

Recombinant human erythropoietin: effects on frataxin expression *in vitro*

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Abstract

Background Friedreich's ataxia (FRDA) is a neurodegenerative disorder caused by decreased expression of the protein frataxin, recently described to be an iron chaperone for the assembly of iron-sulphur clusters in the mitochondria, causing iron accumulation in mitochondria, oxidative stress and cell damage. Searching for compounds that could possibly influence frataxin expression, we found that the cytokine recombinant human erythropoietin (rhuEPO) significantly increases frataxin expression by a still unknown mechanism.

Materials and methods Isolated lymphocytes from FRDA patients, isolated human cardiac cells (fibroblasts and myocytes) from patients undergoing heart transplantation and P19 mouse cells (neuronal type), were incubated with different concentrations of rhuEPO, and immunoblot was carried out for the detection of frataxin.

Results We show for the first time that the cytokine recombinant human erythropoietin (rhuEPO) can, additionally to its reported neuro- and cardioprotective properties, increase frataxin expression *in vitro*. We show that rhuEPO significantly increases frataxin expression in primary lymphocytes from patients with Friedreich's ataxia. Further we show that rhuEPO can also increase frataxin expression in many other cell types; among them the most affected cell types in FRDA such as neurones and cardiac cells.

Conclusion Our results provide a scientific basis for further studies examining the effectiveness of this agent for the treatment of FRDA patients.

Keywords Frataxin, Friedreich's ataxia, recombinant human erythropoietin.
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Introduction

Friedreich's ataxia (FRDA) is the most common of the inherited ataxias, affecting 1 : 50 000 people [1]. Clinically, FRDA is characterized by multiple symptoms including

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progressive gait and limb ataxia, dysarthria, diabetes mellitus and hypertrophic cardiomyopathy [2]. There is currently no effective treatment for FRDA.

Friedreich's ataxia is caused by a GAA-trinucleotide expansion in the frataxin gene located on chromosome locus 9q13, resulting in a reduced expression of frataxin, a small mitochondrial protein [3,4]. Owing to the mitochondrial localization of frataxin, the neurological and cardiological degenerations observed in FRDA are thought to be the result of a mitochondrial defect [5]. The exact physiological function of frataxin is unknown, but it may be involved in mitochondrial iron homeostasis and/or assembly of iron-sulphur (FeS) proteins and heme synthesis [6]. Intra-mitochondrial iron accumulation has been postulated to initiate the production of hydroxyl radicals by Fenton chemistry, leading to inactivation of FeS enzymes, lipid peroxidation and damage to nucleic acids, proteins and finally resulting in cell death. There is some debate whether mitochondrial iron accumulation within mitochondria is the result or the cause of the oxidative stress responsible for

mitochondrial damage. Studies with conditional knockout mouse models and FRDA-patient cells indicate that deficiencies in FeS enzymes precede iron accumulation [7,8]. Clinically there is an intramitochondrial iron accumulation in heart, liver, nervous system and spleen of FRDA-patients, as well as a reduction of mitochondrial DNA, the FeS cluster-containing subunits of the mitochondrial electron transport chain (complex I–III) and of the enzyme aconitase [9,10]. The presence of increased levels of soluble transferrin receptor as indicator for cytosolic iron deficiency is controversial [11–13] but in general FRDA patients have normal serum iron and ferritin concentrations [14].

Frataxin is implicated to be necessary for normal heme biosynthesis, but there are no reports that FRDA is commonly associated with anaemia [15].

There is currently no effective treatment for FRDA available, especially for neurological deficits. However, the improved understanding of the role of frataxin has led to the consideration of antioxidants such as Idebenone and iron chelators as potential therapeutic agents. Recently, Seznec *et al.* reported a cardioprotective function of Idebenone in a mouse model [16]. These drugs may have a potential to reduce some clinical features of FRDA, but they cannot cure the disease itself. Another approach to treat FRDA is by gene therapy, which will not be readily available within the near future.

Stimulation of frataxin with exogenous substances was shown with hemin and butyric acid [17], and with substances generating reactive oxygen species (such as 3-nitropropionic acid) [18] or those which are cytotoxic like cisplatin [19]. These findings indicate that the cells can increase frataxin-expression in response to various types of stress to protect the cell.

