Polyol Pathway Hyperactivity Is Closely Related to Carnitine Deficiency in the Pathogenesis of Diabetic Neuropathy of Streptozotocin-Diabetic Rats

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ABSTRACT

To investigate the relationship between polyol pathway hyperactivity and altered carnitine metabolism in the pathogenesis of diabetic neuropathy, the effects of an aldose reductase inhibitor, [5-(3-thienyl) tetrazol-1-yl]acetic acid (TAT), and a carnitine analog, acetyl-L-carnitine (ALC), on neural functions and biochemical and hemodynamic factors were compared in streptozotocin-diabetic rats. Significantly delayed motor nerve conduction velocity, decreased R-R interval variation, reduced sciatic nerve blood flow and decreased erythrocyte 2,3-diphosphoglycerate concentrations in diabetic rats were all ameliorated by treatment with TAT (administered with rat chow containing 0.05% TAT, ~50 mg/kg/day) or ALC (by gavage, 300 mg/kg/day) for 4 weeks. Platelet hyperaggregation activity in diabetic rats was diminished by TAT but not by ALC. TAT decreased sorbitol accumulation and prevented not only myo-inositol depletion but also free-carnitine deficiency in diabetic nerves. On the other hand, ALC also increased the myo-inositol as well as the free-carnitine content without affecting the sorbitol content. These observations suggest that there is a close relationship between increased polyol pathway activity and carnitine deficiency in the development of diabetic neuropathy and that an aldose reductase inhibitor, TAT, and a carnitine analog, ALC, have therapeutic potential for the treatment of diabetic neuropathy.

The pathophysiological characteristics of diabetic neuropathy are complicated, and a variety of hypotheses, including biochemical and vascular factors for the pathogenesis of this complication, have been proposed (Greene et al., 1992; Cameron and Cotter, 1993). However, the precise mechanism remains unclear. Hyperglycemia-induced polyol pathway hyperactivity has been a leading metabolic contender for the last two decades (Greene et al., 1987; Hotta and Sakamoto, 1990). Although the clinical validity of an aldose reductase inhibitor, which inhibits the rate-limiting enzyme of the polyol pathway, has not been established yet, evidence has accumulated to support the importance of this pathway, especially from the viewpoint of its interrelationship with vascular and other pathogenic factors in diabetic neuropathy (Cameron and Cotter, 1992; Cameron et al., 1994).

Alterations in fatty acid metabolism have been suggested as one of the metabolic factors for the development of diabetic neuropathy (Cameron et al., 1991). The accumulation of long-chain fatty acid esters disturbs cellular metabolism and membrane functions (Brecher, 1983). Carnitine plays an important role in long-chain fatty acid oxidation in mitochondria (Bremer, 1983). Carnitine metabolism is altered in diabetes, and it has been reported that the free l-carnitine content in myocardium is diminished in diabetic animals (Vary and Neely, 1982). Treatment with l-carnitine or carnitine analogs such as ALC and propionyl-l-carnitine improve various abnormalities in cardiac function and biochemistry after ischemia or reperfusion (Rodrigues et al., 1988; Ferrari et al., 1989). Recently, carnitine deficiency in diabetic tissues has received considerable attention concerning its role in the pathogenesis of not only diabetic neuropathy (Ido et al., 1994; Cotter et al., 1995; Hotta et al., 1996b; Malone et al., 1996; Stevens et al., 1996) but also retinopathy (Hotta et al., 1996a; Lowitt et al., 1993), and it has been reported that treatment with carnitine analogs can prevent the delay in motor nerve conduction velocity (Ido et al., 1994; Cotter et al., 1995; Hotta et al., 1996b; Malone et al., 1996; Stevens et al., 1996), electroretinographic abnormalities (Hotta et al., 1996a; Lowitt et al., 1993) and increased permeability of albumin in ocular tissues and nerves of diabetic rats (Ido et al., 1994). In addition, ALC improves nerve regeneration

ABBREVIATIONS: ALC, acetyl-l-carnitine; MNCV, motor nerve conduction velocity; CVr-r, coefficient of variation of the R-R interval; SNBF, sciatic nerve blood flow; 2,3-DPG, 2,3-diphosphoglycerate; PKC, protein kinase C.

