Neurochemical Research, Vol. 28, No. 6, June 2003 (© 2003), pp. 815–823

Pentose Phosphate Pathway, Glutathione-Dependent Enzymes and Antioxidant Defense during Oxidative Stress in Diabetic Rodent Brain and Peripheral Organs: Effects of Stobadine and Vitamin E

Nuray N. Ulusu,[1] Meral Sahilli,[2] Aslihan Avci,[3] Orhan Canbolat,[4] Gülgün Ozansoy,[2] Nuray Ari,[2] Musa Bali,[5] Milan Stefek,[6] Svorad Stolc,[6] Andrej Gajdosik,[6] and Çimen Karasu[7,8]

(Accepted October 18, 2002)

The aim of the present study was to investigate the effects of treatment with antioxidant stobadine (ST) on the activities of enzymes related with pentose phosphate pathway and glutathione-dependent metabolism and the other markers of oxidative stress in brain and peripheral organs of diabetic rats, and to compare the effects of ST treatment alone with the effects of treatments with another antioxidant vitamin E and ST plus vitamin E. Rats were made diabetic by the injection of streptozotocin (STZ; 55 mg/kg IP), and, 2 days later, some control and diabetic rats were left untreated or treated with ST (24.7 mg/kg/day, orally), vitamin E (400–500 U/kg/day, orally), or both substances together. In the brain, although 6-phosphogluconate dehydrogenase activity (6-PGD) did not change, glucose-6-phosphate dehydrogenase activity (G-6PD) was markedly increased in diabetic rats compared with controls; only combined treatment with ST and vitamin E produced a partial prevention on this alteration. The aorta G-6PD and 6-PGD of diabetic rats were 52% and 36% of control values, respectively. Neither single treatments with each antioxidant nor their combination altered the G-6PD and 6-PGD in aorta of diabetic rats. Glutathione peroxidase (GSHPx) activity was increased by STZ-diabetes in brain, heart, and kidney. In diabetic brain, vitamin E alone or combination with ST kept GSHPx at normal levels. Diabetes-induced stimulation in GSHPx did not decrease in response to the treatment with vitamin E in heart and kidney, but was greatly prevented by ST alone. The activity of glutathione reductase (GR) was decreased in brain and heart of diabetic rats. The treatment with each antioxidant or with a combination of both agents completely prevented this deficiency and resulted in further activation of GR in diabetic tissues. Glutathione *S*-transferase (GST) activity did not significantly change in diabetic brain and aorta. GST was stimulated by all treatment protocols in the brain of diabetic rats and was depressed in aorta of control rats. Catalase (CAT) was activated in diabetic heart but depressed in diabetic kidney. Diabetes-induced abnormalities in CAT activity did not respond to vitamin E alone in heart, was moderately ameliorated by the treatment with this vitamin in kidney, and was completely prevented by ST alone in both tissues. Superoxide dismutase (SOD) activity of brain

[1] Department of Biochemistry, Faculty of Medicine, Hacettepe University, Ankara, Turkey.
[2] Department of Pharmacology, Faculty of Pharmacy, Ankara University, Ankara, Turkey.
[3] Department of Biochemistry, Faculty of Medicine, Ankara University, Ankara, Turkey.
[4] Department of Biochemistry, Faculty of Medicine, Gazi University, Ankara, Turkey.
[5] Department of Nephrology, Faculty of Medicine, Gazi University, Ankara, Turkey.
[6] Institute of Experimental Pharmacology, Slovak Academy of Sciences, Bratislava, Slovakia.
[7] Department of Pharmacology, Faculty of Medicine, Gazi University, Ankara, Turkey.
[8] Address reprint requests to: Çimen Karasu, Gazi University, Faculty of Medicine, Department of Pharmacology, Beşevler, 06510, Ankara, Turkey. E-mail: karasu@gazi.edu.tr

0364-3190/03/0600–0815/0 © 2003 Plenum Publishing Corporation

and heart was unchanged by the diabetes but inhibited in diabetic kidney after the treatment ST alone or ST plus vitamin E. The lipid peroxidation (MDA) was increased in diabetic brain and heart. ST or vitamin E alone partly prevented diabetes-induced increase in MDA in brain and heart; however, antioxidant combination achieved a completely amelioration in MDA of these tissues of diabetic rats. Kidney MDA levels were similar in control and untreated diabetic animals. ST and vitamin E treatments, when applied separately or together, significantly reduced kidney MDA in both control and diabetic rats; and the combined effect of antioxidants was greater than that of each alone. These results are consistent with the degenerative role of hyperglycemia on cellular reducing equivalent homeostasis and antioxidant defense, and provide further evidence that pharmacological intervention of different antioxidants may have significant implications in the prevention of the prooxidant feature of diabetes and protects redox status of the cells.

KEY WORDS: Streptozotocin diabetes; rat brain; oxidative stress; pentose phosphate pathway; glutathione-dependent enzymes; superoxide dismutase; catalase; stobadine; vitamin E.

INTRODUCTION

Diabetes mellitus, in left untreated, may initiate degenerative processes in central nervous system (1,2) and peripheral organs (3,4) because of excess production of oxidative free radicals. Role of reactive radicals in the pathogenesis of organ damage induced by insulin-dependent diabetes mellitus has been well documented (2–5). Diabetes produces alterations in tissue NADPH metabolism that is probably related to the alterations in pentose phosphate pathway, which produces NADPH utilizing as cofactor for glutathione reductase, NAD(P)H-dependent oxidases (i.e., nitric oxide [NO] synthase and many other endogen enzyme systems [6,7]). Such a decrease in the NAD(P)H/NAD^+ ratio has been suggested to have direct implications in diabetes, ischemia-reperfusion injury, and other pathologies in which reductive (high NADPH/NAD^+ ratio) and oxidant (excess reactive oxygen species) imbalances are considered major factors contributing to metabolic disorders. On the other hand, NAD(P)H-dependent oxidases are important sources of tissue superoxide in diabetic state, and the activity of this enzyme system and the levels of NAD(P)H oxidase protein have been demonstrated to increase in diabetic tissues, including vessels and kidney (8,9). In normal vessels, endothelial NO synthase produced NO that scavenges superoxide. However, in diabetic vessels, the endothelium was an additional net source of superoxide production because of dysfunctional endothelial NO synthase. The compromises in natural antioxidant defense systems and changes in antioxidant enzyme activities, including glutathione (GSH)-dependent enzymes such as glutathione peroxidase (GSHPx), glutathione reductase (GR), and glutathione *S*-transferase (GST), and superoxide dismutase (SOD) and catalase, have been implicated in the mechanisms of abnormal tissue functions observed in diabetes mellitus (4,5,7,10–14).

