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Polyol pathway, 2,3-diphosphoglycerate in erythrocytes and diabetic neuropathy in rats

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Abstract

The relationship between the 2,3-diphosphoglycerate concentration in red blood cells as a biological indicator of tissue hypoxia and diabetic neuropathy, and the effect of a potent aldose reductase inhibitor, (2*S*,4*S*)-6-fluoro-2′,5′-dioxospiro [chroman-4,4′-imidazolidine]-2-carboxamide (SNK-860), on both were investigated in streptozotocin-induced diabetic rats. Diabetic rats demonstrated significantly delayed motor nerve conduction velocity and reduced sciatic nerve blood flow. Altered biochemical features in the sciatic nerves, including a marked accumulation of sorbitol and fructose, *myo*-inositol depletion and decreased Na^+/K^+-ATPase activity were also detected in diabetic rats. These defects were accompanied by a decrease in the red blood cell 2,3-diphosphoglycerate concentration. Treatment with SNK-860 partially or completely ameliorated these abnormalities. These observations suggest that a decrease in the red blood cell 2,3-diphosphoglycerate concentration is one of the factors contributing to tissue hypoxia, which results in diabetic neuropathy, and that this decrease is mediated through an aldose reductase inhibitor-sensitive pathway.

Keywords: Diabetic neuropathy; 2,3-Diphosphoglycerate; Hypoxia; Polyol pathway; Aldose reductase inhibitor

1. Introduction

Metabolic and vascular factors have been implicated in the pathogenesis of diabetic neuropathy (Greene et al., 1992; Cameron and Cotter, 1994). However, the exact mechanisms of the development of such neuropathy have not been clarified.

Although the concept of a vascular deficit was originally proposed by Fagerberg (1959), who demonstrated the histological abnormalities of endoneurial vessels in the peripheral nerves of diabetic neuropathy, the interpretation of structural changes has been questioned (Llewwlyn et al., 1988). Recently, this hypothesis was revived and has received considerable attention since the demonstration of reduced nerve blood flow and endoneurial hypoxia in patients with diabetic neuropathy (Newrick et al., 1986) as well as in diabetic rats (Tuck et al., 1984).

An increase in the activity of the polyol pathway is one of the major metabolic contenders for the etiology of diabetic neuropathy, which include the formation of advanced glycation endproduct, the alteration of essential fatty acid metabolism and the abnormalities of neurotrophic factors (Greene et al., 1992). Most investigations on the role of the polyol pathway in the development of diabetic neuropathy have been focused on a biochemical approach based on the altered phosphoinositide metabolism related to polyol pathway-induced *myo*-inositol depletion and the decreased Na^+/K^+-ATPase (Greene et al., 1987; Tomlinson et al., 1992). However, there are recent reports on the effects of aldose reductase inhibitors, which inhibit the rate-limiting enzyme of the polyol pathway, on impaired aortic relaxation (Cameron and Cotter, 1992; Tesfamariam et al., 1993). More recently, Stevens et al. (1994) reported linked roles of aldose reductase, Na^+/K^+-ATPase and nitric oxide in the slowing of motor nerve conduction in streptozotocin diabetic rats from the view point of an altered cytosolic redox state. The glyceraldehyde-3-phosphate dehydrogenase reac-

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tion which catalyzes the formation of 1,3-diphosphoglycerate is also regulated by the redox state in the glycolytic pathway. 1,3-Diphosphoglycerate is converted to 2,3-diphosphoglycerate in erythrocytes. Therefore, the synthesis of 2,3-diphosphoglycerate is also coupled with the cytosolic redox state.

