FEBS Letters 580 (2006) 3153–3160

Erythropoietin production: Molecular mechanisms of the antagonistic actions of cyclic adenosine monophosphate and interleukin-1

Chimedtseren Batmunkh, Jochen Krajewski, Wolfgang Jelkmann, Thomas Hellwig-Bürgel*

Institute of Physiology, University of Luebeck, Ratzeburger Allee 160, D-23538 Luebeck, Germany

Received 21 March 2006; accepted 20 April 2006

Available online 2 May 2006

Edited by Veli-Pekka Lehto

Abstract Erythropoietin (Epo) mRNA expression is suppressed by interleukin 1 (IL-1). Cyclic adenosine monophosphate (cAMP) can increase Epo mRNA and Epo protein levels in IL-1 treated HepG2 cells to some extent. To identify molecular mechanisms of this reaction we investigated three transcription factors (NF-κB, GATA-2 and HIF-1) that control the Epo gene. Western blot analyses and electrophoretic mobility shift assays (EMSAs) revealed that IL-1 strongly activated NF-κB, which is a likely suppressor of the Epo promoter. Treatment of the cells with dibutyryl-cAMP (Bt_2-cAMP) inhibited the activation of NF-κB by IL-1. Bt_2-cAMP increased GATA-2 DNA binding. Since GATA-2 is a suppressor of the Epo promoter, GATA-2 activation was unlikely to cause the increase of Epo mRNA expression in IL-1 treated cells. Furthermore, Western blots, EMSAs and reporter gene studies showed that Bt_2-cAMP was without effect on the hypoxia-inducible transcription factor HIF-1. Thus, NF-κB is probably the primary transcription factor by which cAMP counteracts the inhibition of Epo gene expression by IL-1.

Keywords: Cytokines; Cyclic adenosine monophosphate; Erythropoietin; Interleukin 1; NF-κB

1. Introduction

The erythropoietin (Epo) gene possesses several regulatory DNA elements. GATA-2, a zinc finger transcription factor, binds to the GATA motif located in the −30 region relative to the transcriptional initiation site of the Epo promoter, thereby inhibiting Epo gene expression [1,2]. Furthermore, the 5′ region of the Epo gene contains binding sites for NF-κB [3]. The 50 bp hypoxia-responsive element (HRE) of the 3′ enhancer contains a hypoxia-inducible factor (HIF) binding site (HBS), a CACA sequence, and a direct repeat of two steroid hormone receptor-binding half-sites separated by two base pairs (DR-2 element) [4]. Hypoxia-inducible-factor-1 (HIF-1) is a crucial transcription factor for the hypoxic induction of the Epo gene [5]. Activated HIF-1 is composed of two subunits, HIF-1α and HIF-1β [6], with HIF-1α being unstable in the presence of O_2 (for Refs. see [7,8]).

We have recently shown that the proinflammatory cytokines interleukin 1 (IL-1) and tumor necrosis factor-α (TNF-α) activate GATA-2 and NF-κB [9]. IL-1 and TNF-α have proved to inhibit Epo mRNA expression and Epo synthesis in the human hepatoma cell lines, Hep3B and HepG2 [10–12] and in rat kidneys [11,13]. On the other hand, cyclic adenosine monophosphate (cAMP) has been reported to stimulate Epo synthesis in hepatic and renal cell lines [14,15] and to counteract the inhibitory action of IL-1 and TNF-α on Epo synthesis [12].

The present study aimed at investigating molecular mechanisms by which cAMP prevents the suppression of Epo mRNA expression and Epo production by IL-1. Results obtained by Western blotting, electrophoretic mobility shift assays, reporter gene studies, Epo mRNA quantification and Epo immunoassay suggested that cAMP restores Epo production in IL-1 treated HepG2 cells by preventing NF-κB mobilization rather than through modulating GATA-2 or HIF-1 signaling.

2. Materials and methods

2.1. Cell cultures

The human hepatoma cell line HepG2 was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig). Cells were grown in RPMI 1640 medium (Gibco, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS; Gibco). Medium for the HepG2 derivatives HRG1, IκBα-HepG2 and IκBαM-HepG2 [9,16] was supplemented with 50 μg/ml G418, 100 IU/ml penicillin and 100 μg/ml streptomycin (PAA Laboratories, Cölbe, Germany). Recombinant human IL-1β was a gift from Ciba-Geigy (Basel, Switzerland). Dibutyryl-cAMP (Bt_2-cAMP) was obtained from Sigma (Taufkirchen, Germany). Cell cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and subcultivated two to three times a week. Cells received fresh medium the day before the experiments. For study of hypoxia, cells were placed in a humidified atmosphere containing 3% O_2, 5% CO_2 and balanced N_2 (Heraeus incubators, Hanau, Germany). In order to exclude cytotoxic effects of IL-1β and Bt_2-cAMP, the colorimetric tetrazolium salt/formazan method was applied, which is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) to purple formazan in living cells. Confluent HepG2 cultures in 96-well dishes were used for these studies as described [17].

