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Research paper

Participation of membrane calcium channels in erythropoietin-induced endothelial cell migration

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ABSTRACT

Calcium (Ca^{2+}) plays an important role in angiogenesis, as it activates the cell migration machinery. Different proangiogenic factors have been demonstrated to induce transient Ca^{2+} increases in endothelial cells. This has raised interest in the contribution of Ca^{2+} channels to cell migration, and in a possible use of channel-blocking compounds in angiogenesis-related pathologies. We have investigated the ability of erythropoietin (Epo), a cytokine recently involved in angiogenesis, to induce Ca^{2+} influx through different types of membrane channels in EA.hy926 endothelial cells. The voltage-dependent Ca^{2+} channel antagonists amlodipine and diltiazem inhibited an Epo-triggered transient rise in intracellular Ca^{2+}, similarly to a specific inhibitor (Pyr3) and a blocking antibody against the transient potential calcium channel 3 (TRPC3). Unlike diltiazem, amlodipine and the TRPC3 inhibitors prevented the stimulating action of Epo in cell migration and *in vitro* angiogenesis assays. Amlodipine was also able to inhibit an increase in endothelial cell migration induced by Epo in an inflammatory environment generated with TNF-α. These results support the participation of Ca^{2+} entry through voltage-dependent and transient potential channels in Epo-driven endothelial cell migration, highlighting the anti-angiogenic activity of amlodipine.

1. Introduction

Among the different mechanisms underlying the process of cell migration, the involvement of calcium (Ca^{2+}) has previously been reported (Giannone et al., 2004). A migrating cell extends a protrusion, called lamellipodium, toward the source of the stimulus, and retracts its rear end in order to move one "step" forward. Such morphological changes are accompanied by dynamic modifications in cytoskeleton, cell adhesions and localization of membrane and cytosolic proteins which constitute the migration machinery. The distribution of cytosolic Ca^{2+} in migrating cells is also polarized, being higher at the rear end. A lower basal Ca^{2+} concentration at the front allows for the occurrence of Ca^{2+} "flickers" in response to promigratory stimuli (Tsai et al., 2015). These transient elevations of the cation activate the detachment of the cell rear through actin-myosin contractile force and the disassembly of cell adhesions by calcium-dependent phosphatases and proteases (Lee et al., 1999).

The ability to migrate is a key feature of endothelial cells and, not surprisingly, angiogenic factors have been reported to trigger Ca^{2+} rises in this cell type. Human umbilical vein endothelial cells (HUVEC) exhibited an increase in cytosolic Ca^{2+} levels within 5–10 min of exposure to vascular endothelial growth factor (VEGF) (Faehling et al., 2002). Erythropoietin (Epo), the most important erythropoietic growth factor, is also recognized as a proangiogenic cytokine, as demonstrated in different *in vitro* and *in vivo* studies (Ribatti et al., 1999; Jaquet et al., 2002; Kertesz et al., 2004), and it has been shown to induce calcium transient increases in endothelial cells (Vogel et al., 1997; Maltaneri et al., 2017). A previous work by our group revealed the participation of extracellular calcium, as well as that of nitric oxide and reactive oxygen species, as a mediator of the effect exerted by Epo on endothelial cell migration (Maltaneri et al., 2017).

The involvement of calcium in cell motility has prompted

Abbreviations: Aml, amlodipine; Ca^{2+}, free calcium; DHP, dihydropyridine; Dil, diltiazem; Epo, erythropoietin; EpoR, erythropoietin receptor; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PBS, phosphate-buffered saline; Pyr3, TRPC3 inhibitor; Qui, quinine; ROS, reactive oxygen species; SEM, standard error of the mean; Thap, thapsigargin; TNF-α, tumor necrosis factor-alpha; TNFR1, TNF-α receptor 1; TRPC3, transient potential calcium channel 3; VDCC, voltage-dependent calcium channel; VEGF, vascular endothelial growth factor

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researchers to investigate the contribution of Ca^{2+} channels to migration-related pathologies, such as metastasis and tumor vessel proliferation in cancer. In this context, the study of Ca^{2+} channels and their inhibitors may lead to interesting therapeutical applications.

Calcium channels represent an important fraction of ion channels in endothelial cells, and also constitute a varied group. Non-selective cation channels, which may be operated by different agonists, have been demonstrated to mediate Ca^{2+} entry in endothelial cells (Nilius et al., 1993). Store-operated calcium channels (SOCs) are, in contrast, highly selective for this cation, and become activated upon depletion of Ca^{2+} stores in the endoplasmic reticulum (Nilius and Droogmans, 2001). Transient receptor potential channels (TRP) are abundant in the endothelium, though their expression levels are frequently subjected to regulation by environmental stimuli (Cheng et al., 2016). It has been demonstrated that TRP channels of the canonical type (TRPC) mediate the proangiogenic effect of cytokines such as VEGF, and that their suppression leads to deficient cell migration (Hamdollah Zadeh et al., 2008). In addition, the contribution of TRP channels of the vanilloid type (TRPV) has recently been reported in erythropoietin-induced angiogenesis (Yu et al., 2017).

On the other hand, the presence of voltage-dependent calcium channels (VDCCs) in endothelial cells is still a matter of debate, since this is a non-excitable cell type. This family comprises L-type (high voltage activated) and T-type (low voltage activated) channels, which are essential for cardiac and vascular smooth muscle contractility, as they increase cytosolic Ca^{2+} levels through augmented influx and release from intracellular stores (Catterall, 2011).

Inhibition of these voltage-dependent channels in vascular smooth muscle cells as well as in cardiomyocytes not only results in coronary and peripheral artery vasodilation, but also in decreased cardiac contractility. Stemming from this, the clinical use of calcium antagonists has been demonstrated to successfully prevent stroke, ischemic heart disease and mortality in hypertensive patients (Grossman and Messerli, 2004).

According to their chemical structure, calcium antagonists may be classified as phenilalkylamine, benzothiazepine and dihydropyridine drugs. Verapamil and diltiazem, which belong to the first two classes, respectively, are antihypertensive drugs with marked effects on cardiac conduction, for which they are employed as treatment for supraventricular arrhythmias. Dihydropyridines (DHPs), on the other hand, are more potent vasodilators with minimal effect on heart contractility. From the first generation of drugs released in the '60s, such as nifedipine, advances have been made to give rise to more pharmacokinetically-stable, and less cardio-selective compounds. Among the latter group, amlodipine has become a drug of choice to treat hypertension (Fares et al., 2016).

Considering the involvement of calcium in the migration of different cell types, the aim of this work was to investigate the participation of transient potential and voltage-dependent channels in the Ca^{2+} influx triggered by Epo in endothelial cells. In addition to this, and given that the administration of Epo as a proangiogenic compound represents a potential benefit in coronary ischemia (Buemi et al., 2002), we were also interested in investigating its performance in the presence of the calcium antagonists amlodipine and diltiazem —which are used to treat hypertensive patients— both in normal conditions and in a proinflammatory environment, characteristic of cardiovascular disease.

