentration of 1 nM was selected for future experiments.

3.2. CB1 and CB2 mediated AEA-stimulated T-hESC migration

Next, we studied the participation of the endocannabinoid receptors on the AEA-triggered fibroblast migration. For this, we described the expression of CB1 and CB2 in T-hESC cells. Western blot analyses showed that CB1 protein was expressed in T-hESC as a single band at ~58 KDa (Fig. 2a). Immunofluorescence analyses showed specific immunoreactivities of CBs in T-hESC (Fig. 2b). CB1 localization did not change with or without TRITON X-100. When T-hESC cells were not permeabilized, CB1 staining seemed to be localized mainly around the nucleus and it was more diffuse towards the cell edges. TRITON X-100 pre-treatment increased the perinuclear staining. As observed in the merge panels, CB1 might not be localized in the nuclear area when T-hESC were incubated with or without TRITON X-100. In the case of CB2, when T-hESC were not permeabilized, the receptor was mainly localized outside the nucleus. However, when cells were treated with TRITON X-100 a strong CB2 signal appeared in the nuclear area.

Once we detected that both CB1 and CB2 were present in T-hESC, cells were incubated with AEA 1 nM, AEA + AM251 10 nM (a selective CB1 antagonist), AEA + AM630 10 nM (a selective CB2 antagonist), and AM251 or AM630 alone. The concentration of CBs antagonists was selected from previous published data [15]. Also, based on binding and functional data, AM251 and AM630 at the selected concentration are highly potent and selective antagonists for CB1 and CB2 receptors respectively [22,23]. The co-incubation of AEA with AM251 or with AM630 completely prevented AEA-triggered T-hESC migration (Fig. 3a). Treatment with AM251 or AM630 alone did not show any effect.

The degradation pathway of AEA involves the participation of the FAAH enzyme. AEA metabolites could also exert biological effects in the uterus. To confirm the hypothesis that the effect of AEA was mediated by its binding to CB receptors and not by its metabolites, T-hESC were incubated with methanandamide 1 nM. Methanandamide is a non-hydrolysable analog of AEA as it is not a substrate of FAAH. We observed that methanandamide 1 nM also increased stromal cells wound healing closure (Fig. 3b).

These results reinforced the notion that AEA might increase endometrial fibroblast migration through binding to its cannabinoid receptors CB1 and CB2, and not via the generation of metabolites resulting from its degradation.

3.3. COX-2 participated in the migration of endometrial stromal cells

COX-2-derived prostaglandins are downstream mediators of AEA-triggered actions. To determine the participation of the COX-2 pathway, meloxicam was used as it is a highly COX-2 inhibitor. Thus, T-hESC were incubated for 15 h with AEA 1 nM, AEA + meloxicam 1 μM or meloxicam alone, and cells were tested for wound healing closure. AEA + meloxicam completely prevented AEA-triggered migration (Fig. 4a), suggesting that COX-2 participated in this process. Meloxicam alone did not exert any effect. Based on this result, we investigated if AEA modulated COX-2 expression and localization. The incubation of T-hESC with AEA 1 nM did not alter COX-2 localization (Fig. 4b), as COX-2 was localized in the cytoplasm and nucleus of T-hESC in control and AEA conditions. The incubation with AEA 1 nM increased the staining of COX-2 in the cytoplasm and nucleus (Fig. 4b). Moreover, the co-incubation of AEA with AM 251 10 nM (CB1 selective antagonist) or with AM630 10 nM (CB2 selective antagonist) reversed AEA action. This result suggests that AEA stimulated COX-2 expression via CB1 and CB2 receptors.

Afterward, T-hESC were treated with prostaglandin E2 or F2alpha. Prostaglandin E2 1 nM or 10 nM did not modify stromal cell migration (Supplementary Fig. 1a). However, prostaglandin F2alpha 10 nM stimulated T-hESC wound healing closure. Prostaglandin F2alpha 1 nM did not show any effect (Supplementary Fig. 1b).

Altogether, these results suggest that AEA binding to CB receptors triggered the downstream activation of COX-2, and that COX-2-derived prostaglandin F2alpha might mediate AEA-stimulatory effect on endometrial stromal cell migration.

3.4. Endogenous AEA promoted T-hESC migration through CB1 and CB2 receptors

As mentioned, AEA degradation involves the FAAH enzyme. URB-597, a FAAH selective inhibitor, increases the endogenous levels of AEA in the oviduct and other tissues [7,24]. Hence, URB-597 was selected to study the action of endogenous AEA and corroborate the effect of exogenous AEA on T-hESC migration.

FAAH protein was detected by Western blot analyses as a single band with a molecular size of ~55 kDa (Fig. 5a) and it was localized in the cytoplasm (Fig. 5b). To our knowledge it is the first time that the presence of FAAH is described in the T-hESC cell line.

Once we confirmed that FAAH was expressed in T-hESC, cells were incubated with URB-597, the FAAH inhibitor, and assayed for wound healing closure. As exogenous AEA, URB-597 also presented a concentration-dependent effect (Fig. 5c). URB-597 100 nM stimulated endometrial stromal cells migration compared to the control, having an effect similar to that of AEA 1 nM (Fig. 5c). Other URB-597 concentrations as 1 nM and 10 nM did not alter T-hESC wound closure. Also, URB-597 100 nM did not modify COX-2 localization but increased its cytoplasmic and nuclear staining (Fig. 5d). Thus, it seemed that exogenous and endogenous AEA exerted a similar effect on T-hESC migration and COX-2 expression.

