The Function of Endogenous Protective Systems in Patients with Insulin-Dependent Diabetes Mellitus and Polyneuropathy: Effect of Antioxidant Therapy

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α-Lipoic acid is a very efficient antioxidants for the treatment and prevention of diabetic neuropathy. The aim of the present study was to evaluate the function of nitric oxide (NO) and stress proteins (HSP72) in insulin-dependent diabetes complicated by polyneuropathy and possible contribution of these systems to the therapeutic effects of α-lipoic acid. Plasma content of nitrites and nitrates in diabetic patients was almost 2-fold below the normal. The treatment with α-lipoic acid completely normalized the plasma content of these stable NO metabolites. The majority of patients had also low level of HSP72. Positive clinical effects of α-lipoic acid were accompanied by normalization of HSP72 synthesis. Thus, activation of the NO and HSP protective systems is involved in the therapeutic effect of α-lipoic acid in diabetic patients (type 1 diabetes mellitus) with polyneuropathy.

Key Words: *diabetes mellitus; diabetic polyneuropathy; α-lipoic acid; antioxidant; nitric oxide; stress proteins*

Oxidative stress associated with free radical overproduction plays an important role in the development of diabetic complications including diabetic neuropathy (DN) [3,5]. Deficiency of the antioxidant protection systems in patients with diabetes mellitus (DM) increases cell vulnerability to oxidative damage [3]. Therefore, free radical-trapping antioxidants are successfully applied for the treatment and prevention of DN. α-Lipoic acid (α-LA), a lipophilic trap of free radicals [2], is a very efficient antioxidants. However, not all inhibitors of free radical oxidation are so efficient as α-LA [14], which suggests that the molecular mechanisms of α-LA action involves not only its antiradical activity.

When considering the possible targets for drugs with broad spectrum of protective properties, it should be taken into account that apart from cell damage mediated by free radicals, DM is characterized by complex dysfunction of the endogenous protective systems and apoptosis. Nitric oxide (NO) belongs to the most universal protective systems and plays an important role in the regulation of various processes in the body. Experimental and clinical studies confirmed the role of disturbed NO metabolism in diabetic complications [13].

Since NO is a short-living molecule, its protective properties are associated not only with its direct effects, but also with NO-activated endogenous protective systems. In particular, NO-dependent increase in the resistance to oxidative stress and apoptosis can be attributed to enhanced synthesis of HSP72 stress proteins [11,12]. There is accumulating evidence that these proteins play a protective role in DM [8]. In light of this, the analysis of interactions between NO and stress proteins is of special interest.

The aim of the present study was to evaluate the state of the NO/HSP72 system in insulin-dependent

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DM with polyneuropathy and its contribution to the therapeutic effect of α-LA.

MATERIALS AND METHODS

The study included 16 patients (7 men and 9 women, 16-50 years, mean age 32.3±2.1) with compensated DM (mean level of HbA_{1c} 9.10±0.25%). The severity of DN was 7.0±0.7 and 8.0±0.5 according to TSS and DMN scales, respectively [2]. In addition to clinical examination, the function of peripheral nerves was tested by electromyography. The control group comprised 10 healthy volunteers (2 men and 8 women, 25-31 years, mean age 27.6±1.1).

Only DM patients without hyperlipidemia, arterial hypertension, and inflammatory diseases were included in the study. Four days before blood tests the patients followed a diet excluding food possibly containing nitrites or nitrates. Intense physical exercises and drugs except insulin were also excluded.

The patients were examined before the treatment, after 20-day period of intravenous infusions, and after completion of the course (40 days).

α-LA (Thioctacid, Asta Medica) was infused intravenously for 30 min in a dose of 600 mg in 200 ml saline. The dropper was set at 11.00 in the morning, 2 h after the first breakfast. The intravenous course lasted 3 weeks with the infusions only on working days, while during weekends the patients received Thioctacid tablets (600 mg, 30 min before breakfast). For the next 20 days (days 21-40) the patients received 600 mg Thioctacid daily in the morning.

