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Recognition and characterization of Erythropoietin binding-proteins in the brain of mice

Reza Kowsari [1], Rezvan Yazdian-Robati [1], Bibi Marjan Razavi [2], Atena Pourtaji [3], Maryam Ghorbani [4], Hediye Moghadam-Omranipour [1], Hossein Hosseinzadeh [3], Parisa Lari [3], Khalil Abnous [5, 6*]

[1] Department of Biotechnology, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran
[2] Targeted Drug Delivery Research Center, Department of Pharmacodinamy and Toxicology, Mashhad University of Medical Sciences, Mashhad, Iran
[3] Pharmaceutical Research Center, Department of Pharmacodynamy and Toxicology, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran
[4] Department of Pharmacology and Toxicology, Faculty of Medicine, Baqiyatallah University of Medical Sciences, Tehran, Iran
[5] Pharmaceutical Research Center, Mashhad University of Medical Sciences, Mashhad, Iran
[6] Department of Medicinal Chemistry, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran

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ABSTRACT

Objective(s): Erythropoietin (EPO), is a 34KDa glycoprotein hormone, which belongs to type 1 cytokine superfamily. EPO involves in erythrocyte maturation through inhibition of apoptosis in erythroid cells. Besides its main function, protective effects of EPO in heart and brain tissues have been reported. EPO has a critical role in development, growth, and homeostasis of brain. Furthermore EPO has great potential in the recovery of different brain diseases which are still under studying. In this research, EPO binding pattern to brain proteins in animal model was studied.

Materials and Methods: EPO antibody was covalently crosslinked to protein A/G agarose. in order to interact between EPO and its target in brain, about 5µg EPO added to brain homogenates(500ul of 1 mg/ml) and incubate at 4o C for 30 min. brain tissue lysate were added to agarose beads, After isolation of target proteins(EPO - protein) both one and two-dimensional gel electrophoresis were performed. Proteins were identified utilizing MALDI-TOF/TOF and MASCOT software.

Results: This research showed that EPO could physically interact with eightproteins including Tubulin beta, Actin cytoplasmic 2, T-complex protein 1, TPR and ankyrin repeat-containing protein 1, Centromere-associated protein E, Kinesin-like protein KIF7, Growth arrest-specific protein 2 and Pleckstrin homology-like domain family B member 2.

Conclusion: Since EPO is a promising therapeutic drug for the treatment of neurological diseases, identified proteins may help us to have a better understanding about the mechanism of protective effects of EPO in the brain. Our data needs to be validated by complementary bioassays.

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Introduction

Erythropoietin (EPO) or hematopoietin, a member of the type1cytokine superfamily, is a glycoprotein hormone, which is responsible for the regulation of erythropoiesis through inhibiting of apoptosis, proliferation and differentiation of erythroid precursor cells. Discovery of EPO and EPO receptor in neural cells indicated that, in addition to erythropoiesis, EPO has protective effects in the brain (1, 2). Studies over the past years revealed that EPO can protect neurons from injury and has an important role in the survival and proliferation in neural progenitor cells (3, 4).

Administration of recombinant human EPO in a rabbit model of subarachnoid hemorrhage induced acute cerebral ischemia, considerably decreased acute ischemic neuronal damage and increased the EPO concentration in the cerebrospinal fluid (5).

It has been shown that EPO can induce a wide range of cellular responses to protect and repair brain injury in different stress conditions like hypoxia and excitotoxicity (4, 6). Preventive effects of EPO against oxidative damage through increasing antioxidant enzymes such as superoxide dismutase and glutathione peroxidase have also been reported (7). EPO could reduce inflammation by inhibition of inflammatory mediators including TNF-α, interleukin-6 (IL-6), IL-1beta, IL- 1alpha and interferon-γ (8).

*Corresponding author: Khalil Abnous. Pharmaceutical Research Center,, Department of Medicinal Chemistry, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran. Tel: +98-51-3882 3268; Fax: +98-51-3882 3251; email: abnouskh@mums.ac.ir

Moreover EPO is involved in the recovery of traumatic brain and spinal cord injuries by inhibition of apoptosis and anti-oxidant properties, induction of neurogenesis and angiogenesis. According to documents a great potential of EPO in the recovery of stroke, multiple sclerosis, Alzheimer, huntington, Parkinson, traumatic brain and spinal cord injuries, has been shown(4, 9). Affinity chromatography technique has been widely used to isolate specific target proteins from a complex proteome. In order to isolate bound protein targets, small molecules are immobilized on to a solid matrix. The eluted proteins can then separate by gel electrophoresis and analyzed by mass spectrometry (1).