Previous studies provided support for the use of biological antioxidants in prevention of diabetes-induced complications (2,5,13,14). Stobadine (ST), (−)-*cis*-2,8-dimethyl-2,3,4,4a,5,9b-hexa-hydro-1H-pyrido[4,3-b] indole, was shown to be able to scavenge hydroxyl, alkoxyl, and peroxyl radicals and superoxide and to quench singlet oxygen, repair oxidized amino acids, and preserve oxidation of SH groups by one-electron donation (15). It recently has been shown by us or others that the treatment of diabetic rats with a low dose of ST lowers blood glucose, plasma lipid peroxidation, and protein glycosylation; completely prevents calcium accumulation in diabetic tissues (16); and improves morphological and/or functional abnormalities in diabetic heart (17) and aorta (18). Although the response of antioxidant defense systems of diabetic tissues to the effects of vitamin E supplementation has been documented previously, the effects of ST and vitamin E on tissue pentose phosphate pathway have not been documented in a rodent model of experimental diabetes mellitus. There is only one study on the role of ST on tissue antioxidant status showing the beneficial effect of ST in heart of STZ-diabetic rats (17).

The present study investigated the enzymes of pentose phosphate pathway (glucose-6-phosphate dehydrogenase, G-6PD; 6-phosphogluconate dehydrogenase, 6-PGD) in whole brain and some peripheral tissues of 10-week duration diabetic rats untreated or treated with ST, vitamin E, or ST plus vitamin E. We also compared the effects of these antioxidants on lipid peroxidation (malondialdehyde formation, MDA), glutathione-dependent enzymes (GSHPx, GR, GST),

superoxide dismutase (SOD), and catalase (CAT) in diabetic animals. The preliminary results of this study have been presented previously (19,20).

EXPERIMENTAL PROCEDURE

Induction of Diabetes and Treatment Protocols. Male Wistar rats, body weight 250–300 g, were fed a standard rat chow diet and had access to water ad libitum. Diabetes was induced by a single IP injection of streptozotocin (STZ, 55 mg/kg) to animals fasted overnight. Diabetes was verified 48 h later by measuring tail vein blood glucose, and the rats with a blood glucose concentration of 300 mg/dl or more were considered diabetic. Two days after injection of STZ or vehicle, rats were divided into the following groups: (i) untreated diabetic rats (n = 11); (ii) diabetic rats treated with ST (24.7 mg/kg/day, orally) (n = 12); (iii) diabetic rats treated with vitamin E (D,L-alpha-tocopheryl acetate, 400–500 IU/kg/day, orally) (n = 11); (iv) diabetic rats treated with both ST and vitamin E, as given in protocols 2 and 3 (n = 11); (v) untreated control rats (n = 8); (vi) control rats treated with ST as given in protocol 2 (n = 6); (vii) control rats treated with vitamin E, as given in protocol 3 (n = 6); and (viii) control rats treated with ST plus vitamin E as given in protocol 4 (n = 6). The animals were treated for a period of 10 weeks beginning 48 h after either vehicle or STZ injection. The principles of laboratory animal care (NIH publication No. 85–23, revised 1985) were observed.

Tissue and Blood Analysis. Blood glucose concentrations were measured by an Accutrend® GCT meter (Roche Diagnostics, Mannheim, Germany). Glucose-6-phosphate dehydrogenase (G-6PD) activity was determined spectrophotometrically using LKB Ultraspec Plus (4054 UV/visible) spectrophotometer, by monitoring the NADPH production at 340 nm and at 37°C (21). The assay mixture contained 10 mM $MgCl_2$, 0.2 mM $NADP^+$, and 0.6 mM G6P in 100 mM Tris/HCl buffer, pH 8.0. Assays were carried out in duplicate, and the activities were followed for 40 s. The reaction was linear during this time period. One unit (U) of activity is the amount of enzyme required to reduce 1 μmol of $NADP^+$ per min under the assay conditions. Specific activity is defined as units per gram of protein.

6-Phosphogluconate dehydrogenase (6-PGD) activity was measured by substituting 0.6 mM 6-phosphogluconate as substrate in the assay mixture given above for G-6PD measurement (22). Because 6-PGD also catalyzed the production of NADPH, in the earlier steps of the purification, both G-6PD and 6-PGD activities were measured as sum, and the initial velocities of G-6PD were calculated by subtracting the 6-PGD activities.

The method of Lawrence was used to measure glutathione peroxidase (GSHPx) activity (23). The assay mixture consisted of 75 mM phosphate buffer with EDTA and NaN_3 (pH 7.0), 0.150 mg 10,000 × *g* supernatant protein of tissue, 0.1 mM NADPH, 4.0 mM GSH, and 1.5 U glutathione reductase (GR) in a final volume of 500 μl. The reaction was started by the addition of 3.0 mM H_2O_2. The rate of change of absorbance during the conversion of NADPH to $NADP^+$ was recorded spectrophotometrially at 340 nm for 3 min. GSHPx activity was expressed as μmol of NADPH oxidized to $NADP^+$ min^{-1} mg^{-1} tissue protein.

Glutathione reductase (GR) activity was measured according to modified Stall method (24). The incubation mixture contained 100 mM sodium phosphate buffer, pH 7.4; 1 mM GSSG; 100 μM NADPH; liver tissue homogenate sample; and Cd^{2+} ions when indicated. Decrease in the absorbance of NADPH at 340 nm was monitored spectrophotometrically, at 37°C. A unit of activity (U) was defined as the amount of enzyme that catalyses the oxidation of 1 μmol of NADPH in 1 min under these conditions.

Glutathione *S*-transferase (GST) activity was assayed by measuring the conjugation of reduced glutathione with 1-chloro-2,4-dinitrobenzene, as described by Habig et al. (25).

