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The effect of erythropoietin application on erythrocyte Na,K- ATPase activities in patients with diabetic polyneuropathy

[Diyabetik polinöropatili hastalarda eritropoietin uygulamasının eritrosit Na,K-ATPaz aktivitesi üzerine etkileri]

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ABSTRACT

Objectives: In this study we aimed to investigate the effects of erythropoietin administration on Na,K-ATPase enzyme activity in diabetic patients with polyneuropathy.

Methods: The study was carried out at Meram Education and Research Hospital department of Nephrology and Selcuk University Meram School of Medicine Hospital department of Biochemistry, Turkey. Eleven diabetic patients with diabetic polyneuropathy and on erythropoietin therapy and ten healthy control were contributed to this study. Diabetic group was formed from the patients with polyneuropathy and on erythropoietin therapy. Erythropoietin was administered with a dose of 150 U/kg/ week to the patients. Heparinized blood samples were taken from the control group for once and from the patients for twice before and one month after the erythropoietin therapy. Erythrocyte membranes were isolated and erythrocyte membrane Na,K-ATPase enzyme activities were measured. Na,K-ATPase activities were given as $\mu molP_i.mg.protein^{-1}.10\ min^{-1}$.

Results: Na,K-ATPase enzyme activities were 2.91±0.38 μmolPi.mg.prt^{-1}.10min^{-1}, 1.93±0.38 **μmolPi.mg.prt**$^{-1}$.10min^{-1} and 2.40±0.63 μmolPi.mg.prt^{-1}.10min^{-1} in control group, and patient group
before and after therapy, respectively. Na,K-ATPase enzyme activities decreased in patient group before the therapy compared to the control group and significantly increased in patient group after erythropoietin therapy compared to the basal levels of the patients. We determined 24% increase in erythrocyte Na,K-ATPase activities after one month EPO application compared to the basal levels.

Conclusion: It was concluded that erythropoietin therapy could improve neuropathy and this improvement was supported with the increase in Na,K-ATPase activities which could be used as a marker.

Keywords: Erythropoietin, Na-K ATPase, diabetic polyneuropathy

Conflict of Interest: We declare that there is no conflict of interest.

ÖZET

Amaç: Bu çalışmada diyabetik polinöropatili hastalarda eritropoietin uygulamasının eritrosit membranı Na,K-ATPaz aktivitesi üzerine etkisini araştırmayı amaçladık.

Metot: Çalışma Konya Eğitim ve Araştırma Hastanesi Nefroloji kliniği ve Selçuk Üniversitesi Meram Tıp Fakültesi Hastanesi Biyokimya bölümünde yapıldı. Bu çalışma eritropoietin tedavisine başlanacak, 11 diyabetik polinöropatili hasta ile sağlıklı 10 kontrol vakası üzerine gerçekleştirildi. Hastalar diyabetik polinöropati tanısı almış, eritropoietin tedavisine yeni başlanacak hastalardan seçilmiştir. Hastalarda eritropoietin 150 U/kg/hafta dozunda kullanıldı. Sağlıklı kontrollerden ve diyabetik hastalardan eritropoietin uygulamasından önce ve bir ay sonra olmak üzere heparinize kan örnekleri alındı. Eritrosit membranları izole edildi ve eritrosit membranı Na,K-ATPaz aktviteleri ölçüldü. Na,K-ATPaz aktivitesi $\mu molP_i.mg.protein^{-1}.10\ min^{-1}$ olarak verildi.

Bulgular: Na,K-ATPaz enzim aktivitesi kontrol grubunda ve eritropoietin uygulamasından önce ve sonra sırasıyla 2.91±0.38 μmolPi.mg.prt^{-1}.10min^{-1}, 1.93±0.38μmolPi.mg.prt^{-1}.10min^{-1} ve 2.40±0.63 μmolPi.mg.prt^{-1}.10min^{-1} olarak bulundu. Na,K-ATPaz enzim aktivitesi uygulama öncesi hasta grubunda kontrol grubu ile karşılaştırıldığında azalmış olarak, uygulama sonrası grupta hastaların bazal seviyeleri ile karşılaştırıldığında artmış olarak bulundu. Çalışmamızda Eritropoietin tedavisinin 30. günündeki eritrosit zarı Na,K-ATPaz enzim aktivitesinin tedavi öncesindeki Na,K-ATPaz enzim aktivitesine gore %24 arttığını gözlemledik.

