

Adenosine Triphosphatase Activity of Streptozotocin-Induced Diabetic Rat Brain Microsomes. Effect of Vitamin E

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Abstract. Hyperglycemia causes protein glycosylation, oxidation and alterations in enzyme activities, which are the underlying causes of diabetic complications. This study was undertaken to test the role of vitamin E treatment on Ca²⁺-ATPase activity, protein glycosylation and lipid peroxidation in the brain of streptozotocin (STZ)-induced diabetic rats. Male rats weighing about 250–300 g were rendered diabetic by a single STZ injection of 50 mg/kg *via* the tail vein. Both the diabetic and non-diabetic rats were fed a vitamin E supplemented diet (500 IU/kg/day). Ca²⁺-ATPase activity was significantly reduced at week 10 of diabetes compared to the control group ($p < 0.05$), with 0.225 ± 0.021 U/l (mean \pm S.E.M.) in the control group and 0.072 ± 0.008 U/l (mean \pm S.E.M.) in the diabetic group. Vitamin E treatment prevented the enzyme activity from decreasing. The activities observed were 0.226 ± 0.020 U/l and 0.172 ± 0.011 U/l (mean \pm S.E.M.) in the vitamin E-treated control and diabetic group, respectively. STZ-induced diabetes resulted in an increased protein glycosylation and lipid peroxidation. Vitamin E treatment led to a significant inhibition in blood glucose, protein glycosylation and lipid peroxidation, which in turn prevented abnormal activity of the enzyme in the brain. This study indicates that vitamin E supplementation may reduce complications of diabetes in the brain.

Key words: Diabetes — Calcium-ATPase — Vitamin E — Brain

Introduction

The complications of diabetes mellitus involve damage of many tissues including nerves, skin, retina, kidney, heart and brain. The results of the Diabetes Control

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and Complications Trial Group (1993) have shown that strict glycemic control can prevent the onset and progression of diabetic complications. Substantial experimental evidence supports the role of increased accumulation of advanced glycation end products, increased flux through the polyol pathway, oxidant formation, as well as of alterations in enzyme activities and lipoprotein metabolism (Greene et al. 1987; Brownlee et al. 1988).

One of the enzymes that display altered activity in diabetes is the membrane Ca^{2+} -ATPase. The Ca^{2+} -ATPase is responsible for fine-tuning of intracellular calcium levels (Carafoli 1989). The study indicated that the nature of the alterations is tissue specific and may depend on the level of blood glucose, or insulin, or both. Along with other authors, we recently found that, in diabetes, Ca^{2+} -ATPase activity was increased in kidneys (Daş Evcimen et al. 1999) and erythrocytes (Levy et al. 1990), and decreased in liver (Doğru-Pekiner et al. 2002a), retina (Kowluru et al. 1996) and brain cortex (Öner et al. 2000). A decreased enzyme activity was observed also in the heart of insulin-deficient diabetic rats (Hylinger et al. 1987) and in red blood cells of insulin-dependent diabetes mellitus patients (Schaefer et al. 1987).

Treatment of diabetic animals with a mixture of certain antioxidant compounds, such as beta-carotene, vitamin E or its analog trolox C, prevented the development of diabetes-induced defects, such as enhanced lipid peroxidation (Kowluru et al. 2000). Previous studies have suggested that some lipophilic and water-soluble antioxidants, including butylated hydroxytoluene and vitamin E, are able to prevent the effects of oxidative stress on Ca^{2+} -ATPase activity (Tappia et al. 2001). Previously we demonstrated the effect of stobadine and vitamin E on Ca^{2+} -ATPase activity in streptozotocin (STZ)-induced diabetic liver and heart tissues. Vitamin E treatment seems to effectively prevent protein glycation and deranged activity of microsomal liver Ca^{2+} -ATPase (Doğru-Pekiner et al. 2002b).

So far, the effect of antioxidants on the diabetic condition has not been fully clarified. This study was undertaken to test the hypothesis that treatment of diabetic rats with vitamin E can prevent abnormalities in activity of microsomal brain Ca^{2+} -ATPase. Further, it was to show that antioxidant supplementation can also prevent peroxidation and glycosylation, which may result in the development of damage of brain cells in diabetes mellitus.

