Na\textsuperscript{+}/H\textsuperscript{+} exchanger 1 inhibition reverses manifestation of peripheral diabetic neuropathy in type 1 diabetic rats

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Lupachyk S, Watcho P, Shevalye H, Vareniuk I, Obrosov A, Obrosova IG, Yorek MA. Na\textsuperscript{+}/H\textsuperscript{+} exchanger 1 inhibition reverses experimental peripheral diabetic neuropathy. Control and streptozotocin-diabetic rats were treated with the specific Na\textsuperscript{+}/H\textsuperscript{+} exchanger 1 inhibitor cariporide for 4 wk after 12 wk without treatment. Neuropathy end points included sciatic motor and sensory nerve conduction velocities, endoneurial nutritive blood flow, vascular reactivity of epineurial arterioles, thermal nociception, tactile allodynia, and intraepidermal nerve fiber density. Advanced glycation end product; endoneurial blood flow; nerve conduction velocity

diabetic peripheral neuropathy; Na\textsuperscript{+}/H\textsuperscript{+} exchanger-1; advanced glycation end product; endoneurial blood flow; nerve conduction velocity; vascular reactivity

DIABETIC PERIPHERAL NEUROPATHY is a complication of diabetes that has a complex etiology and even with good glycemic control can have severe consequences. To date, there is no effective treatment for diabetic peripheral neuropathy (54). There have been many mechanisms proposed to contribute to diabetes complications, and there is now widespread evidence for an important role for oxidative-nitrosative stress and its downstream effectors in the development of peripheral diabetic neuropathy (1, 16, 26, 40, 51). Hyperglycemia is thought to be an important contributing factor to the pathology of diabetic complications, but the intracellular metabolic pathways linking glucose and oxidative injury are not completely understood (9, 20, 56). Diabetes-associated inhibition or insufficient activation of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase underlies diversion of excessive glycolytic flux toward the formation of methylglyoxal and \(\alpha\)-glycerophosphate (5–7, 28). This in turn has been shown to lead to 1) a decrease in free cytosolic NAD\textsuperscript{+}/NADH ratio and resulting activation of the superoxide-generating enzyme NAD(P)H oxidase and 2) a formation of advanced glycation end products (AGE) that generate free radicals during interaction with their receptors (3, 22, 62). The mechanisms underlying diabetes-associated activation of the upper part of glycolysis remain unidentified. One mechanism we have been investigating is the activation of Na\textsuperscript{+}/H\textsuperscript{+} exchanger 1. Na\textsuperscript{+}/H\textsuperscript{+} exchanger 1 is distributed ubiquitously in mammalian tissues and plays an important role in the regulation of intracellular pH by removing protons that are generated continuously in normal cells (8, 30, 32, 33, 50, 65). Several reports suggest that upregulation of Na\textsuperscript{+}/H\textsuperscript{+} exchanger-1 activity, demonstrated recently in several tissue sites for diabetes complications, leads to an increase in cytosolic pH and consequent activation of glucose transport and all enzymes in the upper part of glycolysis, especially phosphofructokinase (24, 53, 58). Given this information, we propose that diabetes-driven increased expression/activation of Na\textsuperscript{+}/H\textsuperscript{+} exchanger 1 may cause an increase in the formation of advanced glycation end products that may then contribute to increased oxidative stress and development of peripheral diabetic neuropathy. We propose that inhibition of Na\textsuperscript{+}/H\textsuperscript{+} exchanger 1 could be an effective treatment for peripheral diabetic neuropathy.

MATERIALS AND METHODS

Materials. Unless stated otherwise, all chemicals used in these studies were obtained from Sigma Chemical (St. Louis, MO).