Recently recombinant human erythropoietin has received considerable attention because of the unexpected finding that it has also broad neuroprotective and cardioprotective capabilities [20–24] by a still poorly understood mechanism. It has been known for a long time that erythropoietin signalling plays a key role in bone marrow erythrocyte proliferation and haemoglobin synthesis.

The aim of this study was to investigate the influence of rhuEPO on frataxin expression in various cell types. Additionally to its reported broad neuroprotective and cardioprotective capabilities we found that the cytokine recombinant human erythropoietin significantly increases frataxin expression. We could show this effect in primary lymphocytes from FRDA patients, primary human cardiac cells and in a neuronal cell line. Therefore our results provide a scientific basis for further studies examining the effectiveness of this agent for the treatment of FRDA patients.

Materials and methods

Reagents and antibodies

All chemicals were purchased from Sigma (Vienna, Austria) if not cited otherwise. The primary rabbit polyclonal antibody against mature human and mouse frataxin was a kind gift from Prof. Gracia Isaya, Departments of Pediatric &

Adolescent Medicine and Biochemistry & Molecular Biology Mayo Clinic College of Medicine, Rochester, MN; the secondary goat-anti-rabbit horse radish peroxidase conjugated antibody was from DakoCytomation (Vienna, Austria). Recombinant human erythropoietin (epoietin beta) was obtained from Roche, Basel, Switzerland.

Patients

Seven patients with Friedreich's ataxia (GAA repeats in the range from 240 to 800) were included in this study after having given their informed consent.

Ethics

All human materials were obtained and processed according to the recommendations of the hospital's Ethics Committee and Security Board including informed consent. The recommendations of the Hospital's Ethics Committee and Security Board are in accordance with the Declaration of Helsinki on ethical principles for medical research involving human subjects.

Cell culture

Lymphocytes

The lymphocytes from seven FRDA patients were collected from fresh blood samples and isolated with Biocoll Separating Solution, density 1.077 g mL^{-1} (Biochrom AG, Berlin, Germany) according to the manufacturer's procedure. Finally, cells were diluted to a density of 1×10^6 cells and cultured in RPMI media supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics, and were used for experiments.

Cardiac cells

Primary cultures of human adult cardiac myocytes and human adult cardiac fibroblasts from patients not suffering from FRDA but undergoing heart transplantation were isolated as described by Macfelda *et al.* [25]. The cells were cultivated in M199 medium containing 10% fetal calf serum as well as 100 U mL^{-1} penicillin, $100 \mu\text{g mL}^{-1}$ streptomycin, $10 \mu\text{g mL}^{-1}$ transferrin and $10 \mu\text{g mL}^{-1}$ insulin at 37°C in a humidified atmosphere of 5% CO_2 .

Neuronal cells

The P19 clone was obtained from the European Cell Culture Collection (ECACC Cat. Nr. 95102707, Salisbury, UK). Cells were cultured in α -modified Eagle's medium (α -MEM) supplemented with 7.5% calf serum (Euroclone, Vienna, Austria) and 2.5% fetal bovine serum (Gibco, Vienna, Austria), 2 mM L-glutamine, 10 mM essential amino acids and antibiotics in a 5% CO_2 humidified chamber. Cellular differentiation was carried out as described by Santos *et al.* [26].

Immunoblotting of frataxin

Expression of frataxin was detected by Western blot. After treatment with rhuEPO for the indicated periods and after extensive washings the cells were lysed with cell culture lysis reagent (Promega, Vienna, Austria) and transferred to a microcentrifuge tube. Fifty micrograms of proteins were separated on 12% SDS (sodium dodecyl sulphate) – polyacrylamide gel electrophoresis under nonreducing conditions using ProSieve 50 Gel solution (BMA, BioWhittaker from Biozym, Vienna, Austria) and Tris/Tricine-electrode buffer (0.1 M Tris, 0.1 M Tricine, 0.1% SDS, pH 8.3) and electroblotted onto nitrocellulose membranes. Primary antibody was directed against mature frataxin (a kind gift from Dr Gracia Isaya, USA), and as a secondary antibody a goat-anti rabbit HRP antibody (1 : 10 000) (DAKO) was used.

Statistical analysis

Statistical analysis was performed with GraphPad Prism software. Differences were examined for statistical significance using the *t*-test. Significant differences are marked in the figures with **P* < 0.05, ***P* < 0.01 and ****P* < 0.001. Differences with *P* < 0.05 were assumed to be significant.