Although FAAH preferentially hydrolyzes AEA, it could also degrade other fatty acid ethanolamides such as oleoylethanolamide, oleamide and palmitoylethanolamide [25]. However, unlike AEA, these fatty acid ethanolamides are not able to activate CB1 or CB2 [26–29]. Here, we postulate that increased endogenous AEA level due to FAAH inhibition is responsible for T-hESC augmented migration. To corroborate this hypothesis we decided to test if the migration induced by URB-597, the FAAH inhibitor, was prevented by the incubation with CB1 or CB2 antagonists. Therefore, T-hESC cells were incubated for 15 h in the presence of media (control), URB-597 100 nM, URB-597 + AM251 10 nM (CB1 antagonist) or URB-597 + AM630 10 nM (CB2 antagonist), and the wound healing closure was evaluated. We observed that the co-incubation with AM251 or AM630 reversed URB-597-induced T-hESC migration (Fig. 5e), reinforcing the notion that URB-597 action is primarily due to AEA accumulation and not to other FAAH hydrolysable amides.

These results reinforce the notion that a physiological concentration of AEA would promote the migration of the endometrial stromal fibroblasts at the maternal-fetal interface and that both cannabinoid receptors mediate this effect.

3.5. AEA promoted endometrial fibroblasts - endothelial cell interaction

We propose that endometrial fibroblasts migration participates and favors the vascular remodeling of the maternal-fetal interface in early gestation. Part of the vascular remodeling process involves the loss of the endothelium present in the maternal vessels. Thus, soluble factors released by stromal cells of the endometrium might reach the vascular

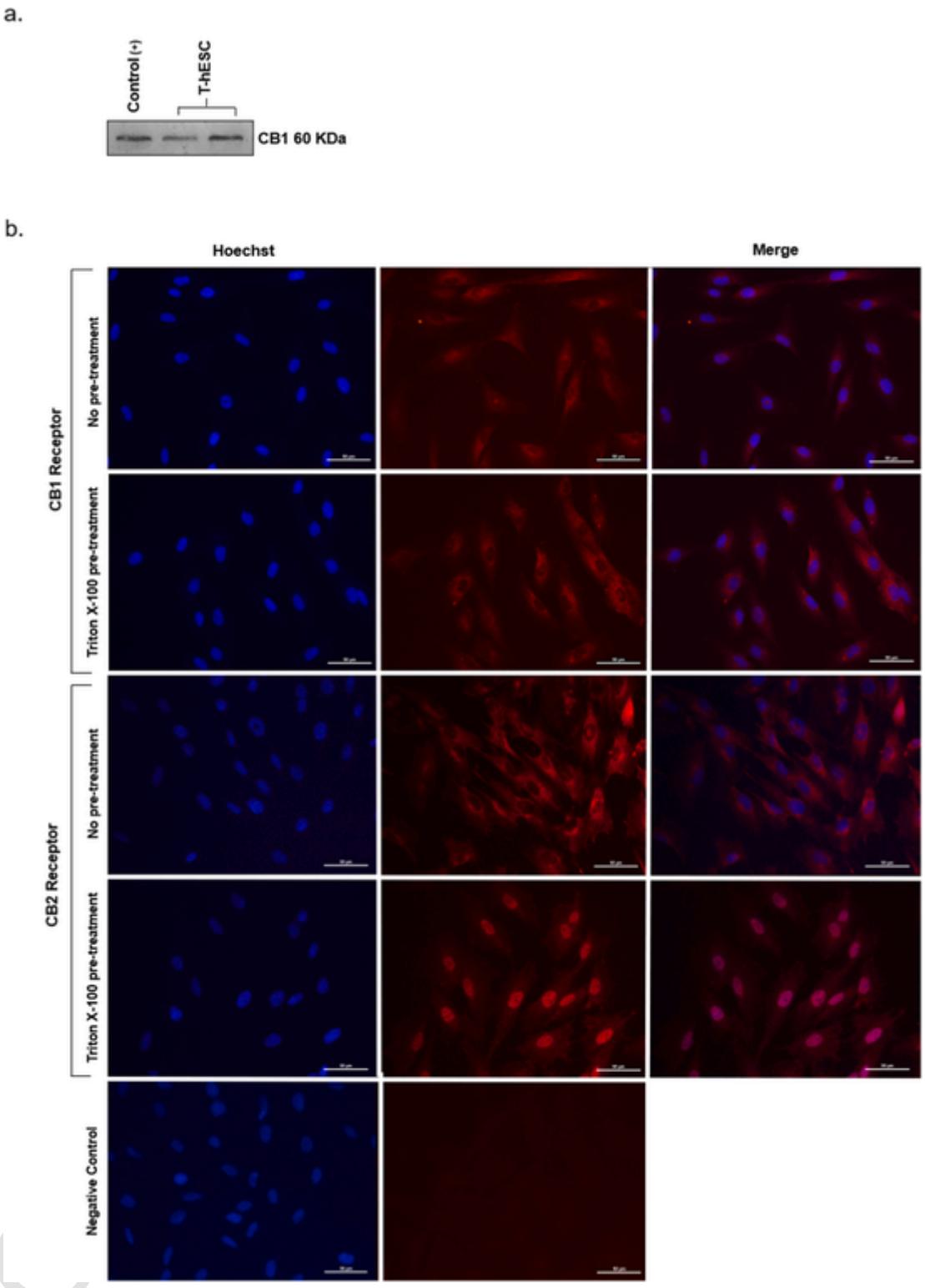

Fig. 2. CB1 and CB2 expression and localization in human endometrial stromal cells (T-hESC). (a) CB1 protein was detected by Western blot in T-hESC. The photograph represents three independent experiments. (b) CB1 and CB2 localization in T-hESC was assessed by immunofluorescence staining. Representative photographs of two independent experiments are shown (40×). Nuclei stained with Hoescht showed blue. CB1 and CB2 appeared as red signals. In the merge panels overlapping blue and red labelings are shown. Negative control: first antibody was omitted.

bed and influence the endothelial cells. To test this hypothesis, we studied whether AEA-driven endometrial fibroblast migration regulated endothelial cells behavior. Therefore, supernatants were collected after T-hESC migration and used as CM in incubations with the EA.hy926 cell line (Fig. 6a).

