Effects of Polyol Pathway Hyperactivity on Protein Kinase C Activity, Nociceptive Peptide Expression, and Neuronal Structure in Dorsal Root Ganglia in Diabetic Mice

Kenji Uehara, Shin-Ichiro Yamagishi, Saori Otsuki, Shyunsuke Chin, and Soroku Yagihashi

We explored the specific impact of polyol pathway hyperactivity on dorsal root ganglia (DRG) using transgenic mice that overexpress human aldose reductase because DRG changes are crucial for the development of diabetic sensory neuropathy. Littermate mice served as controls. Half of the animals were made diabetic by streptozotocin injection and followed for 12 weeks. After diabetes onset, diabetic transgenic mice showed a significant elevation of pain sensation threshold after transient decrease and marked slowing of motor and sensory nerve conduction at the end of the study, while these changes were modest in diabetic littermate mice. Protein kinase C (PKC) activities were markedly reduced in diabetic transgenic mice, and the changes were associated with reduced expression of membrane PKC-α isoform that was translocated to cytosol. Membrane PKC-βII isoform expression was contrariwise increased. Calcitonin gene-related peptide– and substance P–positive neurons were reduced in diabetic transgenic mice and less severely so in diabetic littermate mice. Morphometric analysis disclosed neuronal atrophy only in diabetic transgenic mice. Treatment with an aldose reductase inhibitor (fidaresar 4 mg · kg⁻¹ · day⁻¹, orally) corrected all of the changes detected in diabetic transgenic mice. These findings underscore the pathogenic role of aldose reductase in diabetic sensory neuropathy through the altered cellular signaling and peptide expressions in DRG neurons. 

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Polyol pathway hyperactivity has been extensively studied for the mechanisms of diabetic neuropathy, where aldose reductase is a key regulating enzyme (1,2). In animal models, diabetes-induced peripheral nerve conduction deficits, neurometabolic imbalances, altered nerve blood flow, and morphologic abnormalities are prevented by structurally diverse aldose reductase inhibitors (ARIs) (3–5). Past clinical trials of ARIs were not, however, convincingly successful (6,7), and specific effects of polyol pathway hyperactivity on the clinical and structural aspects of diabetic sensory neuropathy is yet to be clear (8,9). The development of a transgenic animal model provides an ideal tool to identify the specific role of single molecules in disease mechanisms. We have established a strain of transgenic mice that overexpress human aldose reductase (10–12). In this model, we confirmed that polyol pathway hyperactivity was indeed related to the severity of motor nerve conduction delay and nerve fiber atrophy. More recently, a new transgenic model that overexpresses aldose reductase, specifically in Schwann cells by use of myelin protein P0 promoter, has enabled researchers to address more precise mechanisms of polyol pathway in the development of neuropathy in diabetes (13). Nevertheless, from these studies, it was not shown which measures were comparable with those found in human diabetic neuropathy, and it still remains obscure what could be the most appropriate target for the intervention with a potent and specific inhibitor for human aldose reductase.

There is growing evidence that an early involvement in diabetic neuropathy is small nerve fibers, conferring pain sensation and autonomic nerve function (14,15). Careful studies using animals with experimental diabetes demonstrated an early involvement of peripheral sensory nervous system, showing cytoskeletal changes in dorsal root ganglion (DRG) neurons with distal axonal atrophy (16–18). Impaired synthesis and release of neuropeptides exemplified by calcitonin gene-related peptide (CGRP) and substance P detected in streptozotocin (STZ)-induced diabetic rats may derive from defects of metabolic signals, accounting for important aspects of sensory neuropathy (19,20). In this setting, altered protein kinase C (PKC) activity is now proposed to play a central role in the impaired cell function and structure, but its alterations related to polyol pathway hyperactivity are still controversial in diabetic peripheral nerve (21,22). In this study, we therefore investigated time course changes of pain sensation, expression of nociceptive neuronal peptides, and structural changes of DRG neurons and PKC activities in aldose reductase–overexpressing mice. We also examined the effects of an ARI.