NO production was assessed by measuring the total plasma concentration of nitrites and nitrates. Blood samples were centrifuged at 3,000*g* for 15 min without cooling and deproteinized with $ZnSO_4$ (1:20 v/v, 300 g/liter). Supernatant nitrates were reduced to nitrites with Nitralyzer reactors (World Precision Instruments) in 0.5 M NH_4OH buffer (pH 9.0), the concentration of nitrites in plasma aliquots was determined spectrophotometrically at 540 nm using Griss's reaction.

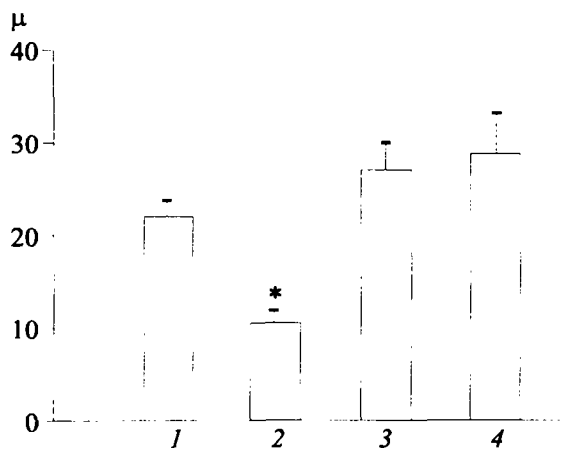

Fig. 1. Plasma level of nitrites and nitrates in healthy individuals (*1*) and patients with diabetic neuropathy before treatment (*2*) after 20 days of treatment with intravenous α-lipoic acid (*3*), and after the following 20 days of treatment with α-lipoic acid in tablets (*4*). *$p<0.05$ in comparison with healthy individuals.

The content of HSP72 in peripheral blood leukocytes from healthy volunteers and diabetic patients was determined by Western blot analysis. After cell lysis in a buffer containing 1% Nonidet P-40, 150 mM NaCl, 10 mM EDTA, and 100 mM Tris-HCl (pH 8.0) and electrophoresis in 12% PAAG, the proteins were transferred to a membrane in accordance with BIO-RAD instructions and the membrane was incubated with monoclonal antibodies to HSP72 (SPA-810, Stree Gen Biotechnologies) diluted 1:1000 (15 h) and then with mouse serum antibodies conjugated with horseradish peroxidase (NA-931, BIO-RAD). Antibodies were visualized with diaminobenzidine.

The data were analyzed statistically using Student *t* and Fisher"s exact probability test.

RESULTS

The treatment with α-LA significantly reduced both clinical and electrophysiological manifestations of polyneuropathy (TSS/DMN scores and EMG indices).

Before the treatment, the total plasma concentration of nitrites and nitrates in patients was almost 2-fold lower than in healthy volunteers (Fig. 1). After intravenous course, plasma concentration of stable NO metabolites returned to normal and remained at the same level after peroral Thioctacid treatment.

Nine of 16 patients had extremely low levels of HSP72 (Fig. 2, upper and lower rows). In other words, DM was accompanied by considerable inhibition of HSP72 synthesis in blood cells. The positive clinical effect of α-LA was accompanied by normalization of HSP72 synthesis. In 6 patients, the content of HSP72 increased to normal after intravenous course and remained at this level throughout the treatment (Fig. 2, upper row). In 7 patients, the initial HSP72 indices were within the normal range and did not change during the treatment (Fig. 2, middle row). In 3 patients with initially low levels of HSP72, no positive effects were noted (Fig. 2, lower row).

Thus, most patients had initially low plasma levels of NO metabolites and HSP content in blood cells. The treatment with α-LA significantly increased the proportion of patients with normal levels of NO and HSP (Table 1). Therefore, the therapeutic effect of α-LA in DM patients with polyneuropathy is mediated by activation of the endogenous NO and HSP systems.

The principal finding of this study is that the positive effect of α-LA in diabetic neuropathy is accom-

panied by pronounced improvement in the functioning of the NO/HSP system. It was interesting to discuss possible mechanisms and consequences of activated synthesis of NO and HSP for the development of diabetic neuropathy and diabetes.