Results

Influence of rhuEPO on frataxin expression in isolated lymphocytes from FRDA patients

We investigated the effects of rhuEPO on primary lymphocytes isolated from seven patients with Friedreich's ataxia (GAA repeats ranging from 240 to 800). The lymphocytes were incubated with various concentrations of rhuEPO for 24 h and frataxin expression was assayed by Western blot analysis. The basal levels of frataxin varied among the patients, but in general the increase in frataxin expression correlated with the concentration of rhuEPO used (Fig. 1b). Figure 1a represents a Western blot for frataxin from one FRDA-patient, where the isolated lymphocytes were treated with rhuEPO for 24 h.

We have strong evidence that this approach could also work *in vivo* owing to the fact that we could find a significant increase in frataxin expression in lymphocytes obtained from dialysis patients 48 h after receiving rhuEPO compared with lymphocytes obtained from the same patients before rhuEPO-administration (unpublished observation).

Effects of rhuEPO on frataxin expression in human cardiomyocytes and cardiofibroblasts

As a next step we assayed whether the effect of rhuEPO on frataxin expression can also be found in other tissues. The heart is one of the most affected organs in FRDA patients, therefore we investigated the effects of rhuEPO on frataxin expression in primary cultures of human adult cardiac

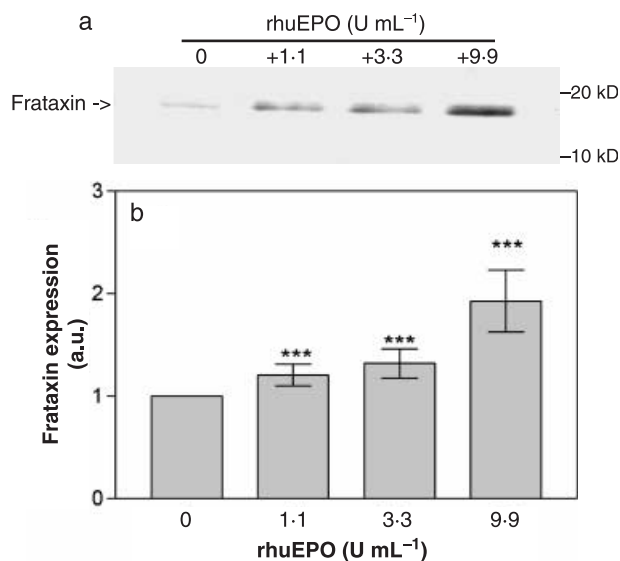


Figure 1 Effects of rhuEPO on frataxin-expression in primary lymphocytes from patients with Friedreich's ataxia. Freshly isolated lymphocytes obtained from seven patients with Friedreich's ataxia were incubated with rhuEPO for 24 h. Cell lysates (50 µg protein) were separated on 12% SDS–polyacrylamide gel electrophoresis under nonreducing conditions using Tris/Tricine-electrode buffer (0.1 M Tris, 0.1 M Tricine, 0.1% SDS) and electroblotted onto nitrocellulose membranes. Western-blot analysis was performed with a polyclonal antibody against human frataxin. (a) Western blot and (b) Western blot densitometric analysis of frataxin expression from three independent experiments. Density of the frataxin band of the control (untreated lymphocytes in the absence of rhuEPO from the same patient) was set as 1 a.u. (arbitrary units). Values represent means ± SEM of three different experiments. Differences were examined for statistical significance using the paired *t*-test. Significant differences vs. control are marked in the figures with ****P* < 0.001.

myocytes (HACMs) and cardiofibroblasts, prepared from ventricular tissue obtained from donor hearts from patients undergoing heart transplantation. We incubated the cells with rhuEPO for 48 h and found in human primary cardiomyocytes (Fig. 2a) and cardiofibroblasts (Fig. 2b) a significant increase in frataxin expression following incubation with rhuEPO. This finding indicates that the increase in frataxin expression by rhuEPO is a more general mechanism and not limited to lymphocytes.