2,3-Diphosphoglycerate has a high affinity for hemoglobin and plays an important role in regulating the binding of oxygen to hemoglobin. It is well-known that a low concentration of 2,3-diphosphoglycerate reduces the ability of red blood cells to release oxygen, resulting in tissue hypoxia, and that the 2,3-diphosphoglycerate concentration in red blood cells is decreased in patients with diabetic ketoacidosis (Alberti et al., 1972a; Hockaday and Alberti, 1972; Ditzel and Standl, 1975). However, conflicting results concerning the 2,3-diphosphoglycerate concentration in non-acidic diabetic patients have been reported (Ditzel, 1972; Alberti et al., 1972b; Rorth et al., 1972; Ditzel et al., 1973; Cauchie et al., 1992) and the interrelationship between red blood cell 2,3-diphosphoglycerate and diabetic complications has not been precisely studied.

The present study was designed to investigate the relation between red blood cell 2,3-diphosphoglycerate and diabetic neuropathy. The effect of a potent aldose reductase inhibitor, (2*S*,4*S*)-6-fluoro-2',5'-dioxospiro [chroman-4,4'-imidazolidine]-2-carboxamide (SNK-860) (Mizuno et al., 1992) on red blood cell 2,3-diphosphoglycerate and diabetic neuropathy was also examined.

2. Materials and methods

2.1. Animals

6-week-old male Sprague-Dawley rats (Chubu Kagakushizai, Nagoya, Japan) with an initial body mass of 200–220 g were allowed to adapt to the experimental animal facility for 7 days. They were housed in an aseptic animal room at a temperature of 20–24°C and a humidity of 40–70%, with a 12-h lighting cycle and 12 fresh air changes/hour, and had free access to rat chow and water. Diabetes was induced by a single injection of streptozotocin (50 mg/kg body mass) (Sigma, St. Louis, MO, USA), freshly dissolved in 50 mmol/l citric acid buffer (pH 4.5), into the tail vein of rats that had been deprived of food overnight. Control rats received an equal volume of citric acid buffer. 1 week after streptozotocin administration, rats with plasma glucose concentrations of > 16 mmol/l were selected as diabetic rats. Both normal and diabetic rats were divided at random into two groups: untreated and SNK-860-treated. SNK-860-treated rats received SNK-860 (Sanwa Kagaku Kenkyusho, Nagoya, Japan) at a dose of 2 mg/kg by gavage everyday for 8 weeks. SNK-860 was suspended in 0.5% tragacanth gum solution (Nakarai Chemical, Kyoto, Japan). Untreated normal and diabetic rats received 0.5% tragacanth gum solution alone.

2.2. Measurement of motor nerve conduction velocity

Rats were placed on a heated pad in a room maintained at 25°C to ensure a constant rectal temperature of 37°C. After intraperitoneal injection of sodium pentobarbital (30–40 mg/kg), motor nerve conduction velocity was determined with a Neuropak NEM-3102 instrument (Nihon-Koden, Osaka, Japan) by the method of Miyoshi and Goto (1973) as described previously (Hotta et al., 1985,1992). Briefly, the tail of the rat was placed in a liquid paraffin bath that was maintained at 37°C by means of a thermostat so as to ensure a constant subcutaneous temperature of the tail. The tail nerve was stimulated at two points: the first was 1 cm from the anus, and the second was 5 cm from the first point. A coaxial needle electrode was inserted into the segmental muscle of the tail, 4 cm from the second stimulus point. The muscle action potential induced by the two-point stimulation of the longitudinal nerve trunk of the tail was recorded, and the conduction velocity was calculated by dividing the distance between the two stimulus points by the latency difference.