2. Materials and methods

2.1. Materials and reagents

All the reagents used in this work were of analytical grade. Human recombinant erythropoietin (specific activity: 125 IU/μg) was kindly provided by Zelltek (Santa Fe, Argentina). The culture media IMDM and M199, the cell dissociation reagent TrypLE Select, the Geltrex growth factor-reduced basal membrane matrix, the Low Serum Growth Kit (LSGS) for culture of primary endothelial cells, the penicillin–streptomycin antibiotic mixture and human recombinant TNF-α (specific activity: $5 \times 10^7 - 2 \times 10^7$ U/mg) were from Gibco – Thermo Fisher Scientific (Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from Natocor (Córdoba, Argentina) and Gibco – Thermo Fisher Scientific. The TRIzol reagent, the BAPTA-AM calcium chelator, the Alexa-Fluor 488-conjugated secondary antibodies, the fluorescent probe Fluo 4-AM (calcium) and the recombinant Taq DNA polymerase, as well as the primers against GAPDH, EpoR and TNFR1, were from Thermo Fisher Scientific (Waltham, MA, USA). The primary antibodies used were: anti-EpoR (sc-697) from Santa Cruz Biotechnologies (Santa Cruz, CA, USA) and anti-TRPC3 (ACC-016) from Alomone Labs (Jerusalem, Israel). The fluorescent probe 2', 7'-dichlorofluorescein diacetate (DCFH-DA, ROS) and the channel blockers amlodipine besylate, diltiazem and quinine were from Sigma Aldrich (Saint Louis, MO, USA). Cytofix/Cytoperm and PermWash buffer were from BD Biosciences (San José, CA, USA). Human recombinant VEGF-165 was purchased from BioLegend (San Diego, CA, USA). The reagents used for reverse transcription, comprising Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase, RNAsin RNAse inhibitor and oligodTs were from Promega Corporation (Madison, WI, USA).The SERCA-pump inhibitor thapsigargin was from Sigma Aldrich, while the TRPC3 inhibitor Pyr3 was from Cayman Chemicals.

2.2. Cell cultures

The endothelial cell line EA.hy926, obtained by fusion of HUVEC cells with the adenocarcinoma cell line A549 (Edgell et al., 1983), was kindly provided by Dr. Fernanda Parborell (IBYME-CONICET, Buenos Aires) with permission from Dr. Gareth Owen (Pontificia Universidad Católica, Chile). Previous experiments by our group have demonstrated the ability of Epo to stimulate migration in EA.hy926 cells (Maltaneri et al., 2017), as observed in HUVEC cells. Cultures were maintained on Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Natocor), 100 U/mL penicillin and 100 μg/mL streptomycin (growth medium), and were used no further than passage 18, due to cell senescence.

Human Umbilical Vein Endothelial Cells (HUVEC; C-003-5C; Life Technologies) were cultured in M199 medium supplemented with Low Serum Growth Kit (Gibco), to the following final concentrations: 1 μg/mL hydrocortisone; 10 ng/mL EGF (Epidermal Growth Factor); 3 ng/mL bFGF (basic Fibroblast Growth Factor); 10 μg/mL heparin, 0.2 μg/mL bovine serum albumin; 1X gentamicin / amphotericin and 2% (v/v) FBS. Cells were used between passages 2 and 5 due to senescence.

Cultures were grown at 37 °C and 5% CO_2, with replacement of culture media every two days. Cells were divided into separate flasks upon reaching 80–90% confluence. In order to reproduce the physiological quiescent state of the endothelium, cells were FBS-deprived before treatment, and experiments were performed in FBS-free medium.

2.3. Cell migration (wound healing) assay

Cells were seeded on 24-well plates (65,000 viable cells/well) and cultured overnight on growth medium. After washing with PBS, cells were FBS-deprived for 8 h before scratching with a pipette tip and adding the corresponding treatments. FBS (10% for EA.hy926 cells, 2% for HUVEC cultures) was used as a positive control of cell migration. Images were acquired at the beginning (t = 0) and at the end of the experiment (t = 15 h for EA.hy926 cells and t = 24 h for HUVEC cells) using an inverted microscope Axiovert 135 (Carl Zeiss) and a Nikon Coolpix 5000 camera, and digitalized with the Axiovision software. For each independent experiment, cell migration quantifications of 4 different fields *per* treatment were averaged. Inhibitors were added at least 10 min before the assays.

2.4. Tube formation assay

EA.hy926 cells (15,000 viable cells/well), previously FBS-deprived for 24 h, were seeded on separate wells of a 96-well multiplate coated with Geltrex™ matrix (50 µL/well). Cells were incubated in FBS-free IMDM medium together with the corresponding treatments, and a positive control using 2% FBS (Gibco) was included in each of the independent experiments. After a 24-h incubation, photographs were taken of ten fields *per* treatment at a 200X magnification. Stimulation of angiogenesis was quantified for each of the treatments by the calculation of an angiogenic score (AS), as described by Aranda and Owen (2009):

$$AS = \frac{[(N° \text{ sprouting cells}) + (N° \text{ connected cells}) + (N° \text{ polygons})]}{\text{Total cell number}} + (0, 1 \text{ or } 2)$$

This scoring system takes into account each of the steps involved in the formation of vessel-like tubules. The addition of 1 or 2 in the equation corresponds to the presence of vessel-like structures with a thickness of 2–3 cells or more than 4 cells, respectively. In each independent experiment, the overall AS of a treatment was obtained by averaging the individual AS of each of the 10 fields.

2.5. Flow cytometry

Determination of intracellular calcium: Cells were FBS-starved for 1 h and incubated with the fluorescent calcium probe Fluo 4-AM (3 µM; 30 min at 37 °C), then harvested with TrypLE and resuspended in HEPES buffer with 1.5 mM $CaCl_2$. Calcium channel inhibitors were added at least 10 min before exposure to Epo for different incubation times. After treatment, cells were immediately fixed in 0.5% paraformaldehyde in PBS for subsequent event acquisition by flow cytometry.

Determination of intracellular ROS: After the corresponding treatment, cells were washed with PBS at room temperature and incubated for 30 min at 37 °C with the fluorescent probe 2', 7'-dichlorofluorescein diacetate (DCFH-DA, 20 µM in PBS). Cells were then collected with TrypLE, centrifuged and fixed with 4% paraformaldehyde before event acquisition.

Determination of EpoR expression: Cells were harvested with TrypLE and washed with PBS prior to fixation/permeabilization with Cytofix/Cytoperm (15 min, 4 °C). After washing with PermWash buffer 1 x (PWB), FC receptors were blocked with 10% FBS in PWB (10 min, 4 °C). Cells were incubated with a primary antibody against EpoR (1:50) for 1 h on ice, then washed with PWB and stained with the corresponding Alexa Fluor 488-conjugated secondary antibody (1:100) for 1 h on ice in the dark. Cells were washed with PWB and resuspended in PBS before acquisition of events by flow cytometry.

In all experiments, events were acquired in a FACS Aria II flow cytometer (BD) and data were analyzed using the FlowJo software (FlowJo LLC).

2.6. RNA isolation, reverse transcription and reverse transcription-PCR

RNA extraction was performed using the TRIzol reagent, according to manufacturer's instructions. Nucleic acid quantification was performed using NanoDrop equipment. A 260/280 nm ratio above 1.80 and a 230/260 nm ratio close to 0.5 were used as the criteria to assess purity of RNA samples from protein and organic contaminants, respectively. cDNAs were obtained by reverse transcription of 1 µg total RNA using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase.

PCR master mixes were prepared with 1.5 mM $MgCl_2$, 0.2 mM dNTPs and the corresponding primers at 0.25 mM (final concentrations). The sequences of the primers used were as follows: EpoR, forward: 5'-TGGTATCTGACTCTGGCAT-3', reverse: 5'-TCCCTGATCATCTGCAGCC-3' (amplicon size: 181 bp); TNFR1, forward: 5'-TCGATTTGCTGTACCAAGTG-3', reverse: 5'-GAAAATGACCAGGGGCAACAG-3' (amplicon size: 492 bp) and GAPDH, forward: 5'-TGATGACATCAAGAAGGTGGTGAAG-3', reverse: 5'-TCCTTGGAGGCCATGTAGGCCAT-3' (amplicon size: 240 bp).

Amplification of EpoR consisted of 35 cycles of denaturation at 95 °C for 30 s, annealing at 64 °C for 30 s and extension at 72 °C for 30 s. The program used for TNFR1 consisted of 35 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 2 min and extension at 72 °C for 2 min. PCR products were resolved on 2% agarose gels stained with 0.05% ethidium bromide.