Superoxide dismutase (SOD) activity in tissue homogenates was measured spectrophotometrically at 560 nm using the previously described method (26). The reaction mixture in 50 mM phosphate buffer (pH 7.8) consisted of the SOD-induced inhibition of the reduction of nitro blue tetrazolium, using a free radical-generating system of 0.1 nM xanthine and an amount of xanthine oxidase to produce a rate of absorbance change of 0.025/min.

Catalase (CAT) activity was measured spectrophotometrically by the method of Aebi (27). Cleaned and minced tissue were homogenized in 3 volumes of 50 mM phosphate buffer, pH 7.0. The final volume of the mixture was made up to 2 ml by adding additional buffer solution. The reaction was started by the addition of 1 ml of freshly prepared 30 mM H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically at 240 nm. The enzyme activity for tissues was expressed as k^{-1} mg^{-1} protein, where *k* is the first order rate constant.

Malonyldialdehyde (MDA), an end product of fatty acid peroxidation, reacts with thiobarbituric acid to form a colored complex that has maximum absorbance at 532 nm (28). For this prupose, 0.2 ml of homogenate was suspended in 0.8 ml phosphate-buffered saline and 0.025 ml butylated hydroxytoluene as described previously (28). Thirty percent trichloroacetic acid was then added. Tubes were vortexed and allowed to stand in ice for at least 2 h. Tubes were centrifuged at 2000 rpm for 15 min. One mililiter each of the supernatant was transferred to another tube. To this was added 0.075 ml 0.1 M EDTA and 0.25 ml of 1% TBA in 0.05 N NaOH. Tubes were mixed and kept in a boiling water bath for 15 min. Absorbance was read at 532 and 600 nm after tubes were cooled to room temperature. Absorbance at 600 nm was subtracted from absorbance at 532 nm. MDA values in nanomoles were determined with the extinction coefficient of MDA-TBA complex at 532 nm = 1.56×10^5 cm^{-1} M^{-1}.

Protein concentrations were determined by the method of Bradford using BSA as standard (29). For scanning the column effluents, the absorbance at 280 nm was measured.

RESULTS

Changes in Hexose Monophosphate Dehydrogenase Activities. In the brain of diabetic rats, G-6PD activity was significantly increased; only combined treatment with ST plus vitamin E partially prevented diabetes-induced increase in G-6PD activity (Table I). 6-PGD activities in the brain tissue were found to be similar in all experimental groups (Table I). The aorta G-6PD and 6-PGD activities of diabetic rats were 52% and 36% of control values, respectively. Neither single treatments with each antioxidant nor their combination altered the G-6PD and 6-PGD activities in aorta of diabetic rats.

Glutathione-Dependent Enzyme Activities. GSHPx activity greatly increased in brain, heart, and kidney homogenates of STZ-diabetic rats (Table II). In dia-

Table I. Changes in Hexose Monophosphate Dehydrogenase Activities

		Brain	Aorta
G-6PD (U/g protein)	Control (n = 8)	15.3 ± 1.1	32.2 ± 2.1
	Control + ST (n = 6)	15.2 ± 1.2	34.4 ± 4.8
	Control + Vit-E (n = 6)	15.1 ± 1.4	29.6 ± 4.9
	Control + ST + Vit-E (n = 6)	15.7 ± 1.1	31.5 ± 6.5
	Diabet (n = 11)	22.5 ± 0.2[c]	16.7 ± 2.1[c]
	Diabet + ST (n = 12)	22.8 ± 1.1[c]	13.3 ± 1.2[c]
	Diabet + Vit-E (n = 11)	23.8 ± 0.9[c]	14.5 ± 3.4[c]
	Diabet + ST + Vit-E (n = 11)	18.9 ± 0.8[b,e]	15.6 ± 1.1[c]
6-PGD (U/g protein)	Control (n = 8)	6.58 ± 0.5	15.9 ± 1.4
	Control + ST (n = 6)	5.84 ± 0.4	14.6 ± 1.4
	Control + Vit-E (n = 6)	6.04 ± 0.6	13.9 ± 1.3
	Control + ST + Vit-E (n = 6)	6.59 ± 0.7	5.80 ± 1.7[c]
	Diabet (n = 11)	6.51 ± 0.5	5.40 ± 0.5[c]
	Diabet + ST (n = 12)	6.42 ± 0.5	5.10 ± 0.5[c]
	Diabet + Vit-E (n = 11)	7.03 ± 0.5	5.90 ± 0.8[c]
	Diabet + ST + Vit-E (n = 11)	6.74 ± 0.9	5.81 ± 0.8[c]

Note: Means ± SEM; n = number of animals.
[a] $P < .05$; [b] $P < .01$; [c] $P < .001$ vs. control; [d] $P < .05$; [e] $P < .01$; [f] $P < .001$ vs. diabet; ANOVA.
G-6PD, glucose-6-phosphate dehydrogenase; 6-PGD, 6-phosphogluconate dehydrogenase.