Animals. The experiments were performed in accordance with regulations specified by the National Institutes of Health Principles of Laboratory Animal Care and the Pennington Biomedical Research Center and Iowa City Veterans Affairs Medical Center; both of these institutions approved the animal study protocols. Male Wistar rats (Charles River, Wilmington, MA) 10–11 wk of age were fed a standard rat chow (PMI Nutrition International, Brentwood, MO) and had access to water ad libitum. Type 1 diabetes was induced by injecting streptozotocin (50 mg/kg body wt ip). Hyperglycemia was verified (nonfasting blood glucose \(\geq 13.8 \text{ mM via tail vein} \) 48 h after the streptozotocin injection. Control rats injected with vehicle and diabetic rats were monitored for 12 wk (weight and blood glucose). After 12 wk the rats were divided into four experimental groups: control and diabetic rats treated with or without cariporide, an Na\textsuperscript{+}/H\textsuperscript{+} exchanger 1 inhibitor (10 mg·kg\textsuperscript{-1}·day\textsuperscript{-1} in the drinking water), for 4 wk (37). Diabetic rats that lost \(>10\%\) of their initial body weight were treated with 1–2 units of insulin every second day until their weight stabilized. Insulin treatments did not correct hyperglycemia.
Behavioral tests. The paw withdrawal latency in response to radiant heat was recorded at a 15% intensity (heating rate of ~1.3°C/s) with a cutoff time of 30 s, using the IITC model 336 TG combination tail-flick and paw algesiometer (IITC Life Sciences, Woodland Hills, CA) (42). Tactile responses were evaluated by quantifying the withdrawal threshold of the hindpaw in response to stimulation with flexible von Frey filaments, as described previously (23). The data were reported in seconds and grams.

Physiological tests. On the day of terminal studies, rats were weighed and anesthetized with Nembutal (50 mg/kg ip; Abbott Laboratories, North Chicago, IL). Nonfasting blood glucose was determined. Sciatic motor nerve conduction velocity and digital sensory nerve conduction velocity were measured as described previously (51). The motor and sensory nerve conduction velocities were reported in meters per second. Sciatic nerve endoneural blood flow was determined as described previously, using the hydrogen clearance method (45). The hydrogen clearance data were fitted to a mono- or biexponential curve using commercial software (Prism; Graphpad, San Diego, CA). Nutritive blood flow (ml·min⁻¹·100 g⁻¹) was calculated using the equation described by Young (64), and vascular conductance (ml·min⁻¹·100 g⁻¹·mmHg⁻¹) was determined by dividing the nutritive blood flow by the average mean arterial blood pressure.

Intraepidermal nerve fiber density. Footpads were fixed in ice-cold Zamboni's fixative for 3 h, washed in 100 mM phosphate-buffered saline (PBS) overnight, and then washed in PBS containing increasing amounts of sucrose, i.e., 10, 15, and 20%, for 3 h in each solution. After washing, the samples were snap-frozen in optimum cutting temperature (OCT) and stored at ~80°C. Three longitudinal 50-μm-thick footpad sections were cut using a Leica CM1950 cryostat (Leica Microsystems, Nussloch, Germany). Nonspecific binding was blocked by 3% goat serum containing 0.5% porcine gelatin and 0.5% Triton X-100 in SuperBlock blocking buffer (Thermo Scientific, Rockford, IL) at room temperature for 2 h. The sections were then incubated overnight with PGP 9.5 antisemur (UltraClone, Isle of Wight, UK) in 1:400 dilution at 4°C, after which secondary Alexa Fluor 488 antibody (Molecular Probes, Life Technologies, Grand Island, NY) in 1:1000 dilution was applied at room temperature for 1 h. Sections were then coverslipped with VectaShield mounting medium (Vector Laboratories, Burlingame, CA). Intraepidermal nerve fiber profiles were counted blindly by three independent investigators using an Axioplan 2 microscope (Carl Zeiss Microscopy, Thornwood, NY) at ×400 magnification, and the average values were reported. The length of epidermis was assessed on the microphotographs of stained sections taken at ≥50 magnification with a 31 Everest imaging system (Intelligent Imaging Innovations, Denver, CO) operated with an Axioplan 2 microscope, using the National Institutes of Health (Bethesda, MD) Image J software. An average of 2.8 ± 0.3 mm of the sample length was investigated to calculate a number of nerve fiber profiles per millimeter of epidermis. Representative images of intraepidermal nerve fibers were obtained by confocal laser scanning microscopy at ×400 magnification, using Leica TCS SP5 confocal system (Leica Microsystems, Nussloch, Germany) (51).