Fluorescence of DNA was detected with a G:BOX Chemi system and digitalized using the GeneSys software (Syngene). Relative quantification of mRNA levels through band densitometry was performed with the ImageJ software, using GAPDH as a reference gene.

2.7. Statistics

Statistical analysis was performed with the Graph Pad Prism software (GraphPad Software Inc.). Results are expressed as mean ± standard error of the mean (Mean ± SEM) of three or more independent experimental replications. Kruskal-Wallis one-way analysis of variance followed by Dunn's test was used for comparison among groups, unless otherwise stated. Least significant difference with $P < 0.05$ was considered the criterion for statistical significance.

3. Results

3.1. Role of intracellular stores in erythropoietin-induced cytosolic calcium transients in endothelial cells

In a previous work we demonstrated the stimulation of an intracellular Ca^{2+} increase upon Epo treatment (peaking at 15 s) on EA.hy926 cultures. This response was dependent on extracellular Ca^{2+}, since the absence of the cation in the resuspension buffer where measurements were performed completely abrogated the generation of Ca^{2+} pulses (Maltaneri et al., 2017).

This emphasizes the role of calcium entry in the response of endothelial cells to Epo, although the possibility of a release of the cation from intracellular stores should not be disregarded. In order to explore this possibility, we performed flow cytometry experiments on cells loaded with the calcium probe Fluo 4-AM and incubated with the cytosolic chelator BAPTA-AM (10 µM) or thapsigargin, an inhibitor of the SERCA pump. As shown in Fig. 1, BAPTA-AM impaired the generation of Ca^{2+} peaks upon Epo treatment in EA.hy926 endothelial cells. The preincubation of these cells with thapsigargin (1 µM), which prevents the refilling of the Ca^{2+} stores in the endoplasmic reticulum, allowed a slightly smaller response to the addition of Epo in a resuspension medium with 1.5 mM calcium, compared with untreated cells.

Together, the results obtained with BAPTA-AM and thapsigargin, as well as those previously reported (Maltaneri et al., 2017) indicate that the Ca^{2+} transient rise observed on EA.hy926 cells upon exposure to Epo has both an intracellular and an extracellular component, which are essential to the response.

3.2. Participation of membrane calcium and potassium channels in the response of endothelial cells to erythropoietin

Intracellular calcium transient increases occur as early as 15 s after incubation with Epo, indicating a fast activation of all the components

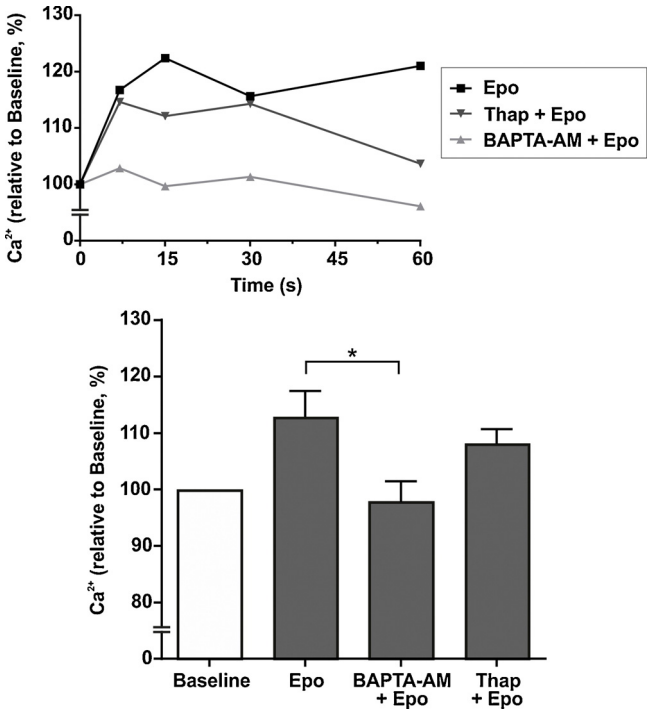

Fig. 1. Participation of intracellular calcium stores in the response of EA.hy926 endothelial cells to erythropoietin. The intracellular calcium concentration was determined by flow cytometry with the fluorescent probe Fluo4-AM (3 μM). Cells were preincubated (10 min) in HEPES buffer without calcium in the presence of the intracellular calcium chelator BAPTA-AM (10 μM) or the SERCA pump inhibitor thapsigargin (Thap, 1 μM). Cells were then resuspended in HEPES buffer with 1.5 mM $CaCl_2$ before being exposed to Epo (200 ng/mL) for the indicated times. Cell suspensions were immediately fixed with 0.5% paraformaldehyde in PBS. Bars represent mean ± SEM of geometric mean of fluorescence (relative to Baseline) at the time of the maximum response obtained with Epo. *P < 0.05, Kruskal Wallis-Dunn, n = 5. The graph above shows the intracellular calcium curves of a representative experiment.

involved. The extracellular contribution to such effect must involve the activation of calcium channels in the plasma membrane. By using pharmacological inhibitors and a blocking antibody we have investigated the participation of voltage-dependent and transient Ca^{2+} channels, respectively, in the response of EA.hy926 cells to Epo.

Flow cytometry experiments on cells loaded with the calcium probe Fluo 4-AM showed that a 10-min preincubation with the L-type calcium channel inhibitors amlodipine and diltiazem abrogated the intracellular Ca^{2+} peak (at 15 s) induced by Epo (Fig. 2A), not only supporting the role of calcium influx in the response of endothelial cells to the cytokine, but also hinting at the presence of VDCCs in this cell type.

In a similar way to amlodipine and diltiazem, a blocking antibody against TRPC3 (*Transient Potential Calcium Channel 3*), as well as the specific inhibitor Pyr3, also inhibited Epo-driven calcium entry in EA.hy926 cells, thus supporting the involvement of these channels (Fig. 2B). In endothelial cells, TRPC channels have previously been shown to allow Ca^{2+} influxes in response to membrane hyperpolarization due to the activation of potassium channels (Ledoux et al., 2006). These Ca^{2+}-induced K^+ channels respond to slight changes in cytosolic Ca^{2+} due to the opening of intracellular stores elicited by the activation of membrane receptors.

Inhibition of K^+ channels by quinine impaired the increase in cytosolic Ca^{2+} caused by Epo (Fig. 2C), further suggesting the involvement of TRPC channels as possible mediators of Epo in this cell type. Additionally, quinine prevented the stimulating effect of Epo on cell migration in wound healing assays (Fig. 2D), thus emphasizing the role of Ca^{2+} and K^+ channels in the cellular response elicited by the cytokine.

Taken together, our results suggest the involvement of membrane calcium channels of the voltage-dependent and transient potential type in the effect of Epo on endothelial cells.

3.3. Inhibition of calcium channels in endothelial cell migration and tube formation

The fact that calcium is essential for the promigratory effect of Epo on endothelial cells, as previously demonstrated by the use of EDTA and EGTA (Maltaneri et al., 2017), in addition to the ability of amlodipine, diltiazem, Pyr3 and the antibody against TRPC3 to inhibit Ca^{2+} influx in this cell type, suggest a possible role for voltage-dependent and transient potential channels in endothelial cell migration.

Migration of EA.hy926 cells was significantly prevented by incubation with the antibody against TRPC3, as well as by the specific inhibitor Pyr3, thus confirming the participation of a transient potential calcium channel in the stimulation of cell motility by Epo (Fig. 3).

Because of their effects on vascular tone, amlodipine and diltiazem, among others, are often prescribed to hypertensive patients, particularly those suffering from comorbidities such as asthma, diabetes, angina pectoris, or peripheral vascular disease. In the light of their clinical applications, it is necessary to investigate their potential impact on angiogenesis, which may represent either a therapeutical advantage or an unwanted effect in certain diseases.