Table II. Glutathione-Dependent Enzyme Activities

Enzyme	Groups	Tissues		
		Brain	Heart	Kidney
Glutathione peroxidase (U/g protein)	Control (n = 8)	6.62 ± 0.5	18.2 ± 0.8	97.1 ± 2.8
	Control + ST (n = 6)	6.40 ± 0.9	18.4 ± 0.2	80.2 ± 5.1
	Control + Vit-E (n = 6)	7.40 ± 1.3	20.8 ± 0.2	88.0 ± 2.0
	Control + ST + Vit-E (n = 6)	6.60 ± 0.5	20.2 ± 3.9	90.0 ± 6.1
	Diabet (n = 11)	14.11 ± 1.6[c]	31.2 ± 1.1[b]	148.0 ± 5.5[c]
	Diabet + ST (n = 12)	9.67 ± 0.6[e]	21.8 ± 1.9[d]	127.1 ± 5.2[b]
	Diabet + Vit-E (n = 11)	7.72 ± 0.8[f]	30.1 ± 3.2[b]	131.4 ± 6.3[c]
	Diabet + ST + Vit-E (n = 11)	6.90 ± 1.0[f]	23.5 ± 2.1[d]	133.3 ± 8.0[c]
		Brain	Heart	
Glutathione reductase (U/mg protein)	Control (n = 8)	15.1 ± 0.6	45.4 ± 1.4	
	Control + ST (n = 6)	14.1 ± 0.4	44.1 ± 1.4	
	Control + Vit-E (n = 6)	15.5 ± 1.4	41.5 ± 1.6	
	Control + ST + Vit-E (n = 6)	15.7 ± 1.0	42.7 ± 1.7	
	Diabet (n = 11)	11.9 ± 0.5[b]	31.7 ± 2.2[b]	
	Diabet + ST (n = 12)	18.1 ± 0.5[b,f]	48.1 ± 2.5[e]	
	Diabet + Vit-E (n = 11)	17.1 ± 0.8[a,f]	47.1 ± 3.8[e]	
	Diabet + ST + Vit-E (n = 11)	17.9 ± 0.6[a,f]	47.8 ± 3.6[e]	
		Brain	Aorta	
Glutathione-S transferase (U/mg protein)	Control (n = 8)	88.6 ± 2.3	55.1 ± 2.9	
	Control + ST (n = 6)	91.1 ± 2.5	35.8 ± 4.4[b,f]	
	Control + Vit-E (n = 6)	90.3 ± 2.7	36.2 ± 4.6[b,f]	
	Control + ST + Vit-E (n = 6)	110.6 ± 6.5[b,e]	38.2 ± 1.9[b,e]	
	Diabet (n = 11)	70.7 ± 5.5	56.9 ± 2.3	
	Diabet + ST (n = 12)	96.7 ± 3.8[d]	55.1 ± 3.9	
	Diabet + Vit-E (n = 11)	97.6 ± 8.2[d]	53.3 ± 2.1	
	Diabet + ST + Vit-E (n = 11)	104.4 ± 7.3[a,e]	49.9 ± 1.8	

Note: Means ± SEM; n = number of animals.
[a] $P < .05$; [b] $P < .01$; [c] $P < .001$ vs. control; [d] $P < .05$; [e] $P < .01$; [f] $P < .001$ vs. diabet; ANOVA.

betic brain, vitamin E treatment alone or combination with ST maintained GSHPx activity in normal levels. Diabetes-induced stimulation in brain GSHPx activity was also prevented by ST treatment, but the effectiveness of ST was relatively less than that of vitamin E. Diabetes-induced stimulation in cardiac GSHPx activity did not decrease in response to treatment with vitamin E; however, it was completely prevented by ST treatment alone. In ST-treated diabetic rat kidney, although GSHPx activity did not greatly increase as shown in untreated-diabetic rats, other treatment protocols exhibited no significant amelioration in GSHPx activity of diabetic kidney (Table II).

The activity of GR was decreased in brain and heart in diabetic rats. The treatment with each antioxidant or with combination of both agents prevented this defect induced by diabetes in cardiac and neuronal GR activity and resulted in slight but significant activation of GR in diabetic tissues (Table II).

GST activity was slightly, not significantly, less in the brain of untreated diabetic rats than in control animals (Table II). Single treatment with ST or vitamin E or combined treatment of these agents stimulated GST activity in the brain of diabetic rats. Combination of ST and vitamin E also produce a significant increase in brain GST activity in control animals. Although there was no significant alteration in GST activity of diabetic aorta compared to controls, both vitamin E and ST, either in singly or in combination treatments, produced a depression in aorta GST activity in control animals. This effect of antioxidants was also observed in diabetic animals without statistical significance (Table II).

Changes in Superoxide Dismutase and Catalase Activities and MDA Level. In diabetic animals, SOD activity of brain, heart and kidneys unchanged, compared to controls. ST treatment also resulted in a reduction in kidney SOD activity when given together with vitamin E to the diabetic animals (Table III).

In diabetic rats, CAT activity of heart increased and activity of kidney decreased. ST treatment alone was found to be more effective than vitamin E treatment alone in the protection of diabetic heart CAT activity at physiological levels (Table III). Similarly, the effect of vitamin E treatment alone was moderate compared with the effect of ST treatment alone on diabetic

Table III. Lipid Peroxidation (MDA) Levels, Superoxide Dismutase (SOD), and Catalase (CAT) Activities

	Groups	Brain	Heart	Kidney
Superoxide dismutase (U/mg protein)	Control (n = 8)	41.2 ± 2.8	6.17 ± 0.37	40.0 ± 0.6
	Control + ST (n = 6)	40.4 ± 4.2	6.14 ± 0.51	37.6 ± 1.3
	Control + Vit-E (n = 6)	39.1 ± 2.8	6.21 ± 0.41	40.2 ± 2.4
	Control + ST + Vit-E (n = 6)	41.9 ± 2.9	5.84 ± 0.44	36.8 ± 1.6
	Diabet (n = 11)	40.3 ± 1.8	6.22 ± 0.67	39.6 ± 0.8
	Diabet + ST (n = 12)	40.3 ± 3.7	6.39 ± 0.18	34.3 ± 0.9[b,e]
	Diabet + Vit-E (n = 11)	43.2 ± 4.9	6.20 ± 0.58	39.4 ± 0.7
	Diabet + ST + Vit-E (n = 11)	46.0 ± 3.7	5.95 ± 0.43	31.8 ± 1.2[c,f]
Catalase (U/mg protein)	Control (n = 8)	ND	5.46 ± 0.24	70.8 ± 2.0
	Control + ST (n = 6)	ND	5.14 ± 0.53[d]	70.8 ± 3.2[f]
	Control + Vit-E (n = 6)	ND	3.96 ± 0.34[e]	70.6 ± 1.8[f]
	Control + ST + Vit-E (n = 6)	ND	4.43 ± 0.42[e]	68.8 ± 4.7[f]
	Diabet (n = 11)	ND	7.49 ± 0.42[a]	41.1 ± 2.7[c]
	Diabet + ST (n = 12)	ND	5.03 ± 0.54[e]	61.4 ± 2.5[f]
	Diabet + Vit-E (n = 11)	ND	6.59 ± 0.65	51.3 ± 2.9[c,d]
	Diabet + ST + Vit-E (n = 11)	ND	5.31 ± 0.58[d]	53.4 ± 3.6[b,e]
MDA (nmol/protein)	Control (n = 8)	0.39 ± 0.018	0.044 ± 0.003	0.216 ± 0.013
	Control + ST (n = 6)	0.35 ± 0.026[f]	0.045 ± 0.003[f]	0.170 ± 0.014[a,e]
	Control + Vit-E (n = 6)	0.32 ± 0.039[f]	0.050 ± 0.007[f]	0.152 ± 0.012[b,f]
	Control + ST + Vit-E (n = 6)	0.29 ± 0.014[f]	0.053 ± 0.008[f]	0.125 ± 0.015[c,f]
	Diabet (n = 11)	1.36 ± 0.164[c]	0.131 ± 0.013[c]	0.213 ± 0.012
	Diabet + ST (n = 12)	0.52 ± 0.049[f]	0.088 ± 0.011[a,f]	0.146 ± 0.006[c,f]
	Diabet + Vit-E (n = 11)	0.45 ± 0.063[f]	0.056 ± 0.007[f]	0.149 ± 0.006[c,f]
	Diabet + ST + Vit-E (n = 11)	0.40 ± 0.059[f]	0.051 ± 0.002[f]	0.117 ± 0.009[c,f]