Effects of rhuEPO on neuronal frataxin expression

Mouse embryonic carcinoma P19 cells were differentiated into neuronal cells. To investigate the influence of rhuEPO on frataxin expression, the cells were incubated with rhuEPO for up to 48 h. After the treatment the cells were washed and lysed and expression of frataxin was detected by Western blotting. In P19 cells (neuronal type) there was a significant increase of frataxin expression following incubation with rhuEPO for 24 h (Fig. 3a) and 48 h (Fig. 3b). Frataxin expression increased up to 2.5 fold when the cells

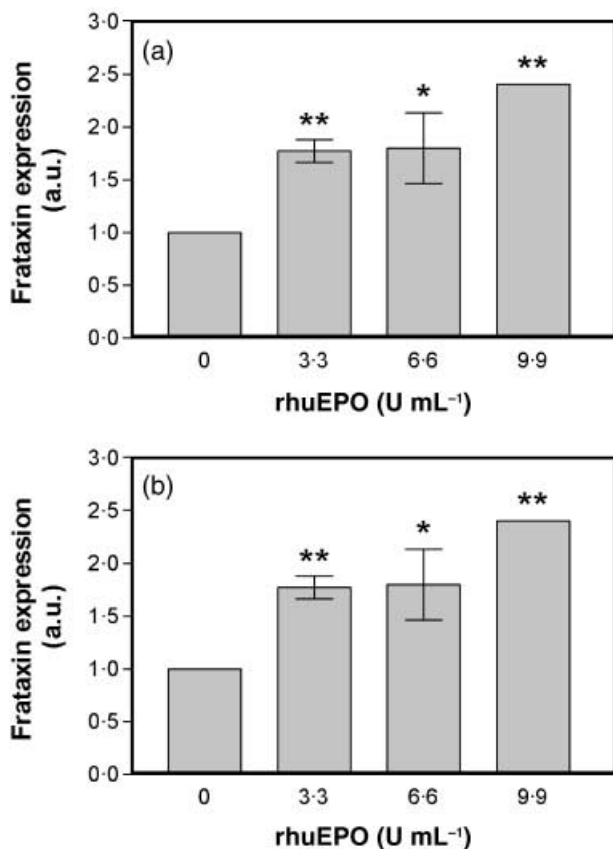


Figure 2 Frataxin-expression in primary human heart cells. Primary cultures of human adult cardiac myocytes (a) and cardiac fibroblasts (b) were incubated with rhuEPO for 48 h. Cell lysates (40 µg protein) were separated on 12% SDS-polyacrylamide gel electrophoresis under nonreducing conditions using Tris/Tricine-electrode buffer (0.1 M Tris, 0.1 M Tricine, 0.1% SDS) and electroblotted onto nitrocellulose membranes. Western-blot analysis was performed with a polyclonal antibody against human frataxin. Densitometric analysis of frataxin expression from three independent experiments is shown. Density of the frataxin band of the control (in the absence of rhuEPO) was set as 1 a.u. (arbitrary units). Values represent means \pm SEM of three different experiments. Some of the error bars are smaller than the symbols. Differences were examined for statistical significance using the paired *t*-test. Significant differences vs. control are marked in the figures with **P* < 0.05 and ***P* < 0.01.

were treated with rhuEPO for 24 h compared with the untreated control cells.

Effects of short-term incubation with rhuEPO on neuronal frataxin expression

Next we studied the effect of short-term incubation with rhuEPO and further cultivation in the absence of rhuEPO on neuronal frataxin expression. We found that short-term incubation (for 1 h) of P19 neuronal cells with rhuEPO and further cultivation in the absence of rhuEPO was sufficient to observe the same increase in frataxin-expression after