Drug target deconvolution is a process in which the biological role of a drug, a small molecule, is characterized through the identification of the proteins that interact with the drug and, so that, initiate the biological effect. Then, the biological relevant targetsareidentified froma mixture of proteins identified in such an approach. Beside the medically desired action of the drug, the identification of other proteins that could interact with the drug, could help to identify the side effects and toxicity at a very early stage of drug development (10). In this project we hypothesized that some of therapeutic effects are through the direct interaction between Erythropoeitin and proteins. The aim of this study we investigated Erythropoietin interacting proteins using affinity chromatography based target deconvolution.

Materials and Methods

Animals and tissue samples

Animal study was approved by MUMS (Mashhad University of Medical Sciences) Ethics Committee. Animals were housed at temperature of 25±2 °C on a 12-hr light/dark cycle with free access to food and water. Twelve male BALB/c mice (6 weeks old, 20–30 g) were sacrificed by decapitation. Mice brains were removed and washed using 0.9% normal saline solution. Brain tissues were frozen in liquid nitrogen and stored at −80 ˚C until use.

Separation of target proteins

Brain tissues (200 mg) were homogenized in 1 ml of lysis buffer, containing 50 mM Tris pH 7.4, 2 mM EGTA, 2 mM EDTA, 2 mM Na_3VO_4, 1% Triton X-100 and 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and complete protease inhibitor cocktail (Sigma P8340, USA) were added just before homogenization of tissues using a Polytron Homogenizer (IKA R T10, Germany) in ice. Homogenates were centrifuged at 10000× g for 15 min at 4 ˚C. Supernatants were collected and in order to interact between EPO and its target in brain, about 5 µg EPO added to brain homogenates (500 ul of 1 mg/ml) and incubate at 4 ˚C for 30 min.

Immunoprecipitation (IP)

EPO protein targets were isolated using IP. According to Pierce Crosslink immune-precipitation Kit protocol (Thermo Scientific, Cat number=26147). Briefly, 5 µg EPO antibody was covalently cross linked to protein A/G resin using disuccinimidylsuberate (DSS). 500 µl of 1 mg/ml tissue lysates were added to control resin, to eliminate proteins with unspecific interactions with beads. After a brief centrifugation, supernatant was added to EPO antibody-cross linked resin (IP step). After few washes, EPO binding proteins were eluted using elution buffer provided in kit (pH 2.8). Concentrated elute was dialyzed against deionized water using a membrane with 2000 Da cutoff (Spectra, USA) for 3 days at 2–8 ˚C and then Freeze dried.

Separation of target proteins on SDS-PAGE

Freeze dried elutes, from IP, step were dissolved in 20 µl of 2X SDS sample buffer, samples were incubated in boiling water for 5 min and then subjected to SDS-PAGE electrophoresis (BioRad). Gels were silver stained and protein bands were excised and collected in microtubes containing 1% acetic acid.

Two-dimensional gel electrophoresis

Freeze-dryed elutes were dissolved in rehydration buffer containing 6 M urea, 2 M thiourea, 2% 3-[(3 cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM dithiothreitol (DTT), 20% Bio-Lyte (BioRad). 125 µl of lysate was loaded to non-linear immobilized pH gradient (IPG) strips (pH range: 3–10, 7cm, BioRad) toseparate EPO target proteins according to their isoelectric point. Following active rehydration at 50V for 12 hrs, isoelectric focusing was performed using PROTEAN IEF CELL (BioRad) at 20 °C in 4 steps as follow: 250 V for 15min; 4000 V for 1 hrs; 35000 V for 1 hrs. IPGs were incubated in equilibration buffer [375 mMTris (pH 8.8), 6 M Urea, 2.5% SDS and 30% glycerol] for 12 min. IPGs were placed on top of 12% SDS-PAGE and sealed with agarose solution [25 mMTris (pH 8.8), 84 mM Glycin, 0.5% agarose, 0.1% SDS and amount of tracking dye bromophenol blue]. Electrophoresis was performed for 80 min at 120 V. After electrophoresis, gels were silver stained.

Protein spots were cut off utilizing sterile scalpel from gels and conveyed to a micro tube containing 1% acid acetic and posted to the Center for Genomic Sciences at the University of Hong Kong for identification and characterization by MALDI-TOF/TOF.

