Research Article

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Proteomic analysis of erythropoietin-induced changes in neuron-like SH-SY5Y cells

Eritropoetin'in Nöron Benzeri SH-SY5Y Hücrelerinde İndüklediği Değişikliklerin Proteomik Analizi

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Abstract

Objective: Erythropoietin (EPO) is widely used for treatment of anemia associated with different diseases; however, its adverse effects limit its use in clinical practice. Therefore, understanding the effects of EPO at the molecular and cellular level is crucial to adjust treatment regimes, and to develop non-hematopoietic EPO derivatives. In this study, we used a proteomics approach to identify how EPO treatment modifies the cellular proteome.
Methods: SH-SY5Y neuroblastoma cells were used as the model system to analyze the effects of EPO treatment at different time points (24 h and 48 h). Proteomic analysis revealed changes in 74 proteins after EPO treatment. Following proteomics analysis, Reactome pathway analysis were carried out to identify the affected cellular pathways.
Results: According to results, EPO alters the levels of 74 protein species (40 were increased, 34 were decreased). The levels of 35 proteins were changed by 24 h EPO incubation, whereas 17 protein species were altered by 48 h EPO incubation. Levels of 22 protein species were altered by both of the incubation periods (24 h and 48 h).
Conclusion: Overall, our results suggest that EPO mainly affects protein species in glucose metabolism, protein and RNA metabolism, cytoskeletal proteins, and mitochondrial protein species.

Keywords: Erythropoietin; Neuron; Proteomics; Neuroprotection; Drug treatment; Signaling pathways.

Özet

Giriş ve Amaç: Eritropoetin (EPO) çeşitli hastalıklar ile ilişkili aneminin tedavisinde yaygın kullanılmaktadır, ancak yan etkileri nedeniyle klinikteki kullanımı sınırlıdır. Bu nedenle, tedavi rejimlerini düzenlemek ve hematopoetik olmayan EPO derivelerini geliştirmek için EPO'nun moleküler ve hücresel düzeydeki etkilerini anlamak çok önemlidir. Bu çalışmada, proteomik yaklaşımla EPO tedavisinin hücresel proteomları nasıl etkilediğini tanımlamayı amaçladık.
Yöntem ve Gereçler: EPO'nun farklı zamanlardaki (24 h ve 48 h) etkisini analiz etmek için sistem model olarak SH-SY5Y nöroblastom hücreleri kullanıldı. EPO tedavisinden sonra değişiklik izlenen 74 protein proteomik analiz ile gösterildi. Proteomik analizden sonra yapılan Reaktom Yolak Analizi ile de etkilenmiş olan hücresel yolaklar tanımlandı.
Bulgular: EPO 74 protein türünün düzeyini değiştirmiştir (40'ı artmış, 34'ü azalmış). 24 saatlik EPO inkübasyonu sonunda 35 proteinin düzeyi değişmiş, 48 saatlik EPO inkübasyonu sonunda ise 17 proteinin düzeyi değişmiştir. 22 protein türünün düzeyi ise her iki inkübasyon süresinde de değişmiştir (24 h ve 48 h).
Tartışma ve Sonuç: EPO'nun başlıca glukoz metabolizmasındaki, protein ve RNA metabolizmasındaki protein

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türlerini, hücre iskeleti proteinlerini ve mitokondrial protein türlerini etkilediği gösterilmiştir.

Anahtar Kelimeler: Eritropoetin; Nöron; Proteomikler; Nöroproteksiyon; Ilaç tedavisi; Sinyal yolakları.

Introduction

Erythropoietin (EPO) is a multifunctional cytokine, which is primarily produced by peritubular cells in the kidney [1]. EPO production is subject to transcriptional regulation, where hypoxia-inducible transcription factors stimulate EPO gene expression [2]. The primary function of EPO is to regulate production of erythrocytes in the bone marrow. EPO receptor is expressed in various tissues [3, 4], including the nervous system, and EPO plays important roles in neurodevelopment [5, 6] and neuroprotection [7–9].

Recombinant EPO is in clinical use for treatment of anemia associated with different diseases, including but not limited to AIDS [10, 11], cancer [12, 13], and renal failure [14, 15]. However, long-term EPO administration is associated with several adverse events, including hypertension [16], cerebral convulsion, hypertensive encephalopathy, thrombo-embolism, iron deficiency, and influenza-like syndrome [17]. Given these findings, it is not only necessary to determine the optimal dose and time for EPO administration, but also to understand how EPO exerts its effects at the molecular level.

Previous studies on EPO has also benefited from proteomics to identify the interactions between cellular proteins and EPO [18], and to determine differentially regulated protein spots after EPO treatment or withdrawal [19–23]. However, the question of how EPO alters the neuronal proteome has not been addressed so far. Therefore, our aim was to use a proteomics approach to identify how EPO modifies the cellular proteome. We used SH-SY5Y neuroblastoma cell line as the in vitro model system to analyze the effects of EPO. Our findings show that EPO treatment alters the cellular proteome, mainly through the proteins related to glucose metabolism, mRNA metabolism, and protein metabolism [24, 25].

Materials and methods

Neuronal cell culture

Human neuroblastoma SH-SY5Y cell line was purchased from Deutsche Sammlung von Mikroorganismen & Zellkulturen (DSMZ). SH-SY5Y cells were maintained in Dulbecco's Modified Eagle Medium: nutrient mixture F-12 (DMEM:F12) (1 : 1) (Gibco, Gaithersburg, MD, USA) supplemented with heat-inactivated fetal bovine serum (10% v/v) and L-glutamine (1% v/v) at 37°C in 5% CO_2.