In wound-healing assays, amlodipine significantly inhibited the promigratory activity of Epo as well as that of VEGF, which was used as a positive control of EA.hy926 endothelial cell migration (Fig. 4A, C). Diltiazem, however, failed to block the effect of both cytokines on cell migration (Fig. 4B, C). Similar results were obtained in primary cultures of HUVEC exposed to Epo in the presence of amlodipine and diltiazem (Fig. 4D).

In line with our previous findings regarding the promigratory effect of Epo on endothelial cells, tube-formation assays performed on an ECM-like matrix (Geltrex™, Gibco) further confirm the angiogenic potential of this cytokine. In Fig. 4E, EA.hy926 cells cultured (24 h) in FBS-free conditions produced a network of 1-or-2-cell-thick tubules, while Epo stimulated the thickening of such structures. Tube formation was highly induced by 2% FBS, a well-known stimulator of endothelial cell motility. These observational changes were quantified by means of an angiogenic score (AS), developed by Aranda and Owen (2009) taking into account the different steps of the tube formation process. As expected, Control cultures obtained a lower AS than cells exposed to 2% FBS (Fig. 4F). In accordance to the wound-healing assays described above, amlodipine significantly inhibited the Epo-stimulated formation of vessel-like structures in cultures of endothelial cells resulting in a lower AS, while diltiazem did not interfere with tubulogenesis.

These findings emphasize the importance of Ca^{2+} entry in endothelial cells exposed to proangiogenic stimuli, and provide evidence for the participation of transient potential and voltage-dependent calcium channels in the migration process. However, we detected a differential effect of amlodipine and diltiazem on cell motility in spite of their ability to block Ca^{2+} influxes.

3.4. Effect of amlodipine and diltiazem on ROS generation in endothelial cells

Although both channel blockers abrogated Epo-induced calcium entry in EA.hy926 endothelial cells, amlodipine and diltiazem exhibited different effects on cell migration. We were therefore interested in exploring other factors that may contribute to the activity of both pharmacological inhibitors in this cell type.

Due to their molecular structure, which confers them the ability to interact with biological membranes, amlodipine and diltiazem have been reported to act as free radical quenchers, with different degrees of effectiveness (Mason et al., 1999). Given that reactive oxygen species

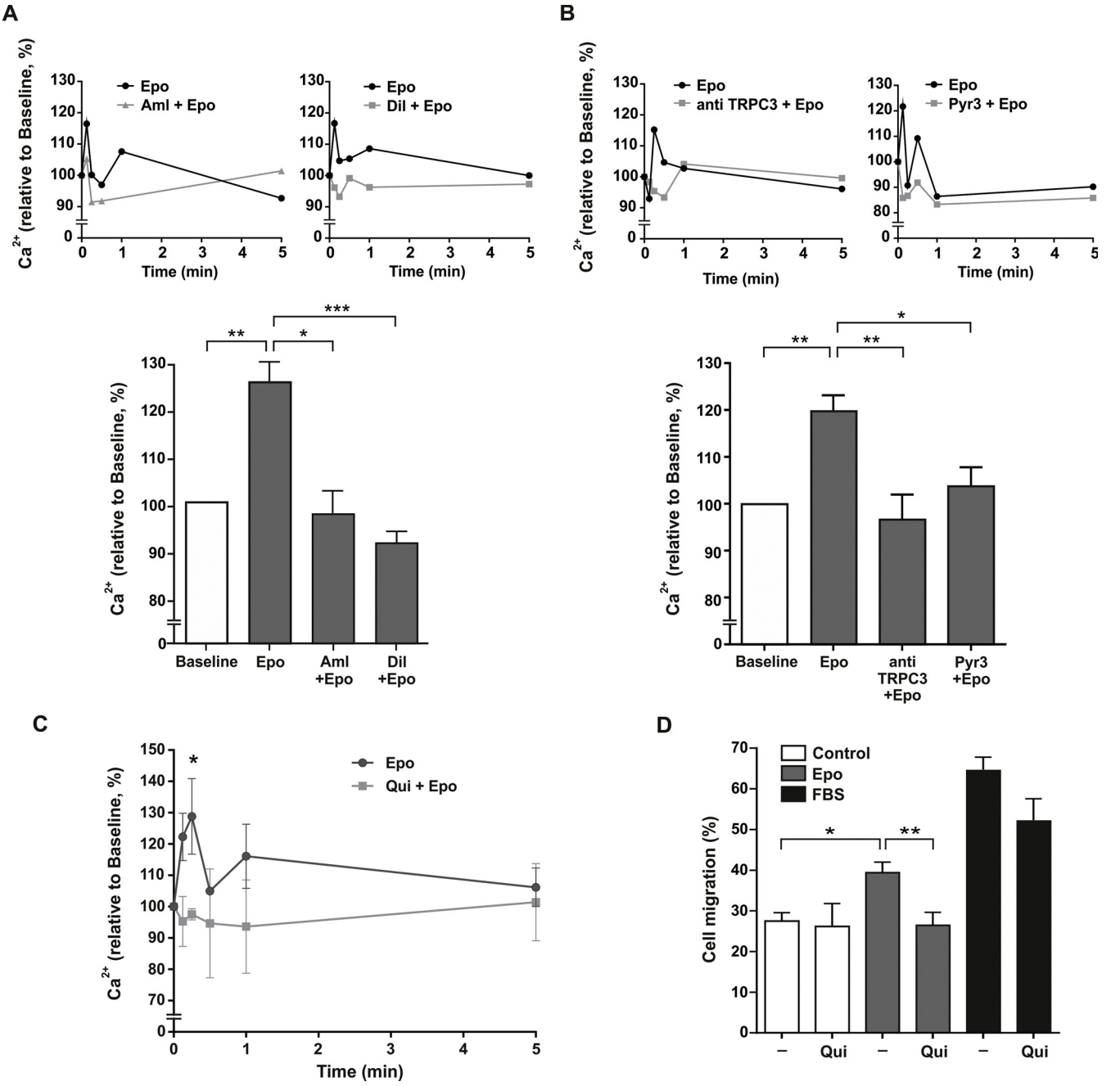

Fig. 2. Participation of membrane calcium and potassium channels in the response of EA.hy926 endothelial cells to erythropoietin. Determination of the intracellular calcium concentration by flow cytometry with the fluorescent probe Fluo4-AM (3 μM). Cells were resuspended in HEPES buffer with 1.5 mM $CaCl_2$ and preincubated (10 min) in the presence of the voltage-dependent calcium channel inhibitors amlodipine (1 μM) and diltiazem (5 μM) (**A**, n = 5), or the TRPC3 inhibitor Pyr3 (1 μM) and an anti-TRPC3 antibody (1:200) (**B**, n = 4), before being exposed to Epo (200 ng/mL) for the indicated times. Cell suspensions were immediately fixed with 0.5% paraformaldehyde in PBS. Bars represent mean ± SEM of geometric mean of fluorescence (relative to Baseline) at the maximum response (15 s). *P < 0.05, **P < 0.01 and ***P < 0.001, Kruskal Wallis-Dunn. The graphs above show representative intracellular Ca^{2+} curves for each of the inhibitors employed. **C)** Determination of intracellular Ca^{2+} in response to Epo (200 ng/mL), in EA.hy926 cells preincubated with the K^+ channel inhibitor quinine (50 μM, 10 min). *P < 0.05, Kruskal Wallis-Dunn, n = 3. **D)** Wound-healing assay in EA.hy926 cells preincubated with quinine (50 μM, 10 min) and then treated with Epo (200 ng/mL) for 15 h. *P < 0.05, **P < 0.01, Kruskal Wallis-Dunn, n = 7. Controls: cultures without FBS (Control) and with 10% FBS.

may play a role in triggering cell migration, as we proposed in a previous work (Maltaneri et al., 2017), we decided to study the effect of these calcium channel blockers on ROS accumulation in endothelial cells.

