Peripheral sensory neuropathy is a major complication of diabetes mellitus. 1 Although the pathogenesis of this complication has not been fully elucidated, biochemical and pathological abnormalities have been observed in the early stages of experimental diabetic neuropathy. 2 - 9 One of the abnormalities is altered neurotropism, and a disturbance of neurotrophic support is reported in the peripheral nervous system of diabetic animals. 2 - 4 The axonal transport of nerve growth factor (NGF) and neurotrophin-3 (NT-3) produced in peripheral tissues was impaired in streptozotocin (STZ)-induced diabetic rats. 10,11 On the other hand, NT-3 treatment improved the nerve conduction velocity deficits, and mitochondrial and calcium homeostasis dysfunction of sensory neurons in the experimental diabetic rats. 13 - 15 Based on these findings, the dysfunction of neurotrophins is considered to be a cause of diabetic neuropathy pathogenesis.

Axonal atrophy is a phenomenon in the peripheral nerves in the diabetic neuropathic state and is thought to be one cause of the slowing of peripheral nerve conduction velocity. 16 - 18 The axon-diameter depends on the integrity of neuronal cytoskeletal proteins, and there is a lot of evidence demonstrating a reduction in axonal transport and the aberrant phosphorylation of cytoskeletal protein in the peripheral nervous system in experimental diabetic animals. 17,19 - 25 Therefore, the correction of these derangements leads to an improvement in diabetic neuropathy.

Aldose reductase (AR) is a rate-limiting enzyme that converts glucose to sorbitol in the polyol pathway. As sorbitol accumulation in peripheral nerve tissue caused by hyperactivation of the polyol pathway is considered to be an etiological mechanism of diabetic neuropathy, 16,20 a number of AR inhibitors (ARIs) have been discovered. 27 ARI has been reported to improve the slowing of peripheral nerve conduction velocity and the axonal atrophy in experimental and clinical diabetic neuropathy. 28 - 32 The aim of this study was to explore whether the inhibition of the polyol pathway affects the deficits in neurotropism and neuronal cytoskeletal protein mRNA expression in the dorsal root ganglion (DRG) in STZ-induced diabetic rats.

MATERIALS AND METHODS

Animal Treatment Six-week-old male Sprague-Dawley rats were purchased from Charles River Japan Inc. (Yokohama, Japan). They were given water and a standard laboratory diet ad libitum, and housed in plastic cages with paper chips to avoid entrapment neuropathy. 33 The temperature was maintained at 23 ± 2 °C with a humidity of 55 ± 10%, with a 12-h light cycle. At seven weeks of age, the rats were rendered diabetic by intravenous injection of STZ (60 mg/kg) after overnight fasting. STZ (SIGMA, St. Louis, MO, U.S.A.) was dissolved in 10 mM citrate buffer (pH 4.5) containing 140 mM NaCl. Normal rats were given citrate buffer only. Two weeks after the injection of STZ, STZ rats were divided into two groups based on body weight and plasma glucose concentration, respectively. Normal rats and a group of STZ rats were administered a vehicle, 0.5% methylcellulose. The other group of STZ rats was given 32 mg/kg zenarestat orally. The vehicle or drug was given daily for two weeks.

Zenarestat, [3-(4-bromo-2-fluorobenzyl)-7-chloro-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-1-yl] was synthesized at our laboratory. After the drug treatment, rats were anesthetized with diethyl ether aspiration. After sampling blood from the aortic abdominalis, rats were killed by bleeding, and lumbar DRG

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The Involvement of Aldose Reductase in Alterations to Neurotrophin Receptors and Neuronal Cytoskeletal Protein mRNA Levels in the Dorsal Root Ganglion of Streptozotocin-Induced Diabetic Rats

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Dorsal root ganglia (DRG) are recognized as one of the organs which are damaged in peripheral sensory diabetic neuropathy. In an experimental animal model, the alteration of the mRNA expression level of neurotrophins, their receptors and neuronal cytoskeletal protein have been reported. In this study, we examined whether these changes are improved by treatment with the aldose reductase inhibitor, zenarestat, in early-stage diabetic neuropathy of streptozotocin (STZ)-induced diabetic rats. Two weeks after the induction of diabetes mellitus by STZ treatment, zenarestat or a vehicle were given orally for two weeks. After the zenarestat treatment, the mRNA expression levels of neurotrophin receptors and neuronal cytoskeletal proteins in dorsal root ganglia were determined with a real-time polymerase chain reaction (PCR) method. Compared with the expression level of normal rats, a significant increase in Trk-C and Tαf α-tubulin and a decrease in neurofilament H mRNA expression level were observed in the DRG of STZ rats, while there were no significant changes in Trk-A, Trk-B, p75, neurofilament L, neurofilament M and βIII tubulin mRNA expression. Zenarestat treatment significantly ameliorated the abnormal increase in Trk-C mRNA expression level. These data suggest that hyperactivation of the polyol pathway induces a deficit in neurotropism on peripheral sensory diabetic neuropathy.