2.3. Measurement of sciatic nerve blood flow

Under anesthesia with sodium pentobarbital, sciatic nerve blood flow was measured by the hydrogen clearance technique with an analog recorder BW-4 (Biochemical Science, Kanazawa, Japan) and an electrolysis tissue blood flow meter RBA-2 (Biochemical Science) as described previously (Hotta et al., 1992). Briefly, after incision of the femur and exposure of the sciatic nerve, the tip of a needle electrode (BE-NS2000-30; Biochemical Science) was inserted into the nerve at about 5 mm proximal to the bifurcation of the tibial and sural nerve and preceded for about 10 mm. A reference electrode was placed in the subcutaneous tissue of the thigh. The hydrogen generated by electrolysis with 5 μA DC current for 20 s at the incision site was analyzed from the disappearance curves during a constant time. The electrode was constructed from Teflon-coated platinum-iridium wire (200 μm diameter). The position of the electrode was kept constant throughout the measurement because slight variations in the disappearance curves were noted that depended on the direction of insertion into the nerve (the tip of the electrode was shaped like an injection needle). Measurements were performed at a constant room temperature in the same room in which motor nerve conduction velocity was measured. Sciatic nerve

blood flow was calculated with the equation of Koshu et al. (1982). Although blood pressure was not monitored during this experiment, there were no significant differences in blood pressure between experimental groups before and after the anesthesia in the preliminary experiment.

2.4. Assay of Na^+/K^+-ATPase activity in sciatic nerve

After the measurements of motor nerve conduction velocity and sciatic nerve blood flow, both sciatic nerves were removed from the sciatic notch to the popliteal fossa and stripped of their epineurial and perineurial tissues. One nerve was frozen in liquid nitrogen and stored at −80°C for assay of the polyol content as described below and another was homogenized at 4°C in 1 ml of 0.2 M sucrose-0.02 M Tris-HCl buffer solution pH 7.5 containing 0.1 mM dithiothreitol, using a Polytron homogenizer for at least three periods, not exceeding 15 s each. The homogenate was centrifuged at $900 \times g$ for 2 min at 4°C. The supernatant was used for determination of ATPase activity and protein content by the methods of Das et al. (1976) and Lowry et al. (1951), respectively. 20 μl of the supernatant was added to 2 ml of the reaction buffer containing 100 mM Tris-HCl, 60 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM ATP and 0.1 mM EDTA with or without 0.1 mM ouabain, and the mixture was incubated at 37°C for 1 h (pH 7.4). At the end of the incubation, 0.5 ml of cold trichloroacetic acid (10% w/v) was added to terminate the reaction and the mixture was then centrifuged at $1700 \times g$ for 10 min at 4°C. Inorganic phosphate liberated by ATPase in the supernatant was measured colorimetrically. The specific activity of Na^+/K^+-ATPase was calculated as the difference in inorganic phosphate liberated with and without ouabain and expressed as μmol inorganic phosphate (Pi)/mg protein per h.

2.5. Assay of polyol content in sciatic nerve

The polyol content in sciatic nerve was determined by gas-liquid chromatography using a modification of the method of Mizuno et al. (1992). Frozen nerve was ground in a mortar containing 1 ml of $ZnSO_4$ (5% w/v) with 10 μg/ml of D-(+)-arabitol as an internal standard and small amounts of sand, and then mixed with 1 ml of 0.15 M $Ba(OH)_2$. After centrifugation at $1700 \times g$ for 10 min at 4°C, the supernatant was lyophilized and treated with 0.6 ml of a 3:2:1 (v/v/v) mixture of pyridine, hexamethyldisilazane and trimethylchlorosilane at 60°C for 1 h. 1 ml of chloroform and 2 ml of distilled water were added and the mixture was centrifuged at $1700 \times g$ for 5 min at 4°C. The chloroform layer was divided into aliquots and dried under a N_2 stream. The residue was solubilized in 0.1 ml of carbon disulfide and analyzed by gas-chromatography (GC-9A, Shimazu, Kyoto, Japan). Area counts of glucose, fructose, sorbitol and *myo*-inositol were calculated with an integrator (Chromatopak CR-4A, Shimazu) and corrected for the internal standard.

