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Dietary Ascorbate Supplementation Reduces Oxidative Tissue Damage and Expression of iNOS in the Kidney of Streptozotocin Induced Diabetic Rats

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Reactive oxygen species (ROS) have been suggested to be contributory factors in complications of diabetes mellitus. In the present study, we investigated the generation of superoxide, the lipid peroxide level measured as thiobarbituric acid reactive substances, the vasorelaxation of isolated thoracic aorta and the iNOS expression in kidney of streptozotocin induced diabetic rats. Sprague Dawley rats were divided into four groups: control, ascorbate (400 mg/kg rat weight daily in drinking water), diabetic (single dose of 50 mg of STZ/kg i.p.) and diabetic simultaneously fed with ascorbate for 12 wk. Rats in groups were studied at tri-weekly intervals (0 to 12 wk). Diabetic rats were evaluated periodically with changes of plasma glucose levels and body weight. The ascorbate supplimentation attenuated the development of hyperglycemia and weight loss induced by STZ injection in rats. In the present experimental condition, the ascorbate supplimentation had no significant effect on plasma glucose levels and changes in body weight of normal rate. The superoxide generation, formation of thiobarbituric acid reactive substance and iNOS expression in kidney were significantly increased in STZ-treated rats that were decreased by ascorbate supplimentation. The ascorbate supplimentation had no effect on vasorelaxation of isolated thoracic aorta. These results indicate that ascorbate supplimentation may exert an inhibitory effect on STZ-induced oxidative tissue damage through protection of pancreatic islet cells by scavanging reactive oxygen species. The ascorbate supplimentation may possibly attenuate the renal complication of diabetes mellitus.

Key Words: Ascorbate, iNOS, Streptozotocin

INTRODUCTION

Streptozotocin (STZ, 2-deoxy-D-glucose derivative of N-methyl-N-nitrosourea) (Herr et al, 1967), shows slective toxicity to pancreatic β-cells (Tjalve et al, 1976; Morgan et al, 1994). Therefore, STZ is widely used as a strong inducer of diabetes in experimental animals as well as the clinical treatment of the pancreatic cancer (Murray-Lyon et al, 1968; Broder & Carter, 1973). STZ is taken up through glucose transporter into pancreatic β-cells (Kawada et al, 1987) and causes impairment of mitochondrial oxidative processes in terms of glucose oxidation, inhibition of protein syntheses and suppression of insulin release (Ledoux & Wilson, 1984; Eizirik et al, 1991; Janjic & Wollheim, 1992; Turk et al, 1993).

Oxidative stress may occur as a result of increased free radical generation, decreased levels of antioxidants and/or impaired regeneration of reduced forms of antioxidants. Evidence for oxidative stress in diabetes includes observations of decreased antioxidant plasma concentrations in both diabetic subjects (Karpen et al, 1985) and animal models of diabetes (McLennan et al, 1988), and reports of increased free radical-generated plasma lipid peroxides (Morel & Chisolm, 1989). There are studies, however, which suggest that plasma and tissue levels of vitamin E may be raised in both insulin-dependent diabetes mellitus (IDDM) (Krempf et al, 1991) and the STZ rat (Thompson et al, 1992), possibly as a compensatory response to increased free radical lipid peroxidation. Free radicals may be responsible for damage to DNA, oxidative modification of lipids including low-density lipoprotein (LDL), and have been implicated in glycation and protein modification reactions that contribute to tissue damage in diabetes (Wolff, 1993). LDL in its native or oxidised state can impair endothelium-dependent relaxation (Jacobs et al, 1990), and oxidised LDL may be toxic to cells (Morel & Chisolm, 1989) and decrease expression of endothelial NO-synthesis (Liao et al, 1995). NO originates from L-arginine in a reaction catalyzed by several different nitric oxide synthase isoenzymes. The endothelial constitutive NOS (ecNOS) is found in the vascular endothelium, depends on calcium and calmodulin, and releases small amounts of NO. The inducible NOS (iNOS) can be expressed by various cell types, including macrophages, vascular smooth muscle cells, and glomerular mesangial cells, leading to the formation of

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ABBREVIATIONS: iNOS, inducible nitric synthase; ROS, reactive oxygen species; STZ, streptozotocin.

large amounts of NO, and can be induced by endotoxins and cytokines.

