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Differential effect of erythropoietin and carbamylated erythropoietin on endothelial cell migration

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1. Introduction

Despite being largely known for its ability to stimulate erythropoiesis, in recent years erythropoietin –a glycosylated cytokine secreted by the adult kidney– has been increasingly recognized for its actions beyond the hematopoietic system. Its pleiotropic activities have been reported to include protection from apoptosis and oxidative/inflammatory damage in different organs and tissues, such as myocardium, endothelium, kidney, retina and brain (Arcasoy, 2008; Chateauvieux et al., 2011).

Recombinant human erythropoietin (rhuEpo) is widely used in the treatment of anemia derived from chronic kidney failure and other pathological conditions. The fact that expression of the Epo receptor (EpoR) was detected in non-hematopoietic tissues (Anagnostou et al., 1994; Masuda et al., 1993), along with the ever-increasing evidence of its pleiotropic activities, have raised concern at the possibility of unwanted targets of Epo treatment. Recent reports of tumour progression and trombo-vascular events have challenged the success of Epo-based therapies (McKinney and Arcasoy, 2011).

In order to prevent the adverse effects related to Epo treatment, research has been focused on the development of modified derivatives of varying biological activity and half-life, many of which are currently being tested in preclinical studies and clinical protocols. One such compound is obtained by carbamylation of erythropoietin on lysine residues (cEpo), which renders the protein incapable of promoting erythropoiesis (Leist, 2004). Although cEpo is unable to bind the classical homodimeric receptor EpoR/EpoR which triggers signals of erythroid cell proliferation (Chamorro et al., 2013), it retains the ability of the native cytokine to bind the heterodimeric receptor consisting of one EpoR subunit and one cytokine β-common (βc) subunit (CD131), shared by the GM-CSF, IL-3 and IL-5 receptors, thus maintaining its cytoprotective effects on non-hematopoietic tissues. In this regard, cEpo was reported to act as a cytoprotective factor in animal models of diseases such as stroke (Villa et al., 2007), diabetic autonomic neuropathy (Schmidt et al., 2008) and cardiac isquemic damage (Moon et al., 2006), where an increase in erythropoiesis would have deleterious consequences.

Given that the endothelium is among the most important non-erythroid targets of Epo, interest has developed in the effects of

Abbreviations: βcR, beta common receptor; cEpo, carbamylated erythropoietin; eNOS, endothelial nitric oxide synthase; Epo, erythropoietin; EpoR, erythropoietin receptor; FBS, fetal bovine serum; NAC, N acetyl-cysteine; L-NMMA, NG-methyl-L-arginine acetate; NO, nitric oxide; PBS, phosphate-buffered saline; PTP1B, protein tyrosine phosphatase 1B; ROS, reactive oxygen species; SEM, standard error of the mean; VEGF, vascular endothelial growth factor.

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cEpo on the vasculature. Although experimental studies in HUVEC and in endothelial progenitor cells (EPCs) indicate little activity of cEpo on cell migration and proliferation (Coleman et al., 2006; Ramirez et al., 2009), some authors have reported proangiogenic effects of cEpo *in vivo* (Imamura et al., 2008; Xiong et al., 2011).

Considering its therapeutic potential, it is necessary to evaluate the possible contribution of cEpo to the angiogenesis process. While treatment of stroke-related pathologies would benefit from angiogenesis stimulation (Ergul et al., 2012), it might have negative consequences on some neurodegenerative diseases where both erythropoietins are being studied for their cytoprotective activities, such as Alzheimer's disease, (Vagnucci and Li, 2003), as well as on cancer.

Therefore, with the purpose of contributing to the current knowledge about the action of Epo in non-erythroid tissues, the aim of our work was to investigate the mechanisms through which Epo exerts its effects on endothelial cells, with special interest in comparing its activity to that of the carbamylated derivative.

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 (Argentina) and Hemax was purchased from Biosidus. All the culture media, the cell dissociation reagent TrypLE Select and the penicillin–streptomycin antibiotic mixture were from GIBCO. Fetal bovine serum (FBS) was purchased from Natocor (Argentina). Primary antibodies against EpoR (sc-697), βcR (sc-21765), PTP1B (sc-14021) and β-actin (sc-47778), as well as the inhibitor CinnGel 2Me were from Santa Cruz Biotechnology. Antibodies against TRPC3 (ACC-016) and TRPC6 (ACC-017) were from Alomone Labs. Horseradish peroxidase-conjugated secondary antibodies against rabbit (A6154) and mouse (A4416) immunoglobulins, the fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA), N-acetyl-cysteine (NAC) and the NOS inhibitor N^G-methyl-L-arginine acetate (L-NMMA) were from Sigma Aldrich. Alexa-Fluor 488-conjugated secondary antibodies, primers against EpoR, βcR and GAPDH, the TRIzol reagent and the fluorescent calcium probe Fluo 4-AM were from Invitrogen. The reverse transcription kit containing Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase, RNAsin RNAse inhibitor and oligodTs was from Promega Corporation. LY294002 and AG490 were obtained from Calbiochem/Millipore. Cytofix/Cytoperm and PermWash buffer were acquired from BD Biosciences.

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). Cultures were grown on Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. Cultures were used no further than passage 20, due to cellular senescence.

The human erythroleukemia cell line (UT-7) was kindly provided by Dr. Patrick Mayeux (Cochin Hospital, France). These cells depend on erythropoietin to grow and are capable of erythroid differentiation. Cultures were maintained in IMDM medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 16 ng/mL recombinant human Epo (Hemax).