2.6. Biochemical assays

After measurement of motor nerve conduction velocity and sciatic nerve blood flow, blood was obtained from the abdominal aorta. A portion of the blood was treated with 0.6 mol/l perchloric acid to precipitate protein, and the mixture was centrifuged at $3000 \times g$ for 10 min. The supernatant was neutralized with 2.5 mol/l potassium carbonate and again centrifuged at $3000 \times g$ for 10 min. The final supernatant was subjected to enzymatic analysis for 2,3-diphosphoglycerate with the 2,3-diphosphoglycerate UV test (Boehringer Mannheim, Mannheim, Germany). The hematocrit was simultaneously measured with microhematocrit tubes centrifuged at $15000 \times g$ for 5 min, and the 2,3-diphosphoglycerate concentration was expressed in μmol/ml of red blood cells. The remaining blood was centrifuged at $3000 \times g$ for 10 min, and the serum was assayed for glucose, total cholesterol and triglyceride by the glucose C test (Wako Pure Chemicals, Osaka, Japan) and Determiner TC-S and TG-S (Kyowa Medex, Tokyo, Japan), respectively. The packed erythrocytes were assayed for sorbitol content in red blood cells as described below.

2.7. Assay of sorbitol content in red blood cells

The sorbitol content in red blood cells was measured by the high-performance liquid chromatographic method described by Tomiya et al. (1992) with a slight modification. 200 μl of packed red blood cells were vigorously mixed with 1 ml of deionized, distilled water containing 50 nmol/ml of D-arabitol as an internal standard. Protein was precipitated and removed from the mixture by adding 2.8 ml of 99% ethanol and centrifuging the samples at $1000 \times g$ for 10 min at 4°C. The supernatant was concentrated to dryness in vacuo and resuspended in 1 ml of deionized, distilled water. These samples were applied to a Sep-Pak Vac C18 cartridge (Millipore Japan, Tokyo, Japan) and to columns of Dowex 50W-X8 (H^+ form, 1 ml) and Amberlite CG-400 (CO_3^{2-} form, 1 ml). These were all washed with 6 ml of deionized, distilled water and the eluate was dried under a vacuum. The residue was dissolved with 1 ml of deionized, distilled water and was filtered (Ultrafree-C3GV, 0.22 μm, Millipore). 25 μl of samples were applied to a SUGAR SC1011 and a SUGAR SP0810 column used in series with an SC10119 column. Elution was with deionized, distilled water at 80°C and a flow rate of 1 ml/min. Detection of sorbitol

Table 1
Body weight, and serum glucose, total cholesterol, triglyceride and insulin concentrations in normal and diabetic rats with or without treatment with SNK-860

Animal group	Body weight (g)	Glucose (mmol/l)	Cholesterol (mmol/l)	Triglyceride (mmol/l)	Insulin (ng/ml)
Normal rats					
Untreated ($n = 11$)	386 ± 10	10.9 ± 1.2	1.10 ± 0.04	1.02 ± 0.12	1.45 ± 0.24
SNK-860-treated ($n = 8$)	419 ± 7	10.2 ± 0.5	1.21 ± 0.08	0.60 ± 0.03	1.20 ± 0.21
Diabetic rats					
Untreated ($n = 9$)	184 ± 10 [a]	22.5 ± 0.9 [a]	3.19 ± 0.40 [a]	3.28 ± 0.62 [a]	0.26 ± 0.02 [a]
SNK-860-treated ($n = 11$)	193 ± 7 [a]	22.8 ± 1.2 [a]	2.56 ± 0.04 [a]	3.96 ± 0.33 [a]	0.24 ± 0.03 [a]

Values are means ± S.E.M. [a] $P < 0.05$ vs. untreated normal rats.

was carried out using a Dionex pulsed electrochemical detector under integrated pulsed amperometry. 0.5 M NaOH was added to the post-column effluent with the Dionex anionic micromembrane suppressor (AMMS-II). The signals were integrated between 0.3 and 0.5 s and the response time of the pulsed electrochemical detector was set to 5 s.