Nitric oxide (NO) at physiological concentrations (i.e., 1~20 nM) exerts several important anti-shock effects which include vasodilation, inhibition of platelet aggregation, attenuation of leukocyte adherence to the vascular endothelium (Davenpeck et al, 1994), and quenching of superoxide radicals (Rubanyi & Vanhoutte, 1986). All these effects are important actions of NO necessary for the maintenance of vascular and microcirculation for the homeostasis. However, NO, like many immunologic mediators, appears to be a "two-edged sword". Diabetic nephropathy is the most common single cause of end-stage renal disease in the United States and in Europe. Increases of renal perfusion and glomerular filtration rate (GFR) occur early in the course of diabetic nephropathy, a feature seen in experimental and clinical diabetes. This was reported to be due to increased formation of nitric oxide (Tolins et al, 1993). Endogenous NO potentiates inflammatory responses and seems to be involved in both acute and chronic inflammation.

In the Cambridge Heart Antioxidant Study, vitamin E supplementation has been shown to be some benefit in non-diabetic patients with coronary artery disease, as it decreased the rate of non-fatal myocardial infarction (Stephens et al, 1996). Some therapeutic potential for dietary antioxidant supplementation has also been inferred from studies of conduit arteries in animal models of diabetes (Cotter et al, 1995).

Theoretically, antioxidant supplementation may be of benefit to vascular function in diabetes. Ascorbate may block propagation of free radical reaction or prevent tissue damages by free radical as an antioxidant. It is well established that ascorbate is also able to perform the role of superoxide anion in metal catalyzed Haber-Weiss reaction and facilitates tissue damage, including lipid peroxidation (Park et al, 1987). Ascorbate may has either an antioxidant or pro-oxidant properties depending on the experimental condition.

Effects of ascorbate on iNOS expression in kidney of streptozotocin induced diabetic rat is not known. Therefore, the aim of this study was to investigate whether this beneficial effect of ascorbate dietary supplementation on superoxide generation, lipid peroxidation, vascular function and renal iNOS expression occurs in an animal model of diabetes.

METHODS

Induction of experimental diabetes

The male Sprague-Dawley rats (200~250 g body weight) were housed in steel cages in a temperature and light controlled room with 12 h cycles of dark and light. Rats were randomly divided into four groups: control group, STZ treated group, ascorbate treated group, and ascorbate +STZ treated group. Diabetes was induced in overnight fasted rats by intraperitoneal injection with a single dose of 50 mg/Kg STZ freshly dissolved in 10 mM citrate buffer, pH 4.0. Age-matched control rats received equivalent amounts of buffer intraperitoneally. Control and STZ-treated rats were given food and water ad libitum. Concentration of ascorbate was based on the observed daily consumption of approximately 50~60 ml water per rat. Blood glucose was checked using a One Touch II Blood Glucose Monitoring System (Lifescan Inc., Milpitas, CA.). If non-fasting blood glucose in the tail vein exceeded 200 mg/dl for two consecutive days, animals were considered as diabetic state. Three hours after the confirmation of diabetes (two days after the STZ injection), ascorbate group and ascorbate+STZ group received ascorbate in drinking water (400 mg/kg rat weight, daily until sacrificed). Body weights were recorded at weekly intervals.

Tissue preparation

The animals were sacrificed by cervical dislocation 6, 9, 12 wk after STZ injection, venous blood were collected, and tissues (kidney) were removed. All tissues were rinsed in cold saline solution and stored at −80°C until analyzed.

