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Carbamoylated erythropoietin induces a neurotrophic gene profile in neuronal cells

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ABSTRACT

Erythropoietin (EPO), a cytokine molecule, is best-known for its role in erythropoiesis. Preclinical studies have demonstrated that EPO has robust neuroprotective effects that appear to be independent of erythropoiesis. It is also being clinically tested for the treatment of neuropsychiatric illnesses due to its behavioral actions. A major limitation of EPO is that long-term administration results in excessive red blood cell production and increased blood viscosity. A chemical modification of EPO, carbamoylated erythropoietin (CEPO), reproduces the behavioral response of EPO in animal models but does not stimulate erythropoiesis. The molecular mechanisms involved in the behavioral effects of CEPO are not known. To obtain molecular insight we examined CEPO induced gene expression in neuronal cells. PC-12 cells were treated with CEPO followed by genome-wide microarray analysis. We investigated the functional significance of the gene profile by unbiased bioinformatics analysis. The Ingenuity pathway analysis (IPA) software was employed. The results revealed activation of functions such as neuronal number and long-term potentiation. Regulated signaling cascades included categories such as neurotrophin, CREB, NGF and synaptic long-term potentiation signaling. Some of the regulated genes from these pathways are CAMKII, EGR1, FOS, GRIN1, KIF1B, NOTCH1. We also comparatively examined EPO and CEPO-induced gene expression for a subset of genes in the rat dentate gyrus. The CEPO gene profile shows the induction of genes and signaling cascades that have roles in neurogenesis and memory formation, mechanisms that can produce antidepressant and cognitive function enhancing activity.

1. Introduction

Erythropoietin (EPO), a 30.4 kDa glycoprotein, is widely prescribed to treat anemia. Progress in understanding EPO's biological actions over the past two decades has shown that it has robust neurotrophic and neuroprotective actions on the brain (Brines and Cerami, 2005). Clinical studies have attempted to harness the neurotrophic properties of EPO to treat schizophrenia and depression. Results from rigorous clinical trials in treatment resistant depression are promising, and indicate that EPO could be developed as an antidepressant and cognition enhancing agent (Miskowiak et al., 2014). Interestingly, neuroimaging and volumetric analyses indicate that the behavioral effects could be due to EPO's actions on the hippocampus (Miskowiak et al., 2015), potentially providing direction for mechanistic understanding and hypothesis testing.

Despite the encouraging results from neuropsychiatry studies and the overall safety of EPO, it should be noted that EPO is a potent inducer of red blood cell production and could produce adverse hematological consequences in non-anemic individuals. Carbamoylated EPO (CEPO), a chemically engineered in vitro post-translational modification of EPO, has emerged as an attractive trophic molecule because it is devoid of erythropoietic activity. Yet, it retains the neurotrophic effects in the central nervous system (Leist et al., 2004). The precise mechanism involved in CEPO's cellular actions are not well understood. It has been proposed that CEPO is non-erythropoietic, as it signals via an EPO receptor (EPOR)- beta common receptor (βcR\CD131) heteromer rather than the classical EPOR-EPOR dimer bound by EPO (Leist et al., 2004). We therefore hypothesized that the downstream signal transduction pathways and gene profile induced by CEPO would differ from EPO due to lack of erythropoietic activity, but exhibit overlap due to their shared neurotrophic activity. We used the neuronal phenotype PC12 cell line in a genome wide microarray analysis of gene expression to gain insight into CEPO's mechanism of action in neurons. This cell line has been used to examine multiple aspects of EPO signaling (Um

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Table 1
Representative top molecules identified by IPA analysis after CEPO treatment.

Gene symbol	Fold change	Genebank accn	Description	Molecular function
Up-regulated genes				
Ddx3x	14.599	NM_001108246	DEAD-box helicase 3, X-linked	Helicase
KAT2B	12.401	XM_003750617	lysine acetyltransferase 2B	Transcription regulator
CIRBP	10.999	NM_031147	cold inducible RNA binding protein	Translation regulator
CHRDL1	10.636	NM_199502	chordin like 1	plays a role in neuronal differentiation
POGZ	10.337	NM_001107693	pogo transposable element derived with ZNF domain	transposase
ALYREF	9.963	NM_001109602	Aly/REF export factor	Transcription regulator
Cts8	9.288	NM_001128216	cathepsin 8	Peptidase
ELAC1	9.193	NM_001107406	elaC ribonuclease Z 1	ribonuclease
PPP1R3A	9.023	NM_001109222	protein phosphatase 1 regulatory subunit 3A	Phosphatase
PAQR7	8.931	NM_001034081	progestin and adipoQ receptor family member 7	Steroid membrane receptor
Down-regulated genes				
CHMP4B*	−6.315	NM_001276456	charged multivesicular body protein 4B	protein homodimerization activity
NPM3	−4.762	XM_001058548	nucleophosmin/nucleoplasmin 3	chaperone protein
GABRA5	−3.948	NM_017295	gamma-aminobutyric acid type A receptor alpha5 subunit	Ion Channel
Raet1l	−3.785	NM_001013063	retinoic acid early transcript 1 L	Peptide antigen binding
KLK1*	−3.73	NM_001005382	kallikrein 1	Peptidase
GJD2	−3.517	NM_019281	gap junction protein delta 2	Transporter
SNW1	−3.497	NM_001109279	SNW domain containing 1	Transcription regulator
KIF1C	−3.353	NM_145877	kinesin family member 1C	microtubule motor activity
GZMB	−3.294	NM_138517	granzyme B	Peptidase
Olr1124	−3.127	NM_001000426	olfactory receptor 1124	G-protein coupled receptor