Vascular reactivity in epineurial arterioles. Videomicroscopy was used to investigate in vitro vasodilatory responsiveness of epineurial arterioles vascularizing the region of the sciatic nerve, as described previously (55). The desired vessels were isolated by exposing the common iliac, and the branch points of the internal pudendal and superior gluteal arteries were identified. The vessels were then clamped, and tissues containing these vessels and the branches of the internal pudendal and superior gluteal arteries were dissected en bloc. The block of tissue was immediately submerged in a cooled (4°C), physiological saline solution (PSS) of the following composition (in mmol/l): 118 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 20 NaHCO₃, 0.026 Na₂EDTA, and 5.5 glucose. Branches of the superior gluteal and internal pudendal arteries (50- to 150-μm internal diam-
Table 1. Effect of diabetes on thermal algasia, IENF density, tactile response, MNCV, and SNCV

<table>
<thead>
<tr>
<th>Determination</th>
<th>Control</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermal response latency, s</td>
<td>9.6 ± 0.5</td>
<td>15.5 ± 0.8*</td>
</tr>
<tr>
<td>IENF, profiles/mm</td>
<td>21.1 ± 2.6</td>
<td>14.1 ± 3.0*</td>
</tr>
<tr>
<td>Tactile response threshold, g</td>
<td>1.4 ± 1.5</td>
<td>6.3 ± 0.5*</td>
</tr>
<tr>
<td>MNCV, m/s</td>
<td>54.9 ± 1.0</td>
<td>48.3 ± 1.3*</td>
</tr>
<tr>
<td>SNCV, m/s</td>
<td>42.4 ± 0.4</td>
<td>35.8 ± 0.3*</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE; n = 8 experimental animals. IENF, intraepidermal nerve fiber; MNCV, motor nerve conduction velocity; SNCV, sensory nerve conduction velocity. Control rats and rats with 12-wk duration of diabetes. *P < 0.05 compared with control.

RESULTS

Baseline changes in diabetic rats prior to treatment. Data in Table 1 demonstrate that after 12 wk of untreated diabetes, thermal sensitivity, tactile response, motor and sensory nerve conduction velocity, and intraepidermal nerve fiber density were all significantly impaired compared with age-matched controls. Data in Fig. 1 demonstrate that expression of Na+/H+ exchanger 1 (NHE-1, control rats; Na+/H+ exchanger 1 content in sciatic nerves of C and D rats. Na+/H+ exchanger 1. Equal protein loading was confirmed with β-actin antibody (Molecular Probes) was used in a working dilution of 1:400. Sections were mounted in VectaShield mounting medium. All sections were processed by a single investigator and evaluated blindly. Color images were captured at ×400 magnification with a 3D Everest imaging system (Intelligent Imaging Innovations) equipped with an Axiosplan 2 microscope (Zeiss). Nitrotyrosine and Na+/H+ exchanger 1 fluorescence intensity of individual dorsal root ganglion neurons was quantified using the Image J software (National Institutes of Health) and normalized per neuronal area. For nitrotyrosine immunofluorescence analysis, nuclei of individual cells were excluded from the regions of interest. Neurons (15–20/rat) were counted, and the average values for each animal were used to calculate group means. Fluorescence intensity was expressed as means ± SE for each experimental group.

Statistical analysis. The results are presented as means ± SE. Data were subjected to equality of variance F test and then to log transformation if necessary before one-way analysis of variance. Where overall significance (P < 0.05) was attained, individual between-group comparisons for multiple groups were made using the Student-Newman-Keuls multiple-range test. When between-group variance differences could not be normalized by log transformation (data sets for body weights and plasma glucose), the data were analyzed by the nonparametric Kruskal-Wallis one-way analysis of variance, followed by the Bonferroni-Dunn test for multiple comparisons. Individual pairwise comparisons in Table 1 and Fig. 1 were made using the unpaired two-tailed Student t-test. Concentration-response curves for acetylcholine were compared using a two-way repeated-measures analysis of variance with autoregressive covariance structure using the proc mixed program of SAS (14, 15). Significance was defined at P < 0.05.

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exchanger 1 in the sciatic nerve is significantly increased after 12 wk of untreated diabetes.

Effect of treatment of diabetic rats with cariporide on weight and blood glucose. Data in Table 2 demonstrate that treating diabetic rats for 4 wk with cariporide, an inhibitor of Na\(^+/\)H\(^+\) exchanger 1, following 12 wk of no treatment did not correct hyperglycemia or weight gain. Treatment of nondiabetic rats with cariporide for 4 wk did not affect blood glucose levels or weight gain.