Superoxide measurement

The superoxide dependent reduction of ferricytochrome c was measured by the method of Markert et al. (1984). The reaction mixtures in plastic microfuge tubes contained kidney homogenate (50 mg/ml), Hanks' balanced salt solution (HBSS), 75 μM ferricytochrome c, and 20 mM HEPES-tris, pH 7.4 in a total volume of 1.0 ml. After a 30 min of incubation at 37°C, the reaction was stopped by placing the tubes in melting ice, and the tissues were rapidly pelleted by centrifuging at 1,500 g for 5 min at 4°C. The supernatants were taken and the amount of reduced cytochrome c was measured at 550 nm. The amount of reduced cytochrome c was calculated by using an extinction coefficient of 2.1×10^4 $M^{-1}cm^{-1}$ at 550 nm (Cohen & Chovaniec, 1978).

Measurement of lipid peroxidation

The sample was homogenized in 10 volume K-H buffer using a Polytron (PCU-2) homogenizer (Kinematica GMBH, Luzern, Switzerland). Homogenates were centrifuged at 12,500 g at 4°C for 30 minutes. The supernatants were then collected. Lipid peroxidation of the kidney was estimated from measurement of malondialdehyde concentration by thiobarbituric acid method. The supernatant of kidney homogenate (1 mg protein/ml) was incubated in the reaction mixture consisting of 150 mM KCl, and 50 mM NaH_2PO_4, pH 7.4. The reaction was started by addition of renal tissue in a final volume of 1.0 ml. After 30 min of incubation, the reaction was stopped by adding 1.0 ml of 1% 2-thiobarbituric acid (TBA) in 50 mM NaOH and 1.0 ml of 2.8% trichloroacetic acid. The chromophore was developed by boiling in a water bath for 10 min. After cooling to the room temperature, the absorbance was measured at 532 nm (Gutteridge, 1981). The concentration of malondialdehyde was expressed as nmol/mg protein, using the molar extinction coefficient of $1.52\times10^6/M^{-1}cm^{-1}$ (Placer et al, 1966).

Vasorelaxation of isolated thoracic aorta

Thoracic aortae isolated from control rats, STZ treated rats, ascorbic acid treated rats, and STZ+ascorbic acid treated rats were immersed in warm oxygenated K-H solution and cleaned of adherent fat and connective tissue. Thoracic aortae were carefully dissected into rings of 2 to 3 mm in length preserving the integrity of the endothelium.

The rings were suspended in oxygenated 15 ml organ bath and subjected to 1.0 g of resting force as previously described (Weyrich et al, 1994). After equilibration, the rings were stimulated with 100 nM U-46619 (9,11-epoxymethano-PGH_2, Biomol Research Laboratories, Plymouth Meeting, PA), a thromboxane A_2 mimetic, to generate about 1.0 g of developed force. Once the contraction reached a stable plateau, ACh, an endothelium dependent vasodilator, was added to the bath in cumulative concentrations of 0.1, 1, 10 and 100 nM. After the response to the highest concentration stabilized, the rings were washed three times with fresh K-H buffer and allowed to equilibrate for 20 min to reach baseline tone. This procedure was repeated with another endothelium-dependent vasodilator, A-23187 (1, 10, 100 and 1,000 nM), as well as with an endothelium-independent vasodilator, acidfied $NaNO_2$ (0.1, 1, 10 and 100 μM), which was titrated to pH 2. Relaxation was calculated as the percent decrease from the peak U-46619 induced precontraction value.

Protein assay

Protein contents of tissue homogenate were measured by the Bradford method as described in the Bio-Rad protein assay kit.

Immunoblotting

Each sample was homogenized in RIPA buffer (1% Triton X-100, 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM Na_3VO_4, 40 mM NaF, 5 mM EGTA, 0.2% SDS, 0.2 mM PMSF, 100 μM Leupepsin). The homogenate was centrifuged at 13,000 g for 30 min. Protein concentrations of supernatant were determined by the method of Bradford using BSA as a standard. Sample buffer was added to an aliquots (50 μg of protein) of supernatant, boiled for 3 min, and then resolved by SDS-PAGE under reducing condition. To assess iNOS activation, Western blotting with anti-iNOS antibodies was performed. The resolved proteins were transferred to PVDF membranes and then blocked in TBST (10 mM Tris, 100 mM NaCl, 0.05% Tween 20, pH 7.5) containing 5% skim milk overnight. The membranes were washed three times in TBST, followed by incubation for 1 hr with goat-anti-rat secondary antibodies conjugated to HRP in TBST containing 5% nonfat dried milk. The blots were washed three times in TBST followed by detection with an ECL detection system (Amersham). In some instances, blots were stripped by incubation in stripping buffer (62.5 mM Tris, pH 6.8, 100 mM β-mercaptoethanol, and 2% SDS) for 30 min at 50°C and then reprobed with other antibodies.