Note: Means ± SEM; n = number of animals.
[a] $P < .05$; [b] $P < .01$; [c] $P < .001$ vs. control; [d] $P < .05$; [e] $P < .01$; [f] $P < .001$ vs. diabet; ANOVA.

kidney CAT activity; ST treatment alone completely prevented the diabetes-induced abnormal enzyme activity (Table III).

The brain and heart of untreated diabetic rats had considerably increased MDA levels compared with controls (Table III). A statistically significant reduction of MDA was found in brain and heart of diabetic rats when the diabetic rats were treated with ST or vitamin E. The combined effect of ST and vitamin E on MDA was slightly greater than that of ST or vitamin E alone, and antioxidant combination achieved a completely amelioration in MDA levels of diabetic brain and heart. Kidney lipid peroxidation levels were similar in control and untreated diabetic animals. However, ST and vitamin E treatments, when applied separately or together, significantly reduced kidney MDA in both control and diabetic rats; and the combined effect of antioxidants was greater than that of ST or vitamin E alone (Table III).

DISCUSSION

As indicated previously (10), the present study has demonstrated that the activity of G-6PD is approximately two-fold more than the 6-PGD activity in brain and aorta. Our results also confirmed that STZ diabetes causes an increase in the activation of G-6PD, whereas 6-PGD is unaltered in the rat brain. The administration of both antioxidants (ST plus vitamin E) together decreased the high activity of G-6PD in diabetic rat brain. In accordance with our findings, other investigators found that G-6PD and/or 6-PGD activities in cytoplasmic and mitocondrial fractions of brain, kidney, heart, sciatic nerve, and lymphoid organs are markedly increased in experimentally diabetic rats (10,30–33). In contrast, there are few reports demonstrating a significant decrease in G-6PD activity in different peripheral organs of diabetic animals (34).

G-6PD and 6-PGD are considered fundamental enzymes of the pentose phosphate pathway. These enzymes produce NADPH, which is required for many reduction systems (6). It has been reported that when G-6PD concentration is reduced to 10% of the normal level, the oxidative pentose phosphate pathway becomes defective, resulting in a lowered content of NADPH (10). NADPH generation is also required for GR activity and GSH production. When NADPH and GSH are deficient, the entrance of oxidizing compounds causes damage to the lipids and proteins and consequent cell destruction. On the other hand, the requirement of NADH and NADPH as cofactors in the cellular reduction of naturally antioxidant compounds such as α-lipoic acid has been reported previously, and now there is direct evidence describing the impaired antioxidant regeneration on the levels of cellular reducing equivalents due to imbalance in the NAD(P)H/NAD^+ ratio in diabetes (7). In previous studies, diabetes-induced stimulation in the pentose phosphate pathway, which pointed out the activation of G-6PD and 6-PGD, has been explained by the activation of glucuronic acid conjugate detoxication system or the activation of nucleotides biosynthesis (10). Increased G-6PD activity in diabetic rat brain could be explained by the rise in substrate level (i.e., glucose-6-phosphate as shown previously [33]). In our study it seems that diabetes raised the capacity of brain cells for the generation of reducing equivalents; this alteration may initially counteract the prooxidant effect of hyperglycemia in diabetes.

In contrast to the observation in the brain, in peripheral systems (i.e., aorta), diabetes affected both enzymes of the penthose phospate pathway negatively. The exact mechanism(s) of the opposite response of G-6PD activity to STZ-induced diabetes in brain and aorta is not clear; however, it may be related to the differences in glucose uptake/utilization between brain and aorta in the presence of insulin deficiency. Recently, it has been reported that in bovine aortic endothelial cells, high glucose stimulated increased cAMP, which leads to increased protein kinase A activity, phosphorylation of G-6PD, and inhibition of G-6PD activity (35). In the polyol pathway, G-6PD deficiency causes hyperglycemia, making more glucose available for the nonenzymatic production of advanced glycosylation end products, which also causes an increase in superoxide anions and quenching of NO (36). G-6PD deficiency has been overlooked as a cause of both oxidative stress and decrease in the generation of NO. It is well known that NO synthase is another enzyme that uses NADPH as cofactor to synthesis of NO. NO is an important endothelium-derived endogen vasodilator and maintains relaxant tonus in physiological range. Diabetes mellitus has been well documented to lead to endothelial dysfunction resulting vasoconstriction and increased blood pressure (4,5,14). NAD(P)H-oxidases, including endothelial NO synthase, are also capable of producing superoxide in some pathological conditions such as diabetes mellitus (37). Among the reactive oxygen species, superoxide anion plays a critical role in vascular biology because it is the source for many other reactive oxygen species. Superoxide promotes peroxynitrite formation, resulting in the tyrosine nitration, as well as a decline in the bioavailability of NO, and leads to various vascular cell function abnormalities that accompany some cardiovascular risk fac-

tors. Recently, G-6PD deficiency has been reported to have an important role in the high prevalence of hypertension in diabetes mellitus (38).