Mass spectrometry analysis

Mass data were acquired in the Genome Research Centre, University of Hong Kong with 4800 MALDI-TOF/TOF analyzer (ABI) in positive ion reflector mode. Data were investigated in both NCBInr and

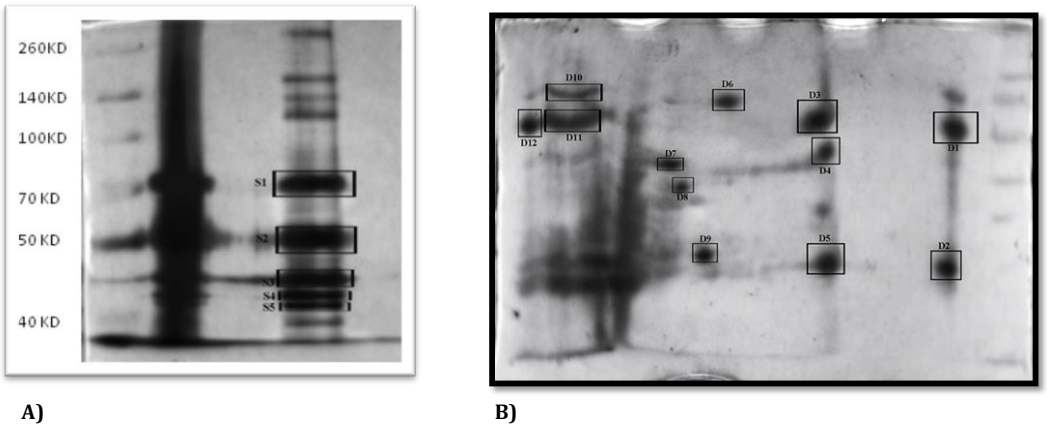

Figure 1. A) Image of cellular targets of EPO in mouse brain separated by SDS-PAGE in a 12 % gel. Gel was stained by Silver. B)Image of cellular targets of EPO in mouse brain separated by IEF in a 7 cm IPG strip containing nonlinear pH gradient 3–10 followed by two-dimensional gel electrophoresis. Protein detection was by Silver-staining

SwissProt databases. For analyzing Mass data MASCOT (version 2.1.0, Matrix Science, London, UK) was applied. MASCOT parameters were set as follow: Taxonomy: Rat, Fixed modification: Carbamidomethyl (Cysteine), Variable modification: Oxidation (Methionine), MS/MS fragment tolerance:±0.2 Da, Precursor tolerance: 75 ppm, peptide charge: +1, monoisotopic. Proteins with a score of > 50 and confidence interval (CI) > 95% were accepted.

Categorization of proteins was performed according to biological function and process using PANTHER classification system (http://www.pantherdb.org).

Results

In order to find cellular targets of EPO in the mice brain tissues, IP was performed. To eliminate unspecific binding of non-target proteins, tissue extracts were incubated with control agarose beads for 30 min, after a brief centrifugation, supernatant was incubated with EPO antibody-cross linked resin. Target proteins were eluted and subjected to 1D (one-dimensional gel electrophoresis) and 2D (two-dimensional gel electrophoresis) and MS analysis respectively. Several bands and spots 1D and 2D gels were detected as targets of EPO, respectively. Bands with enough protein for detection by MASS Spectroscopy were excised, in gel digested and identified (Figure 1).

Eight proteins were identified among the detected spots and bands including Tubulin beta, Actin cytoplasmic 2,T-complex protein 1,TPR and ankyrin repeat-containing protein 1, Centromere-associated protein E, Kinesin-like protein KIF7, Growth arrest-specific protein 2 and Pleckstrin homology-like domain family B member 2.

The information of identified proteins including protein name/gene names, sample name, Swiss-Prot accession number, confidence interval (C.I.%), protein score, sequence coverage (%), theoretical MW (Da)/pI, unique peptides detected, sequence of identified peptide with the highest ion score were listed in Table 1.

Table1. Identified proteins by MALDI/TOF/TOF in mouse brain

spot	Protein name	Gene name	Protein score	C.I %	Data base
S2	Tubulin beta	Tubb	140	100	Swissprot
S3	Actin, cytoplasmic 2	Actg1	531	100	Swissprot
S4	Actin, cytoplasmic 2	Actg1	545	100	Swissprot
S5	T-complex protein 1	Cct8	60	98.512	Swissprot
D11	Pleckstrin homology-like domain family B member 2	Phldb2	66	99.6	Swissprot
	Centromere-associated protein E	Cenpe	55	94.959	Swissprot
D12	Kinesin-like protein KIF7	Kif7	58	97.293	Swissprot
	Growth arrest-specific protein 2	Gas2	53	90.827	Swissprot
D2	TPR and ankyrin repeat-containing protein 1	Trank1	53	91.24	Swissprot