RESEARCH DESIGN AND METHODS

All animal experiments followed the Guidelines for Animal Experimentation of Hirosaki University (approval number M99013). To establish the transgenic...
mice that overexpress human aldose reductase, C57BL/6 was first mated with LmDM and the offspring (BDF1) used for transgene integration. BDF1 female mice under hyperovulation were mated with male littersmates, and full-length human aldose reductase cDNA was injected into the uterus of founder mice. Integration of the transgene into the mouse major histocompatibility antigen class I promoter (H-2Kd) was injected into the fertilized eggs and implanted into the uterus of founder mice. Integration of the transgene into the founder mice (10,11). Both transgenic and littermate mice at 8 weeks of age were made diabetic by an injection of STZ (160 mg/kg i.p.) (Sigma, St. Louis, MO), and diabetic transgenic mice not expressing human aldose reductase were used for comparison.

Both transgenic and littermate mice at 8 weeks of age were made diabetic by an injection of STZ (160 mg/kg i.p.) (Sigma, St. Louis, MO), and diabetic transgenic mice used in this experiment, and littermate mice that did not express human aldose reductase were used for comparison.

Table 1 shows the mean values for body weight, blood glucose levels, and diabetes-induced DRG tissues. The mean values for body weight, blood glucose levels, and diabetes-induced DRG tissues were compared between different groups of mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Initial</th>
<th>End</th>
<th>Body weight (g)</th>
<th>Blood glucose (mmol/l)</th>
<th>DRG tissues</th>
<th>MNCV (m/s)</th>
<th>SNVC (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lm</td>
<td>11</td>
<td>18.6 ± 0.9</td>
<td>27.2 ± 1.8</td>
<td>6.2 ± 0.4</td>
<td>5.9 ± 0.3</td>
<td>0.36 ± 0.03</td>
<td>2.2 ± 0.2</td>
<td>47.4 ± 1.5</td>
</tr>
<tr>
<td>Tg</td>
<td>11</td>
<td>18.8 ± 0.8</td>
<td>26.6 ± 2.0</td>
<td>6.2 ± 0.4</td>
<td>5.9 ± 0.3</td>
<td>0.79 ± 0.13</td>
<td>4.4 ± 0.6</td>
<td>44.1 ± 1.2</td>
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<td>LmDM</td>
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<td>18.9 ± 0.7</td>
<td>17.6 ± 0.9*</td>
<td>6.7 ± 0.3</td>
<td>24.4 ± 1.1*</td>
<td>2.58 ± 0.31*</td>
<td>19.9 ± 2.3°</td>
<td>43.3 ± 2.0</td>
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<tr>
<td>TgDM</td>
<td>11</td>
<td>18.7 ± 1.3</td>
<td>18.3 ± 1.3*</td>
<td>6.3 ± 0.3</td>
<td>23.2 ± 1.4*</td>
<td>4.44 ± 0.41‡</td>
<td>35.3 ± 6.7</td>
<td>31.7 ± 2.2</td>
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<tr>
<td>LmDM+ARI</td>
<td>12</td>
<td>18.4 ± 0.8</td>
<td>18.3 ± 0.8*</td>
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<td>26.3 ± 1.0*</td>
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<td>45.2 ± 3.0</td>
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<tr>
<td>TgDM+ARI</td>
<td>12</td>
<td>18.5 ± 0.8</td>
<td>18.0 ± 1.0*</td>
<td>6.4 ± 0.3</td>
<td>24.8 ± 2.3*</td>
<td>0.55 ± 0.13</td>
<td>9.0 ± 1.0</td>
<td>42.7 ± 1.3</td>
</tr>
</tbody>
</table>

Data are means ± SE. Lm, littermate control mice; Tg, mice transgenic for human aldose reductase; LmDM, diabetic littermate mice; TgDM, diabetic transgenic mice; LmDM+ARI, diabetic littermate mice treated with ARI; TgDM+ARI, diabetic transgenic mice treated with ARI. *P < 0.01 vs. littermate and transgenic mice; †P < 0.05 vs. littermate mice, transgenic mice, diabetic littermate mice treated with ARI, and diabetic transgenic mice treated with ARI; ‡P < 0.01 vs. littermate mice, transgenic mice, diabetic littermate mice, diabetic transgenic mice treated with ARI, and diabetic transgenic mice treated with ARI; ♦P < 0.05 vs. littermate and transgenic mice.