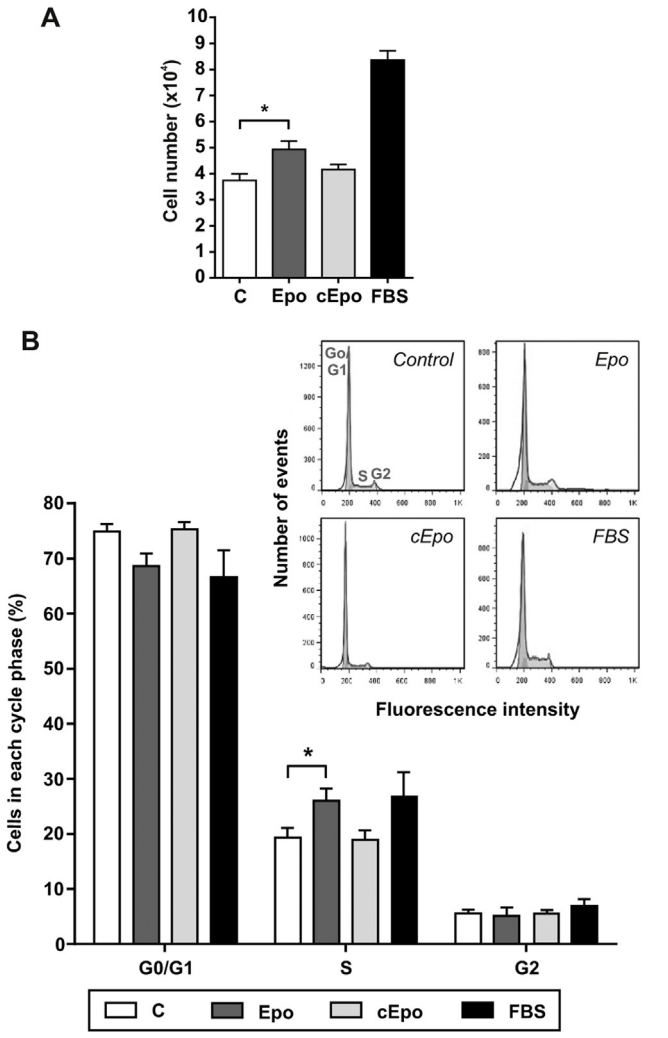

Fig. 1. Effect of Epo and cEpo on EA.hy926 proliferation. A) After a 48-h treatment with Epo or cEpo (200 ng/mL) in FBS-free medium, total cell number was determined in a Neubauer chamber, using the exclusion dye Trypan blue. Controls: cultures without FBS (C) and with 10% FBS.*P < 0.05, Kruskal Wallis-Dunn, n = 7. B) After 15 h of Epo or cEpo stimulation, cell cycle analysis was performed by propidium iodide staining followed by flow cytometry. Histograms show a representative analysis of cell cycle performed with the flow cytometry software FloJo. Shaded areas in the histograms indicate the different G0/G1, S and G2 cell populations identified by the software. *P < 0.05, Kruskal Wallis-Dunn, n = 5.

The human neuroblastoma cell line SH-SY5Y (ATCC; CRL-2266) was grown on 1:1 DMEM/Ham's F-12 medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin and 10% FBS.

All cultures were maintained at 37 °C and 5% CO_2. For adherent cells, media were replaced every 2 days, and cells were divided into separate flasks upon reaching 80–90% confluence. In order to reproduce the physiological quiescent state of the endothelium when performing experiments, endothelial cells were serum-deprived before treatment, and experiments were carried out in serum-free medium unless otherwise stated.

2.3. Preparation and characterization of cEpo

Preparation of cEpo: Carbamylated erythropoietin (cEpo) was prepared as described by Leist (2004) with modifications. Epo (0.3 mg/mL) was mixed with of 0.45 M sodium borate (pH 8.8) and 0.5 M potassium cyanate (KCNO). The mixture was incubated at 37 °C for 48 h. Cyanate was eliminated from the preparation by

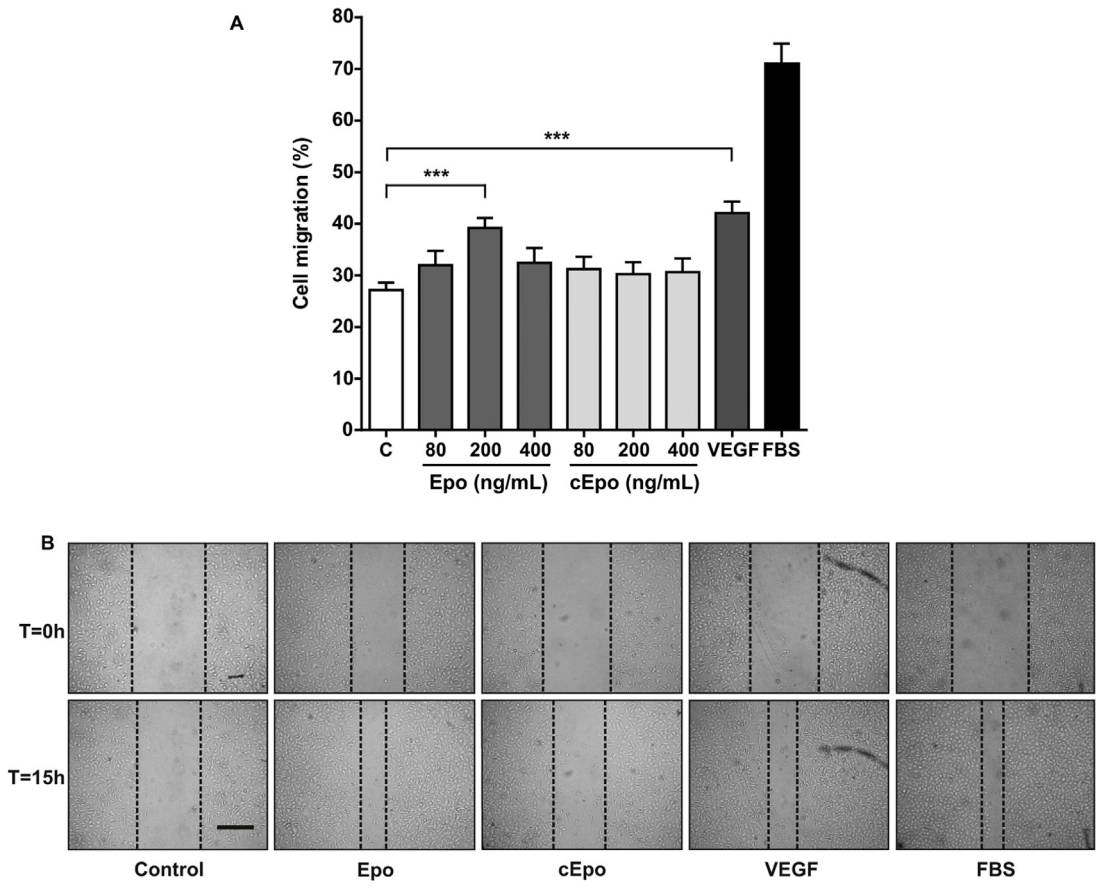

Fig. 2. Effect of Epo and cEpo on cell migration. A) Wound healing assay: After 8 h of serum starvation (t = 0), EA.hy926 cells were exposed to Epo or cEpo (80–400 ng/mL) without FBS for 15 h. Controls: culture without FBS (C), with 10% FBS and with VEGF 10 ng/mL. Cell migration was assessed by measuring wound width in photographs taken at t = 0 h and t = 15 h (***P < 0.001, Kruskal Wallis-Dunn, n = 12). B) Photographs (magnification: 50×) are representative of 12 independent assays (black bar represents 1000 μm).

washing with four times the sample volume of Milli-Q water. Sample washing and concentration were performed in Amicon Ultra-4 centrifugal filters (3 kDa cut-off; Millipore). The same batch of cEpo was used throughout the work after demonstration of its lack of proliferative action on UT-7 cells as well as its protective effect on SH-SY5Y cells induced to apoptosis by staurosporine (Chamorro et al., 2013).

2.4. Electrophoresis and Western blotting

Gel electrophoresis: Cells were lyzed with the Laemmli (1970) sample buffer (2X) at 90 °C, and repeatedly freezed-thawed and vortexed. Cell lysates were boiled for 2 min and resolved by 12% polyacrylamide-SDS gel electrophoresis. Electrophoresis under non-denaturing conditions was run in a Miniprotean III electrophoretic system (BioRad) using 12% polyacrylamide gels.