In our study, neither each antioxidant alone nor their combination achieved the prevention of diabetes-induced decline in the activities of G-6PD and 6-PGD in aorta. Although the roles of some natural antioxidant compounds on specific pathways of reductive equivalents production were documented previously, the effects of a synthetic antioxidant on the activities of G-6PD and 6-PGD have not yet been examined directly in diabetes mellitus. Insulin, vanadate, and selenate have been demonstrated to be able to partially or totally restore the altered activity and the expression of G-6PD and 6-PGD in diabetic animals (30,34). In addition, there are some reports on the G-6PD and 6-PGD regulating effects of various plant products such as Sammo (10), *Capparis decidua* (39), *Mamordica charantia* fruit (40), and *Ginko biloba* extract (41) that have been defined as antihyperglycemic and/or antioxidant. In this regard, the results of our comparable investigation with a synthetic pyridoindole antioxidant, ST, and vitamin E are novel and considerable in the understanding the effects of antioxidants on the imbalance between reductive and oxidative equivalents in diabetes.

We found that in spite of the activation of G-6PD, the GR activity is significantly depressed in diabetic brain. This depression in GR activity was also observed in diabetic heart. The results obtained from the diabetic rats suggest that the production of NADPH, as a consequence of increased activation of G-6PD, not sufficient to maintenance of normal activation of GR, reproduction of GSH, and overcoming the oxidant reactions. Previously, increased or decreased activity was reported in GR activity in different tissues of diabetic rodents (40,42). We also found that the activity of GSHPx is greatly increased in brain, heart, and kidney of diabetic rats. Previously, the activity of GSHPx has been shown to decrease in brain (12) and increase in kidney (11), reticulocytes (38), heart (42), aorta (14), and lyphoid organs (30) of STZ-diabetic rats. These results clearly indicate that the antioxidant enzyme activity is tissue dependent and varies from tissue to tissue and that the duration and the severity of diabetes are major contributing factors for the alterations. GR is responsible for the regeneration of GSH, whereas GSHPx and GST work together with GSH in the decomposition of hydrogen peroxide or other organic hydroperoxides. The induction of GSHPx activity may merely be a manifest of an antioxidant response to the increased peroxidative stress in diabetic state, and the depression in GR activity may reflect the decline of the production and availability of GSH to overcoming hydrogen peroxide. In our study, vitamin E was not found to be effective in the prevention of diabetes-induced activation in GSHPx in peripheral organs (heart and kidney), but it markedly ameliorated GSHPx activity in diabetic brain. Contrary, ST treatment alone was effective in the amelioration of GSHPx activity of both central and peripheral organs of diabetic rats. The treatment with each antioxidant or with combination of both antioxidants completely prevented the defects induced by diabetes in cardiac and neuronal GR activity and resulted in activation of GR in diabetic tissues.

On the other hand, a slight reduction observed in brain GST activity of diabetic rats implies an adaptive response to the increased production of oxidized glutathione (GSSG) in diabetes, as suggested previously (11). A concomitant decrease or increase in tissue GST activity and GSH content has been well documented in diabetic rats (11,43,44). In the brain of diabetic rats, a single treatment with ST, vitamin E, or combined treatment with these agents resulted in activation of GST that may be related to GSH regeneration through increased formation of NADPH. Combination of ST and vitamin E also produced a significant increase in brain GST activity in normal animals. Both antioxidants significantly inhibited GST activity in aorta of normal control rats. Previously, it has been demonstrated that the treatment with some plant extracts having antioxidant properties led to an increase in GST activity and GSH level (40–42).

In our study, although CAT was activated in diabetic heart, it was depressed in diabetic kidney. Diabetes-induced abnormalities in CAT activity did not respond to vitamin E treatment alone in heart, was moderately ameliorated by the treatment with this vitamin in kidney, and was completely prevented by ST treatment alone in both tissues. Diabetes-related and tissue-specific alterations in CAT activities have also been reported (30,39,45). As we indicated previously (45,46), long-term peroxidative stress may result in compensatory elevation in activity of GSHPx and CAT. The mechanism of the different response of heart and kidney CAT to diabetes, although largely unknown, may be related to the concentration of hydrogen peroxide and/or location of the enzyme (45–47). Recently, it has been demonstrated that increased CAT activity in the heart of diabetic rats remained unchanged by treatment with ST (17). This controversy between our results and those of others is most likely a result of the differences between the duration and severity of experimental diabetes or the

treatment dose of ST. Other than that, the in vivo inhibitory effect of ST on diabetes-induced aberration in GSHPx and CAT also supports the finding of a recent study showing the protective effect of in vivo ST supplementation on exogenous hydrogen peroxide–induced abnormal vascular response and integrity (18).

The treatment with ST alone or ST plus vitamin E, but not vitamin E alone, led to a marked decrease in kidney SOD activity in diabetic rats, which is consistent with the ability of ST to scavenge the superoxide in vivo (15), and confirms a previous study demonstrating SOD activity lowering effect of chronically ST administration in diabetic heart (17). The various effects of vitamin E supplementation on oxidative stress–induced detrimental effects in diabetes mellitus and the benefits of this vitamin on tissue antioxidant defense have been well documented (5,48). Our study provided the first evidence on the comparable effects of in vivo treatments with a new antioxidant, ST, and a lipid-soluble antioxidant, vitamin E, on the enzymes of the pentose phosphate metabolic pathway and antioxidant status in brain of diabetic animals. These results are in consistent with the degenerative role of hyperglycemia on cellular reducing equivalent homeostasis and antioxidant defense, and provide further evidence that pharmacological intervention of different antioxidant combinations may have significant implications in the prevention of the prooxidant feature of diabetes and protect redox status of the cells.

ACKNOWLEDGMENTS

The Turkish Scientific and Research Council (TUBITAK-SBAG-2397) and Ankara University Research Foundation (ARFO-2001 08 03 031) supported this study.