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after crush injury (De Angelis et al., 1992) and neuromuscular dysfunction in normal rats (Scarfo et al., 1992). The precise mechanism of carnitine deficiency in diabetic tissues is not clear. However, carnitine uptake into cells is partially dependent on Na⁺/K⁺-ATPase (Virmanni et al., 1994; Burlina et al., 1989), which is generally decreased in diabetic nerves (Kim et al., 1991a; Greene and Lattimer, 1983; Nakamura et al., 1995). On the other hand, it is well known that decreased Na⁺/K⁺-ATPase activity in nerves can be ameliorated by treatment with aldose reductase inhibitors in diabetic rats (Hotta and Sakamoto, 1990; Nakamura et al., 1995). These facts suggest that increased polyol pathway activity and carnitine deficiency may be closely related to each other in the development of diabetic neuropathy. Although previous studies have investigated the effect of carnitine analogs on the nerve myo-inositol content (Ido et al., 1994; Hotta et al., 1996b; Malone et al., 1996; Stevens et al., 1996), the depletion of which would be mediated through increased polyol pathway activity, the effect of aldose reductase inhibitors on the nerve carnitine content has never been studied.

The present study was conducted to investigate the relationship between altered carnitine metabolism and polyol pathway hyperactivity in the development of diabetic neuropathy. The effects of ALC and an aldose reductase inhibitor, TAT (Hotta et al., 1996b; Inukai et al., 1993), on neural functions and biochemistry, and hemodynamic factors were compared in streptozotocin-induced diabetic rats.

**Materials and Methods**

**Animals.** Six-week-old male Sprague-Dawley rats (Chubu Kagakushiza, Nagoya, Japan) with an initial body weight of 200 to 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° to 24°C and a humidity of 40% to 70%, with a 12-hr lighting cycle and 12 fresh air changes per hour, and had free access to rat chow and water. Diabetes was induced by a single injection of streptozotocin (50 mg/kg) (Sigma Chemical, St. Louis, MO), freshly dissolved in 50 mmol/l citric acid buffer (pH 4.5), into the tail vein of rats that had been fasted overnight. Control rats received an equal volume of citric acid buffer. One week after streptozotocin administration, rats with plasma glucose concentrations of >16 mmol/l were selected as the diabetic rats. Both normal and diabetic rats were divided at random into three groups: untreated, TAT-treated and ALC-treated. TAT-treated normal and diabetic rats received laboratory chow containing 0.05% TAT (50 mg/kg/day) (Wakamoto Pharmaceutical Co., Kanagawa, Japan) for 4 weeks. ALC (Nippon Roche, Tokyo, Japan) was administered to normal and diabetic rats at a dose of 300 mg/kg (dissolved in physiological saline adjusted to pH 7.0 with 2 M Na₂CO₃) by oral gavage every day for 4 weeks. Untreated and ALC-treated normal and diabetic rats had free access to laboratory chow and water. After 4 weeks of treatment, the following parameters were measured.

**Measurement of MNCV.** 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 to 40 mg/kg), MNCV was determined with a Neuropak NEM-3102 instrument (Nihon-Koden, Osaka, Japan) as described previously (Nakamura et al., 1995). Briefly, the tail of the rat was placed in a liquid paraffin bath that was maintained at 37°C by 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.

**Measurement of CVr-r on electrocardiogram.** CVr-r was measured with a Labo-System ZS-501 (Pfukuda ME, Tokyo, Japan) under mild ether anesthesia. The rat was fixed to the instrument for CVr-r measurement after the induction of anesthesia and was again anesthetized with ether for 10 to 15 sec just before commencing electrocardiography. The electrocardiogram was recorded continuously, and the data obtained in the 1-min period just before awakening (after which the heart rate exceeded 350 beats/min) were used for the determination of CVr-r (Hotta et al., 1996b).

**Assay of polyol content in caudal nerves.** Under anesthesia with sodium pentobarbital, SNBF was measured by the hydrogen clearance technique with a BW-4 analog recorder (Biochemical Science, Kanazawa, Japan) and RBA-2 electrolysis tissue blood flow meter (Biochemical Science) as described previously (Nakamura et al., 1995). 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 ~5 mm proximal to the bifurcation of the tibial and sural nerve and advanced for ~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 sec at the incision site was analyzed from the disappearance curves during constant time. The electrode was constructed from a 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 MNCV was measured. SNBF 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 each experimental group before and after the anesthesia in the preliminary experiment.