With this purpose, we generated a prooxidant state by incubating EA.hy926 cells with the proinflammatory cytokine TNF-α (15 h). Amlodipine was able to reduce the ROS burden generated by this cytokine, as shown by flow cytometry with the DCFH-DA probe (Fig. 5). On the other hand, diltiazem boosted the cytosolic accumulation of these chemical entities due to exposure to TNF-α.

According to these findings, amlodipine not only blocks Ca^{2+} influx in Epo-treated endothelial cells, but also prevents ROS formation, which may together have a strong inhibitory effect on cell migration. Conversely, despite being a calcium antagonist in endothelial cells, diltiazem seems to have an opposite action on ROS generation, which may account for its lack of effect on cell migration. These results

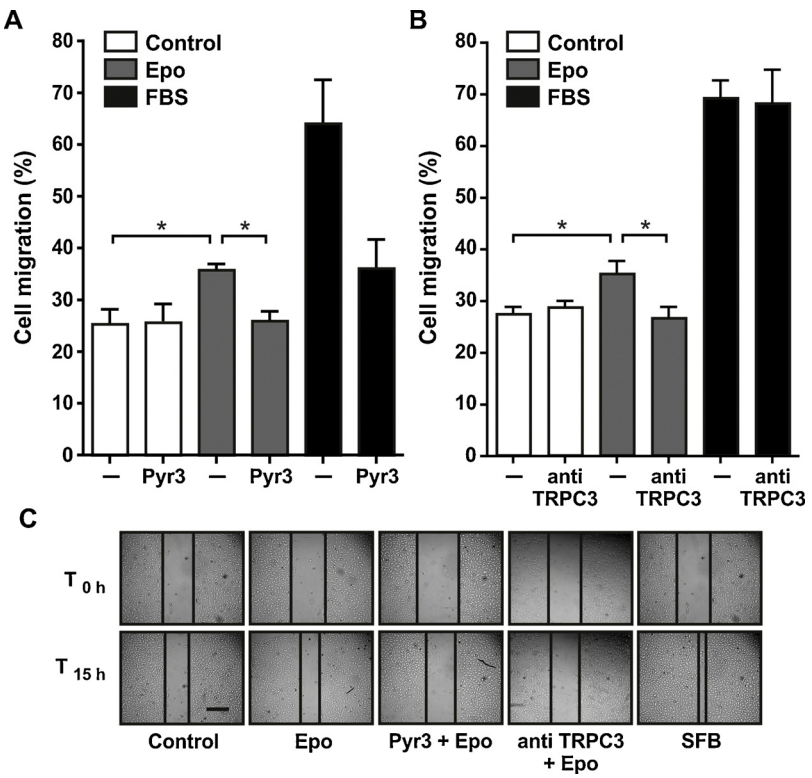

Fig. 3. Inhibition of TRPC3 calcium channels in EA.hy926 cell migration. Wound-healing assays in EA.hy926 cells preincubated (10 min) with the specific TRPC3 inhibitor Pyr3 (1 μM, n = 5) (**A**) or with an anti TRPC3 antibody (1:200, n = 8) (**B**) before the addition of Epo (200 ng/mL, 15 h). *P < 0.05, Kruskal Wallis-Dunn. Controls: cultures without FBS (Control) and with 10% FBS. **C)** Photographs are representative of the different treatments assayed on EA.hy926 cells (black line represents 500 μm).

suggest that Epo-induced cell migration relies on a complex interplay between different factors, such as Ca^{2+} and ROS.

3.5. Effect of amlodipine on the ability of erythropoietin to induce endothelial cell migration in an inflammatory environment

Endothelial cells spend most of the time in a quiescent state, in which they rarely divide or die, although they are metabolically active. In the event of an injury —caused by hypoxia, inflammation, shear stress— the endothelium becomes activated, and quiescent cells switch to a prothrombotic, proinflammatory and proangiogenic phenotype. The proliferation and migration of endothelial cells are vascular outcomes of inflammation. In this sense, an inflammatory environment may prime endothelial cells, thus enhancing the effects of other promigratory factors. Therefore it was important to determine if the activities of TNF-α and Epo on endothelial cells could add up synergically to promote cell migration, and whether amlodipine is able to inhibit cell motility in these conditions.

The proinflammatory cytokine TNF-α was able to induce migration of EA.hy926 endothelial cells in wound-healing assays performed using a concentration range between 30 and 200 ng/mL, with a maximum effect at 100 ng/mL (Fig. 6A). Interestingly, the promigratory effect of TNF-α seemed not to rely strongly on extracellular Ca^{2+}, as determined by coincubation with EGTA.

Following this, we assayed cell migration in response to Epo in a proinflammatory environment generated by TNF-α. We exposed cells to TNF-α 30 ng/mL, a concentration at which little migration is observed, and coincubated them in the presence of Epo. EA.hy926 cultures treated with Epo and TNF-α exhibited a significantly higher migratory activity compared to those treated with Epo alone (Fig. 6B).

Amlodipine significantly abrogated the synergistic effect of Epo and TNF-α bringing cell migration to near-Control levels. Given that Ca^{2+} influx impairment does not significantly affect the ability of TNF-α to promote endothelial cell migration —as demonstrated by incubation with EGTA and amlodipine—, the inhibitory effect of this channel blocker must be related to the Ca^{2+}-mediated signalling pathways involved in Epo-induced migration. On the other hand, diltiazem did not affect the synergistic effect of Epo and TNF-α on cell migration (data not shown).

In order to find an explanation to the synergistic effect between Epo and TNF-α in endothelial cell migration, we studied the expression levels of the Epo receptor, as well as those of the TNF receptor 1 (TNFR1) within the 15 h-incubation period of wound-healing assays. TNF-α and Epo were able to significantly induce TNFR1 when administered together, without much change in mRNA levels due to separate treatment (Fig. 6C). TNF-α and Epo, administered separately, significantly enhanced protein levels of EpoR after a 15-h incubation (Fig. 6D). After a simultaneous treatment with both cytokines, however, EpoR levels returned to baseline, suggesting a faster activation and possibly an earlier signal termination. In order to test this possibility, we performed PCR analysis of EpoR mRNA levels after a 3-h treatment. As shown in Fig. 6E, expression levels of the receptor were significantly increased when cells were simultaneously treated with TNF-α and Epo for 3 h, thus supporting our hypothesis of a faster activation and therefore, a shorter half-life of EpoR.

4. Discussion

The results presented in this work highlight the importance of calcium in the promigratory effect of Epo on endothelial cells, and provide supporting evidence for Ca^{2+} entry inhibition in the treatment of angiogenesis-related diseases.