The incubation of EA.hy926 with control-CM stimulated migration to the same level as basal conditions (Fig. 6b). Notably, AEA-CM induced EA.hy926 migration compared to control-CM. The release of soluble factors triggered by AEA during T-hESC migration promoted EA.hy926 wound healing closure. To confirm that this effect was due to

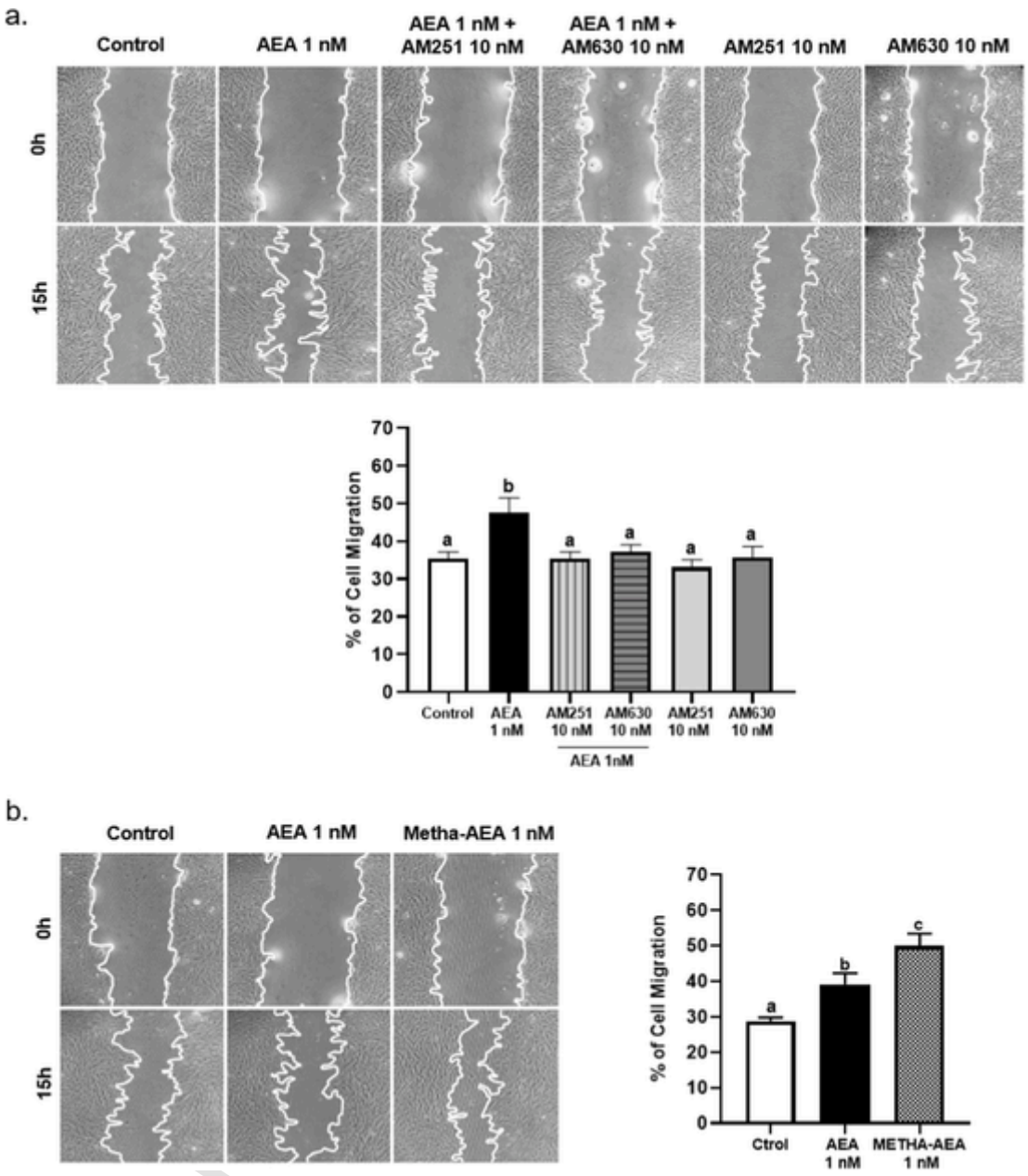

Fig. 3. CB1 and CB2 receptors mediated anandamide (AEA) stimulated human endometrial stromal cells (T-hESC) migration. (a) T-hESC were incubated with medium (control), AEA 1 nM, AEA + AM251 10 nM (selective CB1 antagonist), AEA + AM630 10 nM (selective CB2 antagonist), AM251 10 nM alone or AM630 10 nM alone and assayed for migration (wound healing closure). (b) Action of methanandamide (Metha-AEA, 1 nM) on T-hESC migration. (a) and (b) Representative photographs at 0 and 15 h are shown (4×). Data is expressed as mean ± S.E.M. Three replicates per treatment, each experiment was repeated 3–5 times. Different letters indicate statistical differences ($p < 0.05$).

the release of soluble factors to the culture media and not to a direct action of AEA, EA.hy926 cells were incubated directly with AEA 1 nM and assayed for wound healing. We observed that the addition of AEA 1 nM directly to EA.hy926 stimulated the migration to a higher level compared to AEA-CM (Fig. 6b). Afterward, endothelial cells were assayed for phalloidin staining. We observed that AEA-CM stimulated a migratory phenotype in EA.hy926, denoted by a rearrangement of the actin filaments compared to control-CM (Fig. 6c). AEA-CM induced the elongation of stress fibers in endothelial cells, thus confirming the results obtained in the wound healing closure.

