

Research report

Erythropoietin induces changes in gene expression in PC-12 cells

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Abstract

Erythropoietin (EPO) is the primary modulator of red blood cell production. Recently EPO has received considerable attention for its functions outside of hematopoiesis, including its effects in the nervous system where it has been shown to act as a neuroprotectant. To understand the function of EPO in the nervous system and to determine if EPO functions through the same signaling pathways identified in hematopoietic cells, we used cDNA array hybridization and RT-PCR to investigate the changes in gene expression induced by EPO in the neuronal-like PC-12 cell line. PC-12 cells cultured in the presence of EPO (10 U/ml) showed significant changes in gene expression by 3 h with a return to basal expression levels for the vast majority of genes by 24 h. The genes influenced by EPO included genes with known functions in cell proliferation, differentiation and apoptosis. Semi-quantitative RT-PCR confirmed that 24 h pre-treatment with EPO (10 pM) resulted in a 2.5-fold increase in the expression of the anti-apoptotic gene *bcl_{XL}* and a 4-fold decrease in the expression of the pro-apoptotic gene *bak*. In addition to supporting the current models of EPO function these results suggest previously unidentified mechanisms by which EPO may function in neurons. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Erythropoietin (EPO) is a 34-kDa glycoprotein responsible for regulating erythropoiesis in vivo. EPO function is essential for the production of red blood cells both during development and in the adult. During development EPO is produced primarily in the fetal liver and is necessary for embryonic survival. Embryos that have deleted or non-functional EPO or EPO receptors die by embryonic day 13 due to failure of definitive fetal liver erythropoiesis [12,14,39]. In the adult, EPO production is switched to the kidney where the detection of low arterial oxygen levels results in the production and release of EPO into the circulation to increase the number of red blood cells. Loss of EPO function in the adult is thought to contribute to the

anemia associated with a number of chronic conditions including renal and liver disease [5,36].

EPO elicits its effects by binding to specific, high affinity, cell surface receptors and promoting the survival, proliferation and differentiation of erythroid precursor cells. The EPO receptor (EPOR) is a member of the cytokine receptor family. It is expressed on responsive cells including late stage erythroid precursor cells such as proerythroblasts and erythroblasts and is absent on mature red blood cells [37]. The EPOR has no tyrosine kinase activity of its own but instead uses a variety of cytoplasmic molecules that have kinase activity to transduce a signal [8,38]. Evidence of EPO induced activation of JAK-2/STAT-5 [9,20] MAP kinase [19,23,28], PKB and PI3 kinase [2], and PKC [35] suggests that EPO may make use of several distinct signaling pathways. Which of these EPO uses to control its specific functions such as proliferation, differentiation and cell survival remains unclear. Recent reports suggests that EPO signaling mediated by STAT5 activation may influence cell survival through the control

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of the expression of genes from the *bcl* family [29] and by PKB and PI3 kinase activation [2]. On the other hand, signaling through the MAP kinase pathway may contribute to EPO's influence on cell proliferation [23], and differentiation [19] as well as apoptosis [28].

Receptors for EPO have also been identified on a variety of non-hematopoietic cell types in the adult and neonate including endothelial cells, hepatocytes in the liver and neuronal and non-neuronal cells of the nervous system (for review see Ref. [10]). While the function of EPO in these systems remains unclear, EPO may have effects similar to those observed in hematopoietic cells. For example, recent work suggests that EPO may function to promote cell survival in cells of the nervous system. EPO has been shown to protect neurons *in vitro* from cell death caused by a variety of insults including glutamate toxicity, hypoxia [21,22,24] and growth factor withdrawal [13]. EPO also prevents neuronal cell death *in vivo* resulting from ischemia [22,24] and other forms of neural injury [6]. Furthermore, EPO may function in the control of proliferation and differentiation of neuronal stem cells [32]. Although signaling in these non-hematopoietic tissues has not been as extensively studied as it has been in hematopoietic cells EPO has been shown to induce Jak-2 phosphorylation [11] and MAPK activity [13] in neurons. Therefore, not only is it possible that EPO may have some of the same functions as in hematopoiesis; it may also use some of the same signaling pathways.

To understand the non-hematopoietic functions of EPO as well the signaling mechanisms used in these cells types, we investigated the effect of EPO on gene expression in neuronal-like cells by performing cDNA microarray analysis on EPO treated PC-12 cells that had been differentiated with nerve growth factor (NGF). EPO caused changes in the expression of genes involved in cell proliferation, cell differentiation and cell survival. A number of genes that have been previously implicated in EPO signaling in hematopoietic cells were also regulated by EPO in neuronal cells. The effect of EPO on members of the *bcl-2* family were investigated further using RT-PCR. EPO consistently increased the expression of *bcl_{XL}* and decreased the expression of *bak* in PC-12 cells. These results provide insight into the mechanisms that may be used by EPO to elicit its effects in the nervous system.