2.2. Nuclear protein extraction

Nuclear proteins were extracted as described in detail elsewhere [9,18]. HepG2 cells were washed with ice-cold phosphate-buffered saline (PBS), scraped off and collected. For Western blot and electrophoretic mobility shift assay (EMSA) analysis of NF-κB and GATA-2, the cell suspensions were centrifuged at 4000 × *g* and 4 °C for 5 min. Pelleted cells were resuspended in 350 μl buffer containing 10 mM HEPES

*Corresponding author. Fax: +49 451 5004171.
E-mail address: hellwig@physio.uni-luebeck.de (T. Hellwig-Bürgel).

Abbreviations: ACD, anemia of chronic disease; Bt_2-cAMP, dibutyryl-cyclic adenosine monophosphate; EMSA, electrophoretic mobility shift assay; Epo, erythropoietin; HIF-1, hypoxia-inducible factor-1; HRE, hypoxia-response element; IL, interleukin; TNF, tumor necrosis factor

0014-5793/$32.00
doi:10.1016/j.febslet.2006.04.069

(pH 7.9), 1.5 mM $MgCl_2$ and 10 mM KCl and placed on ice for 15 min. For cell lysis 25 μl of 10% NP-40 solution was added and mixed vigorously for 30 s. Nuclei were collected by centrifugation and resuspended in 60 μl buffer containing 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA and 25% glycerol. The suspension was gently agitated on a shaking platform on ice for 20 min. Nuclei were centrifuged at $12\,500 \times g$ and 4 °C for 5 min and the extracts stored at −80 °C.

For Western blot and EMSA analysis of HIF-1, cell suspensions were centrifuged at $800 \times g$ and 4 °C for 5 min. Cell pellets were washed with 2 ml buffer A (10 mM Tris (pH 7.8), 1.5 mM $MgCl_2$, 10 mM KCl) and subsequently resuspended in 1 ml buffer A and placed on ice for 30 min. Nuclei were collected by centrifugation and resuspended in 100 μl buffer C (10 mM KCl, 20 mM Tris (pH 7.8), 1.5 mM $MgCl_2$, 20% glycerol) by gentle up and down pipetting. Nuclei were pelleted by centrifugation at $13\,000 \times g$ and 4 °C for 30 min. Supernatants were stored at −80 °C. Immediately before use, buffers A and C were supplemented with 2 μg/ml aprotonin, 10 μg/ml leupeptin, 20 μg/ml pepstatin, 1 mM sodium *ortho*-vanadate, 0.5 mM benzamidine, 2 mM levamisole, 10 mM β-glycerophosphate, 0.5 mM dithiothreitol (DDT) and 0.4 mM phenylmethylsulfonyl fluoride (PMSF). Protein concentrations were determined by the Bradford method [19] with bovine serum albumin (BSA) as standard.

2.3. Western blotting

Samples (25 μg of nuclear extracts) were run on denaturing 10% (for NF-κB subunits) or denaturing 7.5% (for HIF-1α) polyacrylamide gels and transferred to nitrocellulose-membranes (Hybond; Amersham, Biosciences). Transfer efficiency was verified by staining with 2% Ponceau S and/or immunodetection of SP1 or β-actin. Membranes were blocked overnight at 4 °C in 5% skim milk/PBS and then incubated with the respective antibody at room temperature for 2 h. Antibodies anti-NF-κB-p50, anti-NF-κB-p65, anti-SP1, anti-β-actin were from Santa Cruz (Heidelberg, Germany) and anti-HIF-1α was from BD Biosciences (Heidelberg, Germany). All antibodies were used in a dilution of 1:1000 in 5% skimmed milk in PBS. For detection, matched horseradish peroxidase conjugated secondary antibodies in a 1:2000 dilution (all from DAKO, Hamburg, Germany) and enhanced chemiluminescence substrate (Amersham, Freiburg, Germany) were used.

2.4. Electrophoretic mobility shift assay

NF-κB and GATA-2 EMSAs were performed as described in [9] and HIF-1 EMSAs as in [18]. Oligonucleotide sequences were as follows: NF-κB sense: 5′-AGT TGA GGG GAC TTT CCC-3′; NF-κB antisense: 5′-GCC TGG GAA AGT CCC CTC-3′; GATA-2 sense: 5′-CAC ACA TGC AGA TAA CAG CCC CGA CC-3′; GATA-2 antisense: 5′-GGT CGG GGC TGT TAT CTG GAT GTG TG-3′; HIF-1 sense: 5′-TTC CTG CAC GTA CAC ACA AAG CGC ACG TAT TTC-3′; HIF-1 antisense: 5′-GAA ATA CGT GCG CTT TGT GTG TAC GTG CAG GAA-3′. Oligonucleotides (MWG, Ebersberg, Germany) were labeled with T4 polynucleotide kinase (MBI Fermentas, St. Leon-Rot, Germany) in the presence of γ-[^{32}P]-ATP (NEN, Köln, Germany). Protein–DNA complexes were resolved by electrophoresis in non-denaturing 6% polyacrylamide gels. For supershift analyses, anti-NF-κB-p50, NF-κB-p65 (all from Santa Cruz, Heidelberg) and anti-HIF-1α (BD Biosciences) were used.