**Assay of platelet aggregation activity.** Blood was obtained from the femoral vein; 4.5 ml of blood was mixed with 1.0 ml of 3.8% trisodium citrate. The platelet suspension was prepared as described previously (Hara et al., 1995). Briefly, citrated blood was centrifuged at 120 × g for 10 min at room temperature. The upper portion of the supernatant was obtained as PRP and recentrifuged at 1100 × g for 10 min at room temperature. The platelet pellet was subsequently suspended in modified Tyrode's balanced salt solution (pH 7.35) containing 0.35% bovine albumin without calcium and magnesium. Platelet concentrations were measured with Celltac MEK-518 (Nihon-Koden, Osaka, Japan) and adjusted to 300,000 platelets/mm² with Tyrode's balanced salt solution. Then, 100 μl of platelet suspension was applied to NBS Hematracer 601 (Niko-Bioscience, Tokyo, Japan), and ADP-induced platelet aggregation was measured by the turbidimetric method by the addition of 2.0 μM ADP (final concentrations) with constant stirring at 1000 rpm. ADP was dissolved in a modified Tyrode's balanced salt solution with calcium and magnesium. Deionized and distilled water was used as PPP, and the percentage of the largest change in light transmittance of the full range between PRP and PPP was calculated and presented as platelet aggregation.

**Assay of polyol content in caudal nerves.** The polyol content in caudal nerves was determined by gas-liquid chromatography. Briefly, frozen caudal nerves were ground in a mortar containing 1 ml of ZnSO₄ (5% wt/vol) 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 × 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 hr. 1 ml of chloroform and 2 ml of distilled water were added, and the mixture was centrifuged at 1700 × g for 5 min.
at 4°C. The chloroform layer was aliquoted and dried under N₂ stream. The residue was solubilized in 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.

**Assay of free carnitine content in sciatic nerves.** For determination of the free carnitine content, sciatic nerves were homogenized in 1 ml of HEPES. After centrifugation at 3000 × g for 10 min at 4°C, the supernatant was heated at 60°C for 30 min and centrifuged again at 3000 × g for 5 min. After extraction of free carnitine, the assay was done by the radioisotope method, as described by McGarry and Foster (1976).

**Biochemical assays.** After measurement of MNCV and SNBF, 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 × g for 10 min. The final supernatant was neutralized with 2.5 mol/l potassium carbonate and again centrifuged at 3000 × g for 10 min. The supernatant was subjected to enzymatic analysis for erythrocyte 2,3-DPG concentration with the 2,3-Diphosphoglycerate UVT Test (Boehringer-Mannheim, Mannheim, Germany). The hematocrit was simultaneously measured with microhematocrit tubes centrifuged at 15,000 × g for 5 min, and the 2,3-DPG concentration was expressed in μmol/ml of red blood cells. The remaining blood was centrifuged at 3000 × g for 10 min, and the serum was assayed for glucose, total cholesterol, triglyceride and insulin by the glucose C test (Wako Pure Chemicals, Osaka, Japan), Determiner TC-S and TG-S (Kyowa Medex, Tokyo, Japan) and radioimmunoassay (Insulin Raabeads; Dainabot, Tokyo, Japan), respectively. Serum inorganic phosphorus determination was performed by the method of Fiske and Subbarow (1925). Blood pH was measured by the ABL300 acid-base laboratory system (Radiometer, Copenhagen, Denmark).

**Statistical analysis.** Results are presented as mean ± S.E.M., differences between experimental groups were detected by analysis of variance and the significance of differences between groups was assessed by Scheffe’s S test. Significance was defined as a P value of < .05.

**Results**

**Body weight and biochemical data.** Body weight and biochemical characteristics in each experimental group are shown in table 1. Untreated diabetic rats demonstrated no body weight gain and remarkable hyperglycemia and hyperlipidemia compared with normal rats. Treatment with either TAT or ALC did not alter any of these parameters in normal rats. However, in diabetic rats, TAT had no effects on body weight, serum glucose or lipid concentrations, and ALC decreased both serum cholesterol and triglyceride concentrations without changing the body weight or serum glucose concentration. The serum insulin concentration was reduced in diabetic rats and was not affected by either TAT or ALC.