2. Materials and methods

2.1. Cell culture and EPO treatment

PC-12 cells from American Type Culture Collection were cultured in DMEM with 10% Horse serum and 5% fetal bovine serum at 37 °C, 5% CO₂. The cells were grown on tissue culture plastic coated with poly-D-lysine (Biocoat from Beckton Dickson). NGF was added to the culture media at a final concentration of 100 ng/ml for 7

days to differentiate the PC-12 cells to the neural phenotype. Differentiation was confirmed by morphological analysis before the start of the experiments. Differentiated PC-12 cells were then left un-treated or treated with recombinant human Erythropoietin (hereafter referred to as EPO) at a final concentration of 10 U/ml. Cells were treated with EPO for 3, 6, or 24 h prior to the isolation of total RNA.

2.2. RNA isolation and probe preparation

Total RNA was isolated from PC-12 cells using the RNeasy mini kit (Qiagen) as described in the manufacturer's instructions. RNA was then subject to one round of T7 polymerase-based linear RNA amplification by reverse transcription with a T7 promoter oligo(dT) primer. Cy3-dCTP labeled fluorescent cDNA probe was synthesized from the amplified RNA as described [26], except that to degrade the amplified RNA template, RNase H (10 Units) and RNase A (10 Units) were added and incubated at 37 °C for 20 min. Probes were then purified with a PCR purification kit (Qiagen), vacuum-dried, and resuspended in 55 µl of hybridization buffer [Version 2 hybridization buffer (Amersham Pharmacia Biotech) with 50% formamide] containing Mouse Cot1 DNA (Life Technologies).

2.3. Microarrays

Clones used for printing on the microarrays were obtained commercially from Research Genetics (IMAGE consortium), Incyte Genomics as well as internal sources. All clones were verified by DNA sequencing. The cDNA clones were PCR amplified and purified with a Qiagen 96 PCR purification kit, then mixed 1:1 with a 10-M NaSCN printing buffer. A contact pin microarrayer (Generation III Array Spotter, Molecular Dynamics) was used to spot the clones in duplicate on amino silane-coated slides (Corning). Microarrays were hybridized and washed as described [26], then scanned with a confocal laser scanner (Array Scanner, Molecular Dynamics). Genes that were included in the analysis were determined to be significantly different from background levels ($P \leq 0.05$, Student's *t*-test, comparing highest expression level of individual genes to background expression levels). When comparing changes in gene expression between untreated and EPO treated cells a ratio of 2.0 or higher was considered significant based on the standard error analysis of the chip hybridization experiments.

2.4. RT-PCR

Culturing and differentiation of PC-12 cells and RNA isolation was performed as described above. Cells were treated with EPO (10 pM) and RNA was isolated at 3, 6 or 24 h after EPO treatment. Real time RT-PCR was performed in a single step using a light cycler with an RNA

Table 1
Genes induced or repressed in PC-12 cells following 3 h treatment with erythropoietin (10 U/ml.)

