Neuropathy in diabetic mice overexpressing human aldose reductase and effects of aldose reductase inhibitor

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Summary

The present study was designed to examine the effect of aldose reductase (AR) overexpression on the development of diabetic neuropathy by using mice transgenic for human AR. At 8 weeks of age, transgenic mice (Tg) and non-transgenic littermates (Lm) were made diabetic with streptozotocin. After 8 weeks of untreated diabetes, plasma glucose levels and the reduction in body weight were similar between the groups of diabetic animals. Despite the comparable levels of hyperglycaemia, levels of sorbitol and fructose were significantly greater in the peripheral nerve of diabetic Tg than in diabetic Lm (both P < 0.01). Ouabain sensitive Na+/K+-ATPase activity was similarly decreased in both diabetic Tg and Lm. Protein kinase C activity in the sciatic nerve membrane fraction was unaffected by diabetes in Lm, but was reduced by nearly 40% in the diabetic Tg. Although both groups of diabetic animals exhibited a significant decrease in tibial nerve motor nerve conduction velocity (MNCV), this decrease was significantly more severe (P < 0.01) in diabetic Tg than in diabetic Lm. Consistent with these findings, nerve fibre atrophy was significantly more severe in diabetic Tg than in diabetic Lm (P < 0.01). These findings implicate increased polyol pathway activity in the pathogenesis of diabetic neuropathy. In support of this hypothesis, treating diabetic Tg with an aldose reductase inhibitor (WAY121-509, 4 mg/kg/day) for 8 weeks significantly prevented the accumulation of sorbitol, the decrease in MNCV and the increased myelinated fibre atrophy in diabetic Tg.

Keywords: diabetes; aldose reductase; transgenic mice; neuropathy

Abbreviations: AR = aldose reductase; ELISA = enzyme-linked immunosorbent assay; Lm = littermate mice; MNCV = motor nerve conduction velocity; PKC = protein kinase C; STZ = streptozotocin; Tg = transgenic mice

Introduction

The peripheral nervous system is commonly affected in diabetic patients and the sequelae of nerve dysfunction often influence the final outcome of the disease. Enhanced polyol pathway activity (Greene et al., 1993; Cameron et al., 1997; Carrington and Litchfield, 1999) and increased non-enzymatic glycation (Brownlee et al., 1988; Yagihashi, 1995) have been implicated in the aetiology of diabetic neuropathy. However, the precise mechanisms that link enhanced polyol pathway activity and non-enzymatic glycation to peripheral nerve damage are still unknown. A large number of independent laboratories have shown that aldose reductase (AR) inhibitors, agents that block substrate flux through the polyol pathway, can protect nerve function and structure in diabetic animals (Yagihashi et al., 1990a; Stevens et al., 1994; Cameron et al., 1996, 1997). Clinical trials of AR inhibitors have produced inconsistent results on diabetic neuropathy (Greene et al., 1999; Pfeifer, 1997), or adverse effects have led to the interruption of the trials (Anon, 2000; Foppiano and Lombardo, 1997). Although AR is a highly conserved protein, there are species differences in the regulation of polyol metabolism (Stribling et al., 1985; Nishimura et al., 1991; Bhatnagar and Srivastava, 1992). An understanding of the
human enzyme and development of AR inhibitors specific for human AR may therefore be important for preventing or interrupting the progressive development of human diabetic neuropathy.

We have established a strain of transgenic mice that overexpress human AR (Yamaoka et al., 1995; Yagihashi et al., 1996). This increase in AR expression and activity was implicated in the development of diabetic-like pathology when the transgenic animals were fed a diet with 30% galactose (Yagihashi et al., 1996). Neuropathic changes in galactosaemic animals do not, however, completely mimic those found in hyperglycaemia. Streptozotocin (STZ)-induced diabetic rats, for example, develop a deficit in nerve Na⁺,K⁺-ATPase activity, while this activity nearly doubles in rats given a galactose diet (Lambourne et al., 1987; Calcutt et al., 1990). Differences between the effects of STZ-induced diabetes and galactosaemia have prompted us to further examine the effects of STZ-induced diabetes in our transgenic animals.