EPO preconditioning

Twenty-four hour prior to treatment, 3×10^6 SH-SY5Y cells (Passage #15) were seeded in 75 cm^2 cell culture flasks in triplicate. Cells were treated with EPO (1 U/mL final concentration) for 24 h, 48 h or left untreated. Cells were maintained in reduced serum medium (OptiMEM, Gibco, Gaithersburg, MD, USA) during EPO treatment.

Sample preparation for 2DE electrophoresis

At the end of incubation time, sample preparation was performed according to Proteome Factory's 2DE sample preparation protocol for cell culture. Total protein was isolated from plated SH-SY5Y cells using TriPure Isolation Reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Urea, ampholytes and DTT were added to a final concentration of 9 M urea, 2% ampholytes and 70 mM DTT during thawing of the protein-pellet (200 µg). After incubation for 30 min at room temperature and centrifugation for 45 min at $15{,}000\times g$ the supernatant was removed and frozen in new tubes at – 80°C.

Two dimensional gel electrophoresis

Two dimensional gel electrophoresis (2DE) was performed by Proteome Factory based on the 2D electrophoresis technique according to Klose and Kobalz [26]. Hundred microgram of protein was applied to vertical rod gels [9 M urea, 4% acrylamide, 0.3% PDA, 5% glycerol, 0.06% TEMED and 4% carrier ampholytes (pH 2–11), 0.02% APS] for isoelectric focusing (IEF) at 8820 Vh in the first dimension. After focusing, the IEF gels were incubated in equilibration buffer, containing 125 mM Tris/phosphate (pH 6.8), 40% glycerol, 65 mM DTT, and 3% standard deviation score (SDS) for 10 min and subsequently frozen at – 80°C. The second dimension SDS-PAGE gels (20 cm × 30 cm × 0.1 cm) were prepared, containing 375 mM Tris-HCl buffer (pH 8.8), 12% acrylamide, 0.2% bisacrylamide, 0.1% SDS, 0.02% APS and 0.03% TEMED. After thawing, the equilibrated IEF gels were immediately applied to SDS-PAGE

gels. Electrophoresis was performed using a 140 mA for 5.5 h until the front reached the end of the gel. After 2DE separation the gels were stained with silver (Fire Silver staining kit, Proteome Factory).

Gel image analysis

The 2DE gels used for comparison analysis were digitized at a resolution of 150 dpi using a PowerLook 2100XL scanner with transparency adapter. Two-dimensional image analysis was performed using the Proteomweaver software (Definiens AG, Munich, Germany). Protein spots with different spot intensities were selected according to two parameters:

- a minimal significant factor which was evaluated as follow with a replicate quality test: "Based on the 500 highest intensity spot-pairs, an average replicate deviation of 68.25% was found. The standard deviation of the average intensities for a group with three gels is 23.67%. The regulation factor between two such groups has a standard deviation of 35.04%. The selected confidence level (0.05) results in a trust factor of 1.96. Exponentiating the standard deviation of the regulation factors with the trust factor results in a minimal significant regulation factor of: 1.802. A minimal significant factor of 1.802 was therefore applied for the selection of changed protein spots.

Trypsin-digestion/nanoLC-ESI-MS/MS

Protein identification using nanoLC-ESI-MS/MS was performed by Proteome Factory (Proteome Factory AG, Berlin, Germany). The HPLC system was coupled to MS detection via Qstar XL mass spectrometer (ABI, Foster City, CA, USA). Peptides from enzymatic cleavage were acified with formic acid and applied to nanoLC-ESI-MS/MS. After trapping and desalting the peptides on enrichment column (Zorbax SB C18, 0.3×5 mm, Agilent) using 1% acetonitrile/0.5% formic acid solution for 5 min peptides were separated on Zorbax 300 SB C18, 75 μm×150 mm column (Agilent, Waldbronn) using an acetonitrile/0.1% formic acid gradient from 5% to 40% acetonitrile within 40 min. MS overview spectra were automatically taken with a tolerance of ± 50 ppm according to manufacturer's instrument settings for nanoLC-ESI-MS/MS analyses, peptide fragmentation and detection was accomplished with an accuracy of ± 0.5 Da. Proteins were identified using MS/MS ion search of the Mascot search engine (Matrix Science, London, England) and nr protein database (National Center for Biotechnology Information, Bethesda, MD, USA). Ion charge in search parameters for ions from ESI-MS/MS data acquisition were set to "1 +, 2 + or 3 +" according to the instrument's and method's common charge state distribution.

Pathway analysis

Reactome pathway database (access: http://www.reactome.org/) was used to identify which cellular pathways are affected by EPO treatment. Two time points (24 h and 48 h) were individually analyzed.

Statistical analysis

Mann-Whitney U-test has been used to compare the differences in spot intensities. p-Values < 0.05 were considered to be statistically significant.