| Gene ID | DNA chip no. | Annotation | Ratio |
|------------------|--------------|---|-------|
| <i>Increased</i> | | | |
| 80178 | 20667 | <i>Thymosin beta-10</i> | 5.6 |
| D26598 | 39370 | <i>Proteasome beta 3 subunit</i> | 4.9 |
| R60006 | 17418 | <i>Triiodothyronine receptor (THRA1, ear1)</i> | 4.9 |
| D76432 | 39402 | <i>EF1 delta</i> | 4.9 |
| 105910 | 38604 | Unknown | 4.7 |
| 111106 | 38621 | Unknown | 4.5 |
| X05137 | 39506 | <i>Low affinity NGF receptor</i> | 4.5 |
| 580795H1 | 21664 | <i>NMDA channel-like</i> | 4.4 |
| M19645 | 6332 | <i>BiP/GRP78/ER resident HSP70</i> | 4.3 |
| M21186 | 14814 | <i>Neutrophil cytochrome b light chain p22</i> | 4.3 |
| AJ000519 | 13244 | <i>Conjugating enzymes</i> | 4.2 |
| U46923 | 5957 | <i>GPCR8 (Orphan, public)</i> | 4.1 |
| 101693 | 38602 | <i>Ribosomal protein (Ke-3)</i> | 4.0 |
| K01911 | 14620 | <i>Neuropeptide Y</i> | 3.9 |
| 100455 | 20298 | <i>Cystatin M</i> | 3.8 |
| D17390 | 6443 | <i>ADAM11</i> | 3.8 |
| M55040 | 14925 | <i>Acetylcholinesterase</i> | 3.8 |
| AF035207 | 39395 | <i>Nip2l</i> | 3.8 |
| 100721 | 38761 | Unknown | 3.8 |
| Y13148 | 39396 | <i>Zinc finger protein</i> | 3.7 |
| 66707 | 38519 | Unknown | 3.7 |
| 80637 | 20669 | <i>Pro alpha 1 collagen type II</i> | 3.6 |
| U07132 | 6202 | <i>Nuclear receptor/orphan Ner-1/UR</i> | 3.6 |
| 111108 | 38503 | Unknown | 3.6 |
| AJ225122 | 39262 | <i>Hyperpolarization-act cation channel HAC1</i> | 3.6 |
| F05184 | 13320 | <i>cAMP dependent</i> | 3.6 |
| J04791 | 39494 | <i>DCOR</i> | 3.6 |
| U19596 | 6060 | <i>P18-INK6</i> | 3.5 |
| 105908 | 20431 | <i>ORF for P-glycoprotein</i> | 3.5 |
| 102748 | 20379 | <i>Cytochrome c1</i> | 3.5 |
| 101112 | 20343 | <i>Hsp27</i> | 3.4 |
| M22488 | 14820 | <i>BMP1</i> | 3.4 |
| M88279 | 6133 | <i>FKBP52</i> | 3.4 |
| X75821 | 5792 | <i>Mitochondrial Hsp10 chaperonin</i> | 3.4 |
| L37296 | 14721 | <i>Bad</i> | 3.3 |
| 111638 | 20504 | <i>Annexin VI</i> | 3.3 |
| M69293 | 39234 | <i>Id2</i> | 3.3 |
| M11931 | 39399 | <i>Neuron specific enolase</i> | 3.2 |
| X12740 | 19941 | <i>c-Jun of the transcription factor AP-1</i> | 3.2 |
| X56065 | 39324 | <i>Dopamine D2 receptor</i> | 3.2 |
| U27767 | 39412 | <i>RGS4</i> | 3.2 |
| 66703 | 39220 | Unknown | 3.2 |
| M94335 | 6150 | <i>Akt1/PKB/RAC</i> | 3.2 |
| U51278 | 39287 | <i>Thymus Bcl-xL</i> | 3.1 |
| D10706 | 39260 | <i>OAZ</i> | 3.1 |
| X64840 | 39393 | <i>ALF1A; ALF1B</i> | 3.1 |
| J03236 | 38625 | <i>Mouse jun-B mRNA</i> | 3.1 |
| Y00371 | 5838 | <i>HSP70</i> | 3.1 |
| M65128 | 6260 | <i>FKBP13/rapamycin-binding protein</i> | 3.1 |
| U04270 | 6193 | <i>Voltage gated potassium channel</i> | 3.1 |
| <i>Decreased</i> | | | |
| 66531 | 38508 | Unknown | 18.1 |
| 109542 | 38486 | <i>Alpha-tubulin</i> | 11.4 |
| 113323 | 39092 | <i>Light molecular-weight neurofilament</i> | 10.5 |
| 108512 | 20461 | <i>Glutamate/aspartate transporter</i> | 10.2 |
| 109001 | 20471 | <i>Ribosomal protein S24</i> | 8.3 |
| 101153 | 20352 | <i>NF-M middle molecular weight neurofilament</i> | 7.5 |
| 80905 | 38952 | Unknown | 6.2 |
| 102718 | 38784 | <i>SCG10</i> | 6.2 |
| X53337 | 20021 | <i>Cathepsin D</i> | 6.1 |

amplification kit (Roche Molecular Biochemicals). Quantitative analysis of individual RT-PCR reactions was performed using the data analysis software included with the light cycler instrument.

3. Results

3.1. EPO induced changes in gene expression

The effect of EPO on gene expression in neurons was examined using PC-12 cells. PC-12 cells are multipotent cells from a rat pheochromocytoma that can be reversibly induced to a neuronal phenotype by treatment with NGF. Neural-like PC-12 cells were cultured in the absence or presence of EPO (10 U/ml) for 3 h and then analyzed for

changes in gene expression using micro-array analysis. Micro-array analysis was performed using a gene chip containing approximately 2500 clones from human, rat and mouse.

Treatment of PC-12 cells for 3 h with EPO (10 U/ml) resulted in significant changes in the expression of several genes. The expression of specific genes was increased as much as 5.6-fold following treatment with EPO. The genes that showed the largest increases in expression have a wide variety of functions (Table 1). For example, treatment with EPO increased the expression of genes for signaling molecules such as *neuropeptide Y* and *BMP-1*, genes that code for transmembrane receptors such as the *dopamine D2 receptor* and genes coding for several channel proteins including an NMDA-like channel, a hyperpolarization activated cation channel and a voltage gated potassium