As mentioned, we observed that COX-2 participated in the migration of stromal fibroblasts (Fig. 4). Therefore, we investigated if the COX-2 pathway participated in the fibroblast-endothelial interaction. We observed that the AEA + meloxicam-CM prevented AEA-CM stimulatory action on endothelial migration (Fig. 6b). As a control, EA.hy926 cells were incubated with meloxicam 1 μM for 15 h and assayed for wound healing. We observed that meloxicam 1 μM did not alter EA.hy926 migration compared to basal and control-CM.

It is postulated that endothelial cells are removed from the maternal vessels by migration and/or apoptosis. Thus, to deepen the analysis of the activation of the endothelial cells by AEA conditioned medium of fibroblasts, we studied the effect of AEA-CM on EA.hy926 proliferation and DNA damage. AEA-CM did not alter endothelial cell proliferation (Fig. 6d). On the contrary, when DNA damage was studied, the treatment of EA.hy926 with AEA-CM augmented the expression of γH2AX, a marker of this type of process (Fig. 6e). The incubation with AEA + meloxicam-CM did not show differences compared to AEA-CM in γH2AX protein level.

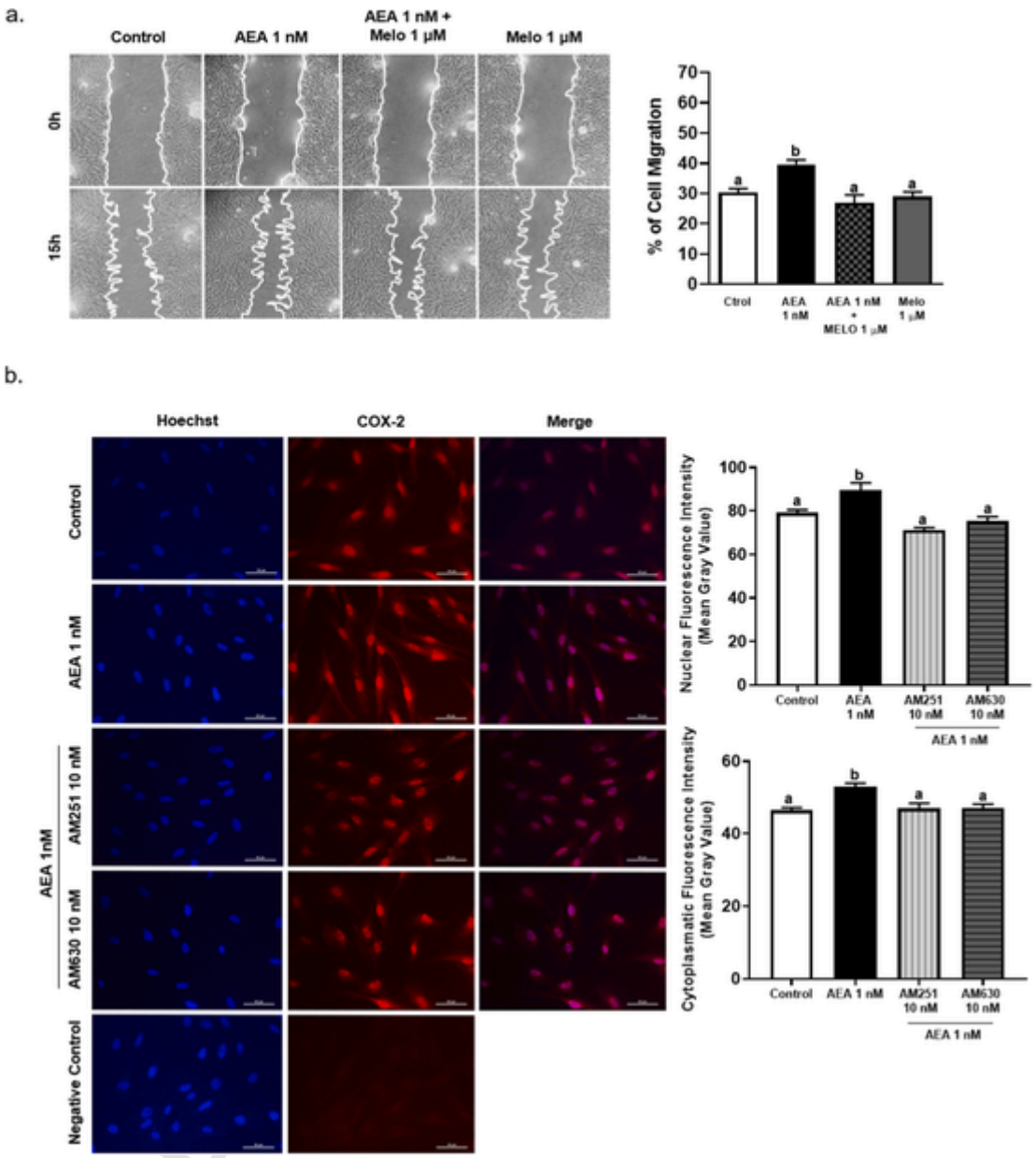

Fig. 4. Cyclooxygenase-2 (COX-2) participated in anandamide (AEA) induced human endometrial stromal cells (T-hESC) migration. (a) T-hESC were incubated with medium (control), AEA 1 nM, or AEA + meloxicam 1 μM (Melo, highly selective COX-2 inhibitor) and assayed for migration (wound healing closure). Representative photographs at 0 and 15 h are shown (4×). Three replicates per treatment, each experiment was repeated 3–5 times. (b) T-hESC were treated with medium (control), AEA 1 nM, AEA + AM251 10 nM (CB1 antagonist) or AEA + AM630 10 nM (CB2 antagonist) and COX-2 localization was assessed by immunofluorescence staining. Representative photographs of three independent experiments are shown (40×). Nuclei stained with Hoescht showed blue. COX-2 appeared as a red signal. In the merge panels overlapping blue and red labelings are shown. Negative control: first antibody was omitted. (a) and (b) Data represents mean ± SEM. Different letters indicate statistical differences ($p < 0.05$).