Erythropoietin has been linked to the stimulation of the different processes involved in angiogenesis, such as endothelial cell migration and proliferation as well as recruitment of endothelial progenitor cells (Ribatti et al., 1999; Sautina et al., 2010). In a previous work we demonstrated that the promigratory effect of Epo on EA.hy926 endothelial cells is mediated by Ca^{2+}, and that Epo is able to trigger a transient rise in intracellular Ca^{2+} (Maltaneri et al., 2017). By performing cytosolic Ca^{2+} measurements in cells resuspended in a calcium-free medium, we showed that these Epo-induced Ca^{2+} pulses are dependent on an extracellular source of the cation, although the type of

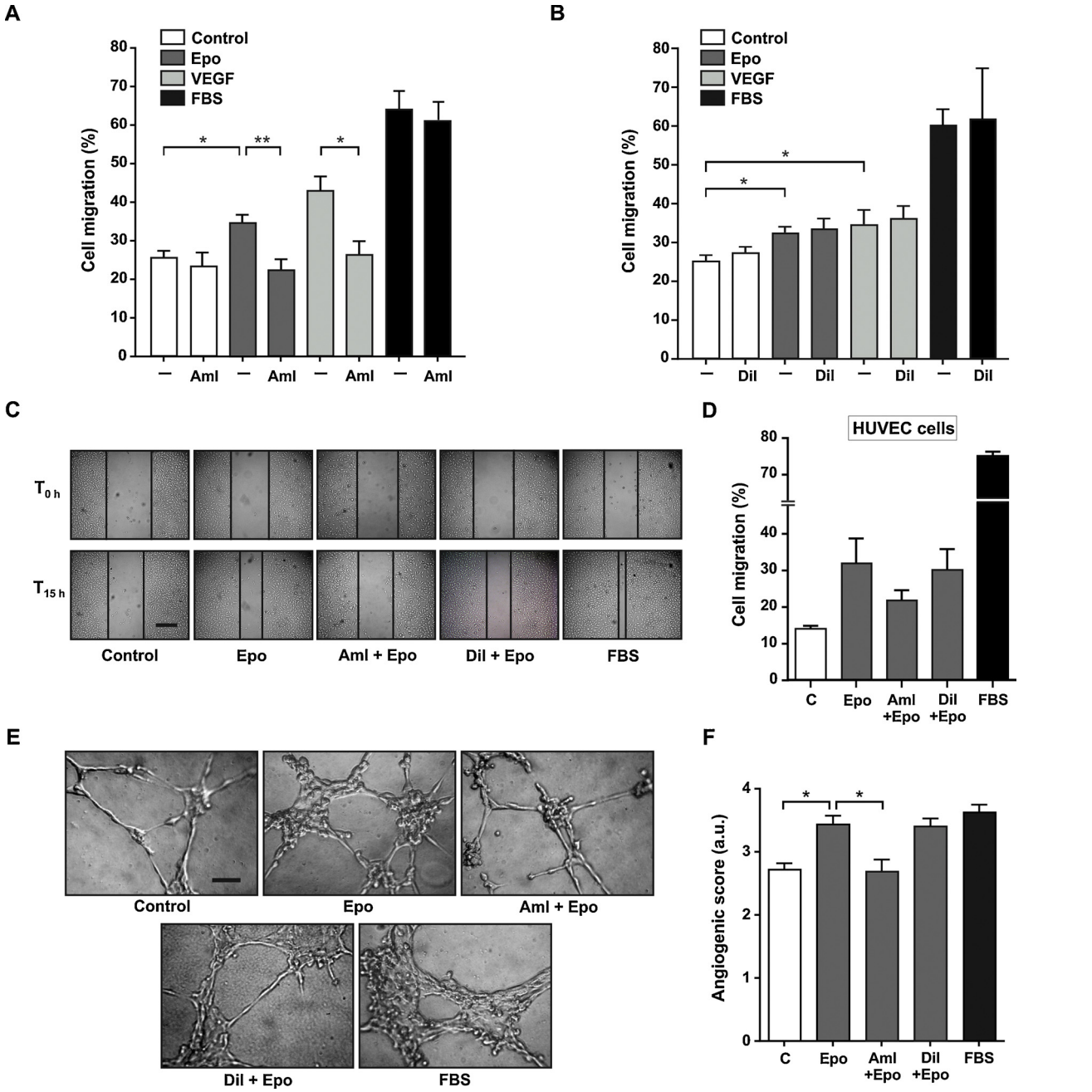

Fig. 4. Inhibition of voltage-dependent calcium channels in cell migration and tube formation assays performed on endothelial cells. Wound-healing assays in EA.hy926 cells in the presence of the calcium channel inhibitors amlodipine (1 μM; n = 13) (**A**) and diltiazem (5 μM; n = 9) (**B**) Cells were preincubated (10 min) with the corresponding inhibitors before the addition of Epo (200 ng/mL) or VEGF (10 ng/mL) for 15 h. Controls: cultures without FBS (Control) and with 10% FBS. *P < 0.05 and **P < 0.01, Kruskal Wallis-Dunn. **C)** Photographs are representative of the different treatments assayed on EA.hy926 cells (black line represents 500 μm). **D)** Wound-healing assay in HUVEC cells preincubated (10 min) with amlodipine (1 μM) or diltiazem (5 μM) before the addition of Epo (200 ng/mL) for 24 h (n = 3). Controls: cultures without FBS (C) and with 2% FBS. **E)** Tube formation on an ECM-like matrix. EA.hy926 cells were incubated with Epo (200 ng/mL) in the presence of amlodipine (1 μM) or diltiazem (5 μM) for 24 h. Controls: cultures without FBS (C) and with 2% FBS. Representative photographs of the fields used for angiogenic score (AS) calculation are included (black line represents 100 μm). **F)** Bars represent mean ± SEM of the AS calculated for each of the experimental groups according to the method described by Aranda and Owen (2009). *P < 0.05, Kruskal Wallis-Dunn, n = 5.

channels involved still remained to be determined. The present work highlights the importance of intracellular calcium sources in the response of EA.hy926 cells to Epo, as shown in the experiments performed with BAPTA-AM and thapsigargin (Fig. 1).

Endothelial cells display a wide variety of ion channels, of which calcium channels represent an important fraction. However, the characterization of their expression patterns and biological impact has proven to be difficult, as their presence may vary according to cell isolation methods, culture and growth conditions (Nilius and Droogmans, 2001).

Given that endothelial cells are non excitable, unlike vascular smooth muscle cells, the existence of calcium channels capable to respond to small changes in membrane potential has been controversial. Electrophysiology experiments carried out in bovine aortic endothelial cells (Bossu et al., 1992) and capillary cells from bovine adrenal glands (Vinet and Vargas, 1999) demonstrated the presence of DHP-sensitive Ca^{2+} entry dependent on cell hyperpolarization. Our study of Ca^{2+} dynamics has provided some interesting information regarding the