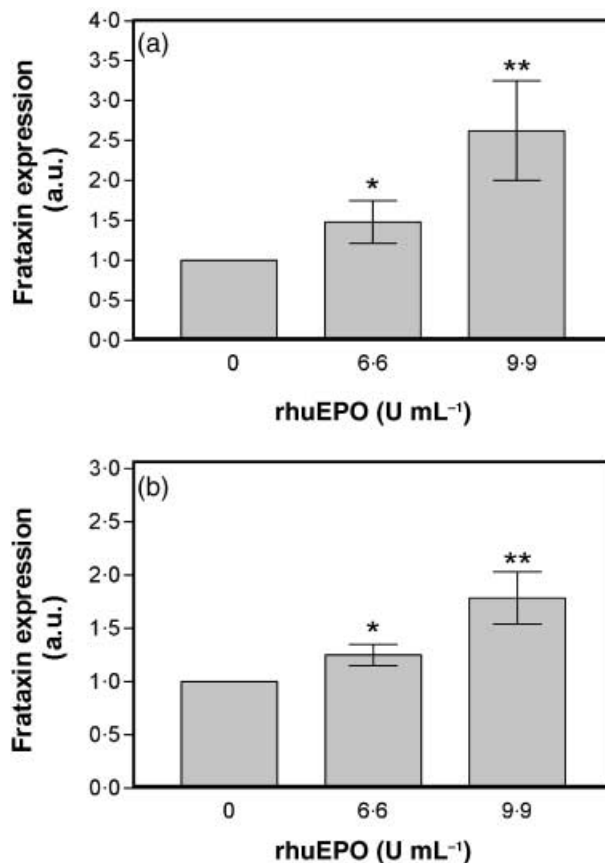


Figure 3 Frataxin expression in neuronal cells increases after treatment with rhuEPO. P19 (neuronal-type) cells were incubated with rhuEPO for 24 h (a) and 48 h (b). Cell lysates (50 µg protein) were separated on 12% SDS-polyacrylamide gel electrophoresis under nonreducing conditions using Tris/Tricine-electrode buffer (0.1 M Tris, 0.1 M Tricine, 0.1% SDS) and electroblotted onto nitrocellulose membranes. Western-blot analysis was performed with a polyclonal antibody against human frataxin, which also detects mouse frataxin due to 94% sequence homology. Western blot densitometric analysis of frataxin expression from three experiments is shown. Density of the frataxin band of the control (in the absence of rhuEPO) was set as 1 a.u. (arbitrary units). Values represent means \pm SEM of 3 different experiments. Differences were examined for statistical significance using the paired *t*-test. Significant differences vs. control are marked in the figures with **P* < 0.05 and ***P* < 0.01.

48 h, as in cells incubated for the whole incubation time with rhuEPO (Fig. 4). These findings indicate that derivatives of erythropoietin with short plasma half-life such as asialoerythropoietin could also be effective to increase frataxin-expression in mammals.

Discussion

There exists a correlation in Friedreich's ataxia patients between age of onset, disease progression and the number

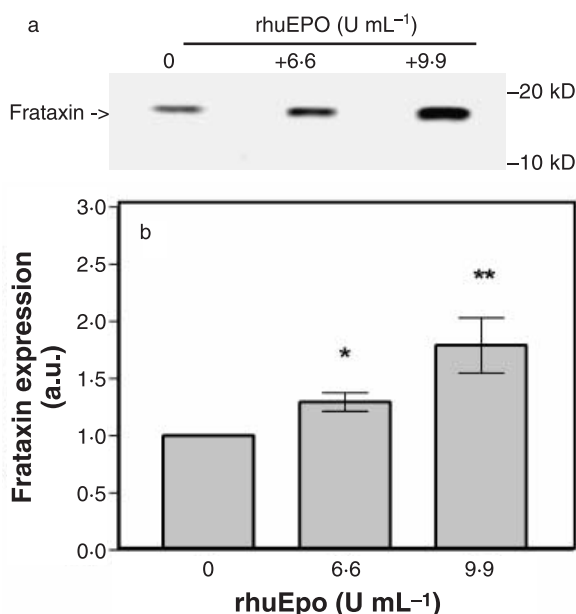


Figure 4 Frataxin expression after short-term treatment with rhuEPO. P19 (neuronal-type) cells were incubated with rhuEPO for 1 h. After washings, the cells were further incubated in the absence of rhuEPO for 48 h. Cell lysates (50 µg protein) were separated on 12% SDS-polyacrylamide gel electrophoresis under nonreducing conditions and electroblotted onto nitrocellulose membranes. Western-blot analysis was performed with a polyclonal antibody against human frataxin. (a) Western blot and (b) Western blot densitometric analysis of frataxin expression from three independent experiments. Density of the frataxin band of the control (untreated lymphocytes in the absence of rhuEPO from the same patient) was set as 1 a.u. (arbitrary units). Values represent means \pm SEM of three different experiments. Differences were examined for statistical significance using the paired *t*-test. Significant differences vs. control are marked in the figures with * $P < 0.05$ and ** $P < 0.01$.

of GAA repeats. This suggests that the length of the expansion modulates the amount of residual frataxin present in patients and hence clinical severity. Peripheral blood leucocytes of FRDA patients have a residual level of frataxin mRNA ranging between 13% and 30%, and FRDA carriers have approximately 40% of controls. Asymptomatic carriers also show reduced frataxin mRNA levels [27]. The amount of residual frataxin in lymphoblastoid cell lines from Friedreich's ataxia patients varies between reports from 4 to 29% [28] and 6 to 8% [18] of the level in the normal controls. To date, no data have shown the actual level of frataxin protein in FRDA patients *in vivo*.