These results suggest that AEA stimulates endometrial fibroblast migration and the release of soluble factors from the COX-2 pathway that participate in the migration of the endothelial cells and apoptosis.

4. Discussion

The present work demonstrates that a physiological concentration of AEA induces the migration of endometrial fibroblasts through a mechanism that involves the participation of CBs receptors and COX-2 in an *in vitro* model. Also, AEA promotes the crosstalk between two crucial players at the maternal-fetal interface, the endometrial fibroblasts and endothelial cells. Understanding the role of AEA on endometrial stromal cell behavior would further shed light on the cellular and molecular basis that governs endometrial pathologies associated with aberrant stromal fibroblasts behavior.

We showed that AEA within the physiological range (1 nM) stimulates stromal fibroblast migration. Conversely, micromolar AEA (1000 nM) has deleterious effects. In line with our findings, Gentilini et al. [30], published that methanandamide stimulates migration and cellular elongation of endometrial fibroblasts primary cultures. Others showed that the incubation of St-T1b with AEA 1 μM does not affect cell viability [31]. Notably, higher concentrations of AEA (10 and 25 μM) do reduce viability and induce apoptosis in St-T1b [31]. The discrepancy with our results might reside in different culture conditions and sensitivity of the cell lines to AEA.

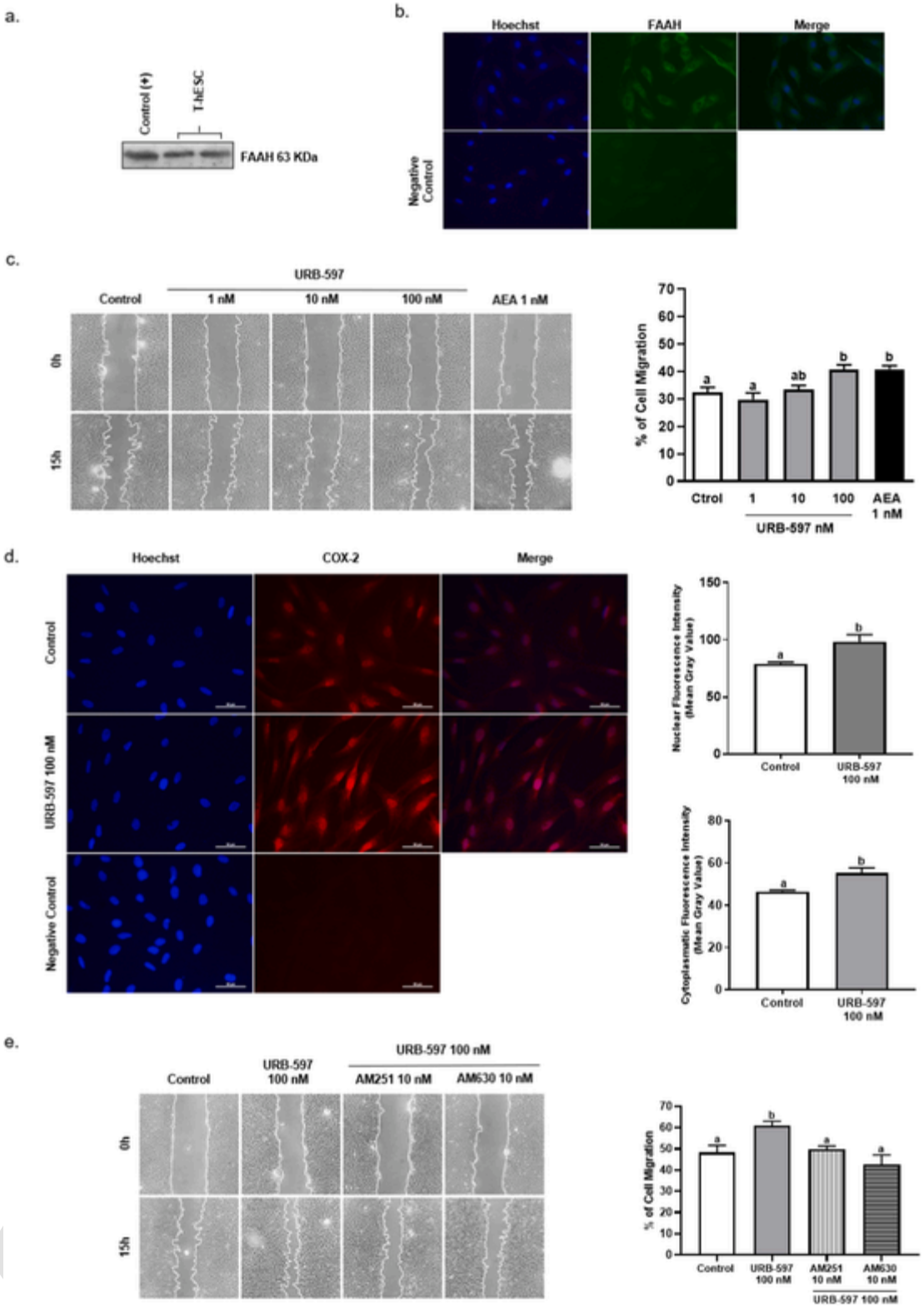

Fig. 5. URB-597, a FAAH inhibitor, increased human endometrial stromal cells (T-hESC) migration via CB1 and CB2 receptors. (a) FAAH protein was detected in T-hESC by Western blot. The photograph represents two independent experiments. (b) FAAH localization in T-hESC was assessed by immunofluorescence staining. Representative photographs of two independent experiments are shown (40×). Nuclei stained with Hoescht showed blue. FAAH appeared as a green signal. In the merge panel overlapping blue and green labelings are shown. Negative control: first antibody was omitted. (c) T-hESC were incubated with medium (control) or URB-597 1 nM and COX-2 localization was assessed by immunofluorescence staining. Representative photographs of three independent experiments are shown (40×). Nucleus stained with Hoescht showed blue. COX-2 appeared as a red signal. In the merge panels overlapping blue and red labelings are shown. Negative control: first antibody was omitted. (e) T-hESC were incubated with medium (control), URB-597 (1, 10 and 100 nM) or with anandamide (AEA, 1 nM) and assayed