During the experimental period, body weight, blood glucose levels, and pain sensation threshold were regularly monitored, and at the end of the experiment, motor nerve conduction velocity (MNCV) and sensory nerve conduction velocity (SNVC) were measured. All animals were maintained in plastic cages in rooms with a constant temperature of 23°C and a 12-h light-dark cycle. All animals were given free access to food and water during the experimental period. All animals were coded, and examinations of pain sensation threshold as well as nerve conduction velocities were conducted in a double-blinded manner. Tissue samples for immunohistochemical analysis on DRG and immunohistochemistry were also coded and evaluated by the examiners, who were unaware of the identity of the samples.

### Pain Sensation Threshold Test

The mechanical threshold for nociceptive flexion was determined by measuring the foot-withdrawal threshold elicited by stimulation of the left hind paw using an algometer (UGP, Basile, Varese, Italy). This device generates a mechanical force that increases linearly with time. Briefly, all mice were anesthetized with isoflurane (Abbot) and placed on a thermostatically controlled heated mat to maintain the body temperature at 37°C. The temperature near the sciatic nerve was also kept constant at 37°C by monitoring with an electronic thermometer (PC-9400 Delta, Sato Keiryoki MFG, Tokyo, Japan) with the aid of a warmed blanket.

For MNCV, the left sciatic nerve was electrically stimulated first at the site of the Achilles tendon using a general evoked response stimulator (MS92 electrolyte myography device; Medelec, London, U.K.) and then at the site of sciatic notch, and the waves were recorded from the second interosseous muscle of the foot. In this case, supramaximal electrical stimulation of 0.1-ms pulses was used to generate M-waves. The point of first negative deflection of M-wave was identified as the period of latency. The latency differences derived from two stimulating sites were divided by the distance between the stimulating sites, yielding the value of MNCV.

For detection of SNVC, the power of electrical stimulation was gradually decreased. With this reduction, the M-wave diminished and instead H-reflex wave appeared. The initial deflection point of H-reflex wave was identified as the latency for SNVC. The identity of the H-reflex was validated by its disappearance on generation of a maximal M- and F-wave. The difference of proximal and distal latency was divided by the distance between the stimulating sites, yielding SNVC. An average of at least five recordings for each was used for measurements.

### Sorbitol and Fructose Contents

Tissue levels of sorbitol and fructose in DRG and the offspring were measured by liquid chromatography with tandem mass spectrometry (LC/MS/MS) method described previously (24). The concentrations were expressed as nanomoles per milligrams of protein.

**PKC activity.** PKC activities were assayed by the method described previously (12). DRG samples were transferred to a tube containing 1.0 ml homogenization buffer (20 mmol Tris-HCl [pH 7.5], 1 mmol CaCl2, 0.5 mmol MgCl2, 0.5 mmol EGTA, 2 mmol EDTA, 2 mmol n-octyl-β-D-glucopyranoside, and 1 mmol phenylmethylsulfonyl fluoride) and homogenized with a Polytron. Homogenate was centrifuged at 50,000 g for 30 min at 4°C. Supernatant was collected and used as cytosolic fraction. The pellet was resuspended in 0.6 ml homogenization buffer containing 1% Triton X-100 and stored on ice for 1 h. Resuspended solution was centrifuged at 50,000 g for 30 min at 4°C, after which supernatant was used as membrane fraction. Phosphorylation assay was carried out in a reaction mixture (20 mmol Tris [pH 7.5], 1 mmol CaCl2, 10 mmol MgCl2, 33 μmol octapeptide [RRKRTLRRL], 5 mmol EGTA, and 10 μmol γ-32P-ATP (5 × 105 cpm)(Perkin Elmer Life Sciences, Boston, MA) in the presence or absence of 6.4 μg/ml diorein and 96 μg/ml phosphatidylserine. The reaction was started by the addition of 30 μl cytosol or membrane fraction, incubated at 30°C for 10 min, and terminated by spotting the reaction mixture onto P-81 paper (Whatman, Maidstone, Kent, U.K.). P-81 paper was washed with 75 mmol phosphate four times for 15 min. The radioactivity was counted by liquid scintillation spectrometer (Aloka, Tokyo, Japan).