Table 1. Continued

| Gene ID | DNA chip no. | Annotation | Ratio |
|----------|--------------|--|-------|
| 80186 | 39175 | Unknown | 6.1 |
| 113301 | 20543 | <i>Matrin 3</i> | 6.1 |
| U13262 | 39437 | <i>MEF-2</i> | 6.1 |
| 113752 | 20563 | <i>Sequence from cosmid U246D9</i> | 6.0 |
| 95332 | 5694 | <i>14-3-3 eta</i> | 6.0 |
| L10284 | 6505 | <i>Calnexin</i> | 6.0 |
| 82475 | 20720 | <i>Mitochondrial genes for 16S rRNA</i> | 5.8 |
| J04509 | 39311 | <i>JUN-D protein</i> | 5.7 |
| 100574 | 20307 | <i>Synaptosomal associated protein 25</i> | 5.6 |
| 100750 | 38894 | <i>Sequence 1 from patent US 5457026.</i> | 5.4 |
| L11667 | 6510 | <i>Cyclophilin-40</i> | 5.3 |
| 113570 | 38816 | Unknown | 5.1 |
| 108620 | 38605 | <i>Receptor for hyaluronan-mediated</i> | 4.8 |
| 100836 | 20321 | <i>Proteasomes C2 component</i> | 4.8 |
| AF091234 | 38455 | <i>Transcription factor</i> | 4.6 |
| U33267 | 18871 | <i>Glycine receptor beta subunit (GLRB)</i> | 4.5 |
| Y00864 | 5844 | <i>SCFR/c-kit</i> | 4.5 |
| S77770 | 6179 | <i>Voltage gated chloride channel</i> | 4.4 |
| 103925 | 20410 | <i>Guanine nucleotide-binding protein G-s, alpha</i> | 4.4 |
| M29548 | 14866 | <i>EF1 A</i> | 4.3 |
| 105894 | 20427 | <i>IgE binding protein</i> | 4.3 |
| 108611 | 39012 | Unknown | 4.1 |
| 81770 | 38458 | Unknown | 4.0 |
| 108687 | 38620 | Unknown | 3.9 |
| L25913 | 14699 | <i>Chaperonin</i> | 3.9 |
| L41840 | 14726 | <i>Nucleoporin (NUP358) gene</i> | 3.9 |
| S73849 | 18135 | <i>Very low density lipoprotein receptor</i> | 3.9 |
| 81747 | 38859 | <i>Beta 1 integrin isoforms A, C</i> | 3.8 |
| AI071095 | 12678 | <i>vgr</i> | 3.8 |
| 109492 | 20475 | <i>Peripherin</i> | 3.8 |
| AF016583 | 6610 | <i>Chk1</i> | 3.7 |
| 103933 | 38822 | <i>Gene for testicular cell adhesion</i> | 3.7 |
| AF038564 | 12245 | <i>Ligase</i> | 3.6 |
| 87651 | 20747 | <i>Binding protein for T-cell receptor</i> | 3.5 |
| 103830 | 20403 | <i>Carboxypeptidase B</i> | 3.5 |
| AA115076 | 5046 | <i>msg1-related gene 1 mrg1</i> | 3.5 |
| X59417 | 39508 | <i>Proteasome alpha 6 subunit</i> | 3.4 |
| 112215 | 39068 | <i>Leucine zipper protein p40</i> | 3.4 |
| AF077302 | 38493 | <i>Bcl-2-inter protein beclin</i> | 3.3 |
| 82462 | 20717 | <i>Annexin V</i> | 3.2 |
| 515944H1 | 20984 | <i>Hrad17</i> | 3.2 |

RNA was isolated from PC-12 cells treated with EPO and used for microarray analysis as described in Section 2. The top 50 genes either induced or repressed following EPO treatment are shown. The Gene ID number refers to the clone accession number or the reference to an internal database.

channel, along with several genes with functions ranging from cell structure to protein modification to cell signaling. In addition, EPO increased the expression of a number of genes that have been implicated in apoptosis including members of the bcl-2 family of proteins, *bad*, *bax* and *bcl_{XL}*, and the heat shock proteins *HSP-70* and *HSP-27* (Table 2). EPO also increased the expression of the immediate early genes *fos*, *jun-B* and *ID2* as well as other genes that have been shown to function in cell proliferation and differentiation including additional transcription factors and signaling molecules (Table 3).

Treatment with EPO also resulted in a decrease in the expression of several genes, some as much as 18-fold. As was seen with the genes that had increased expression in response to EPO, genes with decreased expression also had diverse functions (Table 1). They include cytoskeletal proteins such as the light and middle molecular weight neurofilament proteins, a glutamate/aspartate transporter molecule, a voltage-gated chloride channel and the synaptosomal associated protein SNAP25. Exposure to EPO also resulted in a decrease in the expression of several apoptosis related genes including the eta and gamma isoforms of *14-3-3*, the bcl-2 family member *bak*, *APO3L*, a ligand for the death domain receptor Apo3, and *TFAR19* (Table 2). The expression of a number of genes involved in proliferation and differentiation were also decreased in response to EPO including the tyrosine kinase receptor *c-kit*, the protein tyrosine phosphatase *TD14* and several transcription factors and signaling pathway components (Table 3).

Treatment with EPO also resulted in a change in the expression of a number of novel gene sequences with unknown identity or function.

Table 2
Genes with known function in cell survival and apoptosis that were effected by treatment with erythropoietin

| Gene ID | DNA chip no. | Annotation | Ratio |
|------------------|--------------|----------------------------------|-------|
| <i>Increased</i> | | | |
| X05137 | 39381 | <i>Low affinity NGF receptor</i> | 4.5 |
| AF035207 | 39348 | <i>Nip2l</i> | 3.8 |
| 101112 | 20343 | <i>Hsp27</i> | 3.4 |
| L37296 | 39092 | <i>Bad</i> | 3.3 |
| U51278 | 39356 | <i>Thymus Bcl-xL</i> | 3.1 |
| Y00371 | 5838 | <i>HSP70</i> | 3.1 |
| L22472 | 38539 | <i>Bax</i> | 2.7 |
| <i>Decreased</i> | | | |
| 95332 | 17353 | <i>14-3-3 eta</i> | 6.0 |
| Y13231 | 38761 | <i>Bak</i> | 3.1 |
| 880258T1 | 38866 | <i>Apo3/DR3 ligand (APO3L)</i> | 2.4 |
| 100400 | 16 | <i>14-3-3 gamma</i> | 2.1 |
| 82210 | 17561 | <i>TFAR19</i> | 2.0 |

Data from the microarray experiments was subject to further analysis to determine genes that were effected by EPO and that have a demonstrated function in apoptosis. Based on the standard error analysis of the chip hybridization experiments a difference of two-fold in gene expression was considered significant.