Materials and Methods

Materials

Phosphoenolpyruvic acid (PEPA), MgCl_2 , K_2 -ATP, nicotinamide adenine dinucleotide (NADH), pyruvate kinase, lactate dehydrogenase (LDH), ethylene glycoltetraacetic acid (EGTA) were from Sigma Ltd., CaCl_2 and KCl were from Merck Company, Hepes-HCl and dithiothreitol were from Nutritional Biochemical Corporation.

Animals

Wistar albino male rats weighing 250–300 g were rendered diabetic by intravenous injection of STZ (50 mg/kg body weight) *via* the tail vein, while control animals received an injection of normal saline. During the animal study, National Research Council guidelines were observed. Three days after the STZ injection, the diabetic rats were divided into two groups and were fed either a vitamin E supplemented diet or a control diet. The non-diabetic rats (control) were also divided and fed the same two diets. Vitamin E (500 IU/kg/day) was given orally. The rats were maintained under the same conditions and were supplied food and water *ad libitum*. Blood glucose levels and body weights were measured after 3 days of STZ injection. Glucose levels were measured with a glucometer (Accutrend, Roche Diagnostics). STZ-treated rats with blood glucose levels over 500 mg/dl were considered to be diabetic. They were sacrificed 10 weeks following injection. Brain tissues were obtained at the time of death. Ca^{2+} -ATPase activity was determined after isolation from the brain tissue.

Determination of glycosylated protein and lipid peroxidation

Glycosylation was estimated using the thiobarbituric acid (TBA) procedure, based on the reaction of TBA with the released 5-hydroxymethylfurfural (5-HMF) (Fluckiger and Winterhalter 1976).

Lipid peroxidation was assessed by determination of malondialdehyde (MDA), an end product of fatty acid peroxidation (Jain and Levine 1995).

Membrane-enriched microsome preparation

Tissue specimen, cleaned and minced, were homogenized in six volumes of freshly prepared buffer A containing: 0.3 mol/l sucrose, 10 mmol/l Hepes-HCl (pH 7.4) and 2 mmol/l dithiothreitol. The material was homogenized with a teflon/glass homogenizer (Omni mixer homogenizer, model 18074, Omni Int., CT, USA). The homogenate was centrifuged at $85,000 \times g$ for 75 min (Beckman L 7, Beckman Instruments Inc., Fullerton, California). The supernatant was discarded and the pellet was resuspended with the original volume of buffer A containing 0.6 mol/l KCl using four strokes, and centrifuged again at $85,000 \times g$ for 75 min. The pellet was resuspended with the original volume of buffer A and centrifuged again at $85,000 \times g$ for 75 min. Then, the pellet was suspended in buffer A using four strokes at a protein concentration of 2–7 mg/ml. The samples were frozen at -60°C until assayed. The isolation procedure was performed at $+4^\circ\text{C}$ (Borchman et al. 1988).

Determination of Ca^{2+} -ATPase activity

Ca^{2+} -ATPase activity was measured spectrophotometrically (Niggli et al. 1981). The incubation medium contained: 120 mmol/l KCl, 60 mmol/l Hepes (pH 7), 1 mmol/l MgCl_2 , 0.5 mmol/l $\text{K}_2\text{-ATP}$, 0.2 mmol/l NADH, 0.5 mmol/l PEPA, 1 IU pyruvate kinase, 1 IU LDH/ml, 500 $\mu\text{mol/l}$ EGTA. The medium was incubated at 37°C for 4 min. After preincubation of the assay medium, a total volume of

1 ml, 50 μg microsome preparation was added to the medium. After 2 min, the reaction was started by addition of 600 $\mu\text{mol/l}$ CaCl_2 . The enzyme reaction was stopped at the 4th min. ATPase activity was followed by measuring continuously the absorbance at 365 nm.

Statistical analysis

Comparison of Ca^{2+} -ATPase activity and glycosylated protein amounts, lipid peroxidation, blood glucose concentrations and body weights among groups was performed using multiway analyses of variance followed by the Bonferroni test. The results were expressed as mean \pm S.E.M. of five to six individual experiments. Differences between means were considered statistically significant at $p < 0.05$.

Results

The effects of vitamin E supplementation on body weight and blood glucose levels in non-diabetic and diabetic rats are shown in Table 1. Vitamin E supplementation did not significantly change the weight gain of control rats. The final blood glucose concentrations of untreated diabetic rats were about five times higher than those of normal control rats. Treatment with vitamin E produced a significant fall in blood glucose level of diabetic animals, though they were still hyperglycemic when compared with untreated control rats (Table 1).