Sonuç: Eritropoietin tedavisinin nöropatiyi iyileştirebildiği ve bu iyileşmenin, belirteç olarak kullanılabilen Na+-K+ ATPaz enzim aktivitesi artışı ile desteklendiği sonucuna varılmıştır.

Anahtar kelimeler: Eritropoietin, Na,K-ATPaz, diyabetik polinöropati.

Çıkar Çatışması: Bu makalede yazarlar arasında çıkar çatışması bulunmamaktadır.

Introduction

Na,K-ATPase is an active transport system and its structure and enzymatic characteristics are well defined. It is an integral membrane protein which is responsible of providing and preserving the electrochemical gradient of Na and K ions between two sides of the plasma membrane. This gradient is essential for osmoregulation, transport of various organic and inorganic molecules and electrical excitability of nerve and muscle fibers. This enzyme is usually designated as an ion pump [1,2].

Na,K-ATPase enzyme is formed of two subunits: alpha catalytic subunit and beta subunit which is probably responsible of binding of the enzyme to the membrane. α subunit has 4 and beta 3 isoforms. These isoforms are coded by different genes due to tissue type and developmental stages. α_1 isoform is commonly exist in peripheral nerves and erythrocytes [3].

The etiological mechanisms which explain deceleration in transmission of nervous impulses in hyperglycemia indicate the atrophy of large myelinized fibers and the decrease in Na,K-ATPase activities in peripheral nerves [4]. There are reports which claim that the decrease in Na,K-ATPase activities in nervous tissue plays role in development of diabetic neuropathy. It is important that these researches indicate that both the total number of fibers and specific activities of Na,K-ATPase decrease in diabetic patients. It was also stated that diabetic neuropathy results not only from decrease in the number of nerve fibers but also a decrease in functionality of the fibers [5]. It was suggested previously that decrease in erythrocyte Na,K-ATPase activities was related with development of peripheral neuropathy in type I diabetic patients [6]. It was shown in another study performed on diabetic patients that Na,K-ATPase activities were significantly lower in diabetics compared to healthy subjects as well as in diabetics with neuropathy than diabetics without neuropathy [7]. We have stated that Na,K-ATPase activities decreased by duration of diabetes and this decrease in the enzyme activity could be assumed as a marker of poor control of diabetes [8].

Erythropoietin (EPO), which is a glycoprotein with a molecular weight of 34 kDa, has a regulatory function in erythropoesis. EPO has various effects such as modulation of inflammation, slowing down of apoptosis, stimulation of angiogenesis, recovery from vasospasm and limitation in production of reactive oxygen species. Therefore EPO can protect neurons with a combination of these mechanisms [9].

It was previously reported that recombinant human erythropoietin had the potential for the regeneration of the activity loss of Na,K-ATPase in diabetic rats [10].

We aimed to investigate the effects of erythropoietin administration on Na,K-ATPase activities in diabetic patients with polyneuropathy.

Materials and Methods

Subjects

Eleven (3 males and 8 females) diabetic patients with polyneuropathy whose mean ages were 60 years (48-72) and ten (5 males and 5 females) healthy individuals who were at mean age of 51.3 years (48-56) were contributed to this study. Diabetic group was formed from the patients with polyneuropathy and on erythropoietin (EPO) therapy. Erythropoietin beta U (Neorecormon®) was administered with a dose of 150 U/kg/ week to the patients. Routine biochemical and hematological parameters of the patients were as follows: Mean hemoglobin 9.99 g/dl (8.6-10.8 mg/dl), mean hematocrite 29.6% (25.9-34.1%), mean urea 144 mg/dl (86-265), mean creatinine 4.9 mg/dl (2.6-8.3) and mean glucose 193.5 mg/dl (107-334).

Control group was established from the individuals who were not smokers and did not suffer from any chronic illness such as diabetes, hypertension and chronic renal failure. They also reported that they did not take any drug for a long time for any purposes.

The study was approved by the Medical Ethics Committee of Selcuk University (21.09.2007, no:2007/202).

Measurement of Na,K-ATPase Activities

Chemicals and Reagents

All chemicals were purchased from Sigma-Aldrich Company and were reagent grade.