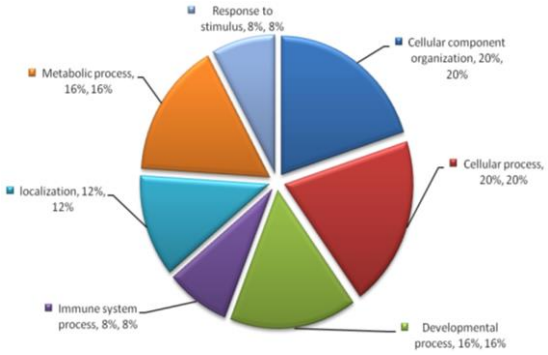

Figure 2. Gene names of EPO target proteins were submitted to PANTHER database and classified according on biological process to seven group. Most of identified proteins were involved in cellular component and cellular process

Gene names of EPO target proteins were submitted to PANTHER database and categorized based on the biological process (Figure 2).

Proteins were divided to seven classes including, cellular component organization, cellular process, immune system process, developmental process, localization, metabolic process and response to stimulus.

Discussion

In the present study, affinity chromatography based target deconvolution was chosen to find potential targets of EPO in the mice brain.Utilizing a proteomics approach and IP, we have identified 8proteins which can be considered as potential EPO target proteins in the mice brain tissue. Neuroprotective effects of EPO have been reported in several studies (1, 2). Different mechanisms including antiapoptotic, increased NO level in the brain vessels, anti-inflammation, maintenance of neuronal structure and survival, regulation of neuronal proliferation and differentiation have been attributed to EPO neuroprotective(1). This study revealed some novel EPO target proteins for the first time in mice brain tissue. These proteins were divided into seven groups based on biological process including cellular component organization, cellular process, immune system process, developmental process, localization, metabolic process and response to stimulus.

Effects of EPO on protein species involved in apoptosis pathway

It is well known that EPO could prevent neuronal apoptosis via several pathways, which include the activation of PKB (protein kinase-B) and the subsequent inhibition of caspase-8, -1, and -3, (11)Furthermore EPO could increase the activity of Akt1(12). Our results showed that EPO can regulate some proteins which involved in apoptosis. Gas2, a component of the microfilament system, belongs to the class of Gas genes whose expression is induced at growth arrest. Gas2 has a role in apoptosis and is considered as a substrate for caspases. This protein is cleaved during apoptosis and could induce actin cytoskeleton rearrangement. Gas2 is a target for caspase-7 and caspase-3 and can induce apoptosis (13).

It was identified that Gas- 2 can be expressed in frontal cortex in cocaine exposed mice. This protein regulates cell shape modifications during apoptosis (14).Gas2 can be up regulated in growth arrested fibroblasts. In addition Gas2, can be acted as a inducer of microfilaments and microtubules modifycations in the growth arrested cells (15).

Due to the direct role of Gas2 protein in several apoptosis pathways, it can be hypothesized that EPO, as an inhibitor of apoptosis, can act through Gas2 and caspase pathways.

Pleckstrin (LL5β) is another protein which is involved in apoptosis pathway. Pleckstrins are part of LL5 proteins which are presented as a branch in Akt/protein kinase-B. One study showed that the activation of pleckstrin branch in Akt/PKB resulted in anti-apoptotic effects in ischemic astrocytes of rat brain hippocampus (16). Pleckstrin is also connected to phosphoinositides and intracellular cytoskeleton proteins. LL5β protein causes accumulation of acetylcholine receptors at the postsynaptic membrane. In addition LL5β can regulate intracellular and membrane skeleton in response to extracellular signaling (17). Considering the role of Pleckstrin in apoptosis and cell survival, organization and maintenance of cell structure and its presence as a part of Akt/protein kinase-B, effective interaction of this protein and EPO in our study is verified.

According to the documents, actin cytoplasmic 2 plays an important role in apoptosis. Transglutaminase enzyme is widely expressed in apoptosis occurring areas. Cytoplasmic actin is one of the intracellular proteins associated with this enzyme. It is recognized that actin acts as a substrate for tissue transglutaminase which can be considered as a regulator of apoptosis biochemical events through transglutaminase dependent cross-linking. It is well known that apoptosis increases the degradation of actin fibers. Actins are substrates of caspases which are responsible for proteolytic breakdown. It is also reported that fragmentation of actins may be associated with induction of apoptosis. ROS mitochondrial generation is considered as one of the main mechanisms involved in apoptosis. Higher actin content is associated with decrease of ROS formation and increase in cell survival. Studies showed that actin inhibitors (eg, Jasplakinolide) could induce ROS production (18).