Fig. 5.—*continued*
for migration (wound healing). (d) T-hESC were incubated with medium (control), URB-597 1 nM, URB-597 + AM 251 10 nM (selective CB1 antagonist) or URB-597 + AM630 10 nM (selective CB2 antagonist) and assayed for migration (wound healing). (c) and (e) Representative photographs at 0 and 15 h are shown (4×). (c), (d) and (e) Data is expressed as mean ± S.E.M. Three replicates per treatment, each experiment repeated 3–5 times. Different letters indicate statistical differences ($p < 0.05$).

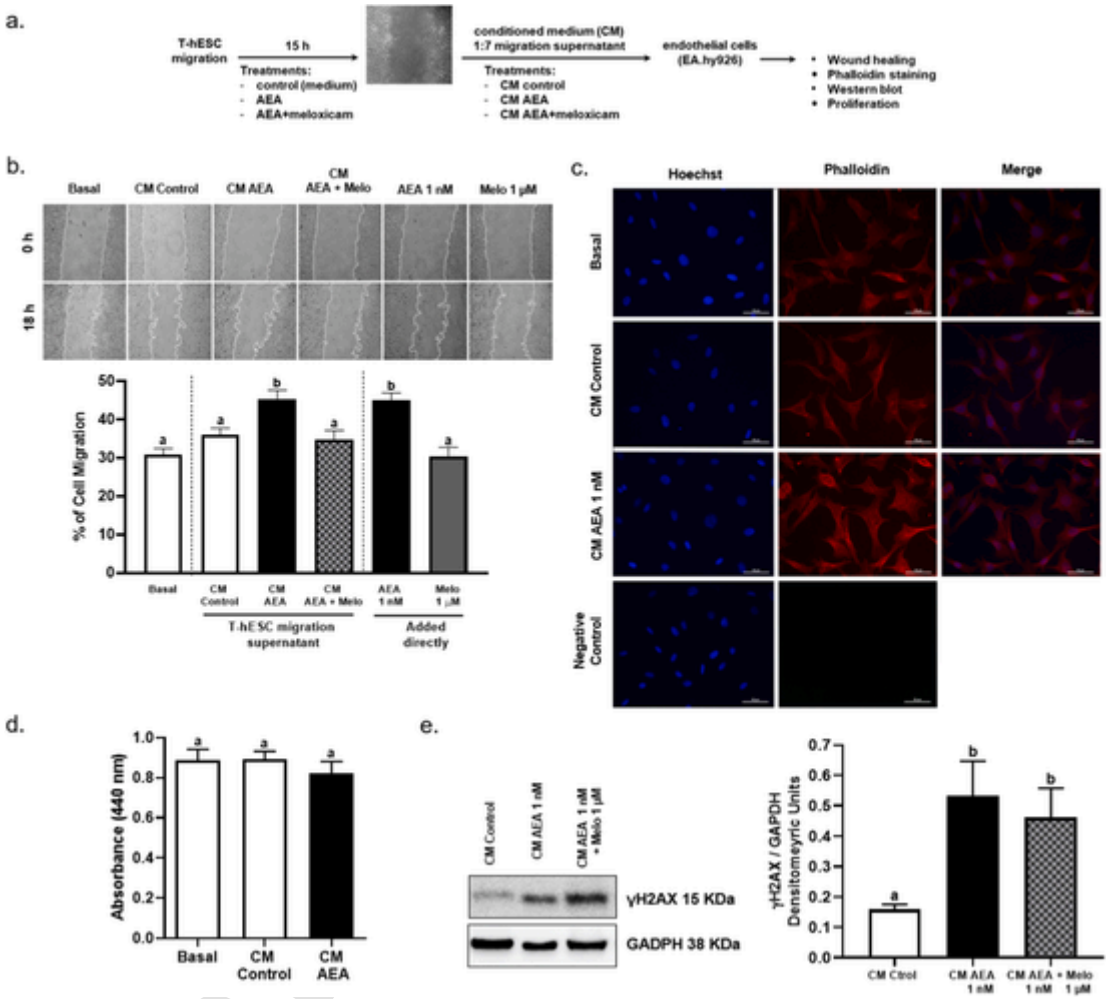

Fig. 6. Anandamide (AEA) promoted the interaction between endometrial stromal cells and endothelial cells. Human endometrial stromal cells (T-hESC) were seeded onto 24 well plates, incubated for 15 h in the presence of medium (control), AEA 1 nM or AEA + meloxicam 1 µM (Melo, a selective COX-2 inhibitor) and assayed for migration (wound healing). Migration supernatants were collected after 15 h and used as condition media (CM, 1:7 of migration supernatants with media). Next, human endothelial cells (EA.hy926) were incubated with medium (basal), control-CM, AEA-CM, or AEA + Melo-CM, or treated directly with AEA 1 nM or meloxicam 1 µM. (a) The scheme represents the experimental procedure. (b) EA.hy926 migration was evaluated by wound healing closure. Representative photographs at 0 and 18 h are shown (10×). (c) EA.hy926 cytoskeleton arrangement was evaluated by phalloidin staining. Actin filaments were stained with TRITC-phalloidin after 18 h of incubation with CM. Representative photographs under a fluorescent microscope are shown (40×). Photographs represent three independent experiments. Negative control: phalloidin was omitted. (d) EA.hy926 proliferation was evaluated by the WST-1 method. (e) The expression of γH2AX in EA.hy926 was evaluated after 24 h. A representative Western blot is shown. (b), (d) and (e) Data is expressed as mean ± S.E.M. Three replicates per treatment, each experiment was repeated 4–6 times. Different letters indicate statistical differences ($p < 0.05$).

