Role of nitrosative stress in early neuropathy and vascular dysfunction in streptozotocin-diabetic rats

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Oxidative stress resulting from increased production of reactive oxygen species (ROS) and downregulation or insufficient upregulation of antioxidant defense is a well-recognized fundamental mechanism in diabetic complications. Superoxide is a primary free radical produced in diabetic and hyperglycemic conditions. Superoxide is rapidly converted to several other ROS, i.e., hydroxyl radicals (Fenton and Haber-Weiss reactions), peroxynitrite (reaction with nitric oxide), and hydrogen peroxide (reaction catalyzed by intrinsic superoxide dismutase). The rate of superoxide reaction with nitric oxide exceeds by at least one order of magnitude the rates of other aforementioned reactions, which makes peroxynitrite the number one oxidant in biological systems (38, 57, 71). Peroxynitrite causes the following numerous cytotoxic effects (nitrosative stress): 1) protein nitration and nitrosylation, 2) DNA single-strand breakage and base modification, 3) activation of poly(A)-ribose) polymerase with resultant changes in transcriptional regulation and gene expression, 4) changes in cell signaling, 5) mitochondrial dysfunction, and, in extreme cases, 6) induction of necrosis and apoptosis (22, 38, 57, 71). Conventional antioxidants have a poor capacity to decompose peroxynitrite, which at least partially explains their low efficacy in clinical trials in diabetic complications (12, 30).

Previous findings (26, 51, 63, 69) have demonstrated beneficial effects of a peroxynitrite decomposition catalyst treatment on diabetes-associated reduction of endothelium-dependent relaxation of aortic rings, myocardial contractility, nerve conduction deficits, and sensory disorders in several mouse models of diabetes. However, the role for peroxynitrite in diabetic vascular dysfunction and neuropathy has not been explored in detail, and no studies in rat models of peripheral diabetic neuropathy (PDN) have been reported so far. The rat model-based approach to studying PDN allows dissection of the role of a pathogenetic factor (in this case, peroxynitrite) in neurovascular changes and, in particular, deficits in endoneurial nutritive blood flow and vascular reactivity of epineurial arteries, as well as bradykinin-induced relaxation by coronary and mesenteric arteries, which were alleviated by FP15 treatment. The findings reveal the important role of nitrosative stress in early neuropathy and vasculopathy and provide the rationale for further studies of peroxynitrite decomposition catalysts in long-term diabetic models.

METHODS

Reagents

Unless otherwise stated, all chemicals were of reagent grade quality and were purchased from Sigma Chemical, St. Louis, MO. Fe(III) tetrakis-(N-triethylene glycol monomethyl ether)pyridyl porphyrin...
(FP15) was synthesized as previously described (63). Rabbit polyclonal anti-nitrotyrosine (NT) antibody was purchased from Upstate, Lake Placid, NY, and mouse monoclonal anti-poly(ADP-ribose) from Trevigen, Gaithersburg, MD. Secondary Alexa fluor 488 goat anti-rabbit and Alexa fluor 488 goat anti-mouse antibodies as well as Prolong Gold Antifade Reagent were purchased from Invitrogen, Eugene, OR. Avidin/Biotin blocking kit, Mouse on Mouse Basic kit, Vectastain Elite ABC kit (standard), 3,3′-diaminobenzidine tetrahydrochloride substrate kit, and 3,3′-diaminobenzidine were obtained from Vector Laboratories, Burlingame, CA. Rabbit polyclonal anti-protein gene product 9.5 (ubiquitin COOH-terminal hydrolase) antibody was purchased from Chemicon International, Temecula, CA. Other reagents for immunohistochemistry have been purchased from Dako Laboratories, Santa Barbara, CA.