Table shows top 10 upregulated and downregulated molecules with their gene symbol, fold change value, gene bank accession number, description and molecular function.

* Duplicate genes in the dataset.

Table 2
Functions related to Nervous system enriched in the dataset.

Functions annotation	p-Value	Activation z-score	Predicted Activation State	# Molecules
Neuronal Number	1.09E−10	2.84	Increased	117
Long-term potentiation	0.00000296	2.506	Increased	66
Neurotransmission	6.79E−10	2.017	Increased	108

The p-values and z scores were generated by IPA for categories neuronal number, long term potentiation and neurotransmission after core analysis. The significance and z scores are calculated by the IPA software as described in the methods section. The $z \geq 2$ predicts increase in activation of that function. Differentially expressed genes in the dataset for each function is represented as number of molecules.

et al., 2007; Wu et al., 2007) and gene profiling analysis (Renzi et al., 2002). We subjected the CEPO gene profile to bioinformatics analysis to examine the functional implications of CEPO regulated genes and to shed light on mechanisms involved in its behavioral effects. We also conducted independent secondary validation of array data using quantitative PCR analysis.

2. Materials and methods

2.1. Carbamoylation of EPO

Erythropoietin was purchased from Prospec Bio (Israel) and carbamoylated in 1 mg aliquots as per (Mun and Golper, 2000) with mild modifications. Briefly, EPO was deprotonated in a high pH (pH = 8.9) borate buffer and then exposed to potassium cyanate for 16 h at 36 °C. CEPO was exhaustively dialyzed for 6 h against PBS. CEPO concentration was determined using the Qubit protein assay (ThermoFisher). CEPO purity was verified by silver staining after electrophoretic gel analysis.

2.2. Cell culture

Rat pheochromocytoma cells (PC-12 cells) were obtained from American Type Culture Collection (ATCC). The cells were grown in suspension in RPMI-1640 (ATCC) with 10% heat inactivated horse serum, 5% fetal bovine serum (Gibco) at 37 °C and 5% CO_2. To differentiate the cells into neuronal cells PC-12 cells were plated in collagen coated dishes (Corning) and were grown in RPMI-1640 with NGF (100 ng/ml, Alomone Labs) and 1% Horse Inactivated serum (Gibco). The cells were grown for 10 days and the medium was changed every 2 days. Neuronal morphology and robust neurite outgrowth was confirmed by microscopy. NGF was removed overnight before the day of experiment. PC-12 cells were treated with CEPO 100 ng/ml for 3 h. Vehicle-treated (PBS) cells were used as control.

2.3. Animals

Adult male Sprague-Dawley rats (n = 6 per group, mass 220–240 g; Envigo) and pair-housed according to treatment group (Vehicle, EPO and CEPO) for the duration of the experiments. Rats were maintained on a standard 12-h light-dark cycle with free access to food and water. All procedures were carried out in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approval by the USD Institutional Animal Care and Use Committee. Every effort was made to minimize the number of animals used. Rats received single daily i.p. injections of either vehicle (PBS), EPO or CEPO (30 μg/kg) for 4 consecutive days. Five hours after the last dose animals were decapitated according to American Veterinary Medical Association guidelines and the brains were frozen on dry ice.

2.4. Laser microdissection

Cryocut hippocampal sections (coronal, 16 μm) were collected on PEN-Membrane slides (Leica) and processed for laser microdissection. Sections were fixed in histochoice fixative (Sigma) for 3 min followed by brief rinses in PBS, 70% ethanol and milliQ water. Sections were stained with cresyl violet (2 min), followed by dehydration in 95% and 100% ethanol. The dentate gyrus was delineated using the free draw tool on a Wacom Cintiq high resolution monitor and microdissected using the Leica LMD 7000 system. Microdissected tissue was collected directly into lysis solution and processed for RNA isolation using the RNAqueous micro kit (ThermoFisher). The RNA samples were then