**Western Blot Analysis of PKC Isoforms.** Western blot analysis was performed using proteins that were extracted as cytosol and membrane fraction for PKC assay. SDS-PAGE was performed using the Xcell SureLock system (Invitrogen, San Diego, CA) in the reducing condition. Aliquots of 100-μg samples of protein were dissolved in the sample buffer (2.5% 2-mercaptoethanol, 62.5 mmol Tris-HCl, 10% glycerol, 2% SDS, 0.0025% bromophenol blue, and 50 mmol reducing agent [dithiothreitol; DTT], pH 6.8) and loaded onto the Novex Tris-glycine Pre-Cast Gel (Invitrogen). After completion of the migration, the proteins were transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore, Bedford, MA) in a transfer buffer (25 mmol Tris, 0.2 mol/l) at 100 V for 2 h. The membrane was probed overnight at 4°C with antibodies against different PKC isoforms. After washing with 0.1% Tween 20 in Tris-buffered saline, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies and visualized with chemiluminescence reagents (ECL, Amersham).
mol glycine, and 20% methanol) using a wet transfer unit of Xcell SureLock system. For blocking, membranes were incubated with 5% skimmed milk in PBS-T (PBS Triton X-100; 137 mmol NaCl, 2.7 mmol KCl, 1.5 mmol KH₂PO₄, 8.0 mmol Na₂HPO₄, pH 7.4, and 1% Triton X-100) overnight at 4°C. After washing with PBS-T, membrane was incubated with polyclonal anti–PKC-α, βII, and βII–specific antibodies (Santa Cruz Bio Tech, Santa Cruz, CA) and β-actin–specific antibody (Santa Cruz) for 1 h at room temperature. The incubation was carried out with peroxidase–conjugated anti-rabbit or anti-goat IgG (Santa Cruz) for 45 min at room temperature. Immunodetection was performed by enhanced chemiluminescence (Amersham-Pharmacia, Buckinghamshire, U.K.). Quantitative analysis of exposed films was performed using National Institutes of Health image software (Version 1.61).

**Immunohistochemistry.** For immunohistochemical analysis, 4–μm thick sections of formalin-fixed tissues were deparaffinized and pretreated with methanol containing 0.3% H₂O₂ to eliminate endogenous peroxidase activity. Polyclonal anti–PKC-α, βII, and βII–specific antibodies (1:200 dilution; Santa Cruz), CGRP (1:1,000 dilution; Affiniti Research Products, Exeter, U.K.), and substance P (1:500 dilution; Affiniti Research Products) were applied to the sections overnight at 4°C. After washing with PBS-T, sections were counterstained with hematoxylin. The specific localization of PKC isoforms on ganglion cells was observed. The number of positively stained nucleated ganglion cells with CGRP– and substance P–specific antibodies was also counted on a single cross section of DRG. The difference in the threshold between nondiabetic transgenic mice (Tg; ○) and transgenic diabetic mice was significant at all time points after diabetes induction. ARI-treated diabetic transgenic mice (Tg+ARI; □) showed complete normalization of the changes. The number of animals per group was 5–11. Data are means ± SE. Statistics were first conducted among all groups by ANOVA and then separately done in each littermate and transgenic group. *P < 0.05 vs. littermate mice, diabetic littermate mice treated with an ARI, and ARI-treated diabetic transgenic mice; †P < 0.05 vs. littermate mice, transgenic mice, and diabetic littermate mice treated with an ARI; ‡P < 0.05 vs. littermate mice, transgenic mice, diabetic littermate mice treated with an ARI, and ARI-treated diabetic transgenic mice; §P < 0.01 vs. littermate mice, transgenic, diabetic littermate mice, ARI-treated diabetic littermate mice, and ARI-treated diabetic transgenic mice.

**Detection of cell apoptosis.** Cell apoptosis was performed by the transferase-mediated dUPT nick-end labeling (TUNEL) method using an apoptosis detection kit (Apop Tag, Chemicon International, Temecula, CA).