After electrophoresis, protein samples were electroblotted onto a nitrocellulose membrane during 1 h and 15 min (transfer buffer: pH 8.3, 25 mM Tris, 195 mM glycine, 0.05% SDS, pH 8.3, and 20% v/v methanol). Membranes were blocked overnight in Tris Buffer Saline (TBS, 25 mM Tris, 137 mM NaCl, 3 mM KCl, pH 7.4) containing 0.1% Tween 20 and 0.5% skimmed-milk powder, and then incubated with specific primary antibodies (1 h). After washing with TBS–0.1% Tween 20, immunoblots were probed with adequate peroxidase-conjugated secondary antibodies (1:3000; 1 h) and washed with TBS-Tween. Antigen-antibody complex signals were detected by enhanced chemiluminiscence in a G:BOX Chemi system and digitalized using the GeneSys software (Syngene).

Capillary zone electrophoresis: Sample analysis was performed at pH 9.9 (150 mM sodium borate buffer) in a P/ACE^{TM} MDQ Capillary Electrophoresis System (Beckman Coulter), equipped with a Photo Diode Array (PDA) detector and a 214 nm filter.

2.5. Flow cytometry

Cell cycle analysis: Cells were harvested with TrypLE, washed and fixed in cold 70% (v/v) ethanol. Samples were kept for at least 24 h at −20 °C, then centrifuged and incubated with ribonuclease A (120 μg/mL) and propidium iodide (0.05 mg/mL) for 1 h at 37 °C. Samples were then diluted with cold PBS, filtered through a piece of voile fabric and kept on ice until acquision of events.

Determination of ROS: After 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 in 0.5% paraformaldehyde/PBS before event acquisition. For treatment times under 5 min, cells were loaded with the probe, then harvested with TrypLE and resuspended in HEPES buffer with $CaCl_2$ (1.5 mM). Cell suspension aliquots were treated with Epo, cEpo or FBS for different periods of time, after which they were immediately fixed with 4% paraformaldehyde for posterior event acquisition.

Determination of the EpoR and βcR receptors and TRPC channel 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

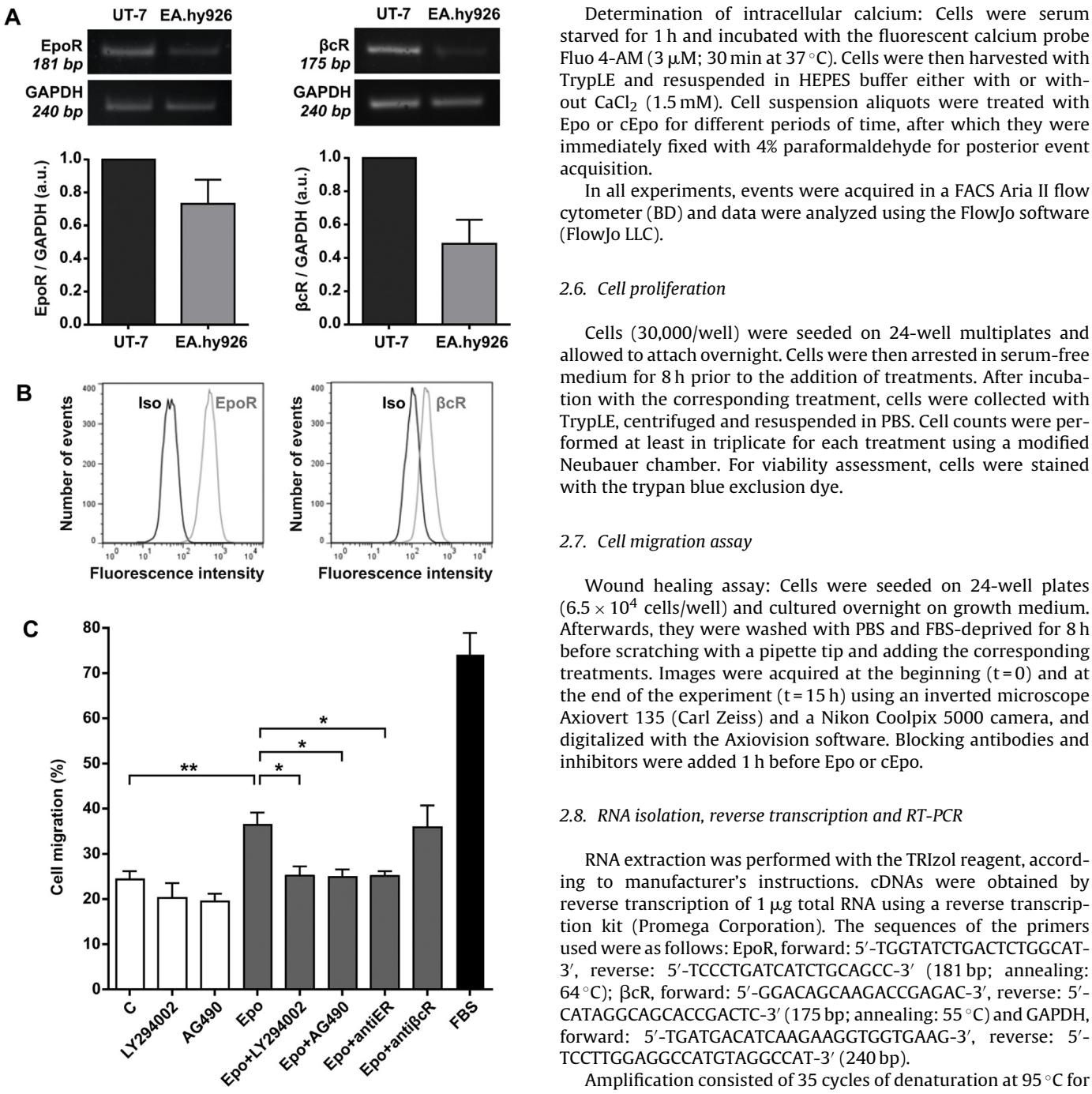

Fig. 3. Receptors and signaling pathways involved in Epo-induced cell migration. A) Detection of EpoR and βcR mRNA levels by endpoint PCR followed by agarose gel electrophoresis and band densitometry. Each bar represents mean ± SEM of band density with respect to GAPDH, used as sample loading control (n = 3). B) Expression of EpoR and βcR in EA.hy926 by flow cytometry with specific antibodies. Graphs are representative of 3 independent determinations. C) Wound healing assays on Epo-treated (200 ng/mL) cultures after a 1 h-preincubation with EpoR- and βcR-blocking antibodies, as well as with the PI3K and JAK2 inhibitors LY294002 (1 mM) and AG490 (1 mM), respectively. *P < 0.05, ANOVA-Dunnet, n = 6. Controls: cultures without FBS (C) and with 10% FBS.

incubated with primary antibodies for 1 h on ice, then washed with PWB and stained with the corresponding Alexa Fluor 488-conjugated secondary antibodies for 30 min on ice in the dark. Cells were washed with PWB and resuspended in PBS before acquisition of events.

Determination of intracellular calcium: Cells were serum starved for 1 h and incubated with the fluorescent calcium probe Fluo 4-AM (3 μM; 30 min at 37 °C). Cells were then harvested with TrypLE and resuspended in HEPES buffer either with or without $CaCl_2$ (1.5 mM). Cell suspension aliquots were treated with Epo or cEpo for different periods of time, after which they were immediately fixed with 4% paraformaldehyde for posterior event acquisition.