Reduced glutathione synthesis, impaired nitric oxide synthesis, reduced Na⁺,K⁺-ATPase activity, increased protein kinase C (PKC) activity, as well as a redox imbalance have all been identified as critical changes secondary to enhanced AR activity that precipitate the development of diabetic complications (Williamson et al., 1993; Stevens et al., 1994; Cameron et al., 1996, 1997, 1999; Keogh et al., 1997). We have now initiated efforts to characterize these biochemical parameters in our human AR-overexpressing mice to better understand how these biochemical abnormalities are linked to the metabolic and structural changes that develop in the peripheral nerve of diabetic animals.

Material and methods

Animals

All animal experiments followed the Guidelines for Animal Experimentation, Hirosaki University (approval number M99013). Female mice transgenic (Tg) for human AR were used in this experiment, and littermate mice (Lm) that did not express human AR were used for comparison. Tg were developed as previously described (Yagihashi et al., 1996) by injecting full-length human AR cDNA (Nishimura et al., 1990) with a mouse major histocompatibility antigen class I promoter into the eggs of B6D2F2 female mice (Yamaoka et al., 1995; Yagihashi et al., 1996). One Tg line designated Kd-AR1 was crossed to B6D2F1 female mice (C57BL6×DBA2). DNA recovered from tail biopsies was used to examine the litters for human AR transgene expression by Southern blot analysis or with the PCR (polymerase chain reaction) using a set of transgene specific primers as described previously (Yagihashi et al., 1996). The sequences of primers for PCR were as follows: upstream primer 5’-CTGCATAACATGTTCATGCC-3’ and downstream primer 5’-TTCACGGCCTCAGTCACCT-3’. PCR was performed with 30 cycles through the following temperature sequence: 94°C for 1 min, 60°C for 1 min and 72°C for 2 min. The PCR reaction mixture consisted of 50 mmol/l KCl and 10 mmol/l Tris–HCl, pH 8.4, with 2.5 mmol/l MgCl₂, 0.2 mmol/l dNTPs, 2.5 U of Taq polymerase, 100 ng of tail DNA and 10 pmol/l of each primer. Mice negative for transgenic expression were used as controls.

After birth all animals were maintained in plastic cages in rooms with a constant temperature of 25°C and a 12-h light and dark cycle. All animals were given free access to food and water during the experimental period.

Experiment I: characterization of neuropathic changes in diabetic transgenic mice

Production of diabetic mice

Both Tg and Lm at 8 weeks of age were made diabetic by an injection of STZ (200 mg/kg, i.p.) (Sigma Co., St Louis, Mo., USA). Within 2 days, they became severely polyuric and polydipsic. The use of diabetic animals was limited to those with blood glucose levels <19.4 mmol/l (350 mg/dl) and with constant glycosuria. Non-diabetic control animals were injected with sterile saline alone. Blood glucose levels were routinely monitored following the induction of diabetes with a reflectance meter using a paper strip (Antisense, Ames-Sankyo, Tokyo). After 8 weeks of uncontrolled diabetes, the animals were killed by an overdose of pentobarbital anaesthesia.

Enzyme-linked immunosorbent assay (ELISA)

Human AR protein levels were quantified in sciatic nerve samples from experimental animals. Only endoneurial tissues from nerve samples were used. These samples were dissected, weighed, homogenized in TSA (Tris-saline-acid)-PMSF (phenylmethylsulfonylfluoride) buffer (pH 8.0) and assayed with an ELISA as previously described (Nishimura et al., 1994). The protein concentration in the nerve samples was determined using the Bradford method, calibrated with bovine-globulin (Bradford, 1976).

Biochemical analysis of carbohydrate levels in the sciatic nerve

Endoneurial carbohydrate concentrations in sciatic nerve samples were determined as previously described (Robinson et al., 1996). Glucose, sorbitol, fructose and myo-inositol were measured as their aldonitrile, alditol and cyclitol acetate derivatives by capillary gas chromatography with mass spectrometric detection (Guerrant and Moss, 1984). Tissue hexose and polyol concentrations were expressed as nmol/mg protein.