2.5. Luciferase assays

HRG-1 cells, which are HepG2 cells stably transfected with a hypoxia responsive luciferase plasmid [16], were used for monitoring HIF-1 activity in reporter gene studies. Cells were grown to ~40% confluence on 24-well plates with 0.5 ml medium per well. Experimental periods were 24 h. Thereafter, cells were washed with PBS and lysed with passive lysis buffer (Promega, Mannheim, Germany). Luminescence was measured with a Micro Lumate (LB 96P; Berthold Technologies, Bad Wildbad, Germany).

2.6. Enzyme-linked immunoassay (ELISA)

Parental HepG2, IκBα-HepG2 or IκBαM-HepG2 cells were seeded in 24-well plates and grown to 90–95% confluence. For study, cultures were washed thoroughly with fresh medium and incubated under hypoxic conditions for 24 h. Epo concentrations were measured in cell culture supernatants by commercial ELISA (Medac, Wedel, Germany).

2.7. Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was isolated according to Chomczynski and Sacchi [20] or with the ABI Prism™ 6100 NucleicAcid PrepStation (Applied Biosystems, Darmstadt, Germany). One microgram of total RNA was reverse transcribed into cDNA using oligo (dT) primers. Target cDNAs were quantified by real-time RT-PCR on an ABI 7000 Sequence detection system (Applied Biosystems) by means of either a commercial SYBR green PCR kit (for the detection of L28; Eurogentec, Seraing, Belgium) or Assays-on-Demand (for the detection of Epo; Applied Biosystems, part number 4331182). Relative expression levels were calculated using the $\Delta\Delta C_T$-method (normalized to L28 and related to the normoxic control). L28 primer sequences were: L28-for-48: 5′-ATG GTC GTG CGG AAC TGC T-3′ and L28-rev-149: 5′-TTG TAG CGG AAG GAA TTG CG-3′.

2.8. Statistics

Results are shown as means + standard deviations (SD). Student's *t*-test was applied to compare mean values in cultures without and with Bt_2-cAMP. A significant difference was assumed at $P \leqslant 0.05$.

3. Results

IL-1 (300 pg/ml) reduced the amount of immunoreactive Epo produced in hypoxically incubated (3% O_2) HepG2 cultures (Fig. 1A). The addition of Bt_2-cAMP (50 μM) partly

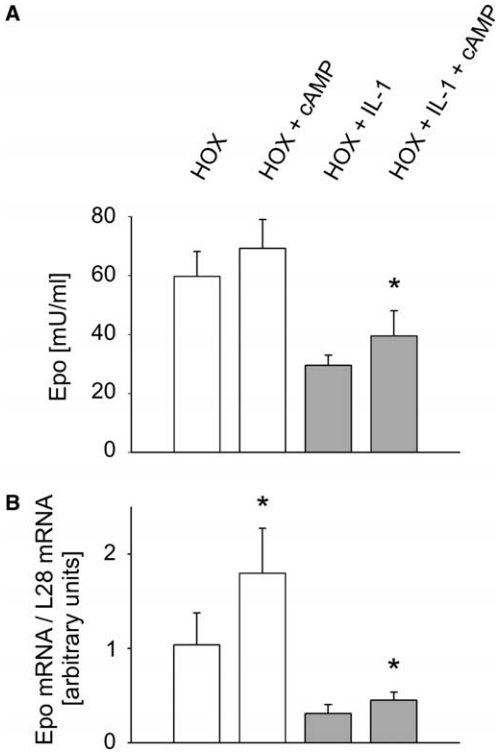

Fig. 1. (A) Epo concentrations in cell culture supernatants of HepG2 cells exposed to 3% O_2 without drugs (HOX) or with 50 μM Bt_2-cAMP (HOX + cAMP), 300 pg/ml IL-1 (HOX + IL-1) or the combination of IL-1 and Bt_2-cAMP (HOX + IL-1 + cAMP) for 24 h. $^*P \leqslant 0.05$ compared to the respective cultures without Bt_2-cAMP, mean + SD, $n = 5$, Student's *t*-test. (B) Epo mRNA levels quantified by real-time RT-PCR of extracts of HepG2 cells exposed to 3% O_2 without drugs (HOX) or with 50 μM Bt_2-cAMP (HOX + cAMP), 300 pg/ml IL-1 (HOX + IL-1) or the combination of IL-1 and Bt_2-cAMP (HOX + IL-1 + cAMP) for 8 h. Expression levels normalized to L28 and related to HOX were calculated with the $\Delta\Delta C_T$ method. $^*P \leqslant 0.05$ compared to the respective cultures without Bt_2-cAMP, mean + SD, $n = 6$, Student's *t*-test.