3.2. Time course of changes in gene expression

Previous experiments investigating EPO's function in the nervous system have shown that the effect of EPO on neurons requires significant pre-treat time suggesting that protein synthesis or gene activation is necessary. For example, EPO's neuroprotective activity against specific insults requires a pre-treatment time of up to 6 h to elicit a maximal effect. To investigate the time frame of EPO mediated changes in gene expression we treated PC-12 cells with EPO (10 U/ml) for 6 and 24 h, in addition to 3 h, and compared the changes in gene expression.

Fig. 1 shows that with few exceptions, the vast majority of genes reached their largest change in gene expression in response to 10 U/ml of EPO at 3 h and had returned to or near basal levels by 6 h.

3.3. EPO increase the expression of *bcl_{XL}* and decreases the expression of *bak*

In order to examine the role of EPO in cell survival we examined more closely EPO's regulation of genes that are known to function in apoptosis. Micro-array analysis showed that the expression of two members of the bcl-2 family of proteins, the anti-apoptotic gene *bcl_{XL}* and the pro-apoptotic gene *bak*, were altered in response to EPO. Quantitative RT-PCR was used to examine the expression of these two genes in PC-12 cells treated with 10 pM EPO, a concentration of EPO that has been shown to be neuroprotective. Treatment of PC-12 cells for 24 h with EPO induced a greater than 2.5-fold increase in the expression of *bcl_{XL}* and a 4-fold decrease in the expression of *bak* (Fig. 2A and B). Unlike the effects observed at higher concentrations of EPO (10 U/ml/25 nM), pre-treatment of PC-12 cells for 3 or 6 h with EPO at 10 pM resulted in no significant changes in the expression of either *bcl_{XL}* or of *bak*.

4. Discussion

Erythropoietin is a growth factor that functions in hematopoiesis to control the proliferation, differentiation and survival of precursor cells belonging to the erythroid lineage. The speculation that EPO may have important functions outside of hematopoiesis is consistent with the expanding role found for a number of other growth factors and cytokines. The expression of EPO receptors on non-hematopoietic cells [10] and the growing evidence that these receptors are functional [1,13,18] supports the idea that EPO might have a function other than producing red blood cells.

The function of EPO in the nervous system has garnered considerable attention since the discovery that subsets of neurons in both the developing and adult nervous system express the EPO receptor [10]. The question of whether the

Table 3
Changes in the expression of genes involved in cell proliferation and differentiation following treatment with erythropoietin