Key words aldose reductase inhibitor; diabetic neuropathy; dorsal root ganglia; neurotrophin; neuronal cytoskeletal protein

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from L3 to L6 was sampled and frozen at −80°C. Animals were treated in accordance with the Guidelines of the Committee for Animal Experiments of Astellas Pharma Inc.

Blood sampled from the tail vein at two weeks after STZ treatment or from the aortic abdominalis after zenarestat-treatment was centrifuged. Plasma glucose was measured by the mutarotase-glucose oxidase method (Glucose CII-Test Wako; Wako Pure Chemicals, Osaka, Japan).

**Measurement of mRNA Expression Level in DRG**

The total RNA in DRG was extracted with an RNeasy Mini Kit (QIAGEN, Hilden, Germany). The mRNA expression levels were determined by a real-time polymerase chain reaction (PCR) method with a 7900HT Sequence Detection System (Applied Biosystems, Darmstadt, Germany). Primers and probes used for the determination of βIII tubulin, neurofilament (NF)-L, NF-M and NF-H mRNA were purchased from Applied Biosystems. Those used for the determination of the other mRNA designed using Primer Express 2.0 software (Table 1) and obtained from SIGMA genosys (St. Louis, MO, U.S.A.). PCR was performed with TaqMan® EZ RT-PCR CORE REAGENTS or TaqMan® Gene Expression Assays (Applied Biosystems). The mRNA expression levels of NF-L, NF-M, NF-H, Tau1 α-tubulin, βIII tubulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified from the reverse-transcribed products of the total RNA in DRG. After extraction of the total RNA in DRG shown above, first-strand cDNA was synthesized from 0.5 μg total RNA, using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, U.S.A.). Total RNA in the whole brain or DRG of normal rats was used to determine the standard curve. After the correction by the amount of GAPDH mRNA, the expression levels of test genes were normalized by the values obtained from normal rats.

**Data Analyses**

Data are expressed as the mean±S.E.M. Statistical analysis was performed by one-way ANOVA followed by Dunnett’s multiple-comparison test. *p<0.05 was considered statistically significant.

**RESULTS**

**Body Weight and Plasma Glucose Levels**

The body weight and plasma glucose levels are shown in Table 2. The body weights of STZ control rats were significantly lower than normal rats throughout the experimental periods at all

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Plasma glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>270.3±4.9</td>
<td>123.9±5.2**</td>
</tr>
<tr>
<td>STZ control</td>
<td>279.5±5.4</td>
<td>148.0±7.0**</td>
</tr>
<tr>
<td>STZ zenarestat</td>
<td>279.6±8.4</td>
<td>520.2±23.5</td>
</tr>
</tbody>
</table>

Table 2. Body Weight and Plasma Glucose in Normal Control, STZ Control and STZ Zenarestat Groups

Fig. 1. Gene Expression of Neurotrophic Factor Receptors in the DRG of the Normal Control, STZ Control and STZ Zenarestat Groups

Data are mean±S.E.M. (n=18—20). *p<0.05 vs. STZ control.
time points measured. The plasma glucose levels in STZ control rats were significantly higher than those of normal control rats at any time point. These parameters were not changed by zenarestat treatment.

**The Expression Levels of Neurotrophic Factor Receptors mRNA**  The expression levels of neurotrophic factor receptors mRNA are shown in Fig. 1. Trk-C mRNA in DRG of STZ control rats had higher expression than that of normal rats, and the change was statistically significant. This increment was significantly suppressed by the treatment with zenarestat. No other gene expressions were affected by either diabetes or zenarestat treatment. The cycle threshold numbers showed a significant linear correlation with concentrations of standard samples (data not shown).

**The Expression Levels of Neuronal Cytoskeletal Proteins mRNA**  The expression levels of neuronal cytoskeletal proteins mRNA are shown in Figs. 2 and 3. No significant differences in gene expression for NF-L and NF-M were observed between normal and STZ control rats. On the other hand, the transcript of NF-H in STZ control rats was significantly lower than that of normal rats (Fig. 2). However, the decrease was not suppressed by zenarestat treatment. The expression levels of neuron-specific tubulin, Tα1α-tubulin and βIII tubulin, are shown in Fig. 3. The transcript of Tα1α-tubulin in STZ control rats was significantly higher than that of normal rats. This increment tended to be suppressed by treatment with zenarestat, but the change was not statistically significant. The gene expression of βIII tubulin was not affected by either diabetes or zenarestat treatment. The cycle threshold numbers showed a significant linear correlation with concentrations of standard samples (data not shown).