Effect of treatment of diabetic rats with cariporide on neural and vascular complications. Data in Table 3 demonstrate that 4 wk of treatment with cariporide significantly improved thermal and tactile responses and motor and sensory nerve conduction velocity compared with untreated diabetic rats. However, each of these neural complications remained significantly impaired compared with control rats. This could be for several reasons, including suboptimal dosing, and a longer duration of treatment may be required to achieve full recovery, or multiple mechanisms may be involved. Data in Fig. 2 demonstrate that treating diabetic rats for 4 wk with cariporide stimulated the recovery of intraepidermal nerve fibers in the hindpaw. Treating diabetic rats for 4 wk with cariporide following 12 wk of no treatment significantly improved endoneurial blood flow (Fig. 3, A and B) and vascular relaxation to acetylcholine by epineurial arterioles of the sciatic nerve compared with untreated diabetic rats (Fig. 4A). In these studies, streptozotocin-induced diabetes did not impair blood pressure or relaxation to sodium nitroprusside (Fig. 4C and data not shown, respectively). Similarly to neural complications that were partially improved with 4 wk of treatment, cariporide vascular relaxation to acetylcholine by epineurial arterioles was not fully corrected. Previously, we had shown that acetylcholine-mediated relaxation by epineural was significantly decreased as early as 1 wk after the induction of diabetes (55). Furthermore, we had shown previously that increased oxidative stress as determined by increased levels of superoxide and nitrotyrosine staining in epineurial arterioles derived from diabetic rats was responsible for vascular impairment (14, 15). In these studies, we demonstrated that treating diabetic rats with cariporide for 4 wk reduced superoxide and nitrotyrosine staining significantly in epineurial arterioles of diabetic rats (Fig. 4, B and C). Treatment of nondiabetic rats with cariporide for 4 wk did not affect neural or vascular end points.

Effect of treatment of diabetic rats with cariporide on AGE, nitrated proteins, and 4-hydroxynonenal in sciatic nerve. Data in Fig. 5 demonstrate that methylglyoxal-derived AGE (Fig. 5A), nitrated proteins (Fig. 5B), and 4-hydroxynonenal (Fig. 5C) are all increased significantly in the sciatic nerve of diabetic rats (16-wk duration). Treating diabetic rats for 4 wk with cariporide significantly reduced these markers associated with advanced glycation end product accumulation and oxidative/nitrosative stress. Treatment of nondiabetic rats with cariporide for 4 wk did not affect the presence of these markers in the sciatic nerve.

**DISCUSSION**

Diabetic neuropathy is a progressive multifactorial complication with no effective treatment other than good glycemic control, and even with intense insulin therapy, diabetic neuropathy develops. Following years of research, many mecha-

### Table 2. Change in body weight and blood glucose in streptozotocin-induced diabetic rats treated with or without cariporide

<table>
<thead>
<tr>
<th>Determination (No. of Experimental Animals)</th>
<th>Control (n = 10)</th>
<th>Control + Cariporide (n = 10)</th>
<th>Diabetic (n = 16)</th>
<th>Diabetic + Cariporide (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial weight, g</td>
<td>314 ± 10</td>
<td>323 ± 3</td>
<td>300 ± 10</td>
<td>316 ± 4</td>
</tr>
<tr>
<td>Final weight, g</td>
<td>570 ± 26</td>
<td>557 ± 9</td>
<td>547 ± 1</td>
<td>530 ± 14</td>
</tr>
<tr>
<td>Initial blood glucose, mM</td>
<td>3.9 ± 0.1</td>
<td>4.5 ± 0.1</td>
<td>3.9 ± 0.1</td>
<td>26.2 ± 1.2*</td>
</tr>
<tr>
<td>Final blood glucose, mM</td>
<td>5.9 ± 0.2</td>
<td>5.9 ± 0.1</td>
<td>6.2 ± 1.2</td>
<td>23.8 ± 0.6*</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. Control rats and rats with 16-wk duration of diabetes maintained with or without cariporide for 4 wk after 12 wk of untreated diabetes. *P < 0.05 compared with control.