REFERENCES

1. Bayners, J. W. 1991. Role ofoxidative stress in development of complications in diabetes. Diabetes 40:405–412.
2. Karasu, Ç., Dewhurst, M., Stevens, E. J., and Tomlinson, D. R. 1995. Effects of anti-oxidant treatment on sciatic nerve dysfunction in streptozotocin-diabetic rats: Comparison with essential fatty acids. Diabetologia 38:129–134.
3. Giugliano, D., Ceriello, A., and Paolisso, G. 1996. Oxidative stress and diabetic vascular complications. Diabetes Care 19:257–267.
4. Karasu, Ç. 2000. Time course of changes in endothelium-dependent and -independent relaxation of chronically diabetic aorta: Role of reactive oxygen species. Eur. J. Pharmacol. 392:163–173.
5. Karasu, Ç., Ozansoy, G., Bozkurt, O., Erdoğan, D., and Ömeroğlu, S. 1997. Antioxidant and triglyceride lowering effects of vitamin E associated with the prevention of abnormalities in the reactivity and morphology of aorta from streptozotocin-diabetic rats. Metabolism 46:872–879.
6. Martins, R. N., Stokes, G. B., and Masters, C. L. 1986. Regulation of liver and brain hexose monophosphate dehydrogenases by insulin and dietary intake in the female rat. Mol. Cell Biochem. 70:169–175.
7. Roy, S., Sen, C. K., Tritschler, H. J., and Packer, L. 1997. Modulation of cellular reducing equivalent homeostasis by alpha-lipoic acid: Mechanisms and implications for diabetes and ischemic injury. Biochem. Pharmacol. 53:393–399.
8. Hink, U., Li, H., Mollnau, H., Oelze, M., Matheis, E., Hartmann, M., Skatchkov, M., Thaiss, F., Stahl, R. A., Warnholtz, A., Meinertz, T., Griendling, K., Harrison, D. G., Forstermann, U., and Munzel, T. 2001. Mechanisms underlying endothelial dysfunction in diabetes mellitus. Circ. Res. 88:E14–E22.
9. Onozato, M. L., Tojo, A., Goto, A., Fujita, T., and Wilcox, C. S. 2002. Oxidative stress and nitric oxide synthase in rat diabetic nephropathy: Effects of ACEI and ARB. Kidney Int. 61:186–194.
10. Abdel-Rahim, E. A., El-Saadany, S. S., Abo-Eytta, A. M., and Wasif, M. M. 1992. The effect of Sammo administration on some fundamental enzymes of pentose phosphate pathway and energy metabolites of alloxanized rats. Die. Nahrung. 36:8–14.
11. Mak, D. H. F., Ip, S. P., Li, P. C., Poon, M. K. T., and Ko, K. M. 1996. Alterations in tissue glutathione antioxidant system in streptozotocin-induced diabetic rats. Mol. Cell Biochem. 162: 153–158.
12. Ramanathan, M., Jaiswal, A. K., and Bhattacharya, S. K. 1999. Superoxide dismutase, catalase and glutathione peroxidase activities in the brain of streptozotocin induced diabetic rats. Ind. J. Exp. Biol. 37:182–183.
13. Kaul, N., Siveski-Iliskovic, N., Hill, M., Khaper, N., Seneviratne, C., and Singal, P. K. 1996. Probucol treatment reverses antioxidant and functional deficit in diabetic cardiomyopathy. Mol. Cell Biochem. 160/161:283–288.
14. Koçak, G., Aktan, F., Canbolat, O., Özoğul, C., Elbeğ, Ş., Yildizoğlu-Ari, N., and Karasu, Ç. 2000. Alpha-lipoic acid treatment ameliorates metabolic parameters, blood pressure, vascular reactivity and morphology of vessels already damaged by streptozotocin-diabetes (The ADIC Study Group). Diabetes Nutr. Metab. 13:308–319.
15. Horakova, L. and Stolc, S. 1998. Antioxidant and pharmacodynamic effects of pyridoindole stobadine. Gen. Pharmacol. 30:627–638.
16. Pekiner, B., Ulusu, N. N., Das-Evcimen, N., Sahilli, M., Aktan, F., Stefek, M., Stolc, S., and Karasu, Ç. 2002. In vivo treatment with stobadine prevents lipid peroxidation, protein glycation and calcium overload but does not ameliorate Ca^{2+}-ATPase activity in heart and liver of streptozotocin-diabetic rats: Comparison with vitamin E. Biochim. Biophys. Acta. 62143, 588:71–78.
17. Stefek, M., Sotnikova, R., Okruhlicova, L., Volkovova, K., Kucharska, J., Gajdosik, A., Gajdosikova, A., Mihalova, D., Hozova, R., Tribulova, N., and Gvozdjakova, A. 2000. Effect of dietary supplementation with the pyridoindole antioxidant stobadine on antioxidant state and ultrastructure of diabetic rat myocardium. Acta Diabetol. 37:111–117.
18. Sotnikova, R., Stefek, M., Okruhlicova, L., Navarova, J., Bauer, V., Gajdosik, A., and Gajdosikova, A. 2001. Dietary supplementation of the pyridoindole antioxidant stobadine reduces vascular impairment in streptozotocin-diabetic rats. Methods Find. Exp. Clin. Pharmacol. 23:121–129.
19. Karasu, Ç., Ulusu, N., Avci, A., Sahilli, M., Canbolat, O., Ozansoy, G., Ari, N., Stefek, S., Stolc, S., and Gojdosik, A. 2001. Effects of ST treatment on pentose phosphate pathway, glutathione-dependent enzymes, superoxide dismutase, and lipid peroxidation in the brain of diabetic rat; comparison with vitamin E. Diabetologia 44:(Suppl. 1) A155:592.
20. Karasu, Ç., Avi, A., Canbolat, O., Bali, M., Ozansoy, G., Ceylan, A., Ari, N., and Stefek, M. 2002. Comparative effects of stobadine and vitamin E treatments on oxidative stress markers in heart and kidney of streptozotocin-diabetic rats. Free Radic. Biol. Med. 33(Suppl. 1):S188, 494.