Results

Differentially regulated protein spots after EPO treatment

To identify how EPO affects global protein levels in SH-SY5Y cells, we treated the cells with EPO (1 U/mL) for 24 h and 48 h. This dose is consistent with previous studies, which characterized the effects of EPO in vitro [27, 28]. Proteomic analysis revealed a total of 74 differentially speciated proteins after EPO treatment (Figures 1–3). The encircled spots are normally the regulated or the analyzed spots. The pH range is from 3 to 10 and the molecular weight is from 10 to 150 kDa. Among all differentially speciated proteins, 40 proteins were upregulated, and 34 proteins were downregulated. Thirty-five proteins showed differential speciation after 24 h EPO treatment (Tables 1 and 2), whereas only 17 proteins showed differential expression after 48 h EPO treatment (Tables 3 and 4). Twenty-two proteins showed differential speciation in both 24 h and 48 h EPO treatment (Tables 5 and 6).

At 24 h time point, neuroleukin was the most upregulated protein (3.19-fold compared to control), whereas pyruvate kinase (PK) was the most downregulated protein (0.19-fold compared to control). At 48 h time point, heterogeneous nuclear ribonucleoprotein R isoform 2 was the most upregulated protein (2.35-fold compared to control), whereas peroxiredoxin-4 was the most downregulated

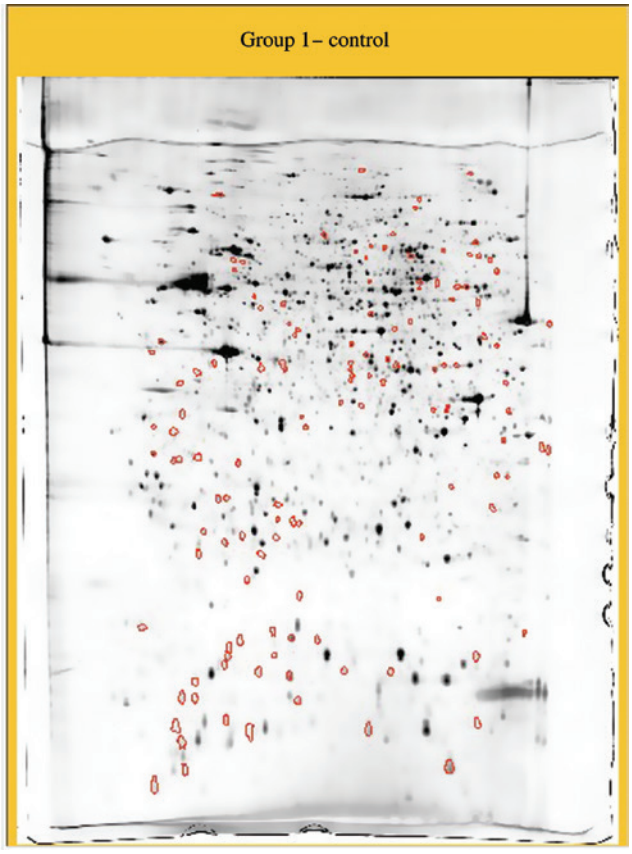

Figure 1: Two-dimensional gel of proteins from control SH-SY5Y cell cultures without application of EPO.
Protein spots circled in control gel represent alteration in 74 protein species after EPO preconditioning.

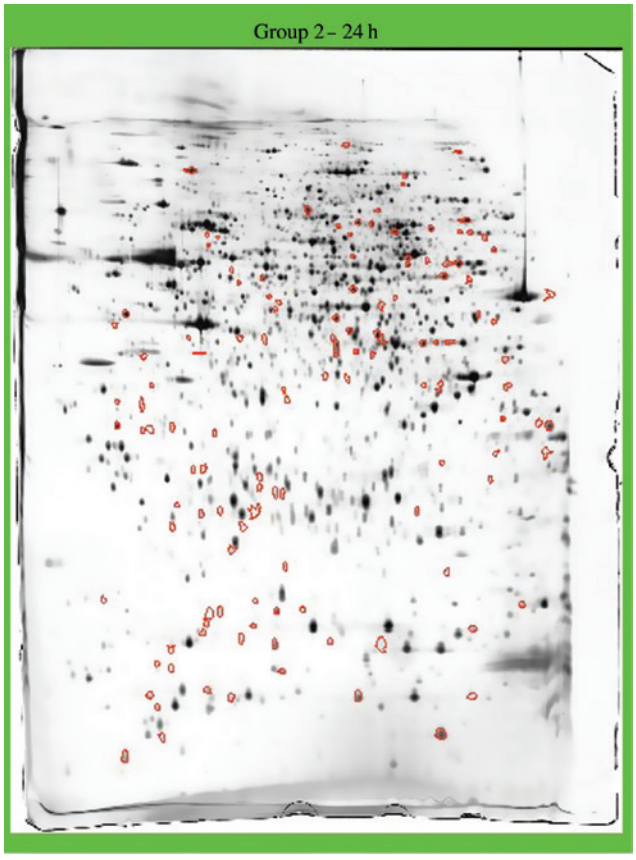

Figure 2: Two-dimensional gel of proteins from 24 h EPO preconditioned SH-SY5Y cell cultures.
Protein spots circled in gel represent altered protein species (18 protein upregulated, 17 protein downregulated).

protein (0.32-fold compared to control). The complete list of differentially speciated proteins is shown in SI Figures.

EPO affects proteins in different cellular pathways

In the next step, we used Reactome pathway database to identify the cellular pathways which are most affected by EPO treatment. The results indicate that EPO treatment affects proteins in glucose metabolism, protein and mRNA metabolism, cytoskeletal and mitochondrial proteins (Figures 4 and 5).