Disturbed glucose metabolism leads to protein glycosylation. Glycosylation end-products are considered to be the principal mediators of late diabetic complications. Their binding to the corresponding receptors activates free radical processes in vascular endothelial and muscle cells, neurons, macrophages, and other cells. This activation is a trigger factor leading to NO deficiency in DM. Free oxygen radicals activate nuclear transcription factor NFkB, which induces the expression of various proteins such as leukocyte adhesion molecule, endothelin-1, cytokine, and tissue factor disturbing vascular homeostasis and damaging endothelium. Under these conditions, endothelial cells can be destroyed via apoptosis. These disturbances manifest themselves in vascular complications such as diabetic angiopathy, neuropathy, and nephropathy [3].

The earliest disturbances of endothelial functions and vasodilation can be largely associated with reduced synthesis, release, and/or effects of endothelial NO. Free radicals directly destroy NO. In addition, membrane lipid peroxidation can deteriorate the structure of endothelium and disturb NO production. Inhibition of guanylate cyclase, activation of cGMP metabolism, or inhibition of Na^+/K^+-ATPase can reduce muscle responsiveness to NO. Finally, DM is accompanied by L-arginine deficiency (substrate for endothelial NO synthase, and its compensation partially normalizes endothelial NO production. Enhanced production of endothelial constrictor factors (endothelin-1, thromboxane A_2, prostaglandin $F_{2\alpha}$) characteristic of DM contributes to the development of endothelium-dependent disturbances of the vascular tone. All these processes lead to absolute or relative NO deficiency and impair regulation of the vascular tone. Attenuation of NO-dependent vasodilations in DM patients increased vascular tone, activates thrombogenesis, and reduces blood supply to nerves, which is an important factor for DN pathogenesis. Normalization of NO synthase activity or compensation for NO deficiency can delay the development of DN. Interestingly, inhibition of NO synthase blocks the positive effects of antioxidants on nerve conductivity and endoneural blood flow suggesting that the effects of antioxidant therapy in polyneuropathy can be mediated by NO [3,5,13,15]. However, these data were obtained only under experimental conditions, and there are no relevant clinical data on the role of NO in the therapeutic effects of antioxidants.

The maintenance of cell structure and function during oxidative stress requires urgent gene expression and synthesis of repair proteins. The most universal and powerful mechanism of cell protection is the HSP system. The possibility of using these proteins for prevention and treatment of different diseases now at-