**Morphometric analysis of ganglion cells.** The fixed samples of left lumbar DRG (L5) were postfixed with 4% paraformaldehyde and dehydrated through an ascending series of ethanol concentrations and embedded in epoxy resin. One-micron thick sections of DRG were stained with toluidine blue. In morphometric analysis, the cellular area of ganglion cells containing nuclei and their nuclear area were measured at a magnification of ×200 in each animal and expressed as a percentage of positive cells to total nucleated ganglion cells counted. For the objective comparison of the staining results among all groups, DRG sections from each group were randomly mounted on a single slide (i.e., six DRG tissues on one slide) and stained under the same condition.

**Statistical analysis.** Data were expressed as means ± SE. Statistical analysis was carried out on a Macintosh computer (Apple, Cupertino, CA) using a commercially available statistical program (Statview, version 4.11 J; Hulinks, Tokyo, Japan). Comparison of the values among the groups was carried out.
using one-way ANOVA, followed by Bonferroni’s corrections for multiple comparisons. For analysis of the values of pain sensation threshold, after the comparison among all groups with the above method, specific comparisons among each littermate or transgenic group were made separately. P values <0.05 were considered to be significant.

RESULTS

Transgene expression did not affect the general condition and behavior of the animals. Body weight values in transgenic mice were comparable with those of littermate mice throughout the experimental period (Table 1). At the end of the study, body weight values of diabetic mice were significantly lower than those of nondiabetic mice (P < 0.01), but there were no significant differences between transgenic and littermate mice groups. Blood glucose levels were markedly elevated following the induction of diabetes but were not different between transgenic and littermate mice after diabetes induction. ARI treatment had no effect on body weight or blood glucose level.

There were no significant differences in both sorbitol and fructose contents between nondiabetic transgenic and nondiabetic littermate mice (Table 1). Carbohydrate contents in diabetic groups were remarkably elevated compared with nondiabetic groups and were significantly greater in diabetic transgenic mice than in diabetic control mice (P < 0.01). ARI treatment corrected the rise of sorbitol (P < 0.01 for both untreated groups) but only partially of fructose in both diabetic transgenic mice and diabetic littermate mice (P < 0.05 for both).

At the end of the experiment, diabetic transgenic mice showed a significant decrease in MNCV compared with nondiabetic groups (P < 0.01), while the decrease of MNCV in diabetic littermate mice was not significant (Table 1). The difference in MNCV between diabetic transgenic mice and diabetic littermate mice was also significant (P < 0.01). ARI treatment significantly improved SNCV in diabetic transgenic mice (TgDM) than diabetic littermate mice (LmDM). Treatment with an ARI (fidaestat) corrected the changes in both diabetic littermate and diabetic transgenic mice. By contrast, PKC activity in cytosolic fraction was significantly increased only in diabetic transgenic mice. ARI treatment corrected this change. The number of animals per group was five to seven. Data are means ± SE. *P < 0.05 vs. littermate mice, transgenic mice, diabetic littermate mice treated with an ARI (LmDM + ARI), and diabetic transgenic mice treated with an ARI (TgDM + ARI); †P < 0.01 vs. littermate mice, transgenic mice, diabetic littermate mice treated with an ARI, and diabetic transgenic mice treated with an ARI.

FIG. 2. PKC activity in membrane and cytosolic fractions of DRG in experimental animals. Membrane PKC activity was significantly reduced in diabetic groups compared with nondiabetic littermate mice (Lm) and transgenic mice (Tg), and the changes were more severe in diabetic transgenic mice (TgDM) than diabetic littermate mice (LmDM). Treatment with an ARI (fidaestat) corrected the changes in both diabetic littermate and diabetic transgenic mice. By contrast, PKC activity in cytosolic fraction was significantly increased only in diabetic transgenic mice. ARI treatment corrected this change. The number of animals per group was five to seven. Data are means ± SE. *P < 0.05 vs. littermate mice, transgenic mice, diabetic littermate mice treated with an ARI (LmDM + ARI), and diabetic transgenic mice treated with an ARI (TgDM + ARI); †P < 0.01 vs. littermate mice, transgenic mice, diabetic littermate mice treated with an ARI, and diabetic transgenic mice treated with an ARI.