Sample Collection and Preparation

Na,K-ATPase activities were measured for once in control and for twice (before and after erythropoietin application) in patient group. Erythrocyte Na,K-ATPase activities were measured with the modified form of Kitao-Hattori method [11].

Inorganic phophorus and microprotein contents of these mixtures were measured with original Beckman reagents on Synchron LX20 (Beckman Coulter, USA) analyzer. Na,K-ATPase activities were given as $\mu molP_i.mg.protein^{-1}.10\ min^{-1}$.

Statistical Evaluation

SPSS for Windows (version 15.0) was used for statistical evaluation of the data. The distribution characteristics of Na-K, ATPase activity was tested with Shapiro Wilk test and showed parametric distribution. Paired t test and independent t test were used to test the differences within the diabetic group before and after EPO therapy and between the groups. The results are given as mean±SD and $p<0.05$ is considered as statistically significant.

Results

Na,K-ATPase activities were given in Table 1. Prediabetic Na,K-ATPase activities were significantly lower than the control group (p=0.0001). It was demonstrated

Table 1. Na^+-K^+ ATPase activities of control and diabetic groups

Parameter	n	Group	Mean±SD	p
Na^+-K^+ ATPase activity ($\mu molP_i.mg.protein^{-1}.10\ min^{-1}$)	10	Control	2.91±0.38	0.0001[1]
	11	Diabetic group before EPO	1.93±0.38	0.0001[2]
	11	Diabetic group after EPO	2.60±0.49	>0.05[3]

p^1: Control versus diabetic group pretreatment
p^2: Diabetic group pretreatment versus posttreatment
p^3: Control versus diabetic group posttreatment

that after EPO treatment for one month duration, Na,K-ATPase activities significantly increased compared to the pretreatment values (p=0.0001). Na,K-ATPase activities after EPO treatment were not significantly different from the control values (p>0.05) .

Discussion

It was reported that oxidative stress caused lipid peroxidation in erythrocyte membranes and disturbances in membrane lipid composition in diabetes and as a result of these changes Na,K-ATPase activities and membrane viscosity decreased [12].

Free radical reactions were suggested to be responsible from the inhibition of erythrocyte Na,K-ATPase activities in type II diabetic patients [13,14]. On the other hand, advanced glycation increased in diabetic patients and the glycation of Na,K-ATPase was found to be related with nerve dysfunction in experimental diabetic neuropathy [15].

Erythrocyte Na,K-ATPase activity plays a pivotal role in homeostasis of intra and extracellular cations. It was thought that the changes in structure and the function of this enzyme were related with the complications of diabetes. In a study to investigate this relation, it was found that Na,K-ATPase activities were decreased in diabetics with neuropathy and retinopathy compared to both the diabetics without these complications and the control group [16].

There are researches which point out the role of decreased Na,K-ATPase activities in nerve tissue in the development of diabetic neuropathy. This diminution causes an increase intraaxonal Na+ concentration and blocks the nerve cell membrane depolarization. The decay in sciatic nerve Na,K-ATPase activities can change normal membranous axon repolarization after the depolarization induced by action potential, thus a decrease in rate of signal transduction in nerve fibers can be supposed [17].

We have found that erythrocyte membrane Na,K-ATPase activities were significantly lower in patient group with diabetic neuropathy. This decay in the activity was approximately 34%. Hyperglycemia, uremia and marked anemia were present in patient group. It is known that hypoxia will develop as a result of anemia which was induced by uremia and free radical production will increase and metabolic stress will occur. The decrease in enzyme activity was suggested to be related these phenomena. Membrane structure and function will be damaged during this process and these give rise to activity loss in this enzyme. The tissues where Na,K-ATPase activities are higher, especially the nervous tissue, are more affected from this cellular damage. A significant diminution in the rate of signal transduction in nerve fibers and an alteration in the repolarization after the depolarization induced by axonal potential will develop. Glycosylation caused by hyperglycemia will also cause a decrease in the enzyme activity.

A significant decrease in the rate of signal transduction in nerves in experimental diabetes was reported and it was hypothesized that decreased Na,K-ATPase activity was responsible from this condition. It was demonstrated in both animal and human studies that sciatic nerve and erythrocyte Na,K-ATPase activities were diminished [18].

Because insulin is the natural activator of Na,K-ATPase in peripheral nerves it is suggested that a problem in this activation might lead to the development of neuropathy. It was also stated that insulin resistance will accompany the decrease in the activity of Na,K-ATPase and this would be related with the hyperglycemia and poliol pathway [19].