21. Ulusu, N. N., Kus, M. S., Acan, N. L., and Tezcan, E. F. 1999. A rapid method for the purification of glucose-6-phosphate dehydrogenase from bovine lens. Int. J. Biochem. Cell Biol. 31: 787–796.
22. Pearse, B. M. F. and Rosemeyer, M. A. 6-Phosphogluconate dehydrogenase from human erytrocytes. Page 220, in Colowich, S. R. and Kaplan, N. O. (eds.), Methods Enzymology. XLI. Academic Press Inc., London.
23. Lawrence, R. A. and Burk, R. F. 1976. Glutathione peroxidase activity in selenium-deficient rat liver. Biochem. Biophys. Res. Commun. 71:952–958.
24. Acan, N. L. and Tezcan, E. F. 1989. Sheep brain glutathione reductase: Prufication and general properties. FEBS Lett. 250:72–74.
25. Habig, W. H., Pabst, M. J., and Jakoby, W. B. 1974. Glutathione S-transferase. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249:7130–7139.
26. Durak, I., Yurtarslan, Z., Canbolat, O., and Akyol, O. 1993. A methodological approach to superoxide dismutase (SOD) activity assay based on inhibition of nitroblue tetrazolium (NBT) reduction. Clin. Chim. Acta. 214:103–104.
27. Aebi, H. 1983. Catalase in vitro. Methods Enzymol. 105:121–126.
28. Jain, S. K. and Levine, S. N. 1995. Elevated lipid peroxidation and vitamin E-quinone levels in heart ventricles of streptozotocin-treated diabetic rats. Free Radic. Biol. Med. 18: 337–341.
29. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.
30. Pereira, B., Rosa, L. F., Safi, D. A., Bechara, E. J., and Curi, R. 1994. Superoxide dismutase, catalase and glutathione peroxidase activities in the lymphoid organs of diabetic rats. J. Endocrinol. 142:161–165.
31. McDermott, B. M., Flatt, P. R., and Strain, J. J. 1994. Effects of copper deficiency and experimental diabetes on tissue antioxidant enzyme levels in rats. Ann. Nutr. Metab. 38:263–269.
32. Thurston, J. H., McDougal, D. B., Jr, Hauhart, R. E., and Schulz, D. W. 1995. Effects of acute, subacute, and chronic diabetes on carbohydrate and energy metabolism in rat sciatic nerve: Relation to mechanisms of peripheral neuropathy. Diabetes 44:190–195.
33. Lakhman, S. S., Sharma, P., Kaur, G., and Kaur, G. 1994. Changes in glucose metabolism from discrete regions of rat brain and its relationship to reproductive failure during experimental diabetes. Mol. Cell Biochem. 141:97–102.
34. Berg, E. A., Wu, J. Y., Campbell, L., Kagey, M., and Stapleton, S. R. 1995. Insulin-like effects of vanadate and selenate on the expression of glucose-6-phosphate dehydrogenase and fatty acid synthase in diabetic rats. Biochimie 77:919–924.
35. Zhang, Z., Apse, K., Pang, J., and Stanton, R. C. 2000. High glucose inhibits glucose-6-phosphate dehydrogenase via cAMP in aortic endothelial cells. J. Biol. Chem. 275:40042–40047.
36. Guzik, T. J., Mussa, S., Gastaldi, D., Sadowski, J., Ratnatunga, C., Pillai, R., and Channon, K. M. 2002. Mechanisms of increased vascular superoxide production in human diabetes mellitus: Role of NAD(P)H oxidase and endothelial nitric oxide synthase. Circulation 105:1656–1662.
37. Gupta, B. L. and Baquer, N. Z. 1998. Hexokinase, glucose-6-phosphate dehydrogenase and antioxidant enzymes in diabetic reticulocytes: Effects of insulin and vanadate. Biochem. Mol. Biol. Int. 46:1145–1152.
38. Gaskin, R. S., Estwick, D., and Peddi, R. 2001. G6PD deficiency: Its role in the high prevalence of hypertension and diabetes mellitus. Ethn. Dis. Fall. 1:749–754.
39. Yadav, P., Sarkar, S., and Bhatnagar, D. 1997. Action of capparis decidua against alloxan-induced oxidative stress and diabetes in rat tissues. Pharmacol. Res. 36:221–228.
40. Raza, H., Ahmed, I., John, A., and Sharma, A. K. 2000. Modulation of xenobiotic metabolism and oxidative stress in chronic streptozotocin-induced diabetic rats fed with momordica charantia fruit extract. J. Biochem. Mol. Toxicol. 14:132–139.
41. Agha, A. M., El-Fattah, A. A., Al-Zuhair, H. H., and Al-Rikabi, A. C. 2001. Chemopreventive effect of Ginkgo biloba extract against benzo(a)pyrene-induced forestomach carcinogenesis in mice: Amelioration of doxorubicin cardiotoxicity. J. Exp. Clin. Cancer Res. 20:39–50.
42. Sanders, R. A., Rauscher, F. M., and Watkins, J. B., 3rd 2001. Effects of quercetin on antioxidant defense in streptozotocin-induced diabetic rats. J. Biochem. Mol. Toxicol. 15:143–149.
43. Gupta, D., Raju, J., Prakash, J., and Baquer, N. Z. 1999. Change in the lipid profile, lipogenic and related enzymes in the livers of experimental diabetic rats: Effect of insulin and vanadate. Diabetes Res. Clin. Pract. 46:1–7.
44. Volkovova, K., Chorvathova, V., Jurcovicova, M., Koszeghyova, L., and Bobek, P. 1993. Antioxidative state of the myocardium and kidneys in acute diabetic rats. Physiol. Res. 42: 251–255.
45. Karasu, Ç. 1999. Increased activity of H_2O_2 in aorta isolated from chronically streptozotocin-diabetic rats: Effects of antioxidant enzymes and enzymes inhibitors. Free Radic. Biol. Med. 27:16–27.
46. Zobali, F., Avci, A., Canbolat, O., and Karasu, C. 2002. Effects of vitamin A and insulin on the antioxidative state of diabetic rat heart: A comparison study with combination treatment (antioxidants in diabetes-induced complications) The ADIC Study Group. Cell Biochem. Funct. 20:75–80.
47. Kowluru, R. A., Kern, T. S., and Engerman, R. L. 1997. Abnormalities of retinal metabolism in diabetes or experimental galactosemia: IV. Antioxidant defense system. Free Radic. Biol. Med. 22:587–592.
48. Gokkusu, C., Palanduz, S., Ademoglu, E., and Tamer, S. 2001. Oxidant and antioxidant systems in NIDDM patients: influence of vitamin E supplementation. Endocr. Res. 27:377–386.