PKC activity. PKC activities in membrane fraction were comparable between the two nondiabetic groups, but they were significantly reduced by diabetes (P < 0.05 for diabetic littermate mice and P < 0.01 for diabetic transgenic mice) (Fig. 2). The activities in diabetic transgenic mice were more severely depressed compared with nondiabetic transgenic mice (P < 0.01). ARI treatment significantly improved the activities in both diabetic groups (P < 0.05 for diabetic littermate mice and P < 0.01 for diabetic transgenic mice). By contrast, PKC activities in cytosolic fraction were significantly increased in diabetic transgenic mice compared with other groups (P < 0.01), while those in diabetic littermate mice were not significantly altered. ARI treatment corrected this increase.

Western blot analysis of PKC isoform. The relative intensities of the expression bands of PKC-α of the membrane fraction were markedly reduced in diabetic compared with nondiabetic groups, and the reduction in diabetic transgenic mice (57.9%) was more severe than that in diabetic littermate mice (17.6%) (P < 0.01) (Fig. 3). ARI treatment corrected these changes (P < 0.05 for diabetic littermate mice and P < 0.01 for diabetic transgenic mice). In contrast to the changes in membrane fraction, the expression levels of cytosolic PKC-α isoform
were significantly elevated by 25.8% in diabetic littermate mice (P < 0.05) and by 36.4% in diabetic transgenic mice (P < 0.05) compared with those in the nondiabetic groups, but there was no significant difference between the two diabetic groups. ARI treatment suppressed the elevation of PKC-α isoform expression in diabetic transgenic mice (P < 0.05) but not in diabetic littermate mice.

The levels of protein expression of PKC-βI isoform in membrane fraction were not significantly different among all of the groups. By contrast, the levels of PKC-βII isoform in membrane fraction were significantly increased in diabetic transgenic mice compared with nondiabetic groups (P < 0.05), but there was no significant difference between the two diabetic groups. ARI treatment inhibited this elevation (P < 0.05). The expression levels of both PKC-βI and -βII isoforms in cytosolic fraction were not different between nondiabetic and diabetic groups, and ARI treatment had no effect on the expression of these isoforms.

**Immunohistochemistry.** In nondiabetic groups, strong positive staining reactions for PKC-α were detected on membrane portions of ganglion cells, predominantly of small to medium sizes (Fig. 4). The membrane reactions in the two diabetic groups appeared to be less apparent compared with the nondiabetic groups and instead showed diffuse weak reactions in the cytosol. Membrane reactions were almost diminished in diabetic transgenic mice. ARI treatment restored the membrane positivity in both diabetic transgenic and diabetic littermate mice. By contrast, reactions for PKC-βI and -βII were mostly located in the cytosol, and diabetic condition did not appear to affect the staining patterns in diabetes (pictures not shown).
Positive reactions of CGRP and substance P were found on small- to medium-sized ganglion cells (Fig. 5). The reactions appeared to be reduced in diabetic groups, markedly so in diabetic transgenic mice. Quantitative evaluations revealed a significant reduction in the population of CGRP-positive cells in diabetic compared with nondiabetic groups \((P < 0.05)\) (Table 2). The reduction of CGRP neurons was most marked in diabetic transgenic mice, but the difference between diabetic transgenic and diabetic littermate mice was not significant. By contrast, reduction of substance P–positive cells was only significant in diabetic transgenic mice compared with the other groups \((P < 0.05)\). ARI treatment partially but significantly corrected the decrease in the population of both CGRP and substance P neurons in diabetic transgenic mice \((P < 0.05)\) for both.

**Apoptosis.** There was no TUNEL-positive reaction in DRG, indicating the absence of the cells undergoing breaks of nuclear DNA strands and hence possibly a lack of ongoing apoptosis in ganglion cells in all experimental groups.

**Morphometric analysis.** There was no significant difference in the mean cellular area between transgenic mice and littermate mice without diabetes (Table 3). While the cellular area was not altered in diabetic littermate mice, it was significantly reduced in diabetic transgenic mice compared with the nondiabetic groups \((P < 0.05)\). ARI treatment significantly prevented this reduction \((P < 0.05)\). The mean nuclear area was comparable among groups of littermate, transgenic, and diabetic littermate mice, while the area of diabetic transgenic mice was significantly reduced compared with other groups. This reduction was significantly prevented by ARI treatment \((P < 0.05)\).