Statistical analysis

Results are given as means±S.E. and n represents the number of experiments. The statistical analysis was carried out by Student t-test. p values less than 0.05 were considered to indicate significant differences. The statistical difference between control and STZ-group was shown in the text, but not in figures and tables.

RESULTS

Plasma glucose levels and body weight

The changes in plasma glucose level in the STZ-treated rats are shown in Table 1. Plasma glucose was significantly elevated in the STZ-treated rats, compared to controls and ascorbate treated groups ($p<0.01$), and the level in the ascorbate+STZ group was lower than that in the STZ groups ($p<0.01$ in 3, 6, 9 and 12 wk). There was no significant difference in plasma glucose concentrations among the STZ-treated groups. Rats were intraperitoneally injected with STZ, or orally administered with ascorbate, and sacrificed 3, 6, 9, 12 wk after the injection as described in Materials and Methods. The changes of body weights in four groups were investigated. There was a progressive increase in the body weight in the control and the ascorbate groups, whereas these in the STZ group decreased (Table

Table 1. Changes in blood glucose

		Control (n=9)	Ascorbate (n=9)	STZ (n=9)	STZ+Ascorbate (n=9)
Glucose (mg/dL)	48 Hours	90.6±2.7	92.6±2.7	257.8±11.9	248.8±10.9
	3 Weeks	92.4±2.3	89.1±2.7	499.8±12.3	313.1±11.7*
	6 Weeks	91.6±2.4	91.6±1.9	514.3±9.4	371.1±12.5*
	9 Weeks	92.6±2.7	93.1±2.2	529.3±15.2	402.4±13.2*
	12 Weeks	95.4±3.5	94.7±2.3	507.1±13.1	387.3±11.9*

*$p<0.01$ significantly different from STZ alone.

Table 2. Changes in body weight

		Control (n=9)	Ascorbate (n=9)	STZ (n=9)	STZ+Ascorbate (n=9)
Weight (Kg)	48 Hours	243.9±4.1	240.6±4.5	244.4±5.6	236.7±5.5
	3 Weeks	337.8±4.6	347.8±6.7	219.4±9.9	257.2±6.0*
	6 Weeks	362.8±6.9	356.1±10.1	216.7±7.7	253.9±8.7*
	9 Weeks	402.2±4.3	389.4±6.7	220.6±7.9	265.6±6.3*
	12 Weeks	413.3±8.6	402.2±11.2	226.1±8.4	276.1±7.8*

*$p<0.05$ significantly different from STZ alone.

2), therefore administration of ascorbate attenuated the weight loss induced by STZ injection in rats under the experimental conditions described in Table 1.

Effect of ascorbate on superoxide generation and lipid peroxidation in STZ induced diabetic rat kidney

We measured superoxide generation and lipid peroxidation of kidney as an index of oxidant induced tissue injury, which resulted from the production of reactive oxygen species (i.e. superoxide radical, hydroxyl radical, hydrogen peroxide). Amounts of superoxide generated in the control and ascorbate groups were 98.50 ± 4.82 and 95.60 ± 5.78 nmol/min/g wet tissue, respectively: The superoxide generation significantly increased in the STZ induced diabetic rat kidney compared to the value in the control rats. Ascorbate supplementation significantly inhibited the superoxide generation in rat kidney following STZ treatment (Fig. 1). The lipid peroxidation was low in the control and ascorbate treated groups (2.03 ± 0.19 and 1.88 ± 0.19 nmol/10 mg protein). However, the lipid peroxidation was significantly increased in the STZ-treated rats when compared to the control and ascorbate treated groups ($p < 0.05$), and the lipid peroxidation in the ascorbate+STZ group was lower than that in the STZ group ($p < 0.05$ in 12 wk)(Fig. 2).