### Table 3. Effect of treatment with cariporide of control and diabetic rats on thermal algesia, tactile response, MNCV, and SNCV

<table>
<thead>
<tr>
<th>Determination (No. of Experimental Animals)</th>
<th>Control (n = 10)</th>
<th>Control + Cariporide (n = 10)</th>
<th>Diabetic (n = 16)</th>
<th>Diabetic + Cariporide (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermal response latency, s</td>
<td>11.0 ± 0.7</td>
<td>11.8 ± 0.3</td>
<td>27.3 ± 0.7*</td>
<td>20.4 ± 0.8*+</td>
</tr>
<tr>
<td>Tactile response threshold, g</td>
<td>18.6 ± 1.8</td>
<td>17.0 ± 1.7</td>
<td>7.1 ± 0.3*</td>
<td>11.0 ± 0.8*+</td>
</tr>
<tr>
<td>MNCV, m/s</td>
<td>63.5 ± 1.6</td>
<td>65.2 ± 1.0</td>
<td>44.0 ± 0.9*</td>
<td>55.2 ± 1.6*+</td>
</tr>
<tr>
<td>SNCV, m/s</td>
<td>46.8 ± 1.0</td>
<td>44.7 ± 1.0</td>
<td>40.0 ± 0.5*</td>
<td>42.0 ± 0.5*+</td>
</tr>
</tbody>
</table>

Control rats and rats with 16-wk duration of diabetes maintained with or without cariporide for 4 wk after 12 wk of untreated diabetes. Data are presented as means ± SE. *P < 0.05 compared with control; +P < 0.05 compared with untreated diabetic.
nisms have been proposed to contribute to diabetic neuropathy (29, 39). One of the more highly investigated theories has been increased oxidative stress. Oxidative stress has been documented in animal models of type 1 and type 2 diabetes (13, 15, 41, 43, 44, 52). It is clearly manifest in neurons, Schwann cells, axons, and endothelial cells of the peripheral nervous system (42). Accumulation of nitrotyrosine (a footprint of peroxynitrite-induced protein nitration) has also been documented in the peripheral nerve of diabetic rats, indicating that diabetes creates not just oxidative but nitrosative stress also in the peripheral nervous system (11, 38, 42).

In this study, we investigated the inhibition of Na\(^{+}/H^+\) exchanger 1 as a potential new treatment aimed at reducing oxidative/nitrosative stress in diabetes. The 10 members of the Na\(^{+}/H^+\) exchanger family described so far show a particular tissue distribution pattern (53). In this study, we were interested in the Na\(^{+}/H^+\) exchanger 1 isoform, which is found in the plasma membrane of most mammalian cells and is normally described as the housekeeping isoform (53). Na\(^{+}/H^+\) exchanger 1 plays a critical role in intracellular pH and cell volume homeostasis and regulates a number of cell behaviors, including adhesion, shape determination, migration, and proliferation (8, 48). Another important function of Na\(^{+}/H^+\) exchanger 1 of interest for diabetes complications is regulation of glycolysis. Activation of Na\(^{+}/H^+\) exchanger 1 causes cytosol alkalinization and resulting activation of glycolysis; furthermore, a Na\(^{+}/H^+\) exchange-dependent increase in intracellular pH by ~0.3 units was recently found to cause a one-order magnitude increase in the rate of glycolysis (25, 34, 46, 49). It is well known that upregulation of glycolysis contributes to the formation of by-products of glycolysis, i.e., methylglyoxal, α-glycerophosphate, and diacylglycerol, with concomitant activation of advanced glycation end product formation.

Hyperglycemia is associated with stimulation of Na\(^{+}/H^+\) exchanger 1 (53, 57). In the diabetic kidney, it has been demonstrated that Na\(^{+}/H^+\) exchanger 3 activity is increased (53). Both hyperglycemia and oxidative stress stimulate Na\(^{+}/H^+\) exchanger 3 activity via angiotensin II receptor activation (53). Angiotensin-converting enzyme inhibitors and angiotensin receptor blockers reduce progression of diabetic nephropathy, and we have shown that these drugs also improve diabetic neuropathy (13, 35, 36, 45, 63). It is not known whether angiotensin-converting enzyme inhibitors or angiotensin receptor blockers decrease Na\(^{+}/H^+\) exchanger 1 activity in diabetes. These data imply that
one possible mechanism for the beneficial effects of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers on diabetic complications, including neuropathy, may be through inhibiting the activation of Na+/H+ exchangers.