The density of ganglion cells was comparable among all of the groups; thus, there was no significant loss of ganglion cells in the diabetic groups.

**DISCUSSION**

In this study, the effects of aldose reductase overexpression on the neuropathic changes were well exemplified by marked slowing of nerve conduction, augmented changes of altered pain sensation, and reduced nociceptive peptide
Data are means ± SE. n = 5 in each group. Lm, littermate control mice; Tg, mice transgenic for human aldose reductase; LmDM, diabetic littermate mice; TgDM, diabetic transgenic mice; LmDM + ARI, diabetic littermate mice treated with ARI; TgDM + ARI, diabetic transgenic mice treated with ARI. *P < 0.05 vs. littermate and transgenic mice; †P < 0.05 vs. diabetic transgenic mice; ‡P < 0.05 vs. littermate mice, transgenic mice, diabetic littermate mice treated with ARI, and diabetic transgenic mice treated with ARI. §P < 0.05 vs. littermate mice, transgenic mice, diabetic littermate mice, diabetic littermate mice treated with ARI, and diabetic transgenic mice treated with ARI.

The decrease in PKC activity in diabetic nerve was once proposed to be due to reduced synthesis of DAG from substrate of myo-inositol (32). This is unlikely in our model because we could not detect significant reduction of nerve myo-inositol in diabetic transgenic mice in our previous studies (11). Alternatively, excessive oxidative stress related to reduced glutathione as well as impaired nitric oxide production caused by NADPH reduction may be involved in the translocation of PKC-α isoform, as it is claimed to operate in vascular walls (4,37). In this setting, the role of mitochondria in the production of oxidative stress through increased glucose flux may be crucial (38). In fact, compared with diabetic wild mice, the levels of reduced glutathione were more severely decreased in diabetic mice overexpressing aldose reductase, specifically in Schwann cells, accompanied by reduced MNCV despite the absence of significant sorbitol accumulation (13).

However, interpretation of aldose reductase overexpres-

### Table 2
Population of cells positive for CGRP and substance P

<table>
<thead>
<tr>
<th>Group</th>
<th>CGRP (percentage of positive cells)</th>
<th>Substance P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lm</td>
<td>13.7 ± 0.3</td>
<td>6.9 ± 0.8</td>
</tr>
<tr>
<td>Tg</td>
<td>14.6 ± 1.2</td>
<td>7.4 ± 0.8</td>
</tr>
<tr>
<td>LmDM</td>
<td>9.4 ± 1.7*</td>
<td>5.3 ± 1.1†</td>
</tr>
<tr>
<td>TgDM</td>
<td>6.8 ± 1.1‡</td>
<td>2.8 ± 0.9§</td>
</tr>
<tr>
<td>LmDM + ARI</td>
<td>12.3 ± 1.8</td>
<td>7.6 ± 0.5</td>
</tr>
<tr>
<td>TgDM + ARI</td>
<td>11.8 ± 0.9</td>
<td>6.6 ± 0.8</td>
</tr>
</tbody>
</table>

Data are means ± SE. n = 5 in each group. Lm, littermate control mice; Tg, mice transgenic for human aldose reductase; LmDM, diabetic littermate mice; TgDM, diabetic transgenic mice; LmDM + ARI, diabetic littermate mice treated with ARI; TgDM + ARI, diabetic transgenic mice treated with ARI. *P < 0.05 vs. littermate and transgenic mice; †P < 0.05 vs. diabetic transgenic mice; ‡P < 0.05 vs. littermate mice, transgenic mice, diabetic littermate mice treated with ARI, and diabetic transgenic mice treated with ARI. §P < 0.05 vs. littermate mice, transgenic mice, diabetic littermate mice, diabetic littermate mice treated with ARI, and diabetic transgenic mice treated with ARI.