Discussion

Following its introduction to the clinical practice, the use of recombinant human EPO has increased exponentially in the past 20 years. EPO treatment is currently used for a broad spectrum of diseases. EPO has a direct effect on blood pressure [29], which is independent of its hematopoietic effects [16]. In addition, several in vitro, in vivo, and clinical studies have been carried out to characterize the hypertensinogenic effects of EPO [30–33].

The choice of SHSY-5Y cells was based on previous studies, which have demonstrated the expression of EPO receptor and tissue-protective EPO receptors in this cell line [34, 35]. In addition, several studies have used SHSY-5Y cells to analyze the functional effects of EPO [36, 37]. Therefore, we preferred using this cell line as a model to characterize the effects of EPO on cellular proteome.

Among all differentially speciated proteins after EPO treatment, PK showed the highest level of downregulation (approximately 80%). PK is one of the key metabolic enzymes that regulate glycolysis and gluconeogenesis, and its deficiency is the second leading cause of enzyme-deficient hemolytic anemia [38, 39]. So far, there is no direct experimental evidence on the interplay between EPO and PK deficiency. Only a single study has investigated the

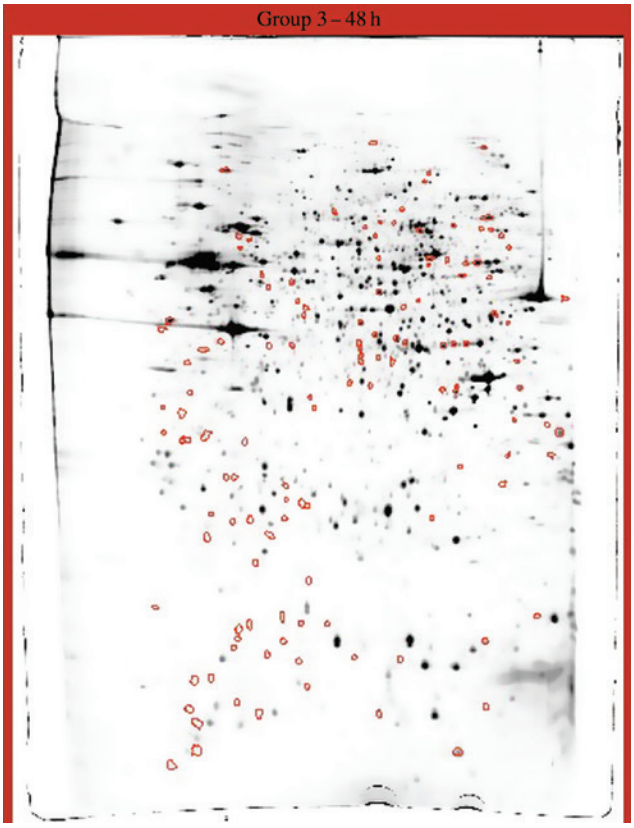

Figure 3: Two-dimensional gel of proteins from 48 h EPO preconditioned SH-SY5Y cell cultures.
Protein spots circled in gel represent altered protein species (eight protein upregulated, nine protein downregulated).

potential link between EPO and PK, which indicates that EPO is an effective treatment option for iron overload [40], a common event in PK deficiency [41, 42].

Stathmin is a small, cytoplasmic protein that is primarily responsible for regulating the dynamics of microtubule formation. Stathmin functions as a relay protein in different signaling pathways (mainly PI3K and MAPK signaling) [43]. The functional roles of stathmin in hematopoiesis have been characterized in several studies. Stathmin speciation increases during chemically induced and EPO-induced erythroid differentiation. In this study, Glucose-6-phosphate isomerase (GPI) encodes an enzyme, which functions as a neurotrophic factor (known as neuroleukin) outside the cell. Neuroleukin is essential for survival of skeletal motor neurons and sensory neurons. In the present study, we have shown that the highest upregulation following 24 h EPO treatment was observed in neuroleukin levels. Thus, it is possible that EPO exerts its neuroprotective effects via neuroleukin.

Sideroflexin-3 is a member of the sideroflexin protein family. Sideroflexin-3 is located on the mitochondria, and functions as an iron transporter to regulate iron homeostasis. In our study, we determined that sideroflexin-3 speciation was the most upregulated protein after EPO treatment at both time points (24 h and 48 h). Given its role in iron homeostasis, sideroflexin-3 may function as a mediator of EPO's hematopoietic effects.

Table 1: List of spots/protein species sensitive to EPO treatment in SH-SY5Y, detected by 2-DE and identified by peptide MS/MS analysis.