Table 1. Data after 10 weeks on the vitamin E supplemented and control diet in non-diabetic and diabetic rats

		Blood glucose (mg/dl)	Body weight (g)
Non-diabetic rats			
Control	6	111.00 \pm 3.50*	323.16 \pm 11.95
Supplemented	6	105.50 \pm 1.72*	300.60 \pm 15.11
Diabetic rats			
Control	6	515.67 \pm 21.17	255.66 \pm 11.47**
Supplemented	6	198.17 \pm 10.36*	252.67 \pm 23.64**

Values are mean \pm S.E.M. * significantly different from the diabetic control group, $p < 0.05$; ** significantly different from the non-diabetic control group, $p < 0.05$.

Fig. 1 shows microsomal Ca^{2+} -ATPase activity measured in the diabetic and non-diabetic stages and the effect of vitamin E on the enzyme activity. The Ca^{2+} -ATPase activity of control animals was 0.225 ± 0.021 U/l (mean \pm S.E.M., $p < 0.05$). A significant decrease to 0.072 ± 0.008 U/l (mean \pm S.E.M., $p < 0.05$) was recorded in the diabetic rat brain after 10 weeks of diabetic condition. Vitamin E

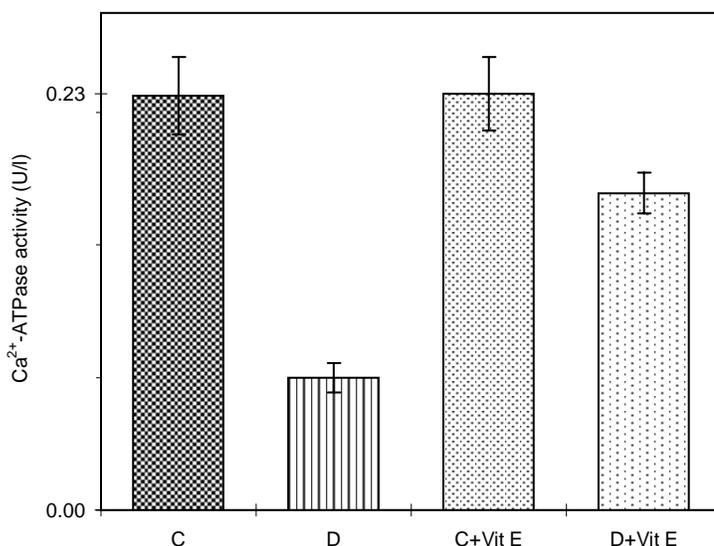


Figure 1. Brain microsomal Ca^{2+} -ATPase activities in control and diabetic rats untreated or treated with vitamin E. Values are the mean \pm S.E.M of 5–6 individual experiments. C, control; D, diabetic; C+Vit E, control group treated with vitamin E; D+Vit E, diabetic group treated with vitamin E.

treatment significantly enhanced the enzyme activity in the diabetic group to nearly non-diabetic levels. The activity observed was 0.226 ± 0.020 U/l and 0.172 ± 0.011 U/l (mean \pm S.E.M., $p < 0.05$) in the vitamin E-treated control and diabetic group, respectively.

Glycosylated protein was determined in the control, diabetic and vitamin E-treated rat brains. In the diabetic stage, high glycosylated protein amounts were observed. Protein glycosylation was decreased to control levels with vitamin E treatment (Table 2).

Lipid peroxidation was assessed by measuring MDA levels. High MDA levels were found in the diabetic group. MDA production was significantly inhibited by vitamin E treatment in the diabetic stage (Table 2).

Discussion

In this study, we found that vitamin E treatment ameliorated the reduced ATPase activity, high lipid peroxidation and high glycosylated protein levels in diabetes.