Animals

The experiments were performed in accordance with regulations specified by the National Institutes of Health “Principles of Laboratory Animal Care, 1985 Revised Version,” and Pennington Biomedical Research Center and University of Iowa protocols for animal studies. They were approved by the Institutional Animal Care and Use Committees at both the Pennington Biomedical Research Center and the University of Iowa. Male Wistar rats (Charles River, Wilmington, MA), 250–300 g body wt, were fed a standard rat chow (PMI Nutrition, Brentwood, MO) and had access to water ad libitum. STZ-diabetes was induced as described (35). Blood samples for glucose measurements were taken from the tail vein at 48 h after the STZ injection and the day before the animals were killed. The rats with blood glucose ≥13.8 mM were considered diabetic. In the first dose-finding study performed at Pennington Biomedical Research Center, the experimental groups comprised control and diabetic rats treated with or without FP15 at 3, 5, or 10 mg·kg⁻¹·day⁻¹ in the drinking water for 4 wk after an initial 2 wk without treatment. The protocol was designed to avoid restoration of normoglycemia or alleviation of hyperglycemia that would occur if a peroxynitrite decomposition administration was started shortly after induction of STZ-diabetes (63). The behavioral tests have been performed in the following order: tactile responses to flexible von Frey filaments (1st day), thermal algesia (2nd day), paw pressure Randall-Selitto test (3rd day), mechanical algesia with rigid von Frey filaments and von Frey anesthesiometer (4th day), and motor (MNCV) and sensory nerve conduction velocities (SNCV; 5th day). Measurements of MNCV and SNCV were performed in rats anesthetized with a mixture of ketamine, acepromazine, and xylazine (80 mg/ml, 1.6 mg/ml, and 5 mg/ml, respectively; Fort Dodge Animal Health, Fort Dodge, IA) administered at 1 ml/kg body wt ip. In the second study performed at University of Iowa, control and STZ-diabetic rats were treated with or without FP15, 5 mg·kg⁻¹·day⁻¹, according to the protocol described above. At the end of experiment, rats were anesthetized with Nembutal (50 mg/kg body wt ip; Abbott Laboratories, North Chicago, IL) and used for assessment of MNCV (index of efficacy of the agent), sciatic endoneurial nutritive blood flow, and mean arterial blood pressure.

Anesthesia, Euthanasia, and Tissue Sampling

The animals were sedated by CO₂ and immediately killed by cervical dislocation. In experiment 1, sciatic nerves, dorsal root ganglia (DRG), and foot pads were fixed in normal buffered 4% formalin for assessment of NT and poly(ADP-ribose) by immunofluorescent histochemistry and intraepidermal nerve fiber density by conventional immunohistochemistry. NT is a footprint of tyrosine nitration induced by peroxynitrite and other ROS (57, 71). Poly(ADP-ribose) is a biopolymer formed from (ADP-ribose) residues that are produced in the poly(ADP-ribose) polymerase (PARP)-catalyzed reaction (33). Poly(ADP-ribose) abundance is considered a measure of PARP activity (1, 33, 36, 72). In experiment 2, epineurial arterioles, coronary, and mesenteric arteries were collected for assessment of responsiveness to vasodilatory stimuli. Part of epineurial arterioles and aorta were used for assessment of superoxide and NT.

Specific Methods

Physiological tests of nerve function and blood flow. Sciatic MNCV, hindlimb digital SNCV, and sciatic endoneurial nutritive blood flow have been measured as described (16, 18). In all measurements, body temperature was monitored by a rectal probe and maintained at 37°C with a warming pad. Nerve temperature during nerve blood flow measurements was maintained at 37°C with mineral oil. Hindlimb skin temperature was also monitored by a thermistor and maintained between 36 and 38°C by radiant heat.

Behavioral tests. Assessment of thermal algesia (by paw withdrawal latencies), mechanical algesia (rigid von Frey filament test and paw pressure Randall-Selitto tests), and tactile allodynia (flexible von Frey filament test) was performed as described (35).

Vascular reactivity. Vasodilatory responsiveness of epineurial arterioles and coronary and mesenteric arteries was assessed in vitro, by videomicroscopy, as described (16, 18). Cumulative concentration-response relationships were evaluated for acetylcholine (10⁻¹⁰ to 10⁻⁴ M), bradykinin (10⁻¹⁰ to 10⁻⁶ M), and sodium nitroprusside (10⁻¹⁰ to 10⁻⁴ M) using vessels from each group of rats. At the end of each dose-response determination, a maximal dose of sodium nitroprusside (10⁻⁴ M) was added. Then papaverine (10⁻⁵ M) was added to determine maximal vasodilation, which was consistently the same as the vascular tone of the resting vessel at 40 mmHg.