Protein	Uniprot accession no.	Control	EPO	Relative expression (EPO/control)
Neuroleukin [Homo sapiens]	P06744	0.246	0.784	3.186992
WD repeat domain 1 [Homo sapiens]	O75083	0.181	0.439	2.425414
40S Ribosomal protein S12 [Homo sapiens]	P25398	0.376	0.864	2.297872
DBH protein [Homo sapiens]	P09172	0.271	0.605	2.232472
Aralar2 [Homo sapiens]	Q9UJS0	0.204	0.453	2.220588
Zinc finger protein 207 isoform a [Homo sapiens]	O43670	0.136	0.299	2.198529
ATP synthase subunit gamma, mitochondrial isoform H (heart) precursor [Homo sapiens]	P36542	0.172	0.375	2.180233
Growth regulated nuclear 68 protein	P17844	0.114	0.241	2.114035
Non-POU domain-containing octamer-binding protein isoform 1 [Homo sapiens]	Q15233	0.509	0.994	1.952849
Chain A, crystal structure of human paics, A bifunctional carboxylase and synthetase in purine biosynthesis	P22234	0.352	0.657	1.866477
HnRNP 2H9B [Homo sapiens]	P31942	0.132	0.246	1.863636
Clongation factor Tu [Homo sapiens]	P49411	0.537	0.999	1.860335
Coiled-coil domain containing 51 [Homo sapiens]	Q96ER9	0.114	0.211	1.850877
Prelamin-A/C isoform 2 [Homo sapiens]	P02545	0.11	0.203	1.845455
Phosphoserine aminotransferase 1 [Homo sapiens]	Q9Y617	0.313	0.577	1.84345
Heterogeneous nuclear ribonucleoprotein A/B isoform a [Homo sapiens]	Q99729	0.255	0.468	1.835294
Non-POU domain containing, octamer-binding [Homo sapiens]	Q15233	0.199	0.365	1.834171
GMP synthase [glutamine-hydrolyzing] [Homo sapiens]	P49915	0.13	0.238	1.830769

Identified protein species from SH-SY5Y samples, 24 h treatment of EPO with upregulated speciation calculated from improved spot density as percentage changes in spot volume against untreated SH-SY5Y samples.

Table 2: Identified protein species from SH-SY5Y samples, 24 h treatment of EPO with downregulated speciation calculated from improved spot density as percentage changes in spot volume against untreated SH-SY5Y samples.

Protein	Uniprot accession no.	Control	EPO	Relative expression (EPO/control)
Pyruvate kinase [Homo sapiens]	P14618	0.25	0.047	0.188
Heat shock cognate 71 kDa protein isoform 1 [Homo sapiens]	P11142	0.255	0.049	0.192157
Glutathione S-transferase-P1c [Homo sapiens]	P09211	0.211	0.045	0.21327
Gamma subunit of CCT chaperonin [Homo sapiens]	P49368	0.112	0.024	0.214286
Chaperonin containing TCP1, subunit 5 (epsilon) [Homo sapiens]	P48643	0.148	0.033	0.222973
Elongation factor 1-alpha 1 [Homo sapiens]	P68104	0.154	0.037	0.24026
MTHSP75 [Homo sapiens]	P38646	0.14	0.04	0.285714
L-lactate dehydrogenase A chain isoform 1 [Homo sapiens]	P00338	0.083	0.024	0.289157
Heat shock cognate 71 kDa protein isoform 1 [Homo sapiens]	P11142	0.079	0.023	0.291139
Polypyrimidine tract-binding protein 1 isoform a [Homo sapiens]	P26599	0.098	0.031	0.316327
Glyceraldehyde-3-phosphate dehydrogenase [Homo sapiens]	P04406	0.383	0.129	0.336815
Polyadenylate binding protein II [Homo sapiens]	P11940	0.106	0.043	0.40566
Hsp89-alpha-delta-N [Homo sapiens]	P07900	0.201	0.084	0.41791
Chain A, human glyoxalase I with benzyl-glutathione inhibitor	Q04760	0.256	0.107	0.417969
KH domain-containing, RNA-binding, signal transduction-associated protein 1 [Homo sapiens]	Q07666	0.087	0.038	0.436782
ATP synthase subunit alpha, mitochondrial precursor [Homo sapiens]	P25705	0.107	0.047	0.439252
HNRPF protein [Homo sapiens]	P52597	0.066	0.035	0.530303

Table 3: Identified protein species from SH-SY5Y samples, 48 h treatment of EPO with upregulated speciation calculated from improved spot density as percentage changes in spot volume against untreated SH-SY5Y samples.

Protein	Uniprot accession no.	Control	EPO	Relative expression (EPO/control)
Heterogeneous nuclear ribonucleoprotein R isoform 2 [Homo sapiens]	Q99729	0.069	0.162	2.347826
CDC10 homolog [Homo sapiens]	Q16181	0.166	0.384	2.313253
ATP synthase, H + transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle [Homo sapiens]	P25705	0.424	0.96	2.264151
S3 ribosomal protein [Homo sapiens]	P23396	0.616	1.0	2.146104
Aconitate hydratase, mitochondrial precursor [Homo sapiens]	Q99798	0.093	0.198	2.129032
Aldehyde dehydrogenase [Homo sapiens]	P05091	0.191	0.386	2.020942
Heterogeneous nuclear ribonucleoprotein A/B isoform a [Homo sapiens]	Q99729	0.149	0.299	2.006711
MLL septin-like fusion protein [Homo sapiens]	Q9UHD8	0.118	0.225	1.90678

Table 4: Identified protein species from SH-SY5Y samples, 48 h treatment of EPO with downregulated speciation calculated from improved spot density as percentage changes in spot volume against untreated SH-SY5Y samples.