Assay of Na⁺,K⁺-ATPase and PKC activities

For the measurement of nerve enzyme activities, sciatic nerves were excised and the perineurial tissues were carefully
removed. Na\(^+\),K\(^+\)-ATPase activity was quantified with a spectrophotometric assay as previously described by Stevens and colleagues (Stevens et al., 1994). The measurement of PKC activity was based on the method of Kim and colleagues (Kim et al., 1991) with slight modification. In brief, the excised nerves were transferred to a tube containing 1.0 ml homogenization buffer [20 mM Tris-HCl (pH 7.5), 330 mM sucrose, 0.5 mM EGTA (ethyleneglycoltetaacetic acid), 2 mM EDTA (ethylenediaminetetraacetic acid), 2 µg/ml aprotinin, 25 µg/ml leupeptin, 1 mM PMSF] and homogenized with a Polytron. The homogenate was centrifuged at 50 000 g for 30 min at 4°C and the supernatant (cytosolic fraction) was collected. The pellet was resuspended in 0.6 ml homogenization buffer containing 1% Triton X-100 and stored at 4°C for 1 h. The resuspended solution was centrifuged at 50 000 g for 30 min at 4°C. The recovered supernatant fraction was defined as the membrane fraction. The phosphorylation assay was carried out in a reaction mixture [20 mM Tris pH 7.5, 1 mM CaCl\(_2\), 10 mM MgCl\(_2\), 33 µM octapeptide, 5 mM EGTA and 100 µM \([γ^{32}P]ATP\) (5–10 × 10\(^5\) c.p.m.)] in the presence or absence of 6.4 µg/ml diorein and 96 µg/ml phosphatidylserine. The reaction, started by the addition of 30I of the cytosol or membrane fraction, was incubated at 30°C for 10 min and terminated by spotting the reaction mixture onto P-81 paper (Whatman, Maidstone, UK). The P-81 paper was washed for 15 min in four changes of 75 mM phosphate. The radioactivity was counted by liquid scintillation spectrometry.

**Motor nerve conduction velocity (MNCV)**

MNCV was examined in all the experimental animals within 48 h before the induction of diabetes and after 8 weeks of untreated diabetes as previously described (Yagihashi et al., 1996). For these measurements, all the mice were anaesthetized with isoflurane. Body temperature was kept constant at 37°C on a thermostatically controlled heated mat, and continuously monitored with an anal temperature probe. MNCV was measured on the left sciatic posterior tibial nerve using a general evoked response stimulator (MS92 electromyogram device; Medelec, London, UK).

**Morphometric analysis**

At the end of the experiment the right sciatic nerves were extirpated and divided into two parts. One portion was used for biochemical analysis and the second portion was fixed overnight at 4°C in 2.5% glutaraldehyde buffered with 0.05 mmol/l sodium cacodylate (pH 7.3). The fixed samples were post-fixed in 1% osmium tetroxide and dehydrated through an ascending series of ethanol concentrations. Mid-portions of the fixed sciatic nerves were embedded in epon and polymerized. One-micron thick semi-thin transverse nerve sections were stained with toluidine blue. For the morphometric analysis, fascicular area, myelinated fibre number and fibre size were measured at a magnification of ×1600 by a computer-assisted image analysing system (NIH image, Agfa Arcus scanner connecting with Macintosh Quadra 700; Cupertino, Calif., USA). The mean values of myelinated fibre size, measured as the area delineated by the outer myelin border, was calculated from at least 800 fibres randomly selected from six to eight micrographs prepared from samples from each mouse. To determine the effect of ageing on the myelinated fibre maturation, sciatic nerve samples from each of five Tg and Lm mice of 8 weeks of age were processed in a similar manner.

**Experiment II: effects of AR inhibitor on neuropathic changes in diabetic Tg and Lm**

**Diabetic mice**

At the age of 8 weeks both Tg and Lm were made diabetic as described above. Tg and Lm diabetic animals were separated into two groups. One group remained untreated and the other was treated with an AR inhibitor, WAY121–509 kindly provided by Wyeth-Ayerst, Princeton, NJ, USA (Malamas and Hohman, 1994), administered for 8 weeks by gavage at a daily dose of 4 mg/kg. MNCV was measured as described above. At the end of the experiment the animals were sacrificed and the sciatic nerves were recovered. The sciatic nerves not used for the MNCV measurements were divided into two sections: one used for biochemical analyses and the other for microscopy.