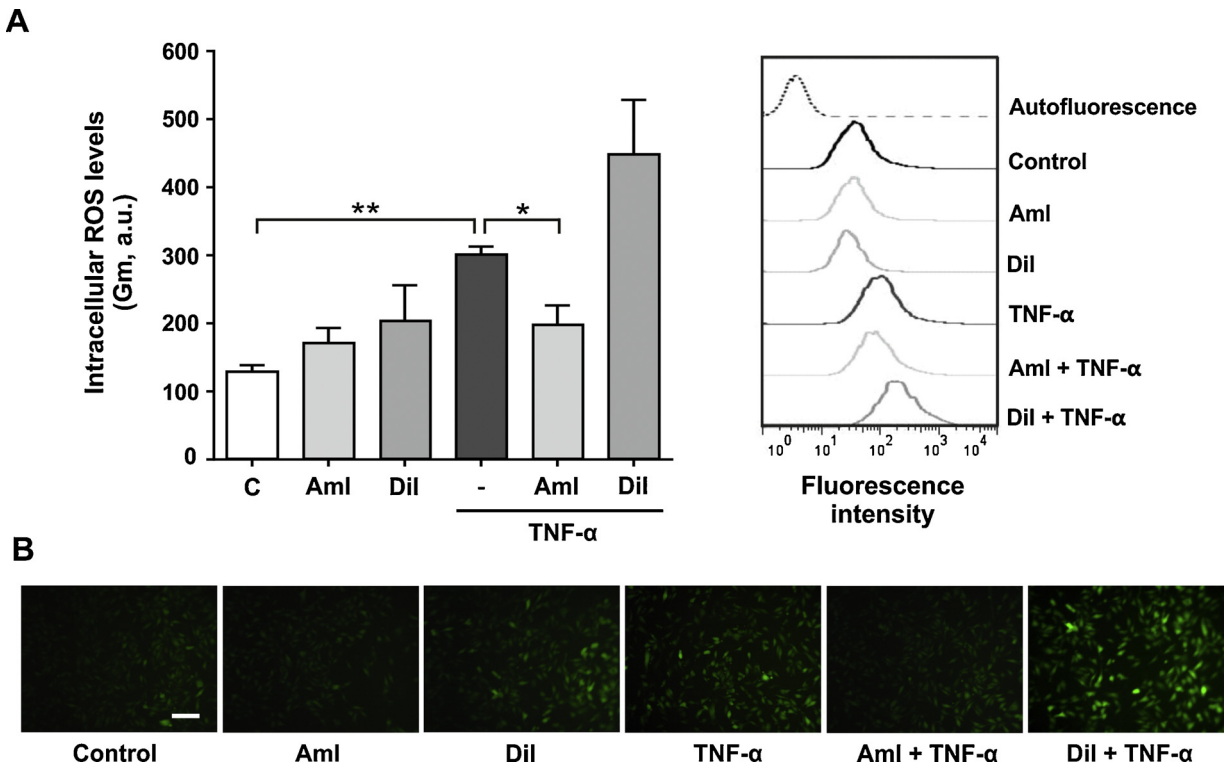

Fig. 5. Amlodipine inhibits oxidative stress produced by a proinflammatory environment in EA.hy926 endothelial cells. Cells were preincubated with either amlodipine (1 μM) or diltiazem (5 μM) for 10 min before the addition of TNF-α (100 ng/mL) for 15 h. Cells were then loaded with the ROS fluorescent probe DCFH-DA (20 μM, 30 min). **A)** Determination of intracellular ROS by flow cytometry. Bars represent mean ± SEM of geometric mean of fluorescence.*P < 0.05 and **P < 0.01, Kruskal Wallis-Dunn, n = 7. Flow cytometry plots of a representative experiment are included on the right. Control (C): cells incubated without FBS. **B)** Representative fluorescence microscopy images of the treatments employed. The white bar represents 200 μm.

existence of voltage-dependent calcium channels (VDCCs) in endothelial cells. In accordance to Yakubu and Leffler (2002), who used nifedipine to inhibit bradykinin-mediated Ca^{2+} influx in microvascular endothelial cells from newborn pig brain, amlodipine and diltiazem were capable of blocking transient increases in cytosolic Ca^{2+} induced by Epo in EA.hy926 cells (Fig. 2).

Transient potential channels have been shown to participate in cytokine-driven angiogenesis (Ge et al., 2009). Yu et al. (2017) recently demonstrated that calcium influx induced by erythropoietin in bovine artery endothelial cells was abrogated by inhibition of TRPV-1, which was also involved in capillary tube formation. In the same work, the authors observed inhibition of Epo-driven Ca^{2+} entry and tubulogenesis by a non-specific TRPC antagonist. Our group has previously demonstrated an enhanced protein expression of TRPC3 channels in EA.hy926 endothelial cells exposed to Epo for 15 h, the period in which wound-healing assays are performed (Maltaneri et al., 2017). Interestingly, TRPC3 is an Epo-regulated Ca^{2+} channel involved in the differentiation of human erythroid progenitors (Tong et al., 2008). In the present study, inhibition of TRPC3 channels by a blocking antibody or with the specific inhibitor Pyr3, not only impaired Epo-driven cytosolic Ca^{2+} transients, but also abrogated the promigratory effect of Epo on the EA.hy926 endothelial cell line (Fig. 3).

The relationship between K^+ and Ca^{2+} channels is tight in blood vessel walls, as it serves as an important regulatory mechanism of vascular tone. In vascular smooth muscle cells, the opening of large conductance (BK) calcium-activated potassium channels leads to K^+ efflux and membrane hyperpolarization, with the consequent closure of VDCCs, a decrease in Ca^{2+} entry and vasodilation. In endothelial cells, however, activation of small (SK) and intermediate conductance (IK) channels leads to a Ca^{2+} influx, also contributing to vasorelaxation through a NO-dependent mechanism (Ledoux et al., 2006). Our results showed an inhibitory effect of quinine on Ca^{2+} transient influxes upon exposure to Epo in EA.hy926 cells (Fig. 2), supporting the collaboration between K^+ and Ca^{2+} channels in the effect of this cytokine.

The relationship between calcium channel antagonists and angiogenesis is yet unclear. Different dihydropyridines have been tested for their effects on angiogenesis. Nifedipine was found to indirectly promote endothelial tube formation through the release of VEGF by vascular smooth muscle cells (Miura et al., 2005), while amlodipine increased capillary density and VEGF expression in a model of dilated cardiomyopathic hamster heart (Kumamoto et al., 1999), suggesting a possible role in the improvement of coronary microcirculation. In a rat model of hypertension, Nishizawa et al. (2010) reported that benidipine increased the density of coronary capillaries in rats fed on a high-salt diet, making it similar to that of control animals. The effect observed with benidipine, however, was not matched by nitrendipine, another compound of the dihydropyridine family. An angiogenesis-suppressing effect was detected in a rat model of retinopathy where nimodipine abrogated the PDGF-induced growth of new blood vessels (Juárez et al., 2000). Although diltiazem was shown to impair neovascularization in a mouse model of oxygen-induced retinopathy (Higgins et al., 1999), a posterior research work suggested an indirect stimulatory effect of this compound on vascular remodeling, through the increase in metalloprotease-1 (MMP-1) expression and collagenolytic activity in PDGF-stimulated vascular smooth muscle cells (Wada et al., 2001).

Our *in vitro* migration and tube formation assays in EA.hy926 cells revealed a different behavior of both calcium channel blockers: while the proangiogenic ability of Epo and VEGF was not affected by diltiazem, it was significantly impaired by amlodipine (Fig. 4). This differential effect could lie on their ability to trigger signalling pathways. Eickelberg et al. (1999) demonstrated that at nanomolar concentrations, all three subclasses of Ca^{2+}-channel blockers were able to induce the activity of certain transcription factors, independently of their Ca^{2+}-blocking action. In this line, Yue et al. (2004) have reported that