Protein	Uniprot accession no.	Control	EPO	Relative expression (EPO/control)
Peroxiredoxin-4 [Homo sapiens]	Q13162	0.122	0.039	0.319672
Actin, cytoplasmic 1 [Homo sapiens]	P60709	0.291	0.101	0.347079
Prohibitin-2 isoform 2 [Homo sapiens]	Q99623	0.157	0.055	0.350318
Cytochrome c oxidase subunit 5A, mitochondrial precursor [Homo sapiens]	P20674	0.307	0.109	0.355049
Keratin 1 [Homo sapiens]/PEA-15 [Homo sapiens]	P04264	0.282	0.113	0.400709
Thioredoxin domain-containing protein 12 precursor [Homo sapiens]	O95881	0.152	0.066	0.434211
Small nuclear ribonucleoprotein F [Homo sapiens]	P62306	0.775	0.375	0.483871
Mutant human thioredoxin and a peptidetarget site in human Nfkb		0.192	0.1	0.520833
Prelamin-A/C isoform 2 [Homo sapiens]	P02545	0.157	0.085	0.541401

Table 5: Identified protein species from SH-SY5Y samples, 24 h as well as 48 h treatment of EPO with upregulated speciation calculated from improved spot density as percentage changes in spot volume against untreated SH-SY5Y samples.

Protein	Uniprot accession no.	Control	EPO 24 h	EPO 48 h	EPO/control 24 h	EPO/control 48 h
Sideroflexin-3 [Homo sapiens]	Q9BWM7	0.099	0.344	0.399	3.474747	4.030303
Cyclophilin [Homo sapiens]	P23284	0.054	0.15	0.137	2.777778	2.537037
Chain A, human mitochondrial nad(P)-dependent malic enzyme	Q16798	0.061	0.157	0.125	2.57377	2.04918
78 kDa gastrin-binding protein [Homo sapiens]	P40939	0.505	1.117	1.197	2.211881	2.370297
2-Phosphopyruvate-hydratase alpha-enolase [Homo sapiens]	P06733	0.099	0.218	0.156	2.20202	1.575758
Lamin A/C [Homo sapiens]	P02545	0.084	0.177	0.159	2.107143	1.892857
ATP: citrate lyase [Homo sapiens]	P53396	0.039	0.079	0.085	2.025641	2.179487
RecName: Full = ES1 protein homolog, mitochondrial; AltName: Full = Protein GT335; AltName: Full = Protein KNP-I; Flags: Precursor	P30042	0.119	0.24	0.24	2.016807	2.016807
Laminin-binding protein [Homo sapiens]	P08865	0.557	1.055	0.934	1.894075	1.67684
Actin-related protein [Homo sapiens]	P61163	0.348	0.657	0.569	1.887931	1.635057
Translation initiation factor eIF3 p40 subunit [Homo sapiens]	O15372	0.142	0.261	0.263	1.838028	1.852113
PAPS sunthetase [Homo sapiens]	O43252	0.143	0.258	0.267	1.804196	1.867133
Aldolase A [Homo sapiens]	P04075	0.208	0.373	0.518	1.793269	2.490385
Poly(ADP-ribose) polymerase [Homo sapiens]	P09874	0.142	0.232	0.372	1.633803	2.619718

Table 6: Identified protein species from SH-SY5Y samples, 24 h as well as 48 h treatment of EPO with downregulated speciation calculated from improved spot density as percentage changes in spot volume against untreated SH-SY5Y samples.

Protein	Uniprot accession no.	Control	EPO 24 h	EPO 48 h	EPO/control 24 h	EPO/control 48 h
Actin, alpha skeletal muscle [Homo sapiens]	P68133	0.584	0.087	0.108	0.148973	0.184932
Mutant beta-actin (beta′-actin) [Homo sapiens]	P60709	0.344	0.063	0.122	0.18314	0.354651
Chaperonin (HSP60) [Homo sapiens]	P10809	0.389	0.126	0.168	0.323907	0.431877
Eukaryotic translation elongation factor 1 alpha 1 [Homo sapiens]	P68104	0.205	0.067	0.073	0.326829	0.356098
Chain A, structure of rho guanine nucleotide dissociation inhibitor	P52565	0.242	0.082	0.094	0.338843	0.38843
Stathmin isoform a [Homo sapiens]	P16949	0.63	0.314	0.081	0.498413	0.128571
Acireductone dioxygenase 1 [Homo sapiens]	Q9BV57	0.114	0.069	0.039	0.605263	0.342105
Calcium-regulated heat stable protein CRHSP-24 [Homo sapiens]	Q9Y2V2	0.122	0.098	0.061	0.803279	0.5