**Statistical analysis**

Data are expressed as the mean ± standard deviation. Mean values for the diabetic and non-diabetic groups were compared using an unpaired t-test. Observations from the initial and terminal time points were compared using a one-way ANOVA (analysis of variance), followed by Bonferroni corrections for multiple comparisons. P values <0.05 were considered to be significant.

**Results**

**Clinical and laboratory findings**

Transgene expression did not affect the general condition and behaviour of the animals. The body weights of Tg were comparable with those of Lm throughout the course of this study (Table 1). At the end of the study the body weights of the diabetic mice were significantly (P < 0.01) lower than those of the non-diabetic mice, but there were no significant differences between the Tg and Lm groups (Table 1). Blood glucose levels were markedly elevated following the induction of diabetes, but were not different between Tg and Lm.

**ELISA**

Human AR contents in sciatic nerves were comparable between the two groups of Tg (959 ± 158 ng/mg protein in
Neuropathy in diabetic transgenic mice

Table 1  Clinical data and nerve carbohydrate levels in experimental animals (Experiment I)

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>Body weight (g)</th>
<th>Blood glucose (mmol/l)</th>
<th>Nerve carbohydrate levels at end (mmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>End</td>
<td>Glucose</td>
</tr>
<tr>
<td>Transgenic (n = 12)</td>
<td>24 ± 2.7</td>
<td>30.3 ± 4.3</td>
<td>5.6 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>P &lt; 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic transgenic (n = 12)</td>
<td>23.8 ± 2.3</td>
<td>20.5 ± 2.0</td>
<td>5.8 ± 0.8</td>
</tr>
<tr>
<td>Littermate (n = 12)</td>
<td>23.6 ± 2.5</td>
<td>31.4 ± 4.2</td>
<td>5.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>P &lt; 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic littermate (n = 12)</td>
<td>24.0 ± 1.8</td>
<td>20.1 ± 1.6</td>
<td>5.9 ± 0.8</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation. *P < 0.01 versus diabetic littermate mice.

Non-diabetic Tg and 898 ± 129 in diabetic Tg, respectively). Human AR expression was not detected in the non-transgenic animals.

Carbohydrate levels of sciatic nerve

Hyperglycaemia in diabetic mice was accompanied by an 8-fold increase in sciatic nerve glucose levels. Compared with the levels in the non-diabetic animal groups, nerve sorbitol levels were increased 5-fold and 2.7-fold in diabetic Tg and diabetic Lm, respectively, while nerve fructose was increased 8.5-fold and 5.7-fold, respectively. The increases in sorbitol and fructose were significantly greater in diabetic Tg than diabetic Lm (P < 0.01 for both). In contrast, diabetes had no effect on nerve myo-inositol levels in either group (Table 1).

Na⁺,K⁺-ATPase and PKC activities

Ouabain-sensitive Na⁺,K⁺-ATPase activity in the sciatic nerve was significantly reduced in both Tg and Lm diabetic animals (Fig. 1). While the activity level was the lowest in diabetic Tg, the difference between diabetic Tg and Lm was not statistically significant. Diabetes had no significant effects on the protein kinase activity of nerve cytosolic fraction in Tg or Lm, but the activity was significantly reduced (P < 0.01) in the nerve membrane fraction of diabetic Tg (Fig. 2).

MNCV

At the initial time point, mean MNCV values ranged from 44–45 m/s in all of the experimental groups. Eight weeks later these values significantly increased to ~48 m/s in the non-diabetic groups (P < 0.05 for both groups) (Table 2). However, after 8 weeks of untreated diabetes MNCV significantly decreased in both groups of diabetic animals compared with the non-diabetic groups (P < 0.01 for both). The 34% decrease in MNCV in diabetic Tg was significantly greater than the 20% decrease observed in Lm (P < 0.01) (Table 2).

Structural and morphometric analysis of sciatic nerve

In a morphometric analysis of sciatic nerves, nerve fascicular area was not altered by diabetes (Table 3). In contrast, the
Fig. 2 Effect of diabetes on PKC activity in human AR Tg and non-transgenic Lm. PKC activity was quantified in the membrane and cytosolic fractions of the sciatic endoneurium from Tg and Lm after 8 weeks of untreated diabetes and in age-matched control animals. Reduction of protein kinase C activity in diabetic Tg was significantly greater than that in diabetic Lm (*P < 0.05). Bar stands for standard deviation. Each group consists of five animals. n.s. = non-significant.