2.8. Statistical analysis

The results are presented as means ± S.E.M., differences between experimental groups were evaluated by analysis of variance and the significance of differences between groups was assessed by Scheffé's *S*-test. Significance was defined as a P value of < 0.05.

3. Results

3.1. Body weight and biochemical data

The changes of body weight, and serum glucose, lipids (total cholesterol and triglyceride) and insulin concentrations of the experimental groups are shown in Table 1. The diabetic rats showed a significant reduction in body weight and a marked hyperglycemia compared with normal rats. Serum lipids (both total cholesterol and triglyceride) and insulin concentrations were significantly higher and lower, respectively, in diabetic rats than in normal rats. Treatment with SNK-860 did not alter any of these parameters either in normal or diabetic rats.

3.2. Motor nerve conduction velocity and sciatic nerve blood flow

The effects of diabetes and treatment with SNK-860 on motor nerve conduction velocity and sciatic nerve blood flow are shown in Table 2. Untreated diabetic rats demonstrated a delayed motor nerve conduction velocity and a reduced sciatic nerve blood flow compared with untreated normal rats. Treatment with SNK-860 markedly improved both the impaired motor nerve conduction velocity and sciatic nerve blood flow, such that there were no significant differences between untreated normal rats and SNK-860-treated diabetic rats. SNK-860 did not affect either motor nerve conduction velocity or sciatic nerve blood flow in normal rats.

3.3. Polyol content and Na^+/K^+-ATPase activity in sciatic nerves

Table 3 shows sorbitol, fructose and *myo*-inositol content, and ouabain-sensitive Na^+/K^+-ATPase activity in sciatic nerves of normal and diabetic rats with or without SNK-860 treatment. Sorbitol and fructose contents were markedly increased in the sciatic nerves of untreated diabetic rats compared with those of untreated normal rats. Treatment with SNK-860 significantly decreased the sorbitol and fructose contents in sciatic nerves of normal rats, and ameliorated the sorbitol and fructose accumulation in sciatic nerves of diabetic rats. Although the fructose content was not completely normalized, there was no significant difference between untreated normal rats and SNK-860-treated diabetic rats. The *myo*-inositol content and Na^+/K^+-ATPase activity in sciatic nerves was signifi-

Table 2
Caudal motor nerve conduction velocity and sciatic nerve blood flow in normal and diabetic rats with or without treatment with SNK-860

Animal group	MNCV (m/s)	SNBF (ml/min per 100 g)
Normal rats		
Untreated ($n = 11$)	36.1 ± 0.9	14.4 ± 0.3
SNK-860-treated ($n = 8$)	34.9 ± 0.8	14.7 ± 0.6
Diabetic rats		
Untreated ($n = 9$)	27.6 ± 0.9 [a]	4.0 ± 0.5 [a]
SNK-860-treated ($n = 11$)	33.0 ± 0.9	14.6 ± 0.4

Values are means ± S.E.M. [a] $P < 0.05$ vs. untreated normal rats. MNCV: motor nerve conduction velocity, SNBF: sciatic nerve blood flow.

Table 3
Polyol content and Na^+/K^+-ATPase in sciatic nerves of normal and diabetic rats with or without treatment with SNK-860

Animal group	Polyol content (nmol/100 mg)			Na^+/K^+-ATPase (μmol Pi/mg protein per h)
	Sorbitol	Fructose	*myo*-Inositol	
Normal rats				
Untreated ($n = 11$)	28.2 ± 2.9	150.8 ± 8.7	485.2 ± 17.2	5.71 ± 0.69
SNK-860-treated ($n = 8$)	15.7 ± 1.4 [a]	66.4 ± 6.4 [a]	462.4 ± 32.2	5.15 ± 0.37
Diabetic rats				
Untreated ($n = 9$)	149.2 ± 14.8 [a]	723.4 ± 49.1 [a]	344.8 ± 26.2 [a]	2.41 ± 0.23 [a]
SNK-860-treated ($n = 11$)	25.3 ± 3.5	250.3 ± 21.3	464.9 ± 21.9	5.03 ± 0.75

Values are means ± S.E.M. [a] $P < 0.05$ vs. untreated normal rats.

cantly decreased in untreated diabetic rats. SNK-860 prevented the *myo*-inositol depletion and the deficit of Na^+/K^+-ATPase activity, but did not have any effect in normal rats.