**Neural functions.** The effects of TAT and ALC on MNCV and CVr-r are shown in table 2. Untreated diabetic rats showed a significantly delayed MNCV compared with untreated normal rats. Both treatment with TAT and ALC normalized the prolongation of MNCV in diabetic rats. A significant decrease in CVr-r was observed in untreated diabetic rats compared with normal rats. The preventive effects of TAT and ALC on decreased CVr-r in diabetic rats were partial but significant, and there were no significant differences in CVr-r between TAT- or ALC-treated diabetic rats and normal rats. TAT and ALC had no effects on either MNCV or CVr-r in normal rats.

**SNBF and platelet aggregation.** In untreated diabetic rats, SNBF was remarkably reduced and platelet aggregation activity was significantly increased compared with normal rats (table 2). This reduction in SNBF was completely prevented by treatment with both TAT and ALC, whereas platelet hyperaggregability in diabetic rats was diminished by TAT but not by ALC.

**Erythrocyte 2,3-DPG and serum inorganic phosphorus concentrations and blood pH.** The erythrocyte 2,3-DPG concentration in untreated diabetic rats was significantly lower than that in untreated normal rats. Treatment with TAT and ALC, which had no effect on 2,3-DPG in normal rats, significantly increased the erythrocyte 2,3-DPG concentration in diabetic rats. There were no significant differences in the serum inorganic phosphorus concentration and blood pH between each experimental group (table 3).

**Polyol Pathway and Free Carnitine**

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Body weight</th>
<th>Glucose</th>
<th>Cholesterol</th>
<th>Triglyceride</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated (n = 11)</td>
<td>412 ± 4</td>
<td>5.8 ± 0.2</td>
<td>1.66 ± 0.08</td>
<td>0.72 ± 0.09</td>
<td>923 ± 124</td>
</tr>
<tr>
<td>TAT-treated (n = 9)</td>
<td>420 ± 6</td>
<td>6.1 ± 0.2</td>
<td>1.71 ± 0.08</td>
<td>0.71 ± 0.06</td>
<td>1104 ± 305</td>
</tr>
<tr>
<td>ALC-treated (n = 8)</td>
<td>407 ± 5</td>
<td>6.0 ± 0.2</td>
<td>1.53 ± 0.05</td>
<td>0.52 ± 0.06</td>
<td>649 ± 166</td>
</tr>
<tr>
<td>Diabetic rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated (n = 13)</td>
<td>243 ± 8′</td>
<td>35.0 ± 1.5′</td>
<td>3.80 ± 0.31′</td>
<td>4.06 ± 0.41′</td>
<td>49 ± 2′</td>
</tr>
<tr>
<td>TAT-treated (n = 14)</td>
<td>227 ± 6′</td>
<td>36.4 ± 1.2′</td>
<td>3.34 ± 0.26′</td>
<td>4.00 ± 0.45′</td>
<td>38 ± 3′</td>
</tr>
<tr>
<td>ALC-treated (n = 13)</td>
<td>225 ± 6′</td>
<td>34.2 ± 1.3′</td>
<td>2.17 ± 0.23′</td>
<td>1.97 ± 0.52′</td>
<td>56 ± 21′</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M.  
*P < .05 vs. untreated normal rats, and untreated and TAT-treated diabetic rats, respectively.
TABLE 2
Effects of TAT and ALC on MNCV, CVR-R on electrocardiogram, SNBF and ADP-induced platelet aggregation activity (PA) in normal and diabetic rats

<table>
<thead>
<tr>
<th>Animal group</th>
<th>MNCV</th>
<th>CVR-R</th>
<th>SNBF</th>
<th>PA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m/sec</td>
<td>%</td>
<td>ml/min/100 g</td>
<td>%</td>
</tr>
<tr>
<td>Normal rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated (n = 11)</td>
<td>34.8 ± 0.9</td>
<td>2.69 ± 0.13</td>
<td>11.3 ± 0.4</td>
<td>36.1 ± 2.8</td>
</tr>
<tr>
<td>TAT-treated (n = 9)</td>
<td>35.4 ± 0.9</td>
<td>2.60 ± 0.16</td>
<td>12.1 ± 1.1</td>
<td>40.3 ± 3.1</td>
</tr>
<tr>
<td>ALC-treated (n = 8)</td>
<td>35.4 ± 0.8</td>
<td>2.64 ± 0.19</td>
<td>10.2 ± 0.6</td>
<td>39.8 ± 2.3</td>
</tr>
<tr>
<td>Diabetic rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated (n = 13)</td>
<td>27.2 ± 0.7*</td>
<td>1.24 ± 0.12*</td>
<td>5.2 ± 0.4*</td>
<td>55.6 ± 0.6*</td>
</tr>
<tr>
<td>TAT-treated (n = 14)</td>
<td>34.7 ± 0.5</td>
<td>1.99 ± 0.15</td>
<td>11.0 ± 0.4</td>
<td>43.5 ± 3.4</td>
</tr>
<tr>
<td>ALC-treated (n = 13)</td>
<td>34.3 ± 0.7</td>
<td>1.99 ± 0.12</td>
<td>10.0 ± 0.6</td>
<td>53.8 ± 1.2*</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M.
* P < .05 vs. untreated normal rats.