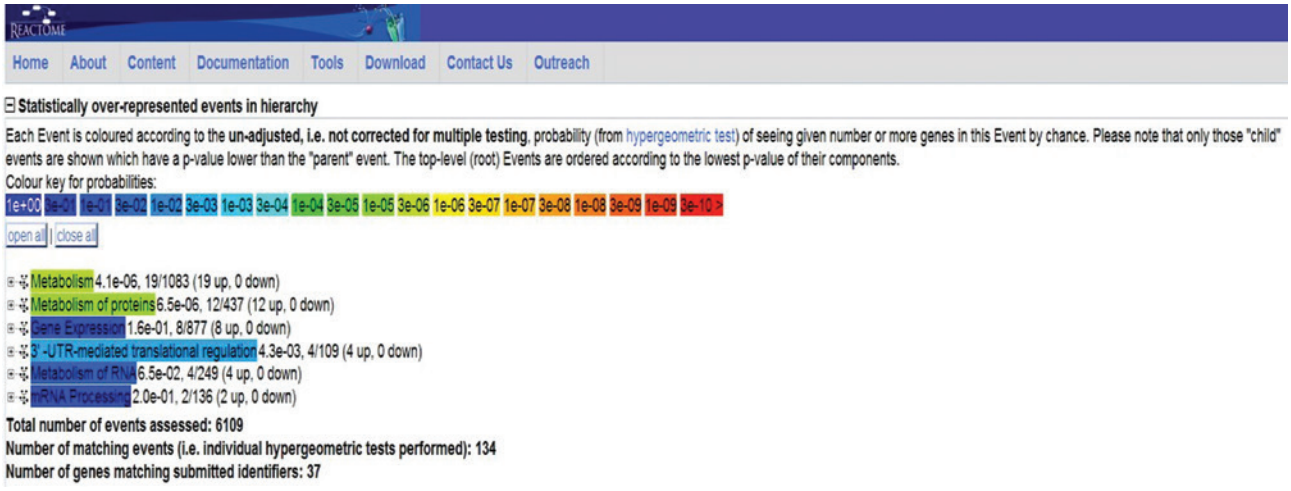

Figure 4: "Reactome pathway analysis" results from 24 h of EPO preconditioning.
Reactome pathway database identified the cellular pathways which were at most affected by EPO treatment. The results indicated that EPO treatment affected protein species in glucose metabolism, protein and mRNA metabolism as well as cytoskeletal and mitochondrial protein species. Each pathway was associated with annotated protein species through colored blocks and Reactome's pathway hierarchy was shown using statistics.

Figure 5: "Reactome pathway analysis" results from 48 h of EPO preconditioning.
EPO high-confidence interacting main cellular pathways were analyzed using the Reactome analysis tool for altered protein speciation. Statistically over-represented interactions of EPO stimulation in hierarchy were demonstrated.

Concluding remarks

Taken together, our findings indicate that EPO affects more than 70 proteins, which play a role in multiple cellular mechanisms in neuronal cells. Functional characterization of these proteins is necessary to further elucidate the mechanism of action of EPO.

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References

1. Koury ST, Bondurant MC, Koury MJ. Localization of erythropoietin synthesizing cells in murine kidneys by in situ hybridization. Blood 1988;71:524–7.
2. Jelkmann W. Regulation of erythropoietin production. J Physiol 2011;589:1251–8.
3. Digicaylioglu M, Bichet S, Marti HH, Wenger RH, Rivas LA, Bauer C, et al. Localization of specific erythropoietin binding sites in defined areas of the mouse brain. Proc Natl Acad Sci USA 1995;92:3717–20.
4. Juul SE, Yachnis AT, Christensen RD. Tissue distribution of erythropoietin and erythropoietin receptor in the developing human fetus. Early Hum Dev 1998;52:235–49.
5. Yu X, Shacka JJ, Eells JB, Suarez-Quian C, Przygodzki RM, Beleslin-Cokic B, et al. Erythropoietin receptor signalling is required for normal brain development. Development 2002;129:505–16.
6. Buemi M, Cavallaro E, Floccari F, Sturiale A, Aloisi C, Trimarchi M, et al. Erythropoietin and the brain: from neurodevelopment to neuroprotection. Clin Sci (Lond) 2002;103:275–82.
7. Ponce LL, Navarro JC, Ahmed O, Robertson CS. Erythropoietin neuroprotection with traumatic brain injury. Pathophysiol 2013;20:31–8.
8. Celik M, Gökmen N, Erbayraktar S, Akhisarogu M, Konakci S, Ulukus C, et al. Erythropoietin prevents motor neuron apoptosis and neurologic disability in experimental spinal cord ischemic injury. Proc Natl Acad Sci USA 2002;99:2258–63.
9. Siren AL, Fratelli M, Brines M, Goemans C, Casagrande S, Lewczuk P, et al. Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. Proc Natl Acad Sci USA 2001;98:4044–9.
10. Rarick MU, Loureiro C, Groshen S, Sullivan-Halley J, Gill PS, Bernstein-Singer M, et al. Serum erythropoietin titers in patients with human immunodeficiency virus (HIV) infection and anemia. J Acquir Immune Defic Syndr 1991;4:593–7.
11. Revicki DA, Brown RE, Henry DH, McNeill MV, Rios A, Watson T. Recombinant human erythropoietin and health-related quality of life of AIDS patients with anemia. J Acquir Immune Defic Syndr 1994;7:474–84.
12. Cascinu S, Fedeli A, Del Ferro E, Luzi Fedeli S, Catalano G. Recombinant human erythropoietin treatment in cisplatin-associated anemia: a randomized, double-blind trial with placebo. J Clin Oncology 1994;12:1058–62.
13. Spivak JL. Recombinant human erythropoietin and the anemia of cancer. Blood 1994;84:997–1004.
14. Nemoto T, Yokota N, Keane WF, Rabb H. Recombinant erythropoietin rapidly treats anemia in ischemic acute renal failure. Kidney Int 2001;59:246–51.
15. Cazzola M, Mercuriali F, Brugnara C. Use of recombinant human erythropoietin outside the setting of uremia. Blood 1997;89:4248–67.
16. Krapf R, Hulter HN. Arterial hypertension induced by erythropoietin and erythropoiesis-stimulating agents (ESA). Clin J Am Soc Nephrol 2009;4:470–80.
17. Singbartl G. Adverse events of erythropoietin in long-term and in acute/short-term treatment. Clin Investig 1994;72:36–43.
18. Kim JY, Kim YG, Baik JY, Joo EJ, Kim YH, Lee GM. A proteomic approach for identifying cellular proteins interacting with erythropoietin in recombinant Chinese hamster ovary cells. Biotechnol Prog 2010;26:246–51.