3.4. Sorbitol content and 2,3-diphosphoglycerate concentration in red blood cells

Untreated diabetic rats demonstrated a marked sorbitol accumulation in red blood cells compared with untreated normal rats (untreated normal: 37.7 ± 6.5, untreated diabetic: 114.2 ± 11.9 nmol/g hemoglobin, $P < 0.05$). Treatment with SNK-860 decreased the sorbitol content in red blood cells from diabetic rats (12.0 ± 1.8, $P < 0.05$) as well as from normal rats (7.8 ± 0.9, $P < 0.05$). The 2,3-diphosphoglycerate concentration in red blood cells was significantly lower in untreated diabetic rats (4.94 ± 0.45 μmol/ ml red blood cell, $P < 0.05$) than in untreated normal rats (7.11 ± 0.18) and this reduction was prevented by the treatment with SNK-860 (Fig. 1).

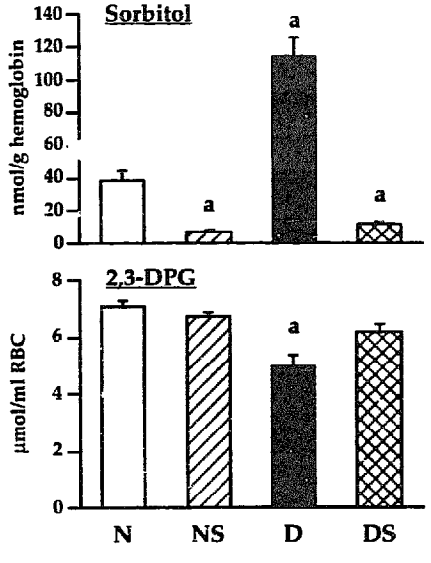

Fig. 1. Sorbitol content and 2,3-diphosphoglycerate concentration in red blood cells of normal and diabetic rats with or without treatment with SNK-860. Values are means ± S.E.M.. [a] $P < 0.05$ vs. untreated normal rats. 2,3-DPG: 2,3-diphosphoglycerate, RBC: red blood cell, N: untreated normal rats ($n = 11$), NS: SNK-860-treated normal rats ($n = 8$), D: untreated diabetic rats ($n = 9$), DS: SNK-860-treated diabetic rats ($n = 11$).

3.5. Correlations between physiological and metabolic parameters

Correlations between parameters measured in untreated and SNK-860-treated diabetic rats are shown in Table 4. Motor nerve conduction velocity correlated well with the red blood cell 2,3-diphosphoglycerate concentration and sciatic nerve blood flow as well as with the polyol content and Na^+/K^+-ATPase activity in sciatic nerves. Na^+/K^+-ATPase activity also showed a significant correlation with the polyol content in sciatic nerves and the red blood cell 2,3-diphosphoglycerate concentration. The red blood cell 2,3-diphosphoglycerate concentration, sorbitol content in red

Table 4
Correlations between physiological and metabolic parameters in untreated and SNK-860-treated diabetic rats

			r	P value
2,3-Diphospho-glycerate	vs.	MNCV	0.578	0.0190
		SNBF	0.662	0.0137
		Na^+/K^+-ATPase	0.865	0.0119
		red blood cell sorbitol	−0.693	0.0060
MNCV	vs.	nerve sorbitol	−0.587	0.0065
		nerve fructose	−0.611	0.0054
		nerve *myo*-inositol	0.531	0.0282
		Na^+/K^+-ATPase	0.953	0.0009
		SNBF	0.662	0.0020
Na^+/K^+-ATPase	vs.	nerve sorbitol	−0.851	0.0151
		nerve fructose	−0.886	0.0079
		nerve *myo*-inositol	0.825	0.0223
SNBF	vs.	red blood cell sorbitol	−0.885	< 0.0001

MNCV: motor nerve conduction velocity, SNBF: sciatic nerve blood flow. ($n = 20$).

blood cells and sciatic nerve blood flow were significantly correlated with each other.