TABLE 3
Effects of TAT and ALC on erythrocyte 2,3-DPG, serum phosphorus concentrations and blood pH in normal and diabetic rats

<table>
<thead>
<tr>
<th>Animal group</th>
<th>2,3-DPG</th>
<th>Phosphorus</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol/ml</td>
<td>mg/dl</td>
<td></td>
</tr>
<tr>
<td>Normal rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated (n = 11)</td>
<td>5.6 ± 0.2</td>
<td>6.53 ± 0.31</td>
<td>7.45 ± 0.02</td>
</tr>
<tr>
<td>TAT-treated (n = 9)</td>
<td>5.7 ± 0.3</td>
<td>6.55 ± 0.43</td>
<td>7.44 ± 0.01</td>
</tr>
<tr>
<td>ALC-treated (n = 8)</td>
<td>5.7 ± 0.1</td>
<td>6.44 ± 0.25</td>
<td>7.48 ± 0.05</td>
</tr>
<tr>
<td>Diabetic rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated (n = 13)</td>
<td>4.1 ± 0.2*</td>
<td>6.31 ± 0.42</td>
<td>7.35 ± 0.01</td>
</tr>
<tr>
<td>TAT-treated (n = 14)</td>
<td>5.1 ± 0.1</td>
<td>6.69 ± 0.34</td>
<td>7.39 ± 0.01</td>
</tr>
<tr>
<td>ALC-treated (n = 13)</td>
<td>5.1 ± 0.1</td>
<td>6.01 ± 0.28</td>
<td>7.38 ± 0.01</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M.
* P < .05 vs. untreated normal rats.

TABLE 4
Effects of TAT and ALC on sorbitol, fructose and myo-inositol contents in caudal nerves of normal and diabetic rats

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Sorbitol</th>
<th>Fructose</th>
<th>myo-Inositol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/100 g wet wt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated (n = 11)</td>
<td>6.7 ± 0.9</td>
<td>34.0 ± 3.5</td>
<td>351 ± 22</td>
</tr>
<tr>
<td>TAT-treated (n = 9)</td>
<td>4.3 ± 0.8</td>
<td>29.1 ± 4.8</td>
<td>328 ± 21</td>
</tr>
<tr>
<td>ALC-treated (n = 8)</td>
<td>6.4 ± 1.0</td>
<td>32.1 ± 6.1</td>
<td>361 ± 20</td>
</tr>
<tr>
<td>Diabetic rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated (n = 13)</td>
<td>39.8 ± 3.2*</td>
<td>232.4 ± 20.6*</td>
<td>220 ± 13*</td>
</tr>
<tr>
<td>TAT-treated (n = 14)</td>
<td>5.5 ± 0.7</td>
<td>67.8 ± 5.6</td>
<td>355 ± 17</td>
</tr>
<tr>
<td>ALC-treated (n = 13)</td>
<td>46.7 ± 2.9*</td>
<td>247.1 ± 14.7*</td>
<td>312 ± 16</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M.
* P < .05 vs. untreated normal rats.

Malone et al., 1996; Stevens et al., 1996). Although the precise mechanisms of this metabolic abnormality remain unknown, administration of carnitine analogs such as L-carnitine, ALC and propionyl-L-carnitine to diabetic animals is able to prevent peripheral nerve dysfunction and biochemical alterations (Ido et al., 1994; Cotter et al., 1995; Hotta et al., 1996b; Malone et al., 1996; Stevens et al., 1996) and histopathological abnormalities (Sima et al., 1996).