19. Christensen B. Serum proteomic changes after randomized prolonged erythropoietin treatment and/or endurance training: detection of novel biomarkers. PloS One 2015;10:e0117119
20. Bader A, Pavlica S, Deiwick A, Lotkova H, Kucera O, Darsow K, et al. Proteomic analysis to display the effect of low doses of erythropoietin on rat liver regeneration. Life Sci 2011;89:827–33.
21. Pellegrin S, Heesom KJ, Satchwell TJ, Hawley BR, Daniels G, van den Akker E, et al. Differential proteomic analysis of human erythroblasts undergoing apoptosis induced by epo-withdrawal. PloS One 2012;7:e38356.
22. Kaindl AM, Sifringer M, Koppelstaetter A, Genz K, Loeber R, Boerner C, et al. Erythropoietin protects the developing brain from hyperoxia-induced cell death and proteome changes. Ann Neurol 2008;64:523-34.
23. Meloni BP, Tilbrook PA, Boulos S, Arthur PG, Knuckey NW. Erythropoietin preconditioning in neuronal cultures: signaling, protection from in vitro ischemia, and proteomic analysis. J Neurosci Res 2006;83:584–93.
24. Jungblut PR, Holzhütter HG, Apweiler R, Schlüter H. The speciation of the proteome. Chem Central J 2008;2:16.
25. Schlüter H, Apweiler R, Holzhütter HG, Jungblut PR. Finding one's way in proteomics: a protein species nomenclature. Chem Central J 2009;3:11.
26. Klose J, Kobalz U. Two-dimensional electrophoresis of proteins: an updated protocol and implications for a functional analysis of the genome. Electrophoresis 1995;16:1034–59.
27. Vittori D, Pregi N, Perez G, Garbossa G, Nesse A. The distinct erythropoietin functions that promote cell survival and proliferation are affected by aluminum exposure through mechanisms involving erythropoietin receptor. Biochimic Biophysic Acta 2005;1743:29–36.
28. Alural B, Duran GA, Tufekci KU, Allmer J, Onkal Z, Tunali D, et al. Epo mediates neurotrophic, neuroprotective, anti-oxidant, and anti-apoptotic effects via downregulation of miR-451 and miR-885-5p in SH-SY5Y neuron-like cells. Front Immunol 2014;5:475.
29. Lundby C, Thomsen JJ, Boushel R, Koskolou M, Warberg J, Calbet JA, et al. Erythropoietin treatment elevates haemoglobin concentration by increasing red cell volume and depressing plasma volume. J Physiol 2007;578:309–14.
30. Raine AE, Roger SD. Effects of erythropoietin on blood pressure. Am J Kidney Dis 1991;18:76–83.
31. Vaziri ND. Mechanism of erythropoietin-induced hypertension. Am J Kidney Dis 1999;33:821–8.
32. Liu Y, Xu Y, Thilo F, Friis UG, Jensen BL, Scholze A, et al. Erythropoietin increases expression and function of transient receptor potential canonical 5 channels. Hypertension 2011;58:317–24.
33. Lee MS, Lee JS, Lee JY. Prevention of erythropoietin-associated hypertension. Hypertension 2007;50:439–45.
34. Wallach I, Zhang J, Hartmann A, van Landeghem FK, Ivanova A, Klar M, et al. Erythropoietin-receptor gene regulation in neuronal cells. Pediatr Res 2009;65:619–24.
35. Um M, Gross AW, Lodish HF. A "classical" homodimeric erythropoietin receptor is essential for the antiapoptotic effects of erythropoietin on differentiated neuroblastoma SH-SY5Y and pheochromocytoma PC-12 cells. Cell Signal 2007;19:634–45.
36. Pregi N, Vittori D, Perez G, Leiros CP, Nesse A. Effect of erythropoietin on staurosporine-induced apoptosis and differentiation of SH-SY5Y neuroblastoma cells. Biochim Biophysic Acta 2006;1763:238–46.
37. Wenker SD, Chamorro ME, Vittori DC, Nesse AB. Protective action of erythropoietin on neuronal damage induced by activated microglia. FEBS J 2013;280;1630–42.
38. Grace RF, Zanella A, Neufeld EJ, Morton DH, Eber S, Yaish H, et al. Erythrocyte pyruvate kinase deficiency: 2015 status report. Am J Hematol 2015;90:825–30.
39. Zanella A, Fermo E, Bianchi P, Valentini G. Red cell pyruvate kinase deficiency: molecular and clinical aspects. Br J Haematol 2005;130:11–25.
40. Vukelja SJ. Erythropoietin in the treatment of iron overload in a patient with hemolytic anemia and pyruvate kinase deficiency. Acta Haematol 1994;91:199–200.
41. Andersen FD, d'Amore F, Nielsen FC, van Solinge W, Jensen F, Jensen PD. Unexpectedly high but still asymptomatic iron overload in a patient with pyruvate kinase deficiency. Hematol J 2004;5:543–5.
42. Marshall SR, Saunders PW, Hamilton PJ, Taylor PR. The dangers of iron overload in pyruvate kinase deficiency. Br J Haematol 2003;120:1090–1.
43. Machado-Neto JA, Saad ST, Traina F. Stathmin 1 in normal and malignant hematopoiesis. BMB Rep 2014;47:660–5.

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