Table 2 MNCV in experimental animals (Experiment I)

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>MNCV (m/s)</th>
<th>Initial</th>
<th>End</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transgenic (n = 12)</td>
<td>44.6 ± 1.8</td>
<td>48.1 ± 2.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>*P &lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>Diabetic transgenic</td>
<td>44.8 ± 2.2</td>
<td>31.7 ± 3.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>P &lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>Littermate (n = 12)</td>
<td>44.1 ± 2.3</td>
<td>48.4 ± 1.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>*P &lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>Diabetic littermate</td>
<td>45.3 ± 2.4</td>
<td>38.8 ± 3.3</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation.

The present study has shown that diabetic mice overexpressing human AR develop severe functional and age-dependent significant (P < 0.01) increase in myelinated fibre size observed in the non-diabetic groups (P < 0.01), was not observed in diabetic Tg or Lm (Table 3). Furthermore, the mean myelinated fibre size of diabetic Tg was significantly smaller than that of diabetic Lm (P < 0.01). Consistently, fibre size frequency histograms showed a trend in the shift of the peak towards small fibre sizes in the diabetic Tg. This shift was not observed for diabetic Lm. The population of large myelinated fibres with a cross-sectional area >70 µm² was significantly reduced in diabetic Tg (2.5 ± 1.1%, n = 5) compared with those in non-diabetic Tg (9.6 ± 1.2 %, n = 5, P < 0.01 versus diabetic Tg), non-diabetic Lm (11.1 ± 3.0%, n = 5, P < 0.01 versus diabetic Tg) and diabetic Lm (6.6 ± 2.9%, n = 5, P < 0.02 versus diabetic Tg) (Fig. 3). Myelinated fibre density was unaffected by diabetes or ageing.

Effects of AR inhibitor on neuropathic changes
AR inhibitor treatment had no effect on body weight or blood glucose levels (data not shown). Nerve sorbitol levels in diabetic Tg and Lm were normalized by AR inhibitor treatment, but nerve fructose levels were reduced by only 55 and 25%, respectively (Table 4).

The effects of diabetes on MNCV in the second experiment closely replicated those observed in the first experiment. MNCV was significantly reduced (P < 0.01) in the untreated diabetic groups and most severely reduced in diabetic Tg (Fig. 4). AR inhibitor treatment significantly reduced the decrease in MNCV by 65% in diabetic Tg. AR inhibitor treatment, however, had no effect on the loss of MNCV in diabetic Lm.

Consistent with the first experiment, a morphometric analysis demonstrated a significant reduction of myelinated fibre size in diabetic Tg compared with non-diabetic Tg (Fig. 5). AR inhibitor treatment significantly prevented this reduction. Consistent with the effects on MNCV, AR inhibitor treatment had no effect on the reduction of myelinated fibre size in diabetic Lm.

Discussion
The present study has shown that diabetic mice overexpressing human AR develop severe functional and
Neuropathy in diabetic transgenic mice

Table 3  Morphometric data on myelinated fibres in experimental animals (Experiment I)

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>Transverse fascicular area (mm²)</th>
<th>Mean myelinated fibre size (µm²)</th>
<th>Mean fibre density (no. × 10³/mm²)</th>
<th>Total fibre number (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (8 weeks of age)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transgenic (n = 5)</td>
<td>0.16 ± 0.02</td>
<td>29.0 ± 0.5</td>
<td>19.9 ± 0.7</td>
<td>4987 ± 829</td>
</tr>
<tr>
<td>Littmate (n = 5)</td>
<td>0.15 ± 0.04</td>
<td>29.5 ± 1.4</td>
<td>20.5 ± 0.8</td>
<td>3798 ± 1144</td>
</tr>
<tr>
<td>End-point (16 weeks of age with 8-week diabetic duration)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transgenic (n = 5)</td>
<td>0.21 ± 0.03</td>
<td>32.8 ± 0.8*</td>
<td>20.5 ± 1.1</td>
<td>4209 ± 596</td>
</tr>
<tr>
<td>Diabetic transgenic (n = 5)</td>
<td>0.19 ± 0.04</td>
<td>26.8 ± 0.6</td>
<td>22.5 ± 2.0</td>
<td>5085 ± 1111</td>
</tr>
<tr>
<td>Littmate (n = 5)</td>
<td>0.21 ± 0.07</td>
<td>33.3 ± 0.8*</td>
<td>20.2 ± 0.9</td>
<td>4011 ± 1254</td>
</tr>
<tr>
<td>Diabetic littmate (n = 5)</td>
<td>0.21 ± 0.05</td>
<td>29.0 ± 0.9</td>
<td>21.9 ± 1.7</td>
<td>4815 ± 1187</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation. *P < 0.01 versus each 8-week-old group.