Actin filament polymerization was used to confirm the migration in T-hESC cells. AEA 1 nM induces a distinctive migratory phenotype in these endometrial cells. Signal transduction pathways that are regulated by AEA include among others activation of mitogen activated protein kinase (MAPK) [32]. Moreover, it has been reported that MAPK controls microfilament remodeling and affects the polymerization and stability of microtubules [33–37]. Therefore, we postulate that AEA could modulate actin turnover and this might play a role in T-hESC migration.

An appropriate tone of AEA is necessary for normal embryo development, oviductal transport and implantation [7,38]. AEA level in mice implantation sites is lower than in inter-implantation zones and the non-receptive uterus [6]. Low levels of AEA are informed in the late luteal phase of the menstrual cycle, coincident with the window of implantation [39]. Furthermore, nanomolar AEA is detected in different reproductive fluids [21] and an imbalance is associated with the onset and progression of pathological conditions [40]. On the contrary, micromolar AEA induces apoptosis in rat primary decidual cell cultures, and interferes with decidualization in rat and human [41–45]. Also, a proper concentration of AEA is needed to guarantee the development of the feto-placental unit and the continuity of pregnancy [7,38,46,47]. High levels of AEA are found in the plasma of women with threatened miscarriage and patients who failed to achieve an ongoing pregnancy after embryo transfer [9,10]. AEA is elevated in systemic and follicular

fluid of women with endometriosis, a pathology associated with dysregulated endometrial fibroblasts migration and infertility [48,49]. Taken together with these findings, our results suggest that within the uterus a proper concentration of AEA is required to guarantee endometrial fibroblast migration and the plasticity necessary to achieve gestation and fertility.

Both CB1 and CB2 were expressed in the T-hESC cell line. Almada and colleagues [44] described that both CB1 and CB2 proteins are detected in fibroblasts isolated from human term placentas. However, only CB1 is expressed in the St-T1b cell line [44]. The fact that CB1 is expressed in T-hESC while it is not observed in St-T1b, could explain the difference in the sensitivity to AEA 1 μM on cell viability.

Immunofluorescence analysis for CB1 and CB2 in T-hESC shows that while CB1 is localized in the membrane and/or the cytoplasm, CB2 could also be found in the nucleus. Although more experiments with specific membrane and cytoplasm markers are needed to elucidate this issue, it is described that cannabinoid receptors have an intense trafficking and internalization. Other authors reported that CB1 localization is not exclusively on the plasma membrane, as active CB1 is localized in the outer membrane of mitochondria [50] and a predominant intracellular localization is observed in diverse cell types and undifferentiated neurons [51]. Also, CB2 binding sites are located intracellularly in prefrontal cortical pyramidal neurons [52] and functional CB2 receptors are demonstrated at the endo-lysosomal level [50]. Interestingly, a marked perinuclear localization is observed for CB2 in a model of striatal neural progenitor cells [53].

The action of AEA is mediated by receptor and non-receptor pathways. Here we demonstrate that AEA stimulates fibroblast migration through CB1 and CB2 receptors. CB1 and CB2 antagonists completely reverse the effect of AEA suggesting that after the binding of AEA, these receptors induce the same signaling cascades. Also, it seems that both receptors should be active simultaneously so that AEA could promote stromal cell migration. One possibility is that AEA controls actin microfilaments and migration in T-hESC cells through its binding to CBs receptors and downstream activation of the MAPK pathway. Other authors also described the relevance of CBs in the action of AEA in the uterus. CB1 mediates the stimulatory action of methanandamide on the chemotaxis of primary cultures of endometrial fibroblasts [30]. AEA within a micromolar range disrupts decidualization and exerts an antiproliferative action via CB1 in the St-T1b cell line [31,44]. Previously, our group demonstrated that both CB1 and CB2 mediate lysophosphatidic acid action in the uterus of pregnant rats during the window of implantation [15]. Also, we observed that CB2 is involved in AEA effect on nitric oxide synthase activity and prostaglandin levels in the receptive rat uterus [16].

FAAH hydrolyses AEA to arachidonic acid and ethanolamine. Arachidonic acid is the precursor for prostaglandins production by COX. Also, AEA is transformed to prostamides being the substrate of COX-2 oxidative metabolism. The incubation of T-hESC with methanandamide, a non-hydrolyzable analog of AEA, shows a similar result compared to AEA, validating the notion that AEA stimulates fibroblast migration through CB1 and CB2, and not via the production of AEA metabolites.