Searching for compounds that could provide a new treatment for FRDA patients, we found that the cytokine recombinant human erythropoietin significantly increases frataxin expression in primary lymphocytes from FRDA patients in a dose-dependent manner, by a still unknown mechanism (Fig. 1). Erythropoietin possesses biological activities in addition to the erythropoietic effects that originally provided its name [29–32]. Recently rhuEPO has received considerable attention owing to its broad neuroprotective and

cardioprotective capabilities [23,29,33] by a still poorly understood mechanism. We suspect that the function of rhuEPO in tissue protection could also be mediated by increasing frataxin expression, but this has to be further investigated. In this study, we demonstrated that rhuEPO increases frataxin expression in various cell types in a dose-dependent manner. We used primary human cardiac fibroblasts and myocytes and tested the influence of rhuEPO on frataxin expression. We found a significant increase in frataxin levels, especially in cardiac fibroblast where a 2.5-fold increase after 48 h of rhuEPO could be obtained. This result is important because the main cause of premature death in FRDA is cardiomyopathy. Increasing frataxin expression in the heart would possibly protect the heart from the development of a cardiomyopathy and could therefore increase life expectancy. Moreover, as frataxin is postulated to function as a tissue protective protein, increasing frataxin expression could also represent a new target to treat cardiomyopathy in the general population.

Many cell types produce erythropoietin and many cells besides erythroid progenitors express the erythropoietin-receptor, including cells in the brain [31,33]. The discovery that neuronal cells produce EPO in response to a variety of insults including ischaemia/hypoxia, trauma, immune-mediated inflammation, and excessive neuronal excitation [29–32,34] further supports the pleiotropic nature of this cytokine. Using mouse embryonic carcinoma P19 cells (neuronal type) we found significant increases in frataxin expression after incubation with 6.6 U mL⁻¹ and 9.9 U mL⁻¹ rhuEPO for 24 and 48 h (Fig. 3a,b). Our experiments with P19 neuronal type cells (Fig. 4) also indicate that EPO-derivatives with shorter plasma half-life than rhuEPO, like asialoerythropoietin, could be a good nonerythropoietic alternative to rhuEPO. This can be explained by the fact that only short-term incubation with rhuEPO already leads to an increase in frataxin expression and that rhuEPO does not have to be present for a long time to stimulate an increase in frataxin. However, a controlled application of erythropoietically active rhuEPO for certain periods eventually accompanied by phlebotomy in the case of increased haematocrit could also be useful to reduce mitochondrial iron accumulation by triggering mitochondrial heme-biosynthesis and erythropoiesis. This approach to reduce mitochondrial iron load is currently successfully used in other diseases with mitochondrial iron accumulation like myelodysplastic syndrome and sideroblastic anaemia [35]. Such a protocol could be useful especially for patients with large iron deposits in the myocardium [36] and in the dentate nucleus [37] because current clinically available iron chelators do not reach mitochondrial iron deposits.

Over the last decade, rhuEPO has proven to be a safe therapeutic agent in haemodialysis patients with minimal adverse effects [38]. To confirm the *in vitro* effects of rhuEPO on frataxin-expression, we measured frataxin levels in lymphocytes obtained from haemodialysis patients undergoing rhuEPO treatment. We could find a significant increase (up to three-fold) in frataxin expression in lymphocytes obtained from dialysis patients 48 h after receiving rhuEPO compared with lymphocytes obtained from the same patients

before rhuEPO-administration. The patients suffered from endstage renal disease and received dosages of EPO ranging from 3000 to 10 000 U (unpublished observation).

This observation indicates that rhuEPO therapy increases frataxin expression in patients. Our data show for the first time that additionally to its neuro- and cardioprotective properties, rhuEPO increases frataxin expression. The results of our study therefore provide a scientific basis for examining the effectiveness of this agent for the treatment of FRDA patients.

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