Hyperglycemia and increased metabolic rate could accentuate proton production and lead to increased proton efflux through Na+/H+ exchange, which would stimulate Na+/H+ exchange and calcium overload (57). Imbalance of sodium and calcium with hyperglycemia is associated with endothelial dysfunction and apoptosis (57), proliferation of vascular smooth muscle cells (31), retinal microangiopathy and ischemic damage (17, 37), and myocardial damage (2, 10, 18). It has been

Fig. 4. A: vascular reactivity of epineurial arterioles of the sciatic nerve in response to acetylcholine. Control rats and rats with 16-wk duration of diabetes maintained with or without cariporide for 4 wk after 12 wk of untreated diabetes. Means ± SE; n = 8/group. B: representative images of epineurial arterioles stained for superoxide anion radicals. C: analysis of fluorescence as relative light units (RLU) for superoxide staining. D: representative images of epineurial arterioles stained for nitrotyrosine. E: analysis of fluorescence as RLU for nitrotyrosine staining. Means ± SE; n = 6. *P < 0.05 vs. controls; +P < 0.05 vs. untreated diabetic group.

Fig. 5. A: Western blot analysis by densitometry of rat sciatic nerve of expression of methylglyoxal-derived advanced glycation end products (AGE). Control rats and rats with 16-wk duration of diabetes maintained with or without cariporide for 4 wk after 12 wk of untreated diabetes. Control was arbitrarily assigned a value of 100%. B: Western blot analysis by densitometry of rat sciatic nerve of expression of sciatic nerve nitratred proteins. Control was arbitrarily assigned a value of 100%. C: ELISA analysis of sciatic nerve of 4-hydroxynonenal adduct. Value is expressed as µg/mg protein. Means ± SE; n = 6. *P < 0.05 vs. controls; +P < 0.05 vs. untreated diabetic group.
shown that inhibition of Na\(^+/\)H\(^+\) exchanger 1 yields beneficial effects on diabetes vascular, retinal, and renal complications, but no information is available on whether inhibiting Na\(^+/\)H\(^+\) exchanger 1 improves diabetic peripheral neuropathy (37, 53).

In this study, we demonstrated that after 12 wk of untreated diabetes, expression of Na\(^+/\)H\(^+\) exchanger 1 is increased in the sciatic nerve and dorsal root ganglion neurons and that cariporide treatment for 4 wk after 12 wk of untreated diabetes improved vascular and neural deficits of diabetic neuropathy, including nerve conduction velocity, thermal and tactile sensitivity, endoneurial blood flow, and regeneration of intraepidermal nerve fibers. Improvement in diabetic neuropathy endpoints with cariporide treatment was associated with improvement in vascular reactivity of epineurial arterioles, reduction of oxidative stress in the sciatic nerve, dorsal root ganglion neurons, and epineurial arterioles, and reduction of a marker of advanced glycation end product in the sciatic nerve. It is unlikely that cariporide treatment reduced the expression of Na\(^+/\)H\(^+\) exchanger 1 since expression of Na\(^+/\)H\(^+\) exchanger 1 remained elevated in dorsal root ganglion neurons from diabetic rats treated with cariporide.

In this study, 12 wk of untreated diabetes resulted in hyperalgesia based upon the response to stimulation with flexible von Frey filaments applied to the hindpaw and hyperalgesia Based upon the latent response to a thermal stimulus. These procedures test the response of different nerve fibers in the hindpaw, so it was not surprising that the behavioral response was different depending on whether a mechanical or heat stimulus was applied. The myelinated A\(\delta\) fibers are responsive to a mechanical stimulus, whereas unmyelinated C-fibers are responsive to a thermal stimulus. Our study suggests that, after 12 wk of untreated diabetes, a state of increased sensitivity exists in response to a mechanical stimulus, but decreased sensitivity when a thermal stimulus is applied. This could be due to the signaling mechanisms propagating these behavioral responses being affected differently by long-term diabetes or to the preferential loss of C-fibers in the skin of diabetic rats. What is interesting is that treatment with cariporide improved both outcomes as well as intraepidermal nerve fiber density in the hindpaw.