Previously, we published that AEA modulates prostaglandins in the rat uterus [15,16]. Here, we describe that AEA-triggered endometrial fibroblasts migration involves COX-2 pathway. Moreover, prostaglandin F2alpha increases fibroblast migration, while prostaglandin E2 does not show any effect. In addition, COX-2 shows cytoplasmic and nuclear localization in T-hESC. AEA does not modify COX-2 localization but induces its expression via CB1 and CB2. The localization of COX-2 seemed to coincide with CB1 and CB2. We postulate that the binding of AEA to CB1 present in the membrane or the cytoplasm stimulates COX-2 expression in the cytoplasm. In the nucleus, once AEA binds to CB2, it stimulates COX-2 expression, and finally promotes fibroblast migration. These observations need further evaluation but suggest that the endocannabinoid receptors might have more complex roles than the classical interaction with extracellular AEA. More experiments are being performed to unravel the regulation of AEA-CBs-COX-2-prostaglandin pathway in endometrial stromal cells. These findings are relevant since deficient AEA-prostaglandins signaling might contribute to endometrial pathologies associated with aberrant endometrial fibroblasts migration.

In the present manuscript, we describe that both exogenous and endogenous AEA exert similar effects on T-hESC migration and COX-2 localization and expression. FAAH degrades endogenous cannabinoid AEA, oleoylethanolamide, oleamide and palmitoylethanolamide [54–56]. Consistent with this idea, pharmacological inhibition of FAAH activity or genetic disruption of the faah gene results in marked increases in the levels of these fatty acid ethanolamides along with anandamide [57,58]. However, FAAH has an alkaline optimal pH and preferentially hydrolyzes anandamide over other fatty acid ethanolamides [25]. It's worth mentioning that unlike AEA, oleoylethanolamide, oleamide and palmitoylethanolamide are not able to activate the two cannabinoid receptor subtypes CB1 and CB2 [59–62]. We demonstrated that the CB1 and CB2 antagonists reversed URB-597 (the FAAH inhibitor) stimulated T-hESC migration. Therefore, it is likely that the pharmacological action of URB-597 is primarily due to endogenous AEA accumulation. In line with our results, Almada et al. [63] described that in URB-597-treated mesometrial decidual cells, AEA levels were significantly elevated, while the levels of oleoylethanolamide and palmitoylethanolamide were not affected.

Endometrial fibroblasts are fundamental for endometrial architecture and plasticity. Their participation in the vascular remodeling of the uterus supposes that they might be in coordinated dialogue with the endothelium. As AEA shows vascular properties, this endocannabinoid could modulate this interaction. We demonstrate that AEA not only has a direct action on stromal cells migration but also modifies endothelial cells migration indirectly and does not alter their proliferation. Also, DNA damage is involved in fibroblast - endothelium interaction as γH2AX expression is increased. γH2AX is a well-known marker of chromatin modifications linked to DNA damage and is used to predict chronic inflammatory conditions that precede cardiovascular disorders. It is postulated that the loss of the endothelium in the spiral arteries involves several processes including vascular cell migration and/or apoptosis [64]. Our data support this hypothesis and demonstrate that the behavior of the endothelial cells in the maternal vessels would be regulated by AEA and soluble mediators released by the endometrial fibroblasts. Whether the action of AEA results in migration or apoptosis could be explained by a unique sensitivity of vascular cells to endometrial fibroblasts-derived factors.

AEA added directly to EA.hy926 stimulates migration to a similar level compared to AEA-CM. Endothelial cells are sensitive to AEA and AEA-angiogenic properties have been documented [11,12]. However, AEA has a very short half-life and it is most likely no longer present in the CM after 15 h of incubation. Then, we speculate that soluble mediators released from T-hESC during AEA-stimulated migration, and not AEA itself, promote EA.hy926 migration. AEA is detectable in the mesometrial decidua, where the remodeling of the maternal vessels occurs [8]. Very elegant studies in mice showed that CB1 in decidual cells, together with CB2-angiogenic activity, promotes the development of the avascular primary decidual zone, which is critical for pregnancy success in mice [65,66]. Altogether, this evidence strongly suggests that AEA is relevant for fibroblast-endothelium interaction during vascular remodeling at the maternal-fetal interface.

As mentioned, we propose that stromal cells-secreted soluble factors under AEA action could diffuse and reach the endothelial cells of the maternal vessels promoting their migration and/or apoptosis and leading to vascular remodeling. Indeed, we show that COX-2 pathway mediates in part this crosstalk. Badimon et al. [67], also demonstrated that COX-2 derived prostaglandins regulate endothelial cells under physiological and pathological conditions. It is well described that COX-2 par-

ticipates in the vascular mechanisms at the implantation sites [13]. Although prostaglandin F2alpha seems to be a candidate as it promotes stromal cells migration, we could not rule out the participation of other soluble factors independent of COX-2. This possibility is of particular relevance in the case of γH2AX expression, where COX-2 seems not to mediate AEA effect. The endothelium plays a predominant role in modulating many aspects of vascular homeostasis at the maternal-fetal interface [64]. Thus, dysfunction of endothelial cells might contribute to a range of obstetric pathologies such as spontaneous abortion and recurrent reproductive failure [68–70].

The highly dynamic nature of the human endometrium is increasingly recognized. The migratory capacity of endometrial stromal cells seems to underpin the intense tissue remodeling associated with the establishment of pregnancy and the development of the feto-placental unit. Endometrial fibroblasts are present in the undifferentiated endometrium and decidua, and remain in the placenta until late gestation [1,2], suggesting a relevant role throughout pregnancy. Our results demonstrate that AEA promotes stromal cell migration and their interaction with the endothelial cells via a cellular pathway that involves CBs receptors and COX-2. This work contributes to gaining more insight into the role of AEA at the maternal-fetal interface, and proposes a new mechanism of interaction between the endometrial fibroblasts and the endothelium of maternal vessels. This mechanism might contribute to vascular adaptations, ensuring an adequate blood flow in response to the increasing metabolic demands of the embryo. Deficiencies in these processes could be implicated in obstetric complications associated with a poor vascular remodeling of the uterus. Overall, we provide new clues about the role of AEA in vascular remodeling at the maternal-fetal interface during early gestation.

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Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2023.06.004.

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