The neurochemical abnormalities in diabetic nerves presented in this study were the accumulation of sorbitol and fructose and the depletion of the myo-inositol and free carnitine contents. The mechanism by which the free carnitine content is diminished in diabetic nerves has not been well elucidated. Ido et al. (1994) suggested that the decreased levels of carnitine in plasma and nerves would be mediated through increased carnitine excretion in the urine. They also found that the urinary carnitine excretion was linked to the

Discussion
Recently, evidence that carnitine deficiency contributes to the development of diabetic neuropathy has been accumulating (Ido et al., 1994; Cotter et al., 1995; Hotta et al., 1996b; Malone et al., 1996; Stevens et al., 1996). Although the precise mechanisms of this metabolic abnormality remain unknown, administration of carnitine analogs such as L-carnitine, ALC and propionyl-L-carnitine to diabetic animals is able to prevent peripheral nerve dysfunction and biochemical alterations (Ido et al., 1994; Cotter et al., 1995; Hotta et al., 1996b; Malone et al., 1996; Stevens et al., 1996) and histopathological abnormalities (Sima et al., 1996).

The neurochemical abnormalities in diabetic nerves presented in this study were the accumulation of sorbitol and fructose and the depletion of the myo-inositol and free carnitine contents. The mechanism by which the free carnitine content is diminished in diabetic nerves has not been well investigated. The decreased myocardial carnitine content in diabetic animals has been attributed to decreased plasma levels of L-carnitine (Vary and Neely, 1982), but the mechanism of this reduction in plasma carnitine levels has not been elucidated. Ido et al. (1994) suggested that the decreased levels of carnitine in plasma and nerves would be mediated through increased carnitine excretion in the urine. They also found that the urinary carnitine excretion was linked to the
urine volume and plasma glucose concentration. However, the most interesting and novel finding in the present study was that an aldose reductase inhibitor also prevented carnitine deficiency in diabetic nerves without altering the serum glucose concentration or urine volume (data not shown), suggesting that, apart from increased urinary excretion of carnitine, there are polyol pathway-related mechanisms that decreased the levels of carnitine in the plasma and nerve tissues. Polyol pathway hyperactivity-induced inhibition of PKC activity has been invoked in the pathogenesis of diabetic neuropathy (Kim et al., 1991a, 1991b; Greene and Lattimer, 1986), although it is still controversial whether PKC activity is increased or decreased in diabetic tissues prone to complications. The decreased activity of PKC results in an inhibition of Na⁺/K⁺-ATPase activity, which is generally considered to be one of the pathogenic factors of diabetic complications (Greene et al., 1987). Carnitine uptake into cells depends on Na⁺/K⁺-ATPase (Virmuni et al., 1994; Burlina et al., 1989). Therefore, the diminished activity of Na⁺/K⁺-ATPase induced by increased polyol pathway activity would result in decreased carnitine uptake into neural cells and carnitine deficiency in diabetic nerves.

Carnitine plays an important role in long-chain fatty acid metabolism by mediating its transport from cytosol into mitochondria (Bremer, 1983). Therefore, carnitine depletion, which results in an inhibition of β-oxidation of fatty acids and energy production, causes the accumulation of long-chain fatty acids and fatty acid esters in nerve tissues of diabetic animals and may further perturb the membrane lipid composition and functions, involving alterations in PKC and Na⁺/K⁺-ATPase activity (Brecher, 1983). On the other hand, myo-inositol transport is thought to be regulated by PKC and/or Na⁺/K⁺-ATPase (Khatami et al., 1990) and to be energy dependent. Therefore, carnitine deficiency in diabetic nerves would mediate further myo-inositol depletion through the decreased activity of PKC and/or Na⁺/K⁺-ATPase in addition to that induced by the polyol pathway. This hypothesis can be supported by the fact that in the present study, the nerve myo-inositol depletion in diabetic nerves was prevented by the treatment with ALC. However, the effect of carnitine analogs on myo-inositol depletion in diabetic nerves was in conflict with previous studies (Ido et al., 1994; Malone et al., 1996). This inconsistency may be due to differences in the analogs themselves, the dosage of the analogs, the duration of diabetes or the study design (prevention or intervention study).

Stevens et al. (1996) measured the ALC and free carnitine contents in sciatic nerves of diabetic rats and reported that both ALC and free carnitine contents were decreased, and that treatment with ALC (50 and 100 mg/kg) increased the nerve ALC content and ameliorated the delay in MNCV without an increment of free carnitine. They concluded that depletion of ALC in the nerves, not free carnitine, contributes to the development of diabetic neuropathy. This hypothesis would be based on the fact that ALC can act as a precursor of acetylcholin synthesis and has neurotrophic properties that increase the responses to and synthesis of growth factors (Tagliatela et al., 1991).