Effect of ascorbate on vasorelaxation of isolated thoracic aorta

Isolated thoracic aortae were employed to determine the integrity of endothelial function, assessed by the ability of the endothelium to release nitric oxide (Fig. 3).

The thromboxane A_2 mimetic, U46619, was used to contract aortic rings, and the rings were tested with endothelium-dependent (i.e., ACh, A23187) and endothelium-independent (i.e., $NaNO_2$) vasodilators. Both ACh and A23187 fully relaxed aortic rings isolated from the control and ascorbate treated groups (Ach; $89.5 \pm 3.5\%$ and $91.2 \pm 3.4\%$, respectively A23187; $90.3 \pm 4.2\%$ and $91.5 \pm 2.9\%$ respectively). However, the vasorelaxation was markedly decreased in the STZ-treated rats, when compared to the control and ascorbate treated groups ($p < 0.01$), and the vasorelaxation of ascorbate+STZ group was not different from that of STZ treated groups. In contrast to these effects, aortic rings obtained from all the groups responded normally to the endothelium-independent vasodilator $NaNO_2$, indicating that the STZ induced diabetic rats did not result in a significant injury to the vascular smooth muscle of the aorta (Fig. 3). Thus, there appears to be a significant defect specific to endothelium-dependent vasodilators (e.g., ACh and A23187) in the aorta vasculature of STZ induced diabetic rats and the ascorbate did not protect an aortic endothelial function.

Effect of ascorbate in expression of diabetic renal iNOS

We examined the expression of renal iNOS to test renal damage in the STZ induced diabetic rat. Expression of renal iNOS was increased in a time dependent manner, and the

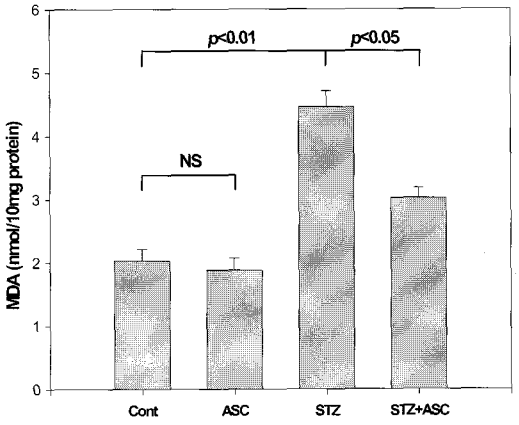

Fig. 2. Effect of ascorbate on lipid peroxidation in kidney of the streptozotocin induced diabetic rats (12 week). Data are expressed as TBARS of nmol/10 mg protein. Bar heights represent means and brackets indicate ±SEM, n=7. ASC, ascorbate; NS, not significant.

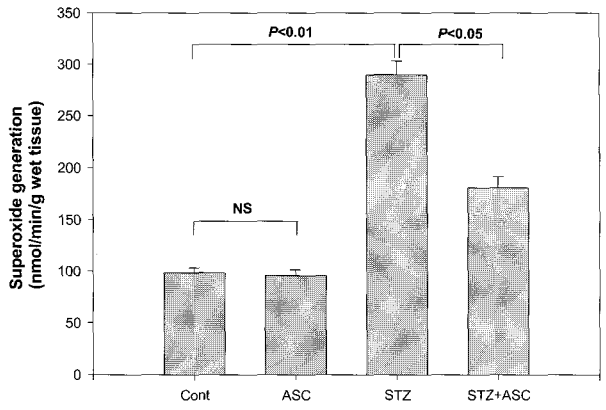

Fig. 1. Effect of ascorbate on superoxide generation in kidney of the streptozotocin induced diabetic rats (12 wk). Data are expressed as superoxide of nmol/min/g wet tissue. Bar heights represent means and brackets indicate ±SEM, n=7. ASC, ascorbate; NS, not significant.