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. Cell proliferation

Cells (30,000/well) were seeded on 24-well multiplates and allowed to attach overnight. Cells were then arrested in serum-free medium for 8 h prior to the addition of treatments. After incubation with the corresponding treatment, cells were collected with TrypLE, centrifuged and resuspended in PBS. Cell counts were performed at least in triplicate for each treatment using a modified Neubauer chamber. For viability assessment, cells were stained with the trypan blue exclusion dye.

2.7. Cell migration assay

Wound healing assay: Cells were seeded on 24-well plates (6.5×10^4 cells/well) and cultured overnight on growth medium. Afterwards, they were washed with PBS and FBS-deprived for 8 h before scratching with a pipette tip and adding the corresponding treatments. Images were acquired at the beginning (t = 0) and at the end of the experiment (t = 15 h) using an inverted microscope Axiovert 135 (Carl Zeiss) and a Nikon Coolpix 5000 camera, and digitalized with the Axiovision software. Blocking antibodies and inhibitors were added 1 h before Epo or cEpo.

2.8. RNA isolation, reverse transcription and RT-PCR

RNA extraction was performed with the TRIzol reagent, according to manufacturer's instructions. cDNAs were obtained by reverse transcription of 1 μg total RNA using a reverse transcription kit (Promega Corporation). The sequences of the primers used were as follows: EpoR, forward: 5′-TGGTATCTGACTCTGGCAT-3′, reverse: 5′-TCCCTGATCATCTGCAGCC-3′ (181 bp; annealing: 64 °C); βcR, forward: 5′-GGACAGCAAGACCGAGAC-3′, reverse: 5′-CATAGGCAGCACCGACTC-3′ (175 bp; annealing: 55 °C) and GAPDH, forward: 5′-TGATGACATCAAGAAGGTGGTGAAG-3′, reverse: 5′-TCCTTGGAGGCCATGTAGGCCAT-3′ (240 bp).

Amplification consisted of 35 cycles of denaturation at 95 °C for 30", annealing for 30" and extension at 72 °C for 30". PCR products were resolved on 2% agarose gels stained with 0.5% ethidium bromide. DNA fluorescence 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 using the ImageJ software.

2.9. Statistics

Statistical analysis was performed with the Graph Pad Prism software (GraphPad Software Inc.). Results are expressed as mean ± standard error (Mean ± SEM). Whenever applied, ANOVA and Kruskal-Wallis one-way analysis of variance were followed by Dunnett's or Dunn's test for comparison among groups, respectively. Least significant difference with P < 0.05 was considered the criterion for statistical significance.

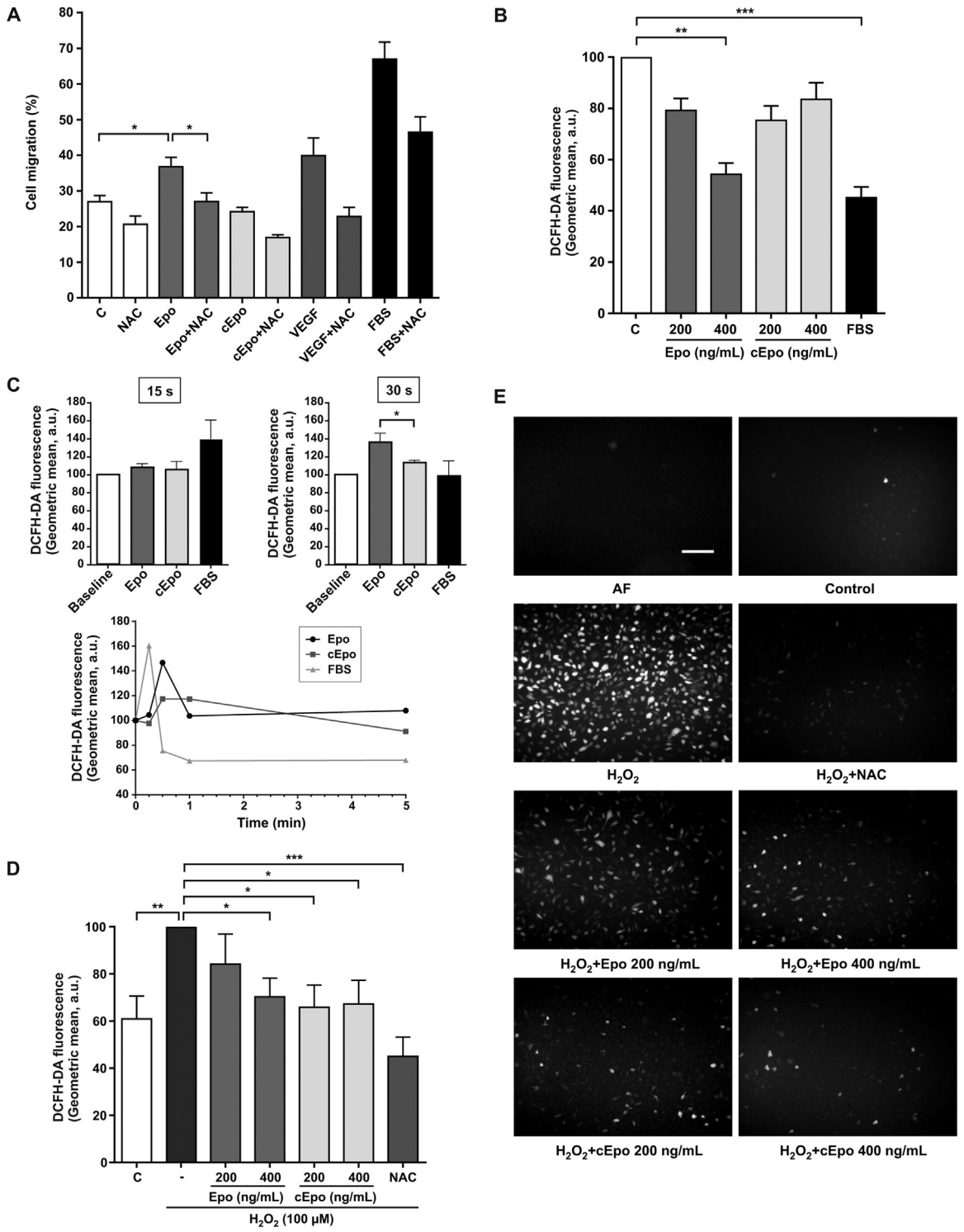

Fig. 4. Participation of ROS and NO in Epo-induced migration signaling. A) Wound healing assay with or without NAC (5 mM) in cells treated with Epo or cEpo (200 ng/mL) and VEGF (10 ng/mL). *P < 0.05, Kruskal Wallis-Dunn, n = 9. Controls: cultures without FBS (C) and with 10% FBS. B) Cells were exposed to Epo or cEpo (200 and 400 ng/mL) for 15 h and the content of ROS was determined by flow cytometry with DCFH-DA. FBS (10%) was used as a control.**P < 0.01 and ***P < 0.001, Kruskal Wallis-Dunn, n = 5. C) Cells previously loaded with DCFH-DA (20 µM, 30 min) were incubated for the indicated times with Epo (200 ng/mL), equal concentration of cEpo or 10% FBS and immediately fixed with 4% PFA in PBS. Intracellular ROS levels at 15 s and at 30 s of treatment are represented as histograms. *P < 0.05, Kruskal Wallis-Dunn, n = 4. A representative flow cytometry analysis is included. D) Preincubation of cells with Epo or cEpo (200 and 400 ng/mL; 30 min) was followed by exposure to hydrogen peroxide (100 µM, 30 min) and evaluation of ROS levels by flow cytometry. NAC (5 mM) was used as a control.*P < 0.05, **P < 0.01 and ***P < 0.001, Kruskal Wallis-Dunn, n = 7. E) Photographs (magnification: 100×) are representative of 3 independent experiments (white bar represents 200 µm).