Previously, we have demonstrated that diabetes-induced vascular dysfunction of epineurial arterioles precedes deficits in nerve conduction velocity, suggesting that vascular impairment is a contributing factor to diabetic neuropathy (12, 44). We have also shown that diabetes-induced impairment of vascular relaxation to acetylcholine, which is endothelium dependent, is in part due to increased oxidative stress and that treating diabetic rats with an antioxidant improves both vascular and neural deficits (14, 15). In this study, treatment of diabetic rats with cariporide improved vascular relaxation to acetylcholine and reduced both superoxide levels and nitrotyrosine staining in epineurial arterioles. Improvement in vascular relaxation of blood vessels that provide circulation to peripheral nerves such as the epineurial arterioles would be expected to reduce ischemia and improve neural function. In aorta and coronary arteries, hyperglycemia was demonstrated to cause impairment in endothelium-dependent vasodilation, and this was prevented by inhibition of Na\(^+/\)H\(^+\) exchanger 1 (57, 59). In mesentery vessels, diabetes-induced hypertrophy was prevented by inhibiting Na\(^+/\)H\(^+\) exchanger 1 (21). In the former studies, the improvement in endothelial function was associated with maintenance of the cytosolic redox potential. Since prevention/reversal of oxidative stress in the endothelium improves vascular relaxation, the increase in oxidative stress in the vasculature may be an indicator of cytosolic redox imbalance.

This study demonstrated that inhibition of Na\(^+/\)H\(^+\) exchanger 1 in diabetic rats reverses oxidative stress and accumulation of advanced glycation end products by the nerve, which has been shown to contribute to diabetic neuropathy. In nerves, calcium overload, a consequence of Na\(^+/\)H\(^+\) exchanger 1 activation, has been shown to cause ischemic like injuries (27). In a model of spinal cord injury, an increase in markers of oxidative stress, nitrotyrosine staining and 4-hydroxynonenal, and calcium overload was shown to activate cysteine protease calpain and the degradation of cytoskeletal proteins (61). In diabetes we have shown that peripheral nerves accumulate the same markers, and with the activation of Na\(^+/\)H\(^+\) exchanger 1 possibly leading to calcium overload, loss of neural function in part could be attributed to axonal
degeneration. Interestingly, in the endothelium the activation of Na\(^+\)/H\(^+\) exchanger 1 by hyperglycemia has been shown to activate calpain, which contributes to hyperglycemia-induced endothelial dysfunction through dissociation of heat shock protein 90 from endothelial nitric oxide synthetase (58). In that study, as in ours, treatment with cariporide attenuated the hyperglycemia-induced impairment of acetylcholine-induced relaxation in streptozotocin diabetic rats.

In summary, it is widely believed that oxidative stress plays an important role in the pathogenesis of peripheral diabetic neuropathy and that several pathways activated by hyperglycemia contribute to the generation of reactive oxygen/nitrogen species. We have shown that Na\(^+\)/H\(^+\) exchanger 1 is overexpressed in peripheral nerve and contributes to the accumulation of markers for oxidative/nitrosative stress and advanced glycation end product. Inhibiting Na\(^+\)/H\(^+\) exchanger 1 in diabetic rats reduced the levels of these markers in nerve and vascular tissue and improved diabetic neuropathy. These studies provide a rationale for further development of Na\(^+\)/H\(^+\) exchanger 1 inhibitors for treatment of diabetic vascular and neural complications.

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**DISCLOSURES**

The authors have no conflicts of interest, financial or otherwise, to declare. The contents of this article are new and solely the responsibility of the authors and do not necessarily represent the official views of the granting agencies.

**AUTHOR CONTRIBUTIONS**

S.L., P.W., H.S., I.V., A.O., I.G.O., and M.A.Y. performed the experiments; S.L., P.W., H.S., I.V., A.O., and M.A.Y. analyzed the data; S.L., A.O., and M.A.Y. prepared the figures; S.L., H.S., A.O., and M.A.Y. edited and revised the manuscript; S.L., P.W., H.S., I.V., A.O., and M.A.Y. approved the final version of the manuscript; I.G.O. and M.A.Y. contributed to the conception and design of the research; I.G.O. and M.A.Y. interpreted the results of the experiments; M.A.Y. drafted the manuscript.

**REFERENCES**


Na+/H+ Exchanger 1 Role in Diabetic Neuropathy


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