| Chip clone ID | Gene ID | User annotation | Ratio |
|------------------|-----------|---|-------|
| <i>Increased</i> | | | |
| 18748 | 80178 | <i>Thymosin beta-10</i> | 5.6 |
| 39354 | R60006 | <i>Triiodothyronine receptor (THRA1, ear1)</i> | 4.9 |
| 39381 | X05137 | <i>Low affinity NGF receptor</i> | 4.5 |
| 38934 | D17390 | <i>ADAM11</i> | 3.8 |
| 39210 | F05184 | <i>c-fos</i> | 3.6 |
| 36698 | M22488 | <i>BMP1 precursor</i> | 3.4 |
| 39126 | M69293 | <i>Id2 protein</i> | 3.3 |
| 39146 | X12740 | <i>C-Jun of the transcription factor AP-1</i> | 3.2 |
| 39131 | M94335 | <i>Akt1/PKB/RAC</i> | 3.2 |
| 39342 | X64840 | <i>ALF1A; ALF1B</i> | 3.1 |
| 38428 | J03236 | <i>jun-B</i> | 3.1 |
| 39306 | D23662 | <i>Ubiquitin-like protein</i> | 3.1 |
| 39237 | U88716 | <i>Nkx-32 homeodomain protein</i> | 3.0 |
| 38745 | L04527 | <i>Hairy 1</i> | 2.9 |
| 38762 | M97200 | <i>Kruempel-like factor</i> | 2.9 |
| 39112 | U89488 | <i>CLIM-2</i> | 2.7 |
| 17719 | 83912 | <i>FK-506 binding protein (fkbp12.6)</i> | 2.7 |
| 38922 | AF009519 | <i>Sry</i> | 2.5 |
| 39363 | M24899 | <i>Nuclear receptor alpha2 for thyroid hormone</i> | 2.5 |
| 39357 | X91753 | <i>SEF2</i> | 2.4 |
| 39176 | Y14296 | <i>BTEB-1</i> | 2.4 |
| 39157 | M95603 | <i>ASH1</i> | 2.3 |
| 20322 | 113529 | <i>Extracellular signal-regulated kinase 1</i> | 2.2 |
| 10680 | U10699 | <i>pH218 GPCR</i> | 2.2 |
| 39111 | U20553 | <i>P57</i> | 2.2 |
| 39223 | U43714 | <i>Hxt</i> | 2.2 |
| 11311 | A1059504 | <i>Parathyroid hormone receptor</i> | 2.1 |
| 39212 | M61909 | <i>NFkB p65 (Rel A)</i> | 2.1 |
| 7986 | AA963299 | <i>Adenosine receptor</i> | 2.1 |
| 20 | 100434 | <i>Bmp-1</i> | 2.1 |
| 39290 | X15052 | <i>NCAM-140</i> | 2.1 |
| 39288 | U48730 | <i>STAT5</i> | 2.0 |
| 39266 | Z36885 | <i>sap1A gene</i> | 2.0 |
| 15122 | U34245 | <i>Fra-1</i> | 2.0 |
| <i>Decreased</i> | | | |
| 341 | 108512 | <i>Glutamate/aspartate transporter</i> | 10.2 |
| 373 | 109001 | <i>Ribosomal protein S24</i> | 8.3 |
| 85 | 101153 | <i>NF-M middle molecular weight neurofilament</i> | 7.5 |
| 39219 | J04509 | <i>JUN-D protein</i> | 5.7 |
| 17304 | Y00864 | <i>SCFR/c-kit</i> | 4.5 |
| 201 | 103925 | <i>Guanine nucleotide-binding protein G-s, alpha</i> | 4.4 |
| 11405 | A1071095 | <i>vgr</i> | 3.8 |
| 38932 | X69292 | <i>Myosin heavy chain</i> | 3.1 |
| 15612 | L13968 | <i>UCRBP transcription factor</i> | 3.1 |
| 38698 | U97281 | <i>Conjugating enzymes</i> | 3.1 |
| 16550 | U12473 | <i>IKKa/CHUK</i> | 3.1 |
| 38808 | 2311441R6 | <i>TD14</i> | 3.0 |
| 38737 | AF042793 | <i>Alpha 2 delta Ca channel sub isoform II</i> | 2.9 |
| 39155 | AF022363 | <i>SP1</i> | 2.7 |
| 39261 | X86368 | <i>Transcription factor</i> | 2.6 |
| 38726 | U44839 | <i>A ubiquitin C-terminal hydrolase</i> | 2.5 |
| 16808 | X63547 | <i>tre-2</i> | 2.4 |
| 38671 | D14425 | <i>Calcineurin</i> | 2.3 |
| 8508 | M74590 | <i>Delta-transcription factor</i> | 2.3 |
| 38720 | M73980 | <i>Neurogenic locus notch homolog protein 1 precursor, NTC1</i> | 2.2 |
| 13708 | L06433 | <i>Nuclear hormone receptor FTZ-F, beta, FTFB</i> | 2.1 |
| 16 | 100400 | <i>14-3-3 gamma</i> | 2.1 |
| 10398 | AF014371 | <i>RhoA</i> | 2.0 |
| 20248 | 112173 | <i>Calcineurin A2</i> | 2.0 |

Genes that were influenced by EPO treatment and that have a known function in cell cycle control, cell fate determination, or other aspects of cell proliferation or differentiation are listed here. Genes that showed a change in expression levels of less than two-fold are not shown (see legend Table 2).

function of EPO in the nervous system is similar to its effects on hematopoietic cells or if it has distinct, novel functions remains unanswered. Recent evidence would

suggest that EPO, at least in part, is able to effect neurons in a manner similar to its function in erythroid precursor cells. A major role for EPO in hematopoiesis is to promote