Immunohistochemical studies. STUDY 1. All sections (except for those stained for PGP 9.5; see below in Intraepidermal nerve fiber density) were processed by a single investigator and evaluated blindly. Low-power observations of skin sections stained for PGP 9.5 were made with a Zeiss Axioskop microscope. Color images were captured with a Zeiss Axiocam HRc charge-coupled device (CCD) camera at 1,300 × 1,030 resolution. Low-power images were generated with a 40X acroplan objective, using the automatic capturing feature of the Zeiss Axiovision software (version 3.1.2.1). Low-power observations of sciatic nerve and DRG sections stained for NT and poly(ADP-ribose) were made with a Zeiss Axioplan 2 imaging microscope. Color images were captured with a Photometric Cool Snap CCD camera at 1,392 × 1,040 resolution. Low-power images were generated with a 40X acroplan objective, using the RS Image 1.9.2 software.

STUDY 2. All sections were processed by a single investigator and evaluated blindly. Low-power observations of vascular segments of epineurial arterioles stained for superoxide or nitrotyrosine were made using an Olympus IX71 inverted imaging microscope. Images were captured with a Hamamatsu digital camera. Optimal settings for the microscope and exposure were determined and left constant for recording of all the samples.

NT immunoreactivity in sciatic nerve and DRG. NT immunoreactivities in the sciatic nerve and DRG were assessed by immunofluorescent histochemistry as described (35). Fluorescence intensity of color low-power images (see above) was quantified using the ImageJ 1.32 software (National Institutes of Health, Bethesda, MD).

Poly(ADP-ribose) immunoreactivity in sciatic nerve and DRG. Poly(ADP-ribose) immunoreactivity was assessed as described (35). At least 10 fields of each section were examined to select one representative image. Representative images were microphotographed and the number of poly(ADP-ribose)-positive nuclei calculated for each microphotograph.

Intraepidermal nerve fiber density. Intraepidermal nerve fiber density was assessed as described (24). Intraepidermal nerve fiber profiles were counted blindly by three independent investigators, under an Olympus BX-41 microscope, and the average values were used. Microphotographs of stained sections were taken on Axioscop 2 microscope (Zeiss) at ×4 magnification, and the length of epidermis...
was assessed with the ImagePro 3.0 program (Media Cybernetics). 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. Because intraepidermal nerve fiber densities in control and diabetic rats with 6-wk duration of STZ-diabetes turned similar, quantitation of intraepidermal nerve fiber profiles in other experimental groups was not performed.

**Superoxide and peroxynitrite in epineurial arterioles and aorta.**

Hydroethidine (Molecular Probes, Eugene, OR), an oxidative fluorescent dye, was used to quantify superoxide anion radicals in epineurial arterioles and aorta. Superoxide and peroxynitrite in epineurial arterioles and aorta. Hydroethidine (Molecular Probes, Eugene, OR), an oxidative fluorescent dye, was used to quantify superoxide anion radicals in epineurial arterioles and aorta.

**Statistical Analysis**

The results are expressed 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 were made using the Student-Newman-Keuls multiple-range test. Significance was defined at P ≤ 0.05. 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 Fisher’s protected least significant difference test for multiple comparisons.

**RESULTS**

In both studies 1 and 2, initial body weights were similar in control and diabetic rats (Table 1). Final body weights were lower by 30 (study 1) and 42% (study 2) in the diabetic groups (P < 0.01 vs. control in both studies). Final blood glucose concentrations were 4.9- (study 1) and 5.8-fold (study 2) higher in diabetic rats compared with controls. FP15, at either 3, 5, or 10 mg·kg⁻¹·day⁻¹, did not affect weight gain or blood glucose concentrations in control or diabetic rats.