A reduction in endoneurial blood flow in diabetic nerves, and the preventive effect of ALC and TAT on this reduction were observed in the present study. Neither ALC nor TAT has a direct vasodilatory action because the nerve blood flow was not affected in the nondiabetic rats. Therefore, the effects of ALC and TAT on nerve blood flow would be specific to diabetes-related abnormalities. One of the action mechanisms increasing nerve blood flow that is common to ALC and TAT may be mediated through Ca⁺⁺ homeostasis. An increased Ca⁺⁺ concentration has been reported in diabetic aorta, which is due to the decreased activity of Na⁺/K⁺-ATPase (Obara et al., 1991). Therefore, TAT and ALC would decrease the Ca⁺⁺ concentration by improving Na⁺/K⁺-ATPase activity, as reported previously (Ido et al., 1994; Stevens et al., 1996). In addition, Van-Hinsbergh and Scheffer (1991) reported that propionyl-l-carnitine decreases the cytoplasmic Ca⁺⁺ level in human endothelial cells, which would also support the notion that the effect of ALC on nerve blood flow may be mediated through Ca⁺⁺ homeostasis.

Serum lipid abnormalities have been considered to contribute to the development of diabetic microangiopathy as well as macroangiopathy. Hyperlipidemia would cause an alteration in the lipid composition of the erythrocyte membrane, resulting in erythrocyte dysfunction. In fact, hemoglobin obtained from patients with hypertriglyceridemia showed an increased oxygen affinity and decreased oxygen release (Ditzel and Dyerberg, 1977). Therefore, the lipid-lowering action of ALC shown in the present study would prevent these abnormalities, resulting in an increase in the oxygen supply and, further, an improvement in MNCV.

In the present study, TAT prevented rheological deficits in diabetic rats such as an increase in platelet aggregation activity and a decrease in the erythrocyte 2,3-DPG concentration, as shown previously (Nakamura et al., 1995). On the other hand, ALC increased the 2,3-DPG concentration but failed to diminish platelet hyperaggregability. The effect of carnitine analogs on these abnormalities has never been reported. The erythrocyte 2,3-DPG concentration can be affected by the serum inorganic phosphorus concentration and blood pH levels, which were not measured in our previous study (Nakamura et al., 1995). There were no significant differences in these parameters between each experimental condition in the present study. Therefore, the decrease in the erythrocyte 2,3-DPG concentration in untreated diabetic rats would be due to hyperglycemia-induced metabolic deficits, and the effect of TAT or ALC also was due to their metabolic actions. We have previously reported a relationship between the erythrocyte 2,3-DPG concentration and the polyol pathway in diabetic rats (Nakamura et al., 1995). The effect of ALC on erythrocyte 2,3-DPG may be mediated through a decrease in hyperlipidemia. Hyperlipidemia causes erythrocyte dysfunction as described above. Erythrocytes with normal functions can increase 2,3-DPG in the hypoxic condition; therefore, the amelioration of membrane function by the lipid-lowering action of ALC may result in a compensatory increase in 2,3-DPG. Platelet aggregation hyperactivity has been invoked in the pathogenesis of diabetic complications (Colwell et al., 1983), but the precise mechanisms remain unclear. In our previous study (Hara et al., 1995), we suggested that platelet hyperaggregability in diabetic rats is mediated through a polyol pathway-sensitive mechanism, as demonstrated by the sorbitol accumulation and myo-inositol depletion in the platelets of untreated diabetic rats and the prevention of these deficits by TAT. Although the sorbitol and myo-inositol contents were not measured in this study, it can be speculated that treatment with ALC increases the
myo-inositol content in platelets without affecting the sorbitol content, as demonstrated in diabetic caudal nerves, or decreasing the platelet aggregation activity, which suggests that sorbitol accumulation rather than myo-inositol depletion may play an important role in platelet hyperaggregation.

In conclusion, the present observation that treatment of diabetic rats with both a carnitine analog, ALC, and an aldose reductase inhibitor, TAT, prevented neural dysfunctions such as delayed MNCV and decreased CVr-r, the reduction in nerve blood flow, and neurochemical abnormalities including myo-inositol and carnitine depletion, suggests that polyol pathway hyperactivity and carnitine deficiency are closely related in the development of diabetic neuropathy.

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References


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