Fig. 3  Fibre size distribution of myelinated fibres in the sciatic nerve of experimental mice. Population of large sized fibres (>70 µm²) was significantly reduced in both diabetic Tg and non-transgenic Lm compared with the non-diabetic groups. This reduction was most severe in diabetic transgenic mice compared with non-transgenic diabetic mice. On the other hand, the population of small sized fibres (<30 µm²) was significantly increased in diabetic Tg compared with other groups. Bar stands for standard deviation. *P < 0.01 versus non-diabetic Lm and Tg; †P < 0.05 versus non-diabetic Lm and Tg.

structural abnormalities in peripheral nerves. The neuropathic changes exemplified by decreased MNCV and myelinated fibre atrophy were similar to those found in rats with STZ-induced diabetes (Yagihashi, 1995, 1997; Ido et al., 1994) and in rats BB (Biobreeding) that spontaneously develop insulin-deficient diabetes (Sima and Hay, 1981; Sima et al., 1987). Diabetic Lm also developed nerve structural and functional changes, although to a lesser extent; i.e. 58% of the decrease in MNCV and 72% of the fibre atrophy observed in diabetic Tg. Non-diabetic Tg did not develop these structural and functional abnormalities. These observations suggest that the enhancement of neuropathic changes in diabetic Tg may be a consequence of the increased flux of glucose through the polyol pathway mediated by human AR expression. These results further suggest that under conditions of diabetic hyperglycaemia, hyperactivity of the polyol pathway is one of the critical determinants of the severity of diabetic neuropathy. The increased severity of the structural and functional damage to the sciatic nerve in diabetic Tg is consistent with recent clinical data that have linked the early onset or frequent occurrence of diabetic retinopathic or neuropathic complications to higher levels of erythrocyte AR activity (Crabbe et al., 1980; Hamada et al., 1991, 1993) or a higher content of erythrocyte AR protein (Nishimura et al., 1994; Ohnishi et al., 1996).

In earlier studies, the neuropathy observed in STZ-induced diabetic mice was rather modest, showing a late onset of a decrease in MNCV when compared with the STZ-rat model (Whiteley and Tomlinson, 1985; Calcutt et al., 1988). This difference between the models was considered to be due to the extremely low content of AR in mice (Gillon and Hawthorne, 1983; Whiteley and Tomlinson, 1985). In our study, even in control mice there was a significant decrease in MNCV and reduced myelinated fibre size after 8 weeks of uncontrolled diabetes, suggesting that AR is not the only determinant for the neuropathy. However, the augmented decrease of MNCV in diabetic Tg may be ascribed to metabolic changes initiated by activation of the polyol pathway mediated by human AR overexpression. In rats with experimental or spontaneous diabetes, increased flux through the polyol pathway has been linked to a reduction of tissue myo-inositol levels, alterations of PKC activity and impaired Na⁺,K⁺-ATPase activity (Kim et al., 1991; Greene et al., 1993; Ido et al., 1994; Tomlinson, 1999). This cascade of biochemical changes is thought to result in the slowing of MNCV (Sima and Brismar, 1985; Sima et al., 1987; Greene...
Table 4 Nerve carbohydrate levels in experimental animals (Experiment II)