4. Discussion

The present study demonstrated that a potent aldose reductase inhibitor, SNK-860, ameliorated the delayed motor nerve conduction velocity and biochemical abnormalities including sorbitol and fructose accumulation, *myo*-inositol depletion and decreased Na^+/K^+-ATPase activity in the sciatic nerves of streptozotocin-induced diabetic rats, and that these effects of SNK-860 were accompanied by an improvement of the reduced sciatic nerve blood flow and decreased 2,3-diphosphoglycerate concentration in red blood cells, which are parameters of peripheral tissue hypoxia.

Although some contradictory results have been reported in the literature (Williamson et al., 1993; Zochodne and Ho, 1992; Tilton et al., 1989; Pugliese et al., 1990), it has been generally accepted by many investigators that nerve blood flow is reduced in diabetic neuropathy (Cameron and Cotter, 1994). Since Low et al. (1984,1986) reported that the exposure of non-diabetic rats to a hypoxic atmosphere impaired motor nerve conduction velocity and that oxygen supplementation of streptozotocin-induced diabetic rats prevented the slowing of nerve conduction, the ischemia/hypoxia hypothesis for the pathogenesis of diabetic neuropathy has attracted much attention. In general, 2,3-diphosphoglycerate is an important regulator of tissue oxygenation and a low concentration of 2,3-diphosphoglycerate results in tissue hypoxia. However, the role of 2,3-diphosphoglycerate in the development of diabetic complications has not been established.

Several conditions that affect the 2,3-diphosphoglycerate concentration in red blood cells have been described (Harkness, 1971) since the demonstration by Benesch and Benesch (1967), and Chanutin and Curnish (1967) that 2,3-diphosphoglycerate influences the binding affinity of hemoglobin for oxygen in red blood cells. Diabetic ketoacidosis is one of the representative disorders in which the 2,3-diphosphoglycerate concentration in red blood cells is decreased. Many factors, including pH, phosphate, pyruvate, inosine, anemia and hypoxia have been identified to influence the 2,3-diphosphoglycerate concentration in red blood cells. The inhibition of glycolysis in the reaction catalyzed by phosphofructokinase at low pH is one of the postulated mechanisms by which the red blood cell 2,3-diphosphoglycerate concentration is decreased in diabetic ketoacidosis (Kono et al., 1981). Because blood pH and ketone body concentrations were not measured in this study, the possibility that the diabetic rats in the present study might have been ketoacidotic cannot be excluded, judging from the severe hyperglycemia of more than 20 mmol/l, the fact that there was no insulin replacement in spite of the low concentration of serum insulin, and the marked reduction of body weight. However, the fact that an aldose reductase inhibitor, SNK-860, which would not alter blood pH, prevented the decrease in the red blood cell 2,3-diphosphoglycerate concentration suggests that this inhibitory effect of a low pH on the phosphofructokinase reaction was not the major cause of the decreased red blood cell 2,3-diphosphoglycerate concentration in this study.