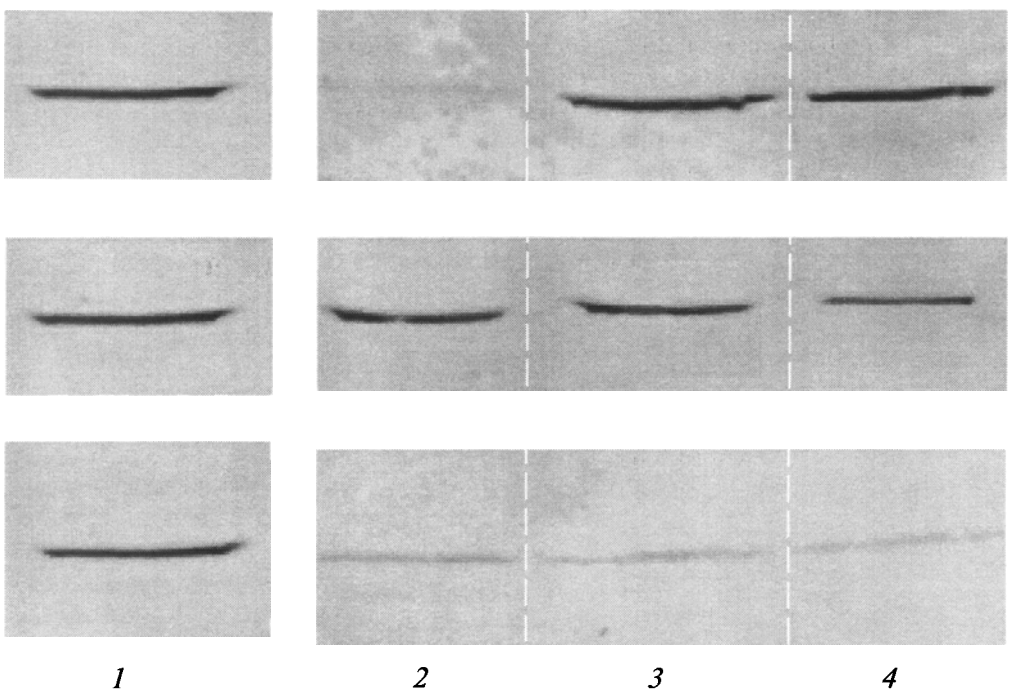

Fig. 2. Deficient synthesis of HSP72 in diabetes and its recovery after treatment with α-lipoic acid. Western blot analysis. Band thickness and staining intensity reflect the level of HSP72 accumulation. *1*) healthy subjects; *2*) patients before treatment; *3*) after 20 days of treatment with intravenous α-lipoic acid; *4*) after the next 20 days of treatment with α-lipoic acid in tablets.

TABLE 1. Effect of Treatment with α-LA on Levels of NO and HSP72 in Diabetic Patients with Polyneuropathy (n=16)

Examination period	Number of patients with normal level			
	HSP72		NO	
	abs.	%	abs.	%
Before treatment	7	44	3	19
Days 1-21 (intravenous course)	11	69	11	69
Days 22-40 (oral course)	13	81	13	81

tracts much attention [11]. There is evidence that cells from DM patients are characterized by impaired HSP accumulation capacity [4]. On the other hand, it was found that cell resistance to autoimmune and, in particular, diabetes-related damages directly depends on their genetically determined ability to activate HSP72 synthesis. In this connection, some attempts were undertaken to compensate HSP deficiency in some diseases (DM) with bacterial HSP. However, the necessity to be extremely careful with HSP vaccination keeping in mind possible complications up to DM aggravation has been emphasized [8]. Therefore, the search for clinically safe compounds capable to stimulate the synthesis of HSP in patients is still very important problem.

α-LA applied in the present study possesses a number of properties responsible for its therapeutic action in DM. It accelerates nerve conductance, stimulates fiber regeneration, and improves blood supply to the nervous tissue. It also exerts favorable effects on energy production and carbohydrate metabolism, possesses pronounced cardioprotective properties, and normalizes endothelium-dependent vascular reactions [3,9].

Administration of exogenous α-LA compensates for its deficiency in DM. It is generally accepted that the favorable effect of α-LA in DM accompanied by neuropathy is attributed to two principal mechanisms: inhibition of free radical processes and improvement of carbohydrate metabolism. In fact, α-LA is a powerful lipophilic antioxidant with especially high efficiency against free radical processes in the nervous tissue; it intensifies endogenous antioxidant protection by normalizing the concentration of reduced glutathione. As for carbohydrate metabolism, α-LA is similar to insulin: it stimulates glucose uptake, intensifies its oxidation, stimulates glycogen synthesis by muscle cells, and inhibits gluconeogenesis, reducing blood glucose level in DM [3,10].

This study showed for the first time the two new mechanisms of the protective action of α-LA: stimulation of the NO and HSP72 syntheses, which determine its advantages in the treatment of DN compared to others [14].

The protective effects of NO is primarily based on direct compensation for its deficiency observed in DM. Normalization of NO production intensifies endothelium-dependent vasodilation and improves nervous fiber blood supply. Moreover, NO is involved in the mechanisms of general protection from free radical damage. Its function is determined by the ability to induce the synthesis of antioxidant enzymes, prevent NFkB activation, and stimulate and stabilize IkB-α, an endogenous inhibitor of NFkB [1]. The important aspect of NO protective activity is its antiapoptotic effect preventing the death of endothelial cells [6].

In addition to the direct protective effects, NO stimulates the synthesis of HSP72 [12], which is an important element in the cell repair system protecting the biosynthesis and structural integrity of proteins in damaged cells. The protective effect of HSP72 is attributed to its ability to disaggregate denaturated protein aggregates, its antioxidant properties, and the ability to interrupt apoptosis [11]. Similarly to NO, HSP72 can reduce the activation of NFkB factor [7].

Thus, apart from antioxidant effects and normalization carbohydrate metabolism, α-LA modulates the activity of the NO/HSP system, one of the principal body protective systems. These mechanisms are probably involved in the therapeutic effects of α-LA in DN. Our findings provide the basis for the development of new approaches to the treatment of DN by directed modulation of NO production and HSP72 synthesis.

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