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>Glucose (nmol/mg)</th>
<th>Sorbitol (nmol/mg)</th>
<th>Fructose (nmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transgenic (Tg) group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tg (n = 5)</td>
<td>$1.4 \pm 1.0$ n.s.</td>
<td>$0.5 \pm 0.1$ n.s.</td>
<td>$0.6 \pm 0.2$ n.s.</td>
</tr>
<tr>
<td>Tg + ARI (n = 6)</td>
<td>$1.2 \pm 0.9$ $P &lt; 0.01$</td>
<td>$0.3 \pm 0.1$ $P &lt; 0.01$</td>
<td>$0.5 \pm 0.2$ $P &lt; 0.01$</td>
</tr>
<tr>
<td>Diabetic Tg (n = 6)</td>
<td>$18.2 \pm 1.8^*$ n.s.</td>
<td>$2.0 \pm 0.7^*$ n.s.</td>
<td>$5.3 \pm 1.8^*$ n.s.</td>
</tr>
<tr>
<td>Diabetic Tg + ARI (n = 5)</td>
<td>$16.8 \pm 3.5^*$ n.s.</td>
<td>$0.5 \pm 0.3$ n.s.</td>
<td>$2.4 \pm 1.3^*$ n.s.</td>
</tr>
<tr>
<td>Littermate (Lm) group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lm (n = 6)</td>
<td>$1.1 \pm 1.1$ n.s.</td>
<td>$0.5 \pm 0.1$ n.s.</td>
<td>$0.6 \pm 0.5$ n.s.</td>
</tr>
<tr>
<td>Lm + ARI (n = 6)</td>
<td>$1.3 \pm 1.2$ $P &lt; 0.01$</td>
<td>$0.3 \pm 0.0$ $P &lt; 0.05$</td>
<td>$0.6 \pm 0.3$ $P &lt; 0.05$</td>
</tr>
<tr>
<td>Diabetic Lm (n = 6)</td>
<td>$17.2 \pm 5.9^*$ n.s.</td>
<td>$1.1 \pm 0.6^*$ n.s.</td>
<td>$3.1 \pm 2.1^*$ n.s.</td>
</tr>
<tr>
<td>Diabetic Lm + ARI (n = 6)</td>
<td>$17.2 \pm 5.9^*$ n.s.</td>
<td>$0.6 \pm 0.4$ n.s.</td>
<td>$2.3 \pm 1.4^*$ n.s.</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation. ARI = aldose reductase inhibitor. $^*P < 0.01$; $^{**}P < 0.05$ versus each non-diabetic group.

Fig. 4 Effects of AR inhibitor-treatment on MNCV. Tibial MNCV was quantified in human AR Tg and non-transgenic Lm after 8 weeks of diabetes with or without AR inhibitor treatment, 4 mg/kg/day AR inhibitor WAY 121–509, and in age-matched non-treated and AR inhibitor-treated non-diabetic control animals. The reduction of MNCV is significantly greater in diabetic Tg compared with that in diabetic Lm ($P < 0.01$), and AR inhibitor-treatment inhibited this reduction only in diabetic Tg. Bar stands for standard deviation. $^*P < 0.01$ versus non-diabetic Tg and AR inhibitor-treated non-diabetic Tg; $^†P < 0.01$ versus untreated diabetic Tg; $^‡P < 0.05$ versus non-diabetic Tg and AR inhibitor-treated non-diabetic Tg; $^§P < 0.01$ versus non-diabetic Lm groups.

et al., 1993). Our results do not appear to be completely consistent with this theory because there was no significant reduction of myo-inositol levels in diabetic Tg. In addition, the decrease in Na$^+$.K$^+$-ATPase activity was not significantly different between diabetic Lm and diabetic Tg.

Recent studies have identified decreased vascular perfusion as a critical factor for the acute decrease in MNCV in short-term STZ-induced diabetic rats (Cameron and Cotter, 1994). This decrease in vascular perfusion can be corrected by a variety of different treatments including AR inhibitors, essential fatty acids, prostaglandins and aminoguanidine (Yasuda et al., 1989; Kihara et al., 1991; Cameron and Cotter, 1994; Cameron et al., 1996). The MNCV deficit in diabetic Tg may also be accounted for by augmented impairment of nerve blood flow. Human AR is expressed not only in the nerve fibres but also in the vessel walls supplying the nerve (data not shown). Increased flux of the polyol pathway is thought to competitively decrease NADPH,
leading to impaired production of nitric oxide (Stevens et al., 1994; Cameron et al., 1996, 1997). This in turn causes impaired dilation of endoneurial microvessels leading to reduced capillary perfusion. Consumption of NADPH by activation of the polyol pathway may also contribute to decreased production of reduced glutathione (Cameron et al., 1996, 1999). As a result, the peripheral nerve as well as endothelial cells are exposed to excessive oxidative stress which appears to further contribute to endothelial dysfunction (Cameron et al., 1995, 1998). In support of this hypothesis, anti-oxidants have been reported to improve the functional and structural deficits in STZ-diabetic rats (Sagara et al., 1996; Kishi et al., 1999; Stevens et al., 2000; Cameron and Cotter, 2001). Our results are consistent with these data showing that high AR expression mediates augmented MNCV delay through decreased protection from reactive oxygen species and impaired blood flow. Prevention of these deficits in AR inhibitor-treated diabetic animals probably contributes to the amelioration of neurovascular function in this study.