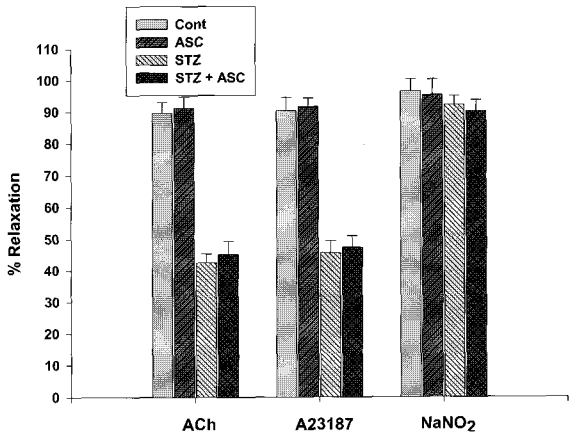

Fig. 3. Summary of the response of thoracic aorta (12 wk) to the heightest concentration of vasodilators: 100 nM ACh, 1 μM A23187, and 100 μM $NaNO_2$. Bar heights represent means and brackets indicate ±SEM for seven individual experiments. ASC, ascorbate.

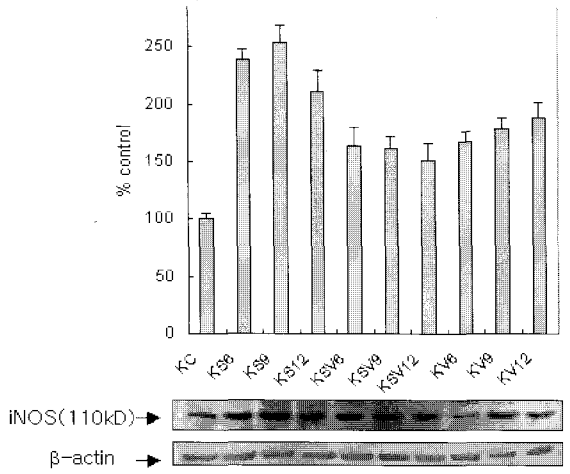

Fig. 4. Immunoblot analysis of iNOS protein level in kidney of the streptozotocin (STZ) induced diabetic rats (6, 9, 12 week). Renal homogenates were prepared, and 50 μg total protein per sample were resolved by SDS-PAGE, and immunoblotted with an anti-iNOS antibody, as described in Methods and Materials. The blots in each panel are representative of three to five independent experiments. KC: control rats. KS: 6, 9, 12 wk after treatment with STZ. KSV: 6, 9, 12 wk after treatment with STZ and ascorbate. KV: 6, 9, 12 wk after treatment with ascorbate.

level of iNOS increased 2.5 fold at 9 wk after the STZ treatment (Fig. 4). Expression of renal iNOS was similarly decreased at 12 week after the STZ treatment, but it was not signficantly different from that of 9 wk treatment. Administration of ascorbate in the STZ induced diabtic rat significantly decreased the expression of renal iNOS, and the level of renal iNOS was similar to ascorbate alone treated group, therefore, indicating that ascorbate may inhibit the expression of renal iNOS.

DISCUSSION

The tissue damage or complications in diabetes mellitus may be due to oxidative stress. Insulin-dependent diabetes mellitus (IDDM) is associated with an increased risk of hypertension, atherosclerosis and disorders of the microcirculation, and there is increasing evidence to suggest that vascular endothelial dysfunction may play a major role. Impaired endothelium-dependent vasodilatation to acetylcholine (ACh) has been reported in aorta and femoral arteries of the STZ treated rat (Taylor et al, 1995), and in diabetic patients (McNally et al, 1994), and has been suggested to be generally associated with reduced availability of nitric oxide (NO). The factors responsible for altered NO synthesis or release are unknown and, although hyperglycemia undoubtedly contributes (Poston & Taylor, 1995), there is increasing evidence that oxidative stress coupled with dyslipidemia (Tribe et al, 1996) may also be important determinants of endothelial dysfunction. Free radicals (which may be generated as a consequence of glucose-induced activation of cyclooxygenase, autooxidation of glucose or from alterations in transition metal metabolism) may cause structural alteration of DNA and modification of proteins within the endothelial cell (Wolff, 1993). Further damage may arise from free radical induced generation of lipid peroxides, particularly oxidised LDL, which has also been implicated directly in reduced endothelial NO synthesis (Liao et al, 1995).