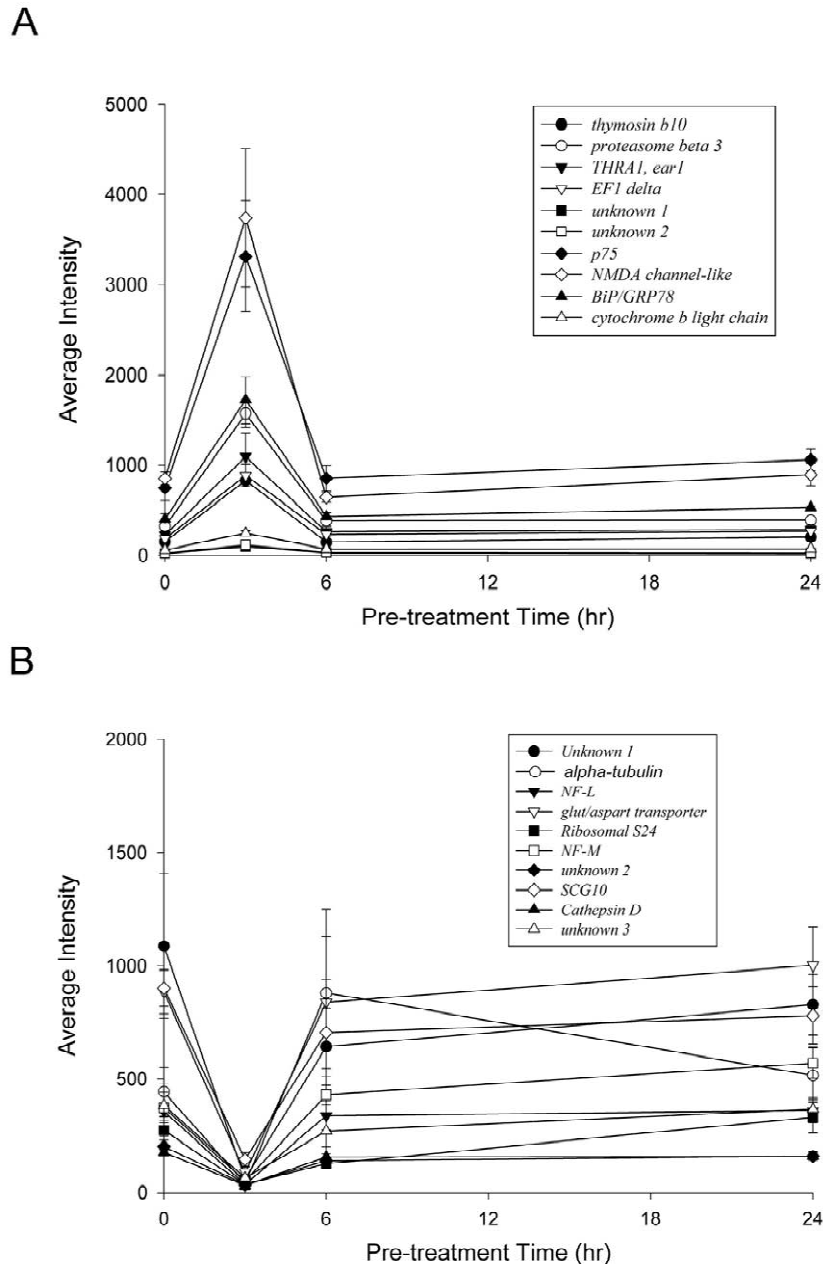


Fig. 1. Changes in gene expression over time in erythropoietin-treated PC-12 cells. The expression of the top 10 induced (A) or repressed (B) genes at 3, 6, and 24 h following EPO treatment is shown. Gene expression is plotted as the average intensity to show the range of expression levels observed for the individual genes. Error bars represent the standard deviation from individual chip hybridizations. All but a few genes showed the greatest change in expression at 3 h following EPO treatment.

the survival of cells belonging to the erythroid precursor pool. Recent reports have shown that EPO can also prevent neurons from undergoing programmed cell death in both in vitro models of neurotoxicity [21], and in vivo models of stroke [22,24] and in other forms of neural injury [6].

The mechanism by which EPO prevents programmed cell death is still unclear. In the erythroid cell line HCD57, the withdrawal of EPO results in a rapid decrease in the expression of *bcl-2* and *bcl_{XL}*, and subsequent cell death. Cells could be rescued by infecting them with a retrovirus expressing *bcl_{XL}* [30]. Thus EPO appears to promote the

survival of these erythroid precursor cells by regulating the expression of the anti-apoptotic *bcl* family members, *bcl-2* and *bcl_{XL}*. A similar increase in *bcl-2* expression has been shown to prevent cell death in neurons exposed to glutamate [4]. The results presented in this paper show that EPO increases the expression of *bcl_{XL}* in neurons. The induction of *bcl_{XL}* expression by EPO in both neurons and erythroid cells suggests that EPO may use a similar mechanism to prevent apoptosis in both of these cell types. The *bcl* family of proteins also contains genes that play a positive role in apoptosis. We have shown here that the

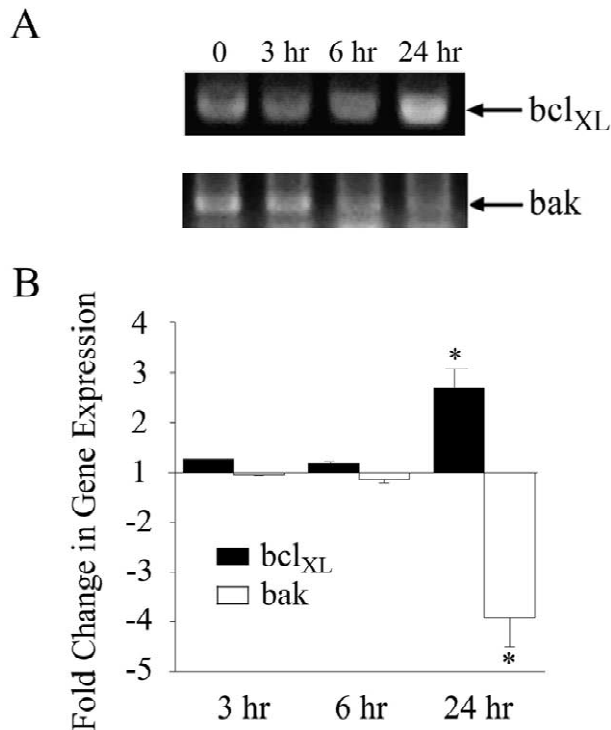


Fig. 2. Real time RT-PCR analysis of erythropoietin-induced changes in the expression of *bcl_{XL}* and *bak*. The mRNA level of *Bcl_{XL}* and *bak* was compared in untreated PC-12 cells and PC-12 cells treated with 10 pM EPO for 3, 6, or 24 h. (A) Shows RT-PCR products run on a 1% agarose gel and (B) shows the mean fold change in gene expression of 4 RT-PCR experiments. The error bars represent the standard deviation of the four experiments. * $P < 0.05$ by Student's two-tailed *t*-test.

treatment of PC-12 cells with EPO causes a decrease in the expression of the pro-apoptotic gene *bak*, suggesting that the neuroprotective effects of EPO may involve controlling the balance of expression between pro- and anti-apoptotic molecules.