A significant alteration of PKC activity was observed only in diabetic Tg, suggesting a specific response to human AR-induced polyol hyperactivity. This effect of diabetes on neural PKC activity is in contrast to those observed in the retina and kidney of diabetic rats where diabetic hyperglycaemia and increased AR activity have been linked to an increase in PKC activity (Keogh et al., 1997; Koya and King, 1998). Our observations on PKC activity are also difficult to interpret in view of the recent report that PKC inhibitors can restore endoneurial perfusion and nerve function in diabetic rats (Cameron et al., 1999; Nakamura et al., 1999). Differential effects of hyperglycaemia on the individual isoforms of PKC may account for these differences (Roberts and McLean, 1998). Examination of expressions of different isoforms of PKC is now underway in our laboratory. Separate measurements of endoneurial and peri-neurial vascular tissues for PKC activities (Borghini et al., 1994) may also be important for further elucidation of the relationship between PKC activities and the polyol pathway.

Consistent with the observations on MNCV, our morphometric analysis demonstrated that myelinated fibre size was the smallest in the diabetic Tg. The population of large myelinated fibres (<70 µm²) was most markedly reduced in this same animal group. These structural changes are consistent with previous findings in peripheral nerves of STZ-induced diabetic rats (Jakobsen, 1976; Yagihashi et al., 1990a, b) and spontaneously diabetic BB rats (Sima and Hay, 1981; Sima et al., 1987). Some investigators have proposed that fibre atrophy in growing STZ-diabetic rats is primarily due to a maturational defect (Sharma et al., 1977; Thomas et al., 1990). Others have proposed that fibre atrophy is primarily caused by long-term metabolic aberrations leading to impaired synthesis and transport of axonal cytoskeletal elements including neurofilaments, and a lack of neurotrophic support (Yagihashi et al., 1990b; Mohiuddin et al., 1995; Scott et al., 1999). In STZ-diabetic mice, Kennedy and Zochdone have implicated impaired fibre
maturation in the delayed recovery of nerve function following nerve crush injury (Kennedy and Zochdone, 2000). In our study, the mean fibre size in diabetic Tg after 8 weeks of untreated diabetes was smaller than the size observed before the induction of diabetes. It is therefore conceivable that fibre atrophy in diabetic Tg may not be simply due to retarded maturation of nerve fibres, but may be confounded by metabolic factors related to impaired cytoskeletal synthesis as well as neurotrophic support. The recovery of fibre atrophy without gaining weight in AR inhibitor-treated diabetic Tg may support this contention. It is apparent, however, that more detailed studies using electron microscopic analyses are needed to confirm the presence of axonal atrophy. Furthermore, future studies of diabetes-induced alterations of axonal cytoskeletal formation may also help to clarify the mechanisms of how AR overexpression affects the structural changes. It would also be of interest to see the structural alterations in this model with a longer duration of diabetes.

Most of the clinically tested AR inhibitors have advanced into the clinical phase of development based upon their activity in animal models of diabetes. These agents not only lack species specificity in their inhibition of AR, many of them are also indiscriminate in their inhibition of structurally related enzymes such as aldehyde reductase (Dvornik, 1987; Bhatnagar and Srivastava, 1992; Greene et al., 1993). The efficacy and safety profile of these inhibitors may be improved if their activity can be limited to the inhibition of human AR. The transgenic mouse that we have established may be a valuable tool in this effort.

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