The International Journal of Biochemistry & Cell Biology 85 (2017) 25–34

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Differential effect of erythropoietin and carbamylated erythropoietin on endothelial cell migration

Romina E. Maltaneri, María E. Chamorro, Agustina Schiappacasse, Alcira B. Nesse, Daniela C. Vittori*

Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas, Instituto del Departamento de Química Biológica de la Facultad de Ciencias Exactas y Naturales (IQUIBICEN), Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 1 September 2016
Received in revised form
27 December 2016
Accepted 26 January 2017
Available online 28 January 2017

Keywords:
Erythropoietin
Carbamylated erythropoietin
EA.hy926 endothelial cells
Cell migration

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.

* Corresponding author at: Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón II, Piso 4, Ciudad Universitaria Ciudad Autónoma de Buenos Aires, C1428EHA República Argentina.
E-mail address: dvittori@qb.fcen.uba.ar (D.C. Vittori).

http://dx.doi.org/10.1016/j.biocel.2017.01.013
1357-2725/

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).

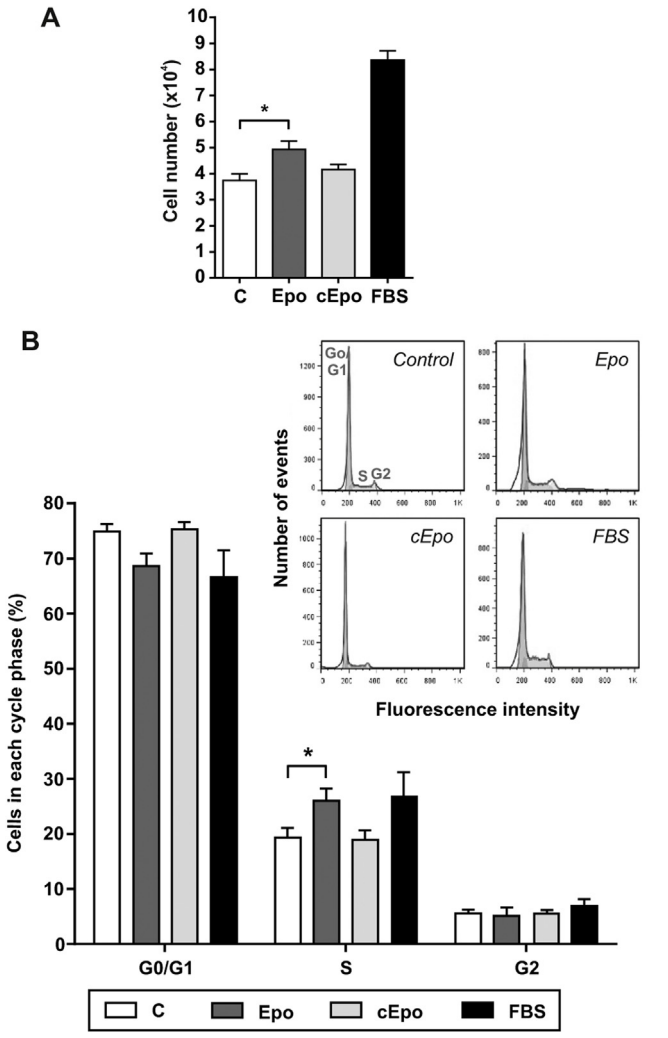

Fig. 1. Effect of Epo and cEpo on EA.hy926 proliferation. A) After a 48-h treatment with Epo or cEpo (200 ng/mL) in FBS-free medium, total cell number was determined in a Neubauer chamber, using the exclusion dye Trypan blue. Controls: cultures without FBS (C) and with 10% FBS.*P < 0.05, Kruskal Wallis-Dunn, 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