The heat shock proteins (HSP) 27 and 70 have also been shown to be neuroprotective. The neuroprotective effect of α -phenyl-*N*-tert-butyl nitron on hippocampal cells of the gerbil in a model of transient ischemia has been reported to be the result of the increased activation of the MAP kinase pathway and subsequent up-regulation of *HSP27* and *HSP70* [33]. *HSP70* inhibits apoptosis by interacting directly with Apaf-1 and preventing the subsequent formation of the Apaf-1-caspase-9 apoptosome [3,25]. Microarray analysis showed that exposure to EPO increased the expression of *HSP27* and *HSP70* by as much as 3-fold in PC-12 cells. The interaction between EPO and HSPs suggests an additional mechanism by which EPO can protect neurons from programmed cell death.

The area of programmed cell death is an active area of research and as such, new molecules that may function in apoptosis are constantly being identified. *APO3L* is a recently identified ligand for the death domain containing receptor Apo3. *APO3L* is related to tumor necrosis factor (TNF) and can induce apoptosis in human breast and

cervical carcinoma cell lines [17]. *TFAR19* (TF-1 cell apoptosis related gene-19) is a pro-apoptotic gene that was cloned from TF-1 cells undergoing apoptosis. The over expression of *TFAR19* in tumor cells has been shown to increase apoptosis resulting from growth factor withdrawal or serum deprivation [15]. EPO decreased the expression of both of these genes suggesting that EPO may control the expression of one or both of these genes as a mechanism for protecting neurons from cell death. In this case EPO may act upstream in the signaling pathway to block the expression of genes that are responsible for initiating earlier events in apoptosis. A mechanism such as this would be appealing as an alternative method for preventing the spread of cell death that occurs following injury to the nervous system, or in the case of prolonged neurodegenerative diseases where the full extent of cell death has not yet been realized.

In addition to its role in promoting cell survival, EPO also promotes the proliferation and differentiation of erythroid precursor cells. While the survival of erythroid precursor cells is an obvious requirement for the proliferation of cells, recent evidence suggest that EPO induces distinct signaling pathways that control cell proliferation (for review see Ref. [27]). Neurons are terminally differentiated cells and are normally not responsive to proliferative signals. PC-12 cells however, are from a cell line derived from tumor (pheochromocytoma) of the adrenal gland and therefore may be able to respond to this type of signal. This appears to be a reasonable assumption as the expression of at least some genes known to mediate the proliferative signal of EPO in hematopoiesis, such as *c-fos*, are increased.

Although the adult population of neurons for the most part then does not replenish themselves recent evidence does support the existence of a population of multipotent cells in the CNS that can give rise to new neurons. These neural stem cells are the target of intense research with the hope of someday being used to treat the loss of neurons in the CNS resulting from acute insult and long-term degenerative conditions. A recent report has suggested that EPO may be responsible for the increase in the survival, differentiation and proliferation of neural stem cells that are cultured in the presence of low oxygen [32]. The ability of EPO to regulate the expression of genes with known functions in proliferation and differentiation in PC-12 cells may provide insight into the mechanisms by which EPO may function in neural stem cells.

EPO has been shown to activate several signaling pathways in neurons. Which of these pathways is used by EPO in neurons to mediate changes in the expression of specific genes remains unclear. EPO has been reported to activate STAT5 [29], a protein kinase C dependent [34] pathway, and the p42/p44 MAP kinase pathway [7,31]. A number of the genes, shown here to be influenced by EPO, have been shown previously to be mediated by the MAP kinase pathway including the expression of *bcl-2* in

neurons [16]. Further investigation is necessary to more accurately describe the use of these pathways in neurons by EPO.

An additional point of interest is that EPO was observed to be effective at mediating the expression of the *bcl_{XL}* and *bak* at 10 pM, a concentration that has previously been shown to be neuroprotective in vivo. Unlike the effects seen in the microarray analysis using higher concentrations of EPO (10 U/ml or 25 nM) these changes in gene expression required a longer pretreatment time of 24 h. One possible explanation for this is that lower concentrations of EPO result in lower levels of receptor occupancy and decreased activation of signaling pathways, therefore requiring a longer period of time to have a full effect on gene expression.

EPO is a growth factor with well-described effects on the proliferation, differentiation and survival of erythroid precursor cells. The recent interest in the function of EPO outside of hematopoiesis has generated many questions concerning the mechanisms by which EPO functions in these non-hematopoietic systems. The results presented here suggest that EPO may make use of some of the same mechanisms (i.e. regulation of *bcl_{XL}*) described in hematopoiesis to elicit its effects in neurons. The results also suggest novel mechanisms (i.e. regulation of *bak*, and HSPs) that might explain the function of EPO in neuron survival. Further study is needed to confirm these mechanisms and to more thoroughly examine the role that they play in the function of EPO outside of hematopoiesis.

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