We found that erythrocyte membrane Na,K-ATPase activities significantly decreased in diabetic group with neuropathy compared to the control group. This can be explained not only with the disturbance in hematopoietic process but also with the energy (ATP) deprivation which occurs due to the abnormality in energy metabolism in diabetic patients. This energy deprivation also causes disturbance in ATPase function. If glycemia of the patient will be controlled recovery can be seen in the enzyme activity. It is because insulin controls the enzyme activity [19].

It was demonstrated that both partial and full recovery in conduction rate in sciatic nerves of diabetic rats after various therapeutic interventions will usually accompany the partial recovery in Na,K-ATPase activity [20]. EPO is suggested as an important choice for these therapeutic interventions because pathways which lead to apoptosis will be activated in most tissues due to long-standing hypoxia resulting from the disturbance in hematopoesis in diabetics with anemia. EPO and IGF-1 are cytokines which inhibit neuronal apoptosis [21].

Campana et al demonstrated that peripheral nerve cells synthesize EPO and EPO-R locally. They stated that EPO-R levels increased in Schwann cells in sciatic nerves after painful neuropathy and JAK2 phosphorylation was induced by EPO [22].

Diabetic patients are prone to neuropathy and especially sensory fibers are more affected in diabetics with uremia. Hassan et al showed that subcutaneous EPO treatment for five months ameliorated motor polyneuropathy in anemic and predialytic patients. They also suggested that nonhematopoetic effect of EPO was related with direct effect of EPO-R in human neurons [23].

In our study we measured Na,K-ATPase activities in diabetic patients with neuropathy who had a mean Hb concentration of 9.99 g/dl and mean hematocrite level of 29,6% and who were under EPO therapy with a dose of 150 U/kg/week after one month treatment. Enzyme activities significantly increased after the treatment compared to the basal levels. This increase was approximately 24%. It was observed by the clinician that significant recovery in pain complaints and neuropathy symptoms in the patients.

Campana and Myers identified that EPO decreased the production of TNF by motor neurons and the role of EPO in the regulation of cytokines in Schwann cells. Moreover they demonstrated that systemic application of EPO decreased dorsal root ganglion apoptosis. According to these data they concluded that rhEPO could be used as an effective treatment of neuropathic pain with a specific neuroprotective mechanism. Possibly, the inhibition of TNF production prevents the apoptotic process. Finally, JAK2 pathway is activated and the process against cell degeneration and death will occur [24].

rhEPO has beneficial effects on brain functions which was demonstrated with the results obtained from neurophysiological and electrophysiological evaluations. This is related with the recovery of the hypoxic state due to the progressive amelioration of hematocrite levels. However in some in vivo and in vitro researches it was demonstrated that EPO would be neuroprotective and neurotrophic. It was observed in unilateral sciatic nerve transaction experiment that systemic EPO application for two weeks significantly prevented the loss of motor neurons [9].

Between the years 2004 and 2009, 4 preclinical studies have been published regarding EPO as potential treatment of diabetic neuropathy [25].

Wu et al. demonstrated that intermittent expression of EPO in DRG (Dorsal Root Ganglion) achieved from a regulatable vector is sufficient to protect against the progression of neuropathy in diabetic animals [26].

The mechanism of neuroprotective effect of EPO was not fully explained. EPO has various effects such as assembly of stem cells, modulation of inflammation, slowing down of apoptosis, stimulation of angiogenesis, recovery from vasospasm and limitation of the production of reactive oxygen species. It can be suggested that EPO protects the neurons with the combination of all these effects [9]. EPO's neuroprotective mechanisms could also include activation of calcium channels to protect from glutamate toxicity, antioxidant enzyme production and neo-angiogenesis that improves blood low and oxygenation in border zones of ischaemic areas [27].

We determined 24% increase in erythrocyte Na,K-ATPase activities after one month EPO application compared to the basal levels. Significant recovery in hematological parameters and hypoxic state were also observed. Blood glucose levels of the patients were also regulated.

We can conclude that EPO therapy will restore neuropathy and this improvement is favored with the increase in erythrocyte Na,K-ATPase activity which can be used as a marker.

Conflict of Interest: We declare that there is no conflict of interest.

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