Rats with 6-wk duration of STZ-diabetes had clearly manifested MNCV and SNCV deficits (Table 2). SNCV deficit was completely corrected by 5 and 10 mg·kg⁻¹·day⁻¹ FP15, whereas MNCV deficit was essentially corrected (study 1) or alleviated (study 2). The dose of 3 mg·kg⁻¹·day⁻¹ tended to improve both MNCV and SNCV deficits in diabetic rats, but the corresponding differences with the untreated diabetic group did not achieve statistical significance. None of the doses of FP15 affected MNCV or SNCV in control rats. Sciatic endothelial nutritive blood flow and vascular conductance were

### Table 1. Initial and final BW and BG concentrations in C and D rats treated with or without the peroxynitrite decomposition catalyst FP15

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>C + 3</th>
<th>C + 5</th>
<th>C + 10</th>
<th>D</th>
<th>D + 3</th>
<th>D + 5</th>
<th>D + 10</th>
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<tr>
<td>Initial BW</td>
<td>300 ± 8</td>
<td>297 ± 6</td>
<td>293 ± 8</td>
<td>298 ± 6</td>
<td>294 ± 2</td>
<td>298 ± 4</td>
<td>292 ± 6</td>
<td></td>
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<tr>
<td>Final BW</td>
<td>462 ± 24</td>
<td>459 ± 15</td>
<td>462 ± 14</td>
<td>478 ± 30</td>
<td>322 ± 12*</td>
<td>304 ± 13*</td>
<td>323 ± 12*</td>
<td>326 ± 12*</td>
</tr>
<tr>
<td>Final BG</td>
<td>5.6 ± 0.3</td>
<td>6.0 ± 0.4</td>
<td>5.1 ± 0.2</td>
<td>5.0 ± 0.2</td>
<td>27 ± 1*</td>
<td>23 ± 1*</td>
<td>26 ± 2*</td>
<td>26 ± 1*</td>
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<tr>
<td><strong>Study 2</strong></td>
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<tr>
<td>Initial BW</td>
<td>346 ± 3</td>
<td>351 ± 3</td>
<td>317 ± 3</td>
<td>315 ± 3</td>
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<td></td>
<td></td>
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<tr>
<td>Final BW</td>
<td>438 ± 5</td>
<td>432 ± 5</td>
<td>254 ± 7*</td>
<td>270 ± 13**</td>
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<tr>
<td>Final BG</td>
<td>4.2 ± 0.2</td>
<td>3.9 ± 0.4</td>
<td>24 ± 0.8*</td>
<td>24 ± 0.6**</td>
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</table>

The data are presented as means ± SE. BW, body weight, g; BG, blood glucose concentration, mM; C, control; D, diabetic; FP15, Fe(III) tetrakis-2-(N-triethyleneglycol monooethyl ether)pyridyl porphyrin; C + 3, C + 5, C + 10, D + 3, D + 5, and D + 10, C and D rats treated with 3, 5, or 10 mg·kg⁻¹·day⁻¹ FP15, respectively. *P < 0.01 vs. controls; **P < 0.01 vs. C.

### Table 2. Variables of peripheral diabetic neuropathy in C and D rats treated with or without the peroxynitrite decomposition catalyst FP15