### Table 3
Morphometric data on ganglion cells

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Cell area (µm²)</th>
<th>Nuclear area (µm²)</th>
<th>Nuclear area-to-cellular area ratio (%)</th>
<th>Density (n/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lm</td>
<td>7</td>
<td>656.0 ± 33.9</td>
<td>102.1 ± 3.9</td>
<td>17.6 ± 0.6</td>
<td>2,685 ± 264</td>
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<tr>
<td>Tg</td>
<td>8</td>
<td>608.3 ± 23.4</td>
<td>104.0 ± 4.2</td>
<td>19.3 ± 0.3</td>
<td>2,716 ± 122</td>
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<tr>
<td>LmDM</td>
<td>8</td>
<td>651.5 ± 26.8</td>
<td>105.3 ± 3.5</td>
<td>18.4 ± 0.5</td>
<td>2,604 ± 87</td>
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<tr>
<td>TgDM</td>
<td>6</td>
<td>469.8 ± 43.9*</td>
<td>86.4 ± 4.4*</td>
<td>20.9 ± 1.0†</td>
<td>2,838 ± 198</td>
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<tr>
<td>LmDM + ARI</td>
<td>9</td>
<td>623.4 ± 44.8</td>
<td>99.7 ± 4.4</td>
<td>18.8 ± 0.6</td>
<td>2,826 ± 111</td>
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<tr>
<td>TgDM + ARI</td>
<td>8</td>
<td>583.3 ± 31.5</td>
<td>98.9 ± 4.1</td>
<td>19.0 ± 0.6</td>
<td>3,167 ± 220</td>
</tr>
</tbody>
</table>

Data are means ± SE. Lm, littermate control mice; Tg, mice transgenic for human aldose reductase; LmDM, diabetic littermate mice; TgDM, diabetic transgenic mice; LmDM + ARI, diabetic littermate mice treated with ARI; TgDM + ARI, diabetic transgenic mice treated with ARI. *P < 0.05 vs. littermate and transgenic mice; †P < 0.05 vs. diabetic transgenic mice, diabetic littermate mice, diabetic littermate mice treated with ARI, and diabetic transgenic mice treated with ARI.
pression on the neuronal changes of PKC activity must be made with caution because polyol flux should also be exaggerated in satellite cells as well as vascular components of DRG in our model, as expected in human diabetic subjects (12,39). Hence, there is a possibility that the changes may not be a primary event in ganglion cells but could be a secondary event exerted by combined effects of the changes in satellite cells as well as vascular tissues in DRG. It would be interesting to examine the changes of DRG in transgenic mice that overexpress aldose reductase only in Schwann cells in order to clarify whether similar neuronal changes are detected under diabetic condition.

Our study revealed that the pain sensation in diabetic animals was initially hyperalgesic followed by late hypoesthesia in the presence of a progressive delay of both MNCV and SNCV. This result argues against the results obtained from the STZ-induced diabetic rat (36) and the diabetic mice with short duration (40). We only conducted the paw withdrawal test under mechanical pressure for the pain sensation and therefore need further evaluation using different methods such as a test for thermal sensitivities. Nevertheless, our results are still in keeping with the data from recent studies on mice (41) and the neuropathic process in human diabetes (42,43).

Reduced expression of nociceptive peptides of CGRP and substance P detected in this study is not specific in this model but has been reproduced demonstrably in STZ-induced diabetic rats with 4–8 weeks’ duration (19,44). The expression of CGRP and substance P was well regulated by neurotrophic support, particularly nerve growth factor, neurotrophin-3, and ciliary nerve trophic factor, which are known to be affected in the diabetic state (45) and corrected by insulin or ARI treatment (19,46). These results strongly suggest that sensory neuropathic symptoms develop based on the metabolic aberration occurring in sensory ganglion cells. The results that augmented structural changes of sensory ganglion cells concurrent with reduced PKC activity and the ARI effects in our model provide strong rationale to use ARIs for diabetic neuropathic pain.

These results strongly suggest that sensory neuropathic changes are associated with nerve growth factor, neurotrophin-3, and related nerve trophic factors. The expression of these factors is altered in diabetic mice under conditions of reduced PKC activity and ARI treatment. The effects of ARIs on sensory nerve conduction velocity in diabetic mice are suggested to be due to changes in satellite cells and vascular tissues, as indicated by the results of our previous study (41).

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