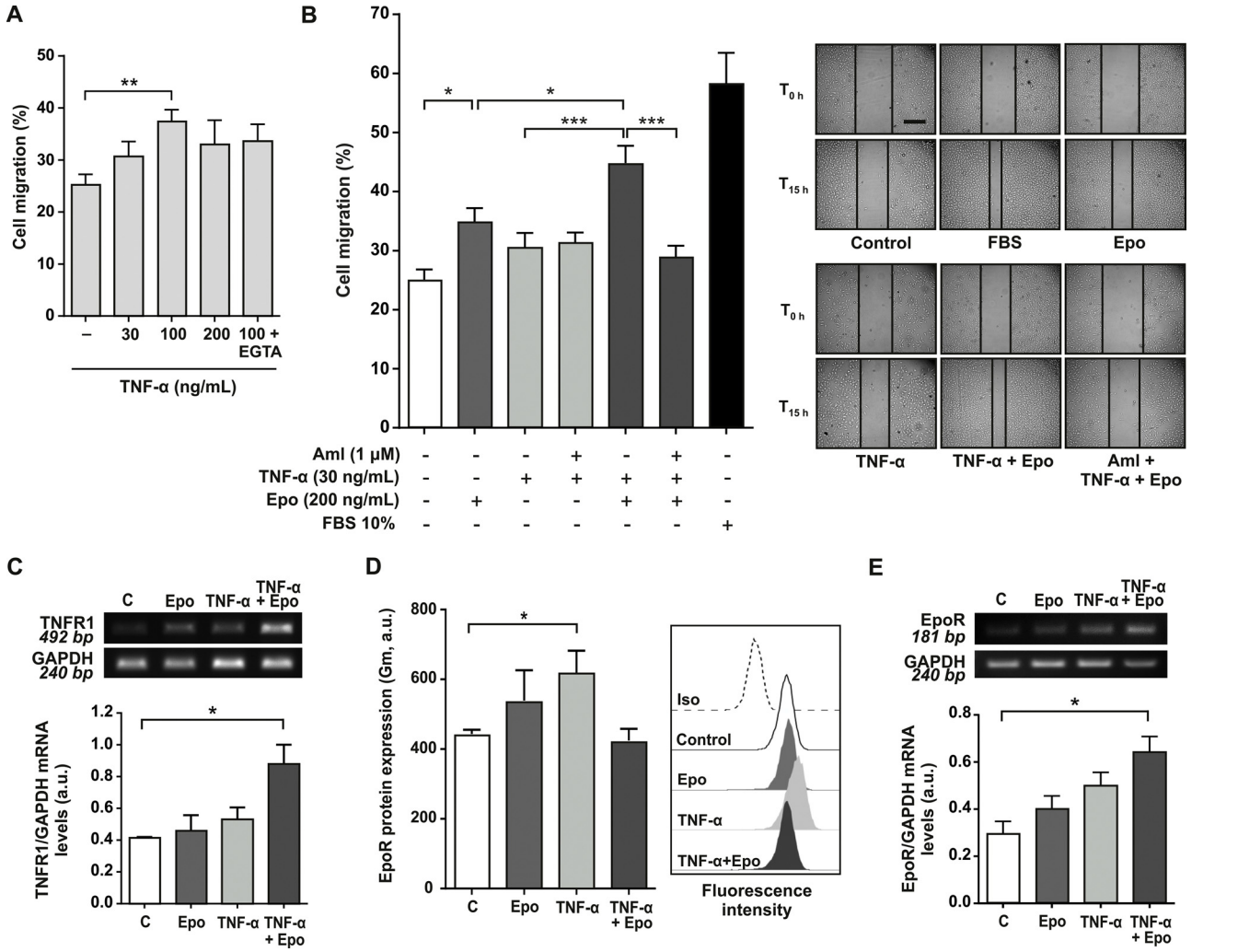

Fig. 6. Amlodipine impairs Epo-induced angiogenesis in a proinflammatory environment. A) Wound-healing assay in EA.hy926 cells incubated with increasing concentrations of TNF-α (30–200 ng/mL, 15 h). EGTA (1 mM) was added at least 10 min before TNF-α. **B)** Wound-healing assay in EA.hy926 cells in the presence of Epo (200 ng/mL) and TNF-α (30 ng/mL) for 15 h. Preincubation with amlodipine (1 μM) was made for 10 min before addition of the cytokines. Controls: cultures without FBS (Control) and with 10% FBS. *P < 0.05 and ***P < 0.001, ANOVA-Tukey; n = 8. Photographs are representative of the different treatments (black line represents 500 μm). **C)** TNFR1 mRNA levels in EA.hy926 cells were determined by reverse transcription-PCR after a 15-h exposure to Epo (200 ng/mL), TNF-α (30 ng/mL) or both. Control (C): cells incubated without FBS. Bars represent media ± SEM of band densitometry (*P < 0.05, Kruskal Wallis-Dunn, n = 3). The photograph of a representative gel was included. **D)** EpoR protein levels in EA.hy926 cells were determined by flow cytometry with a specific antibody. Cells were incubated with Epo (200 ng/mL), TNF-α (30 ng/mL) or both for 15 h. Control (C): cells incubated without FBS. *P < 0.05, Kruskal Wallis-Dunn, n = 4). Representative flow cytometry histograms are included. **E)** EpoR mRNA levels in EA.hy926 cells were assessed by reverse transcription-PCR after a 3-h treatment with Epo (200 ng/mL), TNF-α (30 ng/mL) or both. Control (C): cells incubated without FBS. PCR products were resolved through agarose gel electrophoresis with ethidium bromide staining. Bars represent media ± SEM of band densitometry (*P < 0.05, Kruskal Wallis-Dunn, n = 3).The photograph of a representative gel was included.

nifedipine increased MMP-2 expression in cardiac cells, possibly by means of NO production, while amlodipine —a third generation dihydropyridine— failed to stimulate NO release and provoked a tyrosine-kinase-dependent decrease in MMP-2. Diltiazem, on the other hand, did not affect metalloproteinase expression. These results could explain the inhibitory effect of amlodipine in tube-formation assays where Epo was used as a proangiogenic factor (Fig. 4E, F).

The relationship between calcium antagonists and ROS generation may also account for the differences observed. In this regard, we have previously shown an inhibitory effect of the antioxidant *N*-acetylcysteine on Epo-driven cell migration, as well a transient increase in intracellular ROS upon Epo treatment in EA.hy926 cells (Maltaneri et al., 2017). Dihydropyridines like amlodipine are recognized for their antioxidant effect in the vasculature (Toma et al., 2011), outperforming verapamil and diltiazem in *in vitro* (Mason et al., 1999) and in cell-based (Mak et al., 1992) experiments. Chemical structure differences conferring a differential ability to interact with biological membranes may account for these results. Although both amlodipine and diltiazem exhibit aromatic rings in their structure, which help stabilize free radicals by resonance, amlodipine also contains a transferable hydrogen attached to the aromatic nitrogen in the DHP ring, which represents an additional radical-quenching mechanism. Our present results show that amlodipine reduced the intracellular ROS burden generated by a 15-h exposure to TNF-α in EA.hy926 cells, whereas diltiazem enhanced the generation of these chemical entities (Fig. 5), suggesting an interplay between ROS and Ca^{2+} in endothelial cell migration.

Given that activated endothelial cells display a proangiogenic phenotype, the inflammatory cytokine TNF-α is able to induce endothelial cell migration and angiogenesis, as it has been previously confirmed (Yang et al., 2015; Shu et al., 2012). In our work, coincubation with TNF-α enhanced the promigratory effect of Epo in wound healing assays performed in EA.hy926 cells for 15 h and produced an increase in

TNFR1 mRNA levels (Fig. 6B, C). In a previous report, priming with TNF-α significantly increased the angiogenic behavior of Epo-treated human brain endothelial cells, compared to cultures incubated with Epo alone (Wang et al., 2011). This sensitizing effect of TNF-α was attributed to a TNFR1-mediated induction of EpoR expression, which was also reported in a model of glucose-deprived primary cortical neurons, where upregulation of EpoR and VEGF by TNF-α improved neuroprotection (Taoufik et al., 2008). Our results show that, although EpoR mRNA was found increased at the end of the wound healing assays in the presence of either TNF-α or Epo, no changes were observed in the presence of both cytokines. However, we have detected a significant increase in EpoR mRNA levels in EA.hy926 cells treated with both cytokines for 3 h (Fig. 6E) suggesting that after that period, EpoR expression decreases, although cells treated with Epo or TNF-α alone continue to exhibit higher levels of the receptor. Upon Epo binding, the Epo-EpoR complex is rapidly internalized and degraded by proteasome and lysosomes (Walrafen et al., 2005), which may explain the decrease in receptor levels after the greater induction achieved by the combination of both cytokines.

5. Conclusions

Taken together, our findings support the participation of Ca^{2+} entry through voltage-dependent and transient potential channels in Epo-driven endothelial cell migration and highlight the antiangiogenic properties of amlodipine, which was also effective in a proinflammatory context. These results may have important implications for the treatment of ischemic hearts, which benefit from the proliferation of blood vessels. Ischemia is characterized by the release of TNF-α, the accumulation of ROS and the generation of a proinflammatory environment, which may enhance the proangiogenic effects of Epo and VEGF. The coadministration of amlodipine, a drug of choice for the treatment of ischemic damage through the stabilization of blood pressure, could counter the beneficial effects of an angiogenesis-stimulating therapy.

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Conflicts of interest

The authors declare no conflict of interest

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