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>C + 3</th>
<th>C + 5</th>
<th>D</th>
<th>D + 3</th>
<th>D + 5</th>
<th>D + 10</th>
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</thead>
<tbody>
<tr>
<td><strong>Study 1</strong></td>
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<tr>
<td>MNCV</td>
<td>51.2 ± 2.0</td>
<td>52.8 ± 1.9</td>
<td>51.4 ± 1.6</td>
<td>51.0 ± 2.6</td>
<td>44.7 ± 1.3*</td>
<td>47.7 ± 1.0*</td>
<td>50.1 ± 2.0*</td>
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<tr>
<td>SNCV</td>
<td>41.0 ± 0.9</td>
<td>40.7 ± 0.5</td>
<td>41.2 ± 0.3</td>
<td>41.4 ± 1.5</td>
<td>38.0 ± 0.6*</td>
<td>39.0 ± 0.9</td>
<td>41.3 ± 0.1*</td>
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<tr>
<td>MWT1</td>
<td>44.4 ± 4</td>
<td>48.2 ± 2</td>
<td>45.3 ± 4</td>
<td>47.4 ± 4</td>
<td>24 ± 1**</td>
<td>28 ± 2**</td>
<td>32 ± 2#</td>
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<tr>
<td>MWT2</td>
<td>132 ± 8</td>
<td>135 ± 2</td>
<td>124 ± 9</td>
<td>126 ± 10</td>
<td>81 ± 3**</td>
<td>96 ± 6*</td>
<td>96 ± 5*</td>
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<tr>
<td>TWT</td>
<td>13.2 ± 1.2</td>
<td>13.7 ± 1.0</td>
<td>13.6 ± 1.0</td>
<td>13.4 ± 1.6</td>
<td>5.8 ± 0.6**</td>
<td>7.2 ± 0.9**</td>
<td>8.2 ± 0.7**</td>
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<tr>
<td><strong>Study 2</strong></td>
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<tr>
<td>MNCV</td>
<td>61.8 ± 2.4</td>
<td>55.5 ± 2.0</td>
<td>44.2 ± 1.6**</td>
<td>55.6 ± 2.4#</td>
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<tr>
<td>NBF</td>
<td>13.4 ± 1.3</td>
<td>12.1 ± 2.8</td>
<td>7.5 ± 1.2**</td>
<td>25.3 ± 2.8###</td>
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<tr>
<td>BP</td>
<td>137 ± 6</td>
<td>130 ± 6</td>
<td>72 ± 5</td>
<td>129 ± 5</td>
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<tr>
<td>EVC</td>
<td>0.129 ± 0.014</td>
<td>0.100 ± 0.019</td>
<td>0.058 ± 0.009**</td>
<td>0.191 ± 0.018###</td>
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<tr>
<td>TRL</td>
<td>9.2 ± 2.4</td>
<td>7.7 ± 0.5</td>
<td>9.4 ± 0.7</td>
<td>10.1 ± 0.3</td>
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</table>

The data are presented as means ± SE. MNCV, motor nerve conduction velocity; SNCV, sensory nerve conduction velocity; MWT1, mechanical withdrawal threshold, g; assessed with rigid von Frey filaments and von Frey anesthesiometer; MWT2, mechanical withdrawal threshold, g, assessed by the Randall-Selitto test; TWT, tactile withdrawal threshold, g; NBF, endothelial nutritive blood flow, ml·min⁻¹·100 g⁻¹; BP, mean systemic blood pressure, mmHg; EVC, endothelial vascular conductance, ml·min⁻¹·100 g⁻¹·mmHg⁻¹; TRL, thermal response latency, s. * and **P < 0.05 and <0.01 vs. C; # and ###P < 0.05 and <0.01 vs. untreated D group.

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reduced in diabetic rats, and this reduction was supranormalized by FP15 treatment. The agent did not affect significantly endoneurial blood flow or vascular conductance in control rats. Mean systemic blood pressure was similar in control and diabetic rats treated with or without FP15.

Mechanical withdrawal thresholds assessed by both Randall-Selitto and von Frey filament tests were reduced in diabetic rats compared with controls, which is consistent with the development of mechanical hyperalgesia (Table 2). This disorder was slightly alleviated by FP15 treatment, although mechanical withdrawal thresholds in diabetic rats treated with any of three doses of FP15 still remained markedly lower than in nondiabetic controls. Tactile withdrawal threshold was reduced in diabetic rats, which is consistent with the development of tactile allodynia. It was alleviated by FP15 treatment; however, tactile response thresholds in diabetic rats treated with any of three doses of FP15 remained markedly lower than in nondiabetic controls. The latencies of hindpaw withdrawal in response to radiant heat (study 2) were not different among control and diabetic rats treated with or without 5 mg·kg⁻¹·day⁻¹ FP15.