**DISCUSSION**

Aldose reductase is a rate-limiting enzyme of the polyol pathway, and the sorbitol accumulation induced by the hyperactivation of this pathway in the nerve tissue is thought to be involved in diabetic peripheral neuropathy.16,26) Zenarestat is one of the potent ARIs and improves the slowing of peripheral nerve conduction velocity and the accumulation of sorbitol in peripheral nerve in early stage STZ-induced diabetic rats.31,34) On the other hand, it has been reported that the expression levels of neurotrophins and their receptors were changed in nerves and peripheral tissues in the diabetic state and that diabetic neuropathy was improved by the correction of these changes.5—9) We therefore hypothesized that the suppression of polyol-pathway activation might improve the expression levels of neurotrophin receptors in the peripheral
sensory nerves of experimental diabetic rats, and we examined the expression levels of Trk-A, Trk-B, Trk-C and p75 in the DRG of early stage experimental diabetic rats with or without ARI treatment. One of the important findings of the present study was that the Trk-C mRNA level was significantly increased in the DRG of diabetic rats and this increase was significantly suppressed by ARI treatment. It has been reported that the peripheral production and axonal transport of NT-3, endogenous Trk-C agonist, decreased in the peripheral nervous system of diabetic rats of a longer duration diabetic state than the rats in our study,8,11 and that NT-3 reversed nerve conduction velocity deficits in diabetic rats.13 Moreover, the NT-3 disturbance is considered the cause of alterations in calcium homeostasis and of mitochondrial dysfunction in sensory neuron of diabetic rats.14,15 The increase in Trk-C mRNA level in DRG in our study could be induced by the acute reduction of NT-3 production in the peripheral tissue, for example skeletal muscles,11,35 to compensate for the deficiency in NT-3 support, and it is reasonable to assume that ARI treatment could improve the reduction in NT-3 support in diabetic peripheral nerves.

The reduction in axonal size of peripheral nerves of experimental diabetic rats was reported to be prevented by ARI treatment.6,43 As it has been reported that the aberration of neuronal cytoskeletal proteins mRNA levels in primary sensory neuron of diabetic rats and axonal atrophy are associated with a decrease in neurofilament gene expression,17,37,38 we also studied the effect of ARI on the gene expression level of neurofilaments and tubulins mRNA in STZ-induced diabetic rats. However, ARI treatment did not have any effect on the expression changes of cytoskeletal proteins mRNA observed in the DRG of diabetic rats. Because ARI treatment has been reported to prevent axonal atrophy and slowing of the nerve conduction velocity, which is thought to be induced by the caliber reduction of large fibers in diabetic rats, there must be other mechanisms for the functional improvement in neuronal cytoskeletal proteins by ARI treatment, for example, correction of the aberrant neurofilament phosphorylation in diabetic state.25

In our study, an significant decrease in NF-H mRNA level and increase in Trk-C and Tctpl α-tubulin mRNA level were observed in STZ rats. These changes are similar with that of axotomized and regenerating neurons.39—43 However, the changes observed in our study are very small. Taken together, as Liuzzi et al. have suggested, DRG neurons of early-stage diabetic rats would show the weak response observed in axotomized neurons. Moreover, we could observe neither a clear reduction in NF-L mRNA level, nor an increase in βIIH tubulin mRNA level, as reported in the other report.39 Although further studies are required to address the reason for these discrepancies, the major cause might be the difference in the diabetic duration. In fact, functional and morphological disorders have been reported to get worse when diabetic duration is prolonged.44,45 It is possible that the mild dysfunction of peripheral nerves in early-stage diabetic neuropathy in diabetic rats is induced by the reduction in NF-H and the increase in Tctpl α-tubulin mRNA expression level.

In conclusion, the present study leads to a hypothesis that hyperactivation of the polyol pathway induces a deficiency in the neurotrophic support in peripheral nerves in STZ-induced diabetic rats. Moreover, the well-known improvement with ARI on the slowing of nerve conduction velocity and axonal atrophy in diabetic rats is unlikely to involve the correction of abnormal expression of neuronal cytoskeletal proteins in the DRG of diabetic rats.

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