The important metabolic feature of increased polyol pathway activity is the change of redox state (Williamson et al., 1991) as well as sorbitol accumulation. The polyol pathway consists of aldose reductase and sorbitol dehydrogenase reactions catalyzing sorbitol and fructose formation, respectively. Aldose reductase and sorbitol dehydrogenase are coupled with NADPH: $NADP^+$ and NAD^+: NADH, respectively, thus hyperglycemia-induced activation of the polyol pathway results in diminished NADPH and accumulation of NADH. A pathogenic linking of the diminished NADPH and decreased nitric oxide formation, resulting in reduced endoneurial blood flow for the development of diabetic neuropathy has been proposed recently (Stevens et al., 1994). One of the other sites regulated by NAD^+: NADH in the cytosolic glycolytic pathway is the glyceraldehyde-3-phosphate dehydrogenase reaction. One of the possible mechanisms which mediated the decrease in the red blood cell 2,3-diphosphoglycerate concentration in non-acidotic diabetic rats in the present study may be as follows: the accumulation of NADH due to the increased activity of sorbitol dehydrogenase secondary to the glucose-induced activation of aldose reductase would induce a shift in the equilibrium of the glyceraldehyde-3-phosphate and 1,3-diphosphoglycerate system toward glyceraldehyde-3-phosphate, resulting in a decrease in 1,3-diphosphoglycerate formation which causes the reduction of 2,3-diphosphoglycerate synthesis. The observation that treatment with SNK-860 prevented the decrease in the red blood cell 2,3-diphosphoglycerate concentration of diabetic rats in our study strongly supports this hypothesis.

It is generally accepted that red blood cells have a compensatory function of increasing 2,3-diphosphoglycerate to facilitate oxygen delivery in hypoxic conditions (Valeri and Fortier, 1969). However, such a physiological function of red blood cells might be impaired in diabetes, in which red blood cells exhibit functional abnormalities such as decreased deformability (Schmid-Schonbein and Volger, 1976; Oughton and Barnes, 1981; Robey et al., 1987; Kowluru et al., 1989). Thus the low concentration of 2,3-diphosphoglycerate in red blood cells via the mechanism described above

would be maintained in diabetes in spite of the presence of tissue hypoxia. The decreased 2,3-diphosphoglycerate concentration induces further hypoxia and deterioration of peripheral nerve functions. A previous report that nerve degeneration was inversely correlated with the red blood cell 2,3-diphosphoglycerate concentration in streptozotocin-induced diabetic rats with a genetically determined high and normal red blood cell 2,3-diphosphoglycerate concentration (Farber et al., 1991) supports this hypothesis for the development of diabetic neuropathy. Kowluru et al. (1989) reported a defect in Na^+/K^+-ATPase activity in erythrocytes accompanied by an increase in cell volume and a decrease in filterability, and these defects were reversed by treatment with an aldose reductase inhibitor, sorbinil, presumably through the reduction of sorbitol accumulation. This observation suggests that an aldose reductase inhibitor could improve the physiological functions of erythrocytes. Based on this theory, the interrelation between the red blood cell 2,3-diphosphoglycerate concentration and the red blood cell sorbitol content in the present study would suggest another mechanism that is mediated not through the redox state but through the sorbitol content.

The improvement of motor nerve conduction velocity in SNK-860-treated diabetic rats correlated well with the amelioration of the decreased red blood cell 2,3-diphosphoglycerate concentration and reduced sciatic nerve blood flow, which suggests that the action of SNK-860 is mediated through the correction of ischemia/hypoxia as well as of metabolic abnormalities. The positive correlation between Na^+/K^+-ATPase activity in the sciatic nerve and red blood cell 2,3-diphosphoglycerate suggests that improved oxygenation in the sciatic nerve by increasing red blood cell 2,3-diphosphoglycerate augments energy production, resulting in an amelioration of energy-dependent Na^+/K^+-ATPase.

In conclusion, the decrease in 2,3-diphosphoglycerate concentration in red blood cells of diabetic rats is mediated through increased polyol pathway activity, and this decrease is one of the factors that contribute to endoneurial hypoxia, resulting in motor nerve conduction deficits. An aldose reductase inhibitor, SNK-860, ameliorated the nerve dysfunction by increasing the red blood cell 2,3-diphosphoglycerate concentration as well as by correcting the polyol content in the nerve.

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