Ascorbate is well known as an antioxidant. Since some antioxidants are suggested to have beneficial effects in the treatment of oxidative stress-associated diseases (Philp et al, 1997), the aim of this study was to determine whether such beneficial effect of ascorbate dietary supplementation on lipid peroxidation, vascular function and renal iNOS expression occurs in an animal model of diabetes. In this study, orally administrated ascorbate significantly inhibited the elevation of blood glucose level and weight loss in STZ-induced diabetic rats.

Reactive oxygen species (ROS) have been implicated in the pathogenesis of diabetes mellitus. The ROS in diabetes is produced through glucose autooxidation and non-enzymatic protein glycation (Hunt et al, 1990). Glycation of glomerular basement membrane can alter renal structure and function. Cochrane and Robinson (1995) have demonstrated that in vitro glycation of glomerular basement membrane alters its permeability. Advanced glycation end products have oxidizing potential and can promote reactive oxygen species damage. ROS exert their cytotoxic effects on membrane phospholipids, resulting in the formation of malondialdehyde (MDA). Peroxidation of membrane increases its fluidity and permeability with loss of membrane intergrity. ROS have been implicated in the process of renal injury.

In order to evaluate the protective effect of ascorbate on STZ induced diabetic rats, on the renal superoxide generation and lipid peroxidation were examined: The increased production of superoxide and MDA in the progression of diabetic rats may play a role in tissue damage associated with diabetes. In the present study, superoxide generation and MDA formation were significantly increased in STZ-treated rats, and treatment of rate with ascorbate apparently attenuated the superoxide generation and MDA formation in diabetic kindey. These findings show that ascorbate may protect tissue components from the oxidative stress of STZ.

The defect in ACh-induced endothelium-dependent relaxation and A23187-induced endothelium-dependent relaxation in thoracic aorta from STZ-rat were not reversed by ascorbate dietary supplementation, in agreement with earlier studies (Cotter et al, 1995). $NaNO_2$-induced endothelium-independent relaxation in thoracic aorta from STZ-rat was not changed. The data suggested that the vascular endothelium was damaged however, the vascular smooth muscle was not damaged by STZ-induced diabetes.

Insulin dependent diabetes mellitus(IDDM) is characterized by a series of complications that affect many organs. Diabetic nephropathy is one of the serious complications in patients with IDDM. The kidney exhibits a characteristic pattern of changes in the glomerulus during diabetes. These changes initally lead to hyperfiltration, and eventually to renal insufficiency or complete kidney failure (Michael et al, 1990). Veelken et al, 2000 recently have demonstrated that nitric oxide synthase isoforms affected glomerular hyperfiltration in early diabetic nephropathy, however, renal iNOS levels in late stage diabetic rat have not yet been reported.

Therefore, we investigated the effect of ascorbate on renal iNOS expression in diabetic rats (12 wk). Fig. 4 showed that iNOS levels in diabetic rats was increased about 2 fold and the increased iNOS level in diabetic rats was at-

tenuated by ascorbate supplementation. These effects suggest that ascorbate supplementation may reduce diabetic renal complication. The increased iNOS expression may be involved in diabetic renal complication of diabetic rats. The antioxidant ability of ascorbate could prevent the development and the progression of renal complication in induced STZ diabetes. In conclusion, the exact mechanism of action of ascorbate supplementation is not known, but we postulate that the ascorbate may protect pancreas islet cells from reactive oxygen species, by inhibiting the increased blood glucose, lipid peroxidation, and renal iNOS expression level in diabetic rats.

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