Intraepidermal nerve fiber densities were similar in control and diabetic rats with 6-wk duration of diabetes (Fig. 1). Acetylcholine-induced relaxation of epineurial arterioles was reduced in diabetic rats compared with controls (Fig. 2A), and this decrease was attenuated by FP15 treatment. Similar picture, i.e., blunted acetylcholine-induced endothelium-dependent relaxation in the untreated diabetic group and its essential or complete correction by FP15 treatment, was observed in coronary and mesenteric arteries (Fig. 2, B and C). Bradykinin-induced relaxation was reduced in coronary and mesenteric arteries of diabetic rats, and this reduction was alleviated by a peroxynitrite decomposition catalyst treatment (Fig. 3, A and B). FP15 did not affect acetylcholine- or bradykinin-induced relaxation in any of the three studied vascular beds in control rats. Endothelium-independent relaxation assessed with sodium nitroprusside was not different among the experimental groups (Fig. 4, A and B).

NT immunofluorescence was increased by 22% in the sciatic nerve (Fig. 5, A and B) and by 30% in DRGs (Fig. 5, C and D) of diabetic rats compared with the control group, and this increase was dose-dependently reduced by FP15 treatment. Poly(ADP-ribose) fluorescence was increased by 24% in the sciatic nerve (Fig. 6, A and B), and this increase was dose-dependently reduced by FP15 treatment. DRG poly(ADP-ribose) fluorescence was not different among the experimental groups (Fig. 6, C and D).

Superoxide fluorescence was increased by 57% in epineurial arterioles (Fig. 7, A and B) and by 98% in aorta (Fig. 7, C and D) of diabetic rats compared with controls, and this increase was not reduced by a peroxynitrite decomposition catalyst treatment. Aortic superoxide production assessed by the lucigenin chemiluminescence test was increased by 58% in diabetic rats compared with controls (3.39 ± 0.12 vs. 2.15 ± 0.09 RLU in controls, P < 0.01), and this increase was not affected by FP15 treatment (3.54 ± 0.19 RLU). NT fluorescence was increased by 47% in epineurial arterioles (Fig. 8, A and B) and by 76% in aorta (Fig. 8, C and D) of diabetic rats compared with controls, and this increase in both vascular beds was blunted by FP15 treatment.

**DISCUSSION**

Our study in the STZ-diabetic rat model provides the first evidence of the important contribution of peroxynitrite in diabetes-associated decrease in endoneurial nutritive blood flow, endoneurial vascular conductance, and vascular reactivity of epineurial arterioles as well as coronary and mesenteric arteries. According to the “vascular concept” of PDN (7, 41), diabetes-induced decrease in nerve blood flow and resultant endoneurial hypoxia play a key role in functional and morphological changes in the diabetic nerve. Decrease in nerve blood flow has been demonstrated in both type 1 and type 2 diabetic rodents (7, 8, 16, 18, 44, 53, 54) as well as human subjects with diabetes mellitus (7, 65), although patients with painful diabetic neuropathy displayed a paradoxical increase in sural
nerve epineurial blood flow (29). Despite the fact that the importance of nerve blood flow in PDN in diabetic animal models has been an area of controversy, several lines of evidence, and in particular, beneficial effects of inhibitors of protein kinase C (6, 45), the enzyme activated in diabetic nerve vasculature but not in neural elements of peripheral nervous system (6, 45, 68), as well as endothelial progenitor cell therapy (47), support the key role of neurovascular dysfunction in motor and sensory nerve conduction deficits. Interestingly and surprisingly, nerve blood flow and vascular conductance deficits in diabetic rats were “supranormalized” by FP15 treatment. Similar or more modest supranormalization of endoneurial nutritive blood flow has been caused by treatments with hydroxyethyl starch deferexamine (19), a superoxide dismutase mimetic (17), or a vasopeptidase inhibitor (21) in our earlier studies in STZ-diabetic rats.

Our previous findings (26, 51, 69) revealed a key role for peroxynitrite in neuropathic changes in several mouse models of diabetes. The importance of nitrosative stress in PDN is further supported by the current study in STZ-diabetic rats that demonstrated a dose-dependent correction of MNCV and SNCV deficits with a peroxynitrite decomposition catalyst treatment. A comparison of other findings in rats and mice with similar (6-wk) durations of STZ-diabetes (Ref. 10 and the present study) revealed a number of similarities as well as some striking differences between the two animal models of early PDN. Both STZ-diabetic rats and mice display motor and sensory nerve conduction velocity deficits and tactile allodynia, a condition where a light touch is perceived as painful and is present in a considerable proportion [~30–47% (2, 70)] of human subjects with diabetes mellitus. Mice, but not rats, with 6-wk duration of STZ-diabetes have clearly manifested thermal hypoalgesia (26), i.e., a condition typically present in human subjects with advanced diabetic neuropathy (28). Note that thermal hypoalgesia has also been reported in rats with longer (12-wk) duration of diabetes (5), whereas rats with short-term (4- and even 8-wk) diabetes, like some human subjects with early PDN (28), have been found to be hypersensitive to thermal noxious stimuli (8, 35, 61). Rats with 4- to 8-wk duration of STZ-diabetes display mechanical hyperalgesia (Refs. 8, 35, and 61 and the present study), i.e., a condition (pain on pressure) recently reported in 71% of human subjects with early PDN (56). In contrast, mice with similar duration of STZ-diabetes have increased mechanical withdrawal threshold (26), which is indicative of sensory loss, a factor primarily responsible for foot ulceration and amputation in human subjects with diabetes mellitus (4, 23). Furthermore, mice, but not rats, with 6- to 8-wk duration of STZ-diabetes (Refs. 14, 15, and 26 and the present study) exhibited a reduction of intraepidermal nerve fiber density, a phenomenon present in human diabetic subjects (59, 62). STZ-diabetic rats display epidermal nerve fiber loss at a later stage (12-wk) of disease (50, 67). These important differences between rats and mice with early diabetes should be considered in selecting animal models for future neuropathy studies. The STZ-diabetic rat model provides a characterization of the earliest stage of disease, whereas the mouse model is more suitable for studying advanced PDN with its typical sensory loss and nerve fiber degeneration. A faster development of PDN in type 1 diabetic mouse model is likely attributable to more severe nitrosative stress. Quantitation of nitrotyrosine (a foot-print of peroxynitrite-induced injury) fluorescence in peripheral nerve and DRG neurons revealed the 22 and 30% differences between control and STZ-diabetic rats (present study) and much greater differences, i.e., the 49 and 51% differences between control and STZ-diabetic mice (Drel VR and Obrosova IG, unpublished observations). Taking into consideration that a peroxynitrite decomposition catalyst treatment counteracts nerve conduction deficits and all manifestations of sensory neuropathy in both STZ-diabetic rats and mice, a severity of...
peroxynitrite injury manifested by nitrotyrosine accumulation could be an important factor determining development and progression of PDN. Note that a recent clinical study (74) revealed an association between peroxynitrite retaining in circulation and presence of PDN and cardiac autonomic neuropathy in human subjects with diabetes mellitus.

Our findings are in line with several recent studies (26, 51, 57, 63, 69, 73), suggesting that a key role of reactive nitrogen species in the pathogenesis of diabetes and other diabetic complications is emerging. Accumulation of nitrotyrosine has been documented in vascular endothelium (58, 63), heart (57, 58), retina (27, 52), and kidney (25) of STZ-diabetic rodents as well as circulation (11, 10, 34), cutaneous vasculature (64), myocardium (32), and kidney (66) of human subjects with diabetes mellitus. In peripheral nervous system, increased nitrotyrosine immunoreactivities have been identified in the sciatic nerve, gray matter of spinal cord, DRG, and vasa nervorum of STZ-diabetic rats and mice, Zucker fatty rats, and Zucker diabetic fatty rats as well as leptin-deficient (ob/ob) and high-fat diet-fed mice (13, 16, 24, 26, 48, 51, 52, 54, 60, 69). These findings suggest the presence of peroxynitrite injury in peripheral nervous system at both early and advanced stages of type 1 and type 2 diabetes and, furthermore, at the prediabetic stage. Evidence of the important role of nitrosative stress in diabetic neuropathy in humans is emerging. Increased plasma nitrotyrosine levels have been found to correlate with redistribution of sudomotor responses, an early sign of sympathetic nerve dysfunction, as well as with endothelial dysfunction, an

Fig. 5. Representative microphotographs of immunofluorescent staining of nitrotyrosine in sciatic nerves (A) and dorsal root ganglia (DRG; C) of C and D rats with and without the peroxynitrite decomposition