Studies on T-cells dependent IL-2, indicated that Jasplakinolide diminished the functionality of actin

resulting T-cell apoptosis via increased activity of caspase- 3(19).

It was identified that actin regulatory proteins known as Rsp5p, End3p, sla1p ubiquitin ligase (ubiquitin ligase RSp5p) are primary regulators of oxidative stress associated with actin (20). Function removal of End3p and sla1p led to poor cytoskeleton stability which causes loss of mitochondrial membrane potential and increased oxidative stress (21).Actin is one of the main components of structural cytoskeleton in brain and spinal cord neurons. Many regulatory of actin dependent proteins which are presented in neurons are expressed during axonal growth and dendrite formation. One of these proteins is drebrin. The amount of drebrin is decreased in the frontal and temporal cortex in Alzheimer's disease. In neurodegenerative diseases Actin fragmentation could induce changes in cell morphology, resulting in dysfunction of neuronal receptors signaling (22). It has been indicated that another protein which has a role in actins regulation through attachment to it, is apolipoprotein-E (Apo E). Apo E is also involved in Alzheimer's disease process (23). Regarding the beneficial effects of EPO on Alzheimer disease, it can be concluded that actin could be considered as one of the main proteins which can interact with EPO.

Effects of EPO on protein species involved in cell cycle regulation, development and differentiation.

It has been established that EPO/EPOR systemplay a role both in neurodevelopment and brain homoeostasis. According to the results TPR and ankyrin repeat-containing protein 1 is another EPO target proteins.This protein has six ankyrin repetitive sequences and four TPR (tetra-tricopeptide repeat) repetitive sequences, which is distributed in the brain hippocampus and hypothalamus.

Ankyrin repetitive sequences have been found in many proteins which are involved in cell signaling, maintenance of cytoskeletal structure, cell cycle regulation, inflammatory responses, growth, differentiation and other transport phenomena (24). TPR sequences is also involved in various functions including cell cycle regulation, protein transport and folding proteins (25).

According to the important role of this protein in the cell cycle regulation, growth, differentiation and inflammatory response, it may be considered as one of the functional pathway of EPO in the nervous system.

Kinesin-like protein (KIF7) is also involved in neuronal proliferation, development and microtubule-based transport systems (26). This protein is also considered as a regulator in Sonic hedgehog pathway (Shh pathway) during cell development (27). It has been reported that mutation in the Kif7gene leads to Joubertsyndrome which is a brain abnormality.

Centromere-associated protein-E (CENP-E) is a critical mitotic kinesin which is essential for efficient, stable microtubule capture at kinetochores. It can bind to a kinetochore-associated kinase implicated in the mitotic checkpoint, which is the major cell cycle control pathway. This protein is expressed in the hippocampus (28). One study revealed that CENP-E was over expressed in glioblastoma and astrocytic tumors (29).

Another EPO target protein is T-complex protein 1 which acts as a chaperone that is associated with ATP hydrolysis. It has important roles in actin and tubulin folding, growth and development of nerve cells (30).T-complex protein 1 was identified in rat hippocampus and human cortical neurons (31, 32)

B tubulin, specifically expressed in neurons, is involved in microtubule formation. In mitosis, microtubules have a special role in appropriate chromosomal division in cells (33).

Mesenchymial stem cells (MSCs) may improve symptoms in neurodegenerative diseases. Under normal concentration of oxygen (normoxic), human MSCs can weakly express EPO receptors. EPO administration in normoxic conditions results in expression of tubulin beta-3 and induces cells with neuronal morphology (34).The relevance of tubulin protein and Huntington's disease is also identified (35). It was reported that co- administration of EPO and paclitaxel can inhibit neuronal axonal damage due to the lower level of detyrosinated tubulin (36). Detyrosinated tubulin is a marker for microtubule stabilization. Stabilized microtubules disrupt mitotic processes and arrest the cell division. Considering the role of tubulin in neural cell death, neuronal structure and mitosis, the interaction of EPO and beta tubulin in neurons could be concluded.

Conclusion

EPO is a promising therapeutic drug for the treatment of neurological diseases, in this study we successfully used IP and proteomics techniques for elucidation of the target proteins involved in Epo-induced neuroprotection for potentially powerful therapeutical tool. Identified proteins may help us to have a better understanding about the mechanism of protective effects of EPO in the brain.

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Conflict of interest

The authors have declared no conflict of interest.

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