A great deal of evidence supports the conclusion that hyperglycemia causes adverse effects on cells by a variety of mechanisms (Greene et al. 1987; Brownlee et al. 1988; Daş Evcimen et al. 1999). Along with other authors, we have previously documented that one of these pathways is through alterations of Ca^{2+} -ATPase

Table 2. Protein glycation levels in brain

		Glycosylated protein amount (mg HMF/mg protein)	MDAs (U/mg/mg protein)
Non-diabetic rats			
Control	6	319.80 ± 11.20*	0.40 ± 0.019*
Supplemented	5	283.20 ± 14.76*	0.33 ± 0.040*
Diabetic rats			
Control	6	546.43 ± 13.04	1.38 ± 0.166
Supplemented	6	325.20 ± 14.71*	0.48 ± 0.066*

Values are mean ± S.E.M. * significantly different from the diabetic control group, $p < 0.05$; MDA, malondialdehyde; HMF, hydroxymethylfurfural.

activity (Levy et al. 1990; Daş Evcimen et al. 1999; Kowluru et al. 2000; Öner et al. 2000; Dođru-Pekiner et al. 2002a).

Vitamin E, a membrane-bound, lipid-soluble antioxidant, has been shown to protect biological membranes against injury induced by the reactive oxygen species (Vannucchi et al. 1999) and to block glycation of proteins by inhibiting MDA formation (Jain et al. 1996).

Recent studies established that Ca^{2+} -ATPase became subnormal in the cerebral cortex in hyperglycemic rats and administration of antioxidant diet has prevented the diabetes-induced decreases in enzyme activity, yet had no effect on TBA reactive substances and activities of protein kinase C (PKC) and antioxidant defense enzymes (Kowluru et al. 1999). In our preceding study, we reported that the effect of two antioxidants, vitamin E and stobadine, on cardiac and hepatic Ca^{2+} -ATPase was tissue-specific and unlikely to be directly dependent on their lipid peroxidation and protein glycation lowering effects or on the effect on total calcium levels (Dođru-Pekiner et al. 2002b).

In the present study we found a decrease in the brain microsomal Ca^{2+} -ATPase activity and an increase in protein glycosylation and lipid peroxidation (Fig. 1, Table 2). On the other hand, vitamin E treatment inhibited glycosylation and peroxidation and enhanced the activity of the enzyme.

A number of mechanisms regulate plasma Ca^{2+} -ATPase: conformational transition by calmodulin, acidic phospholipids and fatty acids, PKC-mediated phosphorylation and calcium itself (Kuwahara et al. 1997). Diabetes-related ATPase activity changes in cerebral microvessels may depend on altered blood-brain barrier functions (Mooradian et al. 1994).

The combined results of our data and those cited earlier have clearly established that the decrease in Ca^{2+} -ATPase activity was related to protein glycosylation and lipid peroxidation. Non-enzymatic glycation has been known to occur in a variety of proteins (Brownlee and Cerami 1981; Daş Evcimen and Nebiođlu 1996). Glycosylation was shown to alter protein conformation and function. Moreover,

glucose has been implicated in making pancreatic beta cells more alkaline (Lebrun et al. 1982), hence the alteration observed in ATPase activity in diabetes could be due to a pH change. On the other hand, Ca^{2+} -ATPase is sensitive to its phospholipid milieu and to polyunsaturated fatty acids. The content of these lipids may change in diabetes and may cause alterations in enzyme activity. PKC activates Ca^{2+} -ATPase by phosphorylating the enzyme. Indeed, oxidative stress increases DAG-PKC activity and vitamin E was shown to normalize DAG-PKC activation in diabetic rats (Kunushaki et al. 1994, Way et al. 2001). This can be a different point of view on the effects of vitamin E on the impaired activity of the enzyme, which will be the subject of a further study. Vitamin E is also known to protect ATPase activity in microsomal fraction of the brain and hyperglycemia-induced oxidative damage (Kowluru et al. 2000). Vitamin E has been further shown to protect biological membranes against injury induced by the reactive oxygen species (Jain et al. 1996; Vannucchi et al. 1999; Kinalski et al. 2000), to block glycation of proteins by inhibiting MDA formation (Jain and Palmer 1997; Dođru-Pekiner 2002b), and to inhibit H_2O_2 production effectively (Pathania et al. 1998). In accordance with preceding studies, we also demonstrated a reduction of MDA levels with vitamin E treatment in diabetic rats (Table 2).

It is likely that the changes in Ca^{2+} -ATPase activity, peroxidation and glycosylation induced by hyperglycemia will have biological and possibly pathological importance in the development of diabetic complications in the brain. The reported effect of vitamin E on reversing the adverse effects of hyperglycemia in the brain provides an insight into the pathogenesis of diabetic complications, and may be used to advantage in therapeutic approaches.

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