3. Results

3.1. Carbamylation of Epo

We have previously studied the carbamylated derivative of Epo and characterized its biological effect on erythroid and neuronal cells (Chamorro et al., 2013; Chamorro et al., 2015).

On native gel electrophoresis followed by Western blotting with an erythropoietin-specific antibody, cEpo exhibited a greater mobility than Epo, given that the isotiocyanate groups mask the positive charge of the lysine residues they are covalently bound to. This result was confirmed by capillary zone electrophoresis, where the masking of positive charges caused an increase in the migration time of cEpo compared to that of Epo, with a delay of 21.7 ± 0.1 s.

The erythropoietic ability of carbamylated Epo was evaluated on human UT-7 cells, which were exposed to Epo or cEpo (16 ng/mL) for 48 h cEpo did not support erythroid proliferation, which was in turn significantly increased by Epo. The antiapoptotic effect of both erythropoietins was demonstrated by a 24-h preincubation with either Epo or cEpo (250 ng/mL) in cultures of neuroblastoma SH-SY5Y cells treated with 50 nM staurosporin for 24 h (Chamorro et al., 2013). Based on the functional assays performed, we demonstrated that the chemical modification introduced in Epo effectively allowed us to separate its erythropoetic ability from its cytoprotective effects.

3.2. Effect of Epo and cEpo on endothelial proliferation and cell cycle progression

Although Epo is recognized as a mitogenic stimulus for endothelial cells, the ability of its carbamylated derivative to promote endothelial cell growth remains less certain. With the aim of clarifying this point, we evaluated proliferation, viability and cell cycle progression on cultured EA.hy926 cells exposed to Epo or cEpo.

As shown in Fig. 1A, cell counts revealed a proliferative effect of Epo in EA.hy926 cultures after a 48 h-incubation, while cEpo failed to stimulate cell division in that period. Treatment with Epo or cEpo did not alter cell viability. No differences were observed in cell numbers when treated for less than 24 h (data not shown).

In line with these findings, the assessment of cell cycle progression by propidium iodide DNA staining and flow cytometry showed a significant increase in the number of EA.hy926 cells undergoing the S phase of the cycle when treated with Epo for 15 h, which was not achieved with cEpo (Fig. 1B). Accordingly, the fraction of cells in the G0/G1 phase was also reduced after Epo treatment. A similar pattern was observed in cells maintained in FBS, a strong promoter of angiogenesis *in vitro*.

3.3. Effect of Epo and cEpo on endothelial cell migration

The development of blood vessels from previously existing ones, as well as the repair of injured vessels, does not only rely on the proliferation of endothelial cells, but also on their ability to migrate. In order to complete our assessment of the angiogenic potential of both erythropoietins, we carried out scratching assays on serum-starved EA.hy926 cultures exposed to Epo or cEpo for 15 h. Based on the proliferation assays described in Section 3.2, the choice of a 15-h treatment allowed us to interpret wound closure as a consequence of enhanced cell migration, with a negligible contribution of cell proliferation.

Exposure to 200 ng/mL Epo significantly increased motility of EA.hy926 cells compared to untreated cultures (Fig. 2A and B). Such increase was not achieved by exposure to an equal concentration of cEpo. Interestingly, the ability of Epo to promote endothelial cell migration peaked at 200 ng/mL, to decrease again at higher concentrations.

3.4. Signaling pathways involved in Epo-induced cell migration

A.) Epo receptor and signaling pathways in Epo-stimulated cells

The results obtained from the migration assays described in Section 3.3 show a proangiogenic behavior of Epo. Hence it was interesting to investigate which signaling pathways could be involved in such effect.

Given that the presence of the erythropoietin receptor (EpoR), and particularly that of the β-common receptor (βcR) in the endothelium have been controversial, our first objective was to study the expression of both subunits in EA.hy926 cells. Using the erythroleukemia cell line UT-7 as a positive control, we detected mRNA levels of the EpoR and the βcR subunits in EA.hy926 cells with RT-PCR (Fig. 3A). Regarding protein levels, expression of both receptors was observed through flow cytometry with specific antibodies (Fig. 3B). Even though βcR levels appear to be relatively low, this receptor is able to mediate GM-CSF signaling in endothelial cells, as demonstrated in the scratching assays performed on EA.hy926 in the presence of this growth factor (Control: $26 \pm 1.2\%$, *GM-CSF 10 ng/mL: $33 \pm 2.2\%$; $^*P < 0.05$, Kruskal Wallis-Dunn, $n = 7$).

We carried out scratching assays on Epo-treated cultures after preincubation with EpoR- and βcR-blocking antibodies, as well as with inhibitors of signaling pathways. As shown in Fig. 3C, Epo-induced cell migration was abrogated by treatment with anti-EpoR antibody and with PI3K and JAK2 inhibitors LY294002 and AG490, respectively, but remained unaffected when treated with anti-βcR. These results are consistent with the participation of the homodimeric receptor EpoR-EpoR, and not the heterodimeric receptor, in the endothelial cell migration induced by Epo, as well as with the involvement of signaling pathways related to JAK2 and PI3K/Akt activation.

B.) Participation of ROS and NO in Epo-induced migration signaling

Experimental evidence shows that reactive oxygen and nitrogen species, when in small concentrations, play an important role in endothelial cell motility. In order to determine if ROS mediate the proangiogenic effect of Epo, we performed wound-healing assays in the presence of the antioxidant N-acetyl-cysteine (NAC) (Fig. 4A). NAC inhibited Epo-induced cell migration, as well as that caused by VEGF and 10% FBS to some extent, thus suggesting a possible contribution of Epo to the generation of ROS. Notwithstanding, a 15-h exposure to Epo showed a significant, concentration-dependent protective effect against the generation of ROS caused by serum deprivation (Fig. 4B). In order to explain this incongruence, we measured intracellular ROS at incubation times much shorter than 15 h, by exposing cells to Epo, cEpo or 10% FBS. As shown in Fig. 4C, Epo (200 ng/mL) was able to induce a transient increase in intracellular ROS within the first minute of exposure, peaking at 30 s (DCFH-DA, flow cytometry). An even quicker rise was achieved with 10% FBS. cEpo did not affect intracellular ROS levels throughout the experiment.

Preincubation with Epo (200 ng/mL) also showed a protective effect against the addition of a prooxidant agent such as hydrogen peroxide (Figs. 4D-4E). Interestingly, cEpo exerted a strong protective effect against the prooxidant state generated by H_2O_2 (Figs. 4D and E), which was only mild when ROS were caused by the absence of serum in the culture medium (Fig. 4B).

It is also recognized that the generation of NO by the endothelial nitric oxide synthase (eNOS) stimulates cell motility. In scratching assays performed in the presence of the NOS inhibitor NG-methyl-L-arginine (L-NMMA), stimulation of cell migration by Epo was inhibited, as well as that induced by VEGF (Fig. 5).

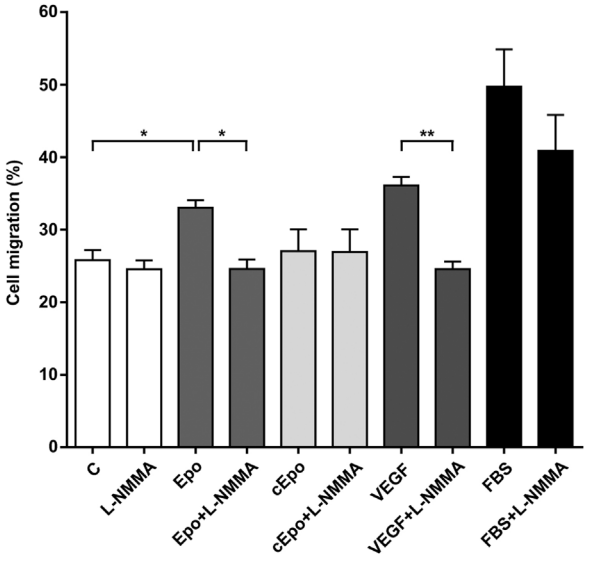

Fig. 5. Participation of the nitric oxide synthase in Epo-induced endothelial cell migration. Wound healing assay on Epo-treated cultures (200 ng/mL) in the presence or absence of the NOS inhibitor L-NMMA (250 μM). *P < 0.05 and **P < 0.01, Kruskal Wallis-Dunn, n = 6.

C.) Calcium signaling in Epo-induced cell migration.

Calcium plays an important role in cell migration, since the turnover of focal adhesions and cytoskeleton dynamics have been shown to depend on this cation (Giannone et al., 2004). In EA.hy926 cells, the Ca^{2+} chelators EGTA and –to a lesser extent– EDTA (1 mM) inhibited migration induced by Epo (Fig. 6A).

In migrating cells, a calcium gradient is established from rear to front, where the basal concentration is low enough to allow rapid calcium transients necessary to induce motility. As shown for other angiogenic factors, Epo (200 ng/mL) was able to generate a calcium transient rise within the first minute of stimulation (Fig. 6B) which was not achieved by cEpo. Interestingly, the generation of such transient by Epo was abrogated by the absence of Ca^{2+} in the suspension buffer, suggesting that the rapid increase in cytosolic Ca^{2+} is caused by entry of this cation and not by its release from intracellular stores. Furthermore, a 15 h-incubation with Epo (200 ng/mL) significantly increased the expression levels of the TRPC3 calcium channels, while those of TRPC6 channels remained unchanged (Fig. 6C). These results support the role of calcium in the stimulation of migration by Epo in endothelial cells.

3.5. *Participation of PTP1B in the differential response of endothelial cells to Epo and cEpo*

The erythropoietin receptor is associated to different phosphatases, which participate in the termination of the signal elicited by this cytokine. In UT-7 cells, capable of erythroid differentiation, we have demonstrated that one such enzyme, the protein tyrosine phosphatase 1 B (PTP1B) is not only more expressed, but also more active, in cEpo-treated cultures compared to those incubated with the native protein. Stemming from this, the incapacity of cEpo to support erythroid proliferation seems to be associated to a premature termination of the signal (Chamorro et al., 2015).

In scratching assays carried out on EA.hy926 cells, treatment with the PTP1B inhibitor CinnGel 2-methyl-ester rendered cEpo (200 ng/mL) capable of stimulating cell migration to levels similar to those achieved by equal concentration of Epo (Fig. 7A). Interestingly, inhibition of PTP1B failed to boost the promigratory effect

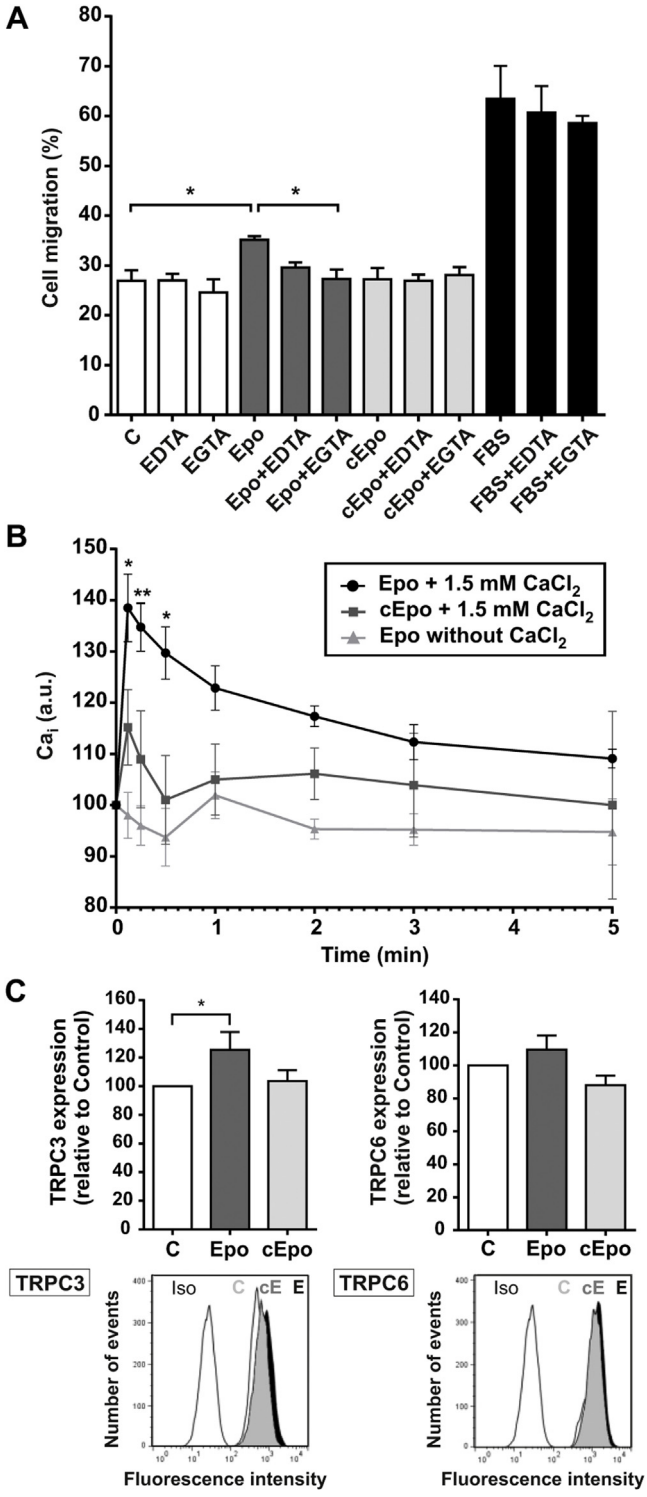

Fig. 6. Calcium as a mediator in Epo-induced endothelial cell migration. A) Wound healing assay in the presence of the calcium chelators EDTA (1 mM) and EGTA (1 mM). *P < 0.05, Kruskal Wallis-Dunn, n = 4. Controls: cultures without FBS (C) and with 10% FBS. B) Determination of the intracellular calcium concentration by flow cytometry with the fluorescent probe Fluo4-AM (3 μM). Cells were incubated for the indicated times with Epo or cEpo (200 ng/mL) in HEPES buffer either with or without 1.5 mM $CaCl_2$, and immediately fixed with 0.5% paraformaldehyde in PBS. *P < 0.05 and **P < 0.01 vs basal Ca_i (time = 0 min), Kruskal Wallis-Dunn, n = 3. C) TRPC3 expression in EA.hy926 cells treated with Epo or cEpo (200 ng/mL; 15 h) by flow citometry. Results are expressed as geometric mean of fluorescence intensity relative to Control. *P < 0.05, Kruskal Wallis, Dunn, n = 5. Histograms show representative analysis of TRPC3 and TRPC6 expression by flow cytometry.

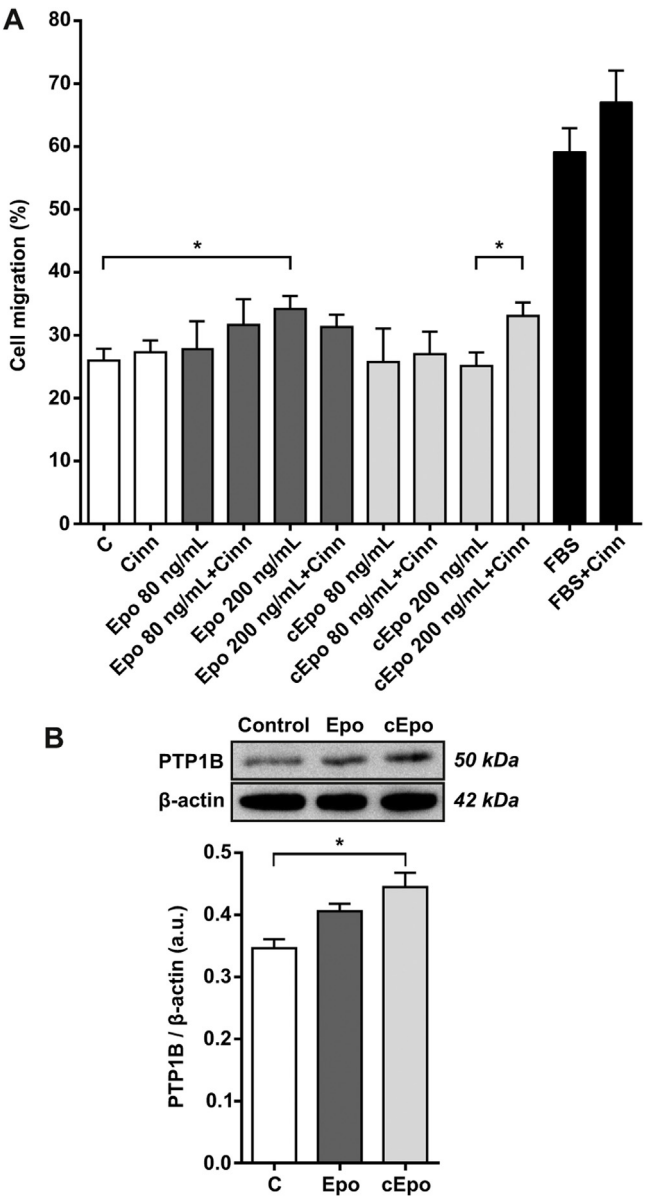

Fig. 7. PTP1B involvement in the differential promigratory response of EA.hy926 to Epo and cEpo. A) Wound healing assay in the presence of the PTP1B inhibitor CinnGel-2Me 20 μM (Cinn) Controls: cultures without FBS (C) and with 10% FBS. *P < 0.01, Kruskal Wallis-Dunn, n = 12. B) PTP1B expression after 15 h of Epo or cEpo treatment assayed by Western blotting and quantified through band densitometry. Each bar represents mean ± SEM of band density with respect to β-actin, used as sample loading control.*P < 0.05, Kruskal Wallis-Dunn, n = 3.

of Epo 200 ng/mL, while it increased migration induced by a lower concentration of the cytokine (80 ng/mL).

In addition, incubation of EA.hy926 cells with cEpo 200 ng/mL for 15 h increased PTP1B expression, which was significantly greater than that induced by Epo (Fig. 7B). These results support our hypothesis that a premature inactivation of the Epo receptor caused by overexpression of this phosphatase could explain, at least partially, the differential effects of both erythropoietins on endothelial cells.

4. Discussion

In accordance to previous research by other authors, we found a proangiogenic effect of Epo on endothelial cells, but we went further to investigate possible mechanisms of action, with special interest in comparing this activity with that of the carbamylated erythropoietin.

The stimulation of angiogenesis by Epo, a complex process which comprises cell migration, cell proliferation and recruitment of circulating endothelial progenitor cells (EPCs), was previously reported in *in vitro* experiments performed on HUVECs (Anagnostou et al., 1990; Du et al., 2013), BAECs (Anagnostou et al., 1990; Su et al., 2011) and EPCs (Sautina et al., 2010).

In this work, we found a promigratory activity of Epo on EA.hy926 cells, which was not matched by treatment with its carbamylated derivative (Fig. 2). Worthy of note is the finding of a concentration-dependent effect of Epo on cell migration, which peaked at 200 ng/mL only to decrease at higher concentrations. This type of bell-shaped concentration-response curve has also been described for other cytokines such as the vascular endothelial growth factor (VEGF), a potent chemotactic agent for endothelial cells within the 5–20 ng/mL concentration range (Rousseau et al., 1997). Such response patterns may indicate the triggering of receptor inhibition mechanisms upon ligand binding.

The effect of Epo on EA.hy926 migration was associated to cell proliferation (Fig. 1). While there is a consensus on the mitogenic ability of the native erythropoietin on endothelial cells and endothelial progenitors (Heeschen et al., 2003; Ribatti et al., 1999), that of the carbamylated derivative remains less certain. While some authors reported a significant protective action of cEpo in rat models of traumatic brain injury (Xiong et al., 2011) and ischemia-reperfusion kidney damage (Imamura et al., 2008) by stimulating angiogenesis, among other effects, we found that cEpo failed to promote EA.hy926 proliferation, in agreement with the results obtained in HUVECs by Coleman et al. (2006). In that same work, cEpo was shown to serve as a mitogenic stimulus for EPCs, although Ramirez et al. (2009) later reported the incapacity of carbamylated darbepoetin to stimulate proliferation of EPCs. These results hint at a differential action of the carbamylated Epo in endothelial progenitors and differentiated endothelial cells.

Our results suggest that the promigratory effect of Epo be mediated by the classical signaling pathway triggered after its binding to the $(EpoR)_2$ receptor, consequently eliciting JAK2 and PI3K activation. Du et al. (2013) reported EpoR participation in the migration of HUVECs stimulated by Epo. Nevertheless, our findings differ from those reported by Sautina et al. (2010) and Su et al. (2011), who demonstrated the involvement of the heterodimeric receptor (EpoR/βcR) in the effects of Epo on EPCs and BAECs.

Expression of the β-common receptor was previously observed in HUVEC and ECV304 endothelial cells responsive to GM-CSF (Colotta et al., 1993). In EA.hy926, we found lower expression levels of βcR (Fig. 3), thus agreeing with the explanation provided by Coleman et al. (2006) about the homodimeric Epo receptor being predominantly expressed in the mature endothelium, thus attributing the differential effect of cEpo on HUVEC and EPC proliferation to different βcR expression patterns. Accordingly, Bennis et al. (2012) reported a βcR-mediated enhancement of the angiogenic potential of EPCs from human umbilical cord blood treated with Epo. It is worth mentioning that, although βcR expression levels are relatively low in EA.hy926 cells, it appears to be functional, as we observed in scratching assays performed with GM-CSF, where wound closure was significantly increased.

Among the possible mechanisms underlying cell motility, we were interested in investigating the participation of ROS in Epo-induced cell migration, as the rapid generation of localized, small concentrations of ROS has been involved in the migratory response of cells stimulated with cytokines such as VEGF (Wang et al., 2011). These chemical entities were shown to inactivate cell migration inhibitors like the protein tyrosine phosphatase PTP1B (Lee et al., 1998). Although we observed an inhibition of Epo-induced wound closure in EA.hy926 cultures treated with the antioxidant N-acetyl-

cysteine, Epo exhibited a protective effect against ROS generated by serum deprivation in the same treatment period of the scratching assay, which is in line with experiments carried out in the presence of hydrogen peroxide (Fig. 4) and with the antioxidant behavior previously reported in different cell types, both *in vitro* and *in vivo* (De Beuf et al., 2010; Maurice et al., 2013; Toba et al., 2012). This apparent inconsistency could be explained by the rapid generation of ROS upon exposure to Epo (Fig. 4C), which was not matched by cEpo. This transient increase in ROS may be important for the promigratory effect of Epo, with its antioxidant effect prevailing at longer exposure times. This would mean that the prooxidant/antioxidant properties of Epo could depend on treatment duration and possibly on the subcellular localization of the reactive species produced.

Regarding the inhibition of Epo-induced cell migration by NAC, this effect could be attributed not only to its antioxidant activity, but also to a direct action of this compound on other intracellular migration effectors (Albini et al., 2001; Zafarullah et al., 2003).

Preincubation with cEpo effectively prevented ROS generation in EA.hy926 exposed to hydrogen peroxide, and was able to reduce the intracellular burden of reactive species generated by serum deprivation, to a lesser extent. Chattopadhyay et al. (2000) suggested the protective action of Epo against oxidative damage would be due –at least partly- to the scavenging power of its carbohydrate moieties, which are also present in the carbamylated derivative.

The role of nitric oxide in the effects of proangiogenic cytokines such as VEGF has been largely documented, comprising stimulation of endothelial cell proliferation, migration and EPC mobilization (Aicher et al., 2003; Fukumura et al., 2001). Epo has been shown to stimulate the expression of the endothelial nitric oxide synthase (eNOS) *in vivo* (Santhanam et al., 2006), as well as its phosphorylation and activity measured as NO production in EPCs and BAECs (Sautina et al., 2010; Su et al., 2011). In the present work, we observed a reduction in Epo- and VEGF-induced wound closure in cells treated with the NOS inhibitor L-NMMA (Fig. 5). This finding is in accordance with Heikal et al. (2016), who reported an increase in endothelial migration caused by Epo in hypoxia, and found this effect of Epo to be "largely mediated by eNOS activation and enhanced cellular NO production". These results further support the participation of NO in the action mechanisms of proangiogenic factors.

As wound healing experiments with the Ca^{2+} chelators EDTA and EGTA suggest, calcium would serve as an intracellular mediator of the effects of Epo on endothelial cell migration (Fig. 6). Hematopoietic growth factors like Epo and GM-CSF have been shown to stimulate a transient rise in intracellular calcium (Ca_i) in early human erythroid precursors within 3–5 min of treatment, which was attributed to release from intracellular stores, and linked to the proliferative and differentiating effect of these cytokines (Miller et al., 1988). Similarly, Vogel et al. (1997) reported a steep increase in Ca_i within the first minute of stimulation with Epo in aortic endothelial cells, which was decreased when measured in Ca-free medium. In EA.hy926 cells, we observed a transient rise in Ca_i within the first minute of Epo treatment, which was not achieved by its carbamylated derivative. Such increase was prevented in the absence of the cation in the resuspension medium (Fig. 6), thus suggesting an extracellular source. Accordingly, expression of the membrane Ca channels TRPC3 and TRPC6, was enhanced by Epo. TRPC3 is an erythropoietin-regulated calcium channel associated to the differentiation of human erythroid progenitors (Tong et al., 2008), while TRPC6 has been implicated in VEGF-induced angiogenesis (Ge et al., 2009) and was reported to participate in the calcium influx generated by Epo in CHO-S cells (Chu et al., 2004).

The results obtained throughout our work show a differential effect of Epo and cEpo on endothelial cell migration and proliferation. In comparison to the native cytokine, cEpo is characterized by its lack of proliferative action in erythroid cells, which appears to be related to a premature inactivation of the Epo receptor by PTP1B among other phosphatases (Chamorro et al., 2015). Previous research by our group demonstrated that PTP1B expression and activation in erythroleukemia UT-7 cells is enhanced by Epo (Callero et al., 2007) and cEpo, the latter to a greater extent (Chamorro et al., 2015). Other authors have also implicated PTP1B in cell migration. Silencing and overexpression experiments demonstrated that PTP1B, as the phosphatase associated to the VEGFR2 receptor, is a negative regulator of VEGF-induced angiogenesis, modulating cell proliferation and cell–cell contacts (Lanahan et al., 2014; Nakamura et al., 2008). Our results show that a 15 h-treatment of EA.hy926 cells with Epo enhanced PTP1B expression, which was significantly higher in cultures incubated with cEpo. Moreover, inhibition of PTP1B by CinnGel 2Me allowed cEpo to overcome its incapacity to stimulate EA.hy926 migration (Fig. 7). Inhibition of PTP1B in cells treated with Epo 200 ng/mL unexpectedly decreased endothelial cell migration. However, preincubation with CinnGel 2Me improved wound closure in cells treated with Epo 80 ng/mL, suggesting that the loss of effect at higher concentrations observed in scratching assays (Fig. 2) may be related to signal termination by receptor-associated phosphatases.

5. Conclusion

For the first time we have demonstrated a differential effect between Epo and its carbamylated derivative on endothelial cell migration. Such difference appears to depend on a premature termination of the cEpo signal due to the enhanced phosphatase activity of PTP1B, as we have previously reported for erythroid cells. Accordingly, Epo-induced migration seems to rely on the classical homodimeric receptor. Although cEpo is devoided of Epo's ability to stimulate migration, it retains its protective action against ROS generation. We believe that the present results provide new insights into the action mechanisms of Epo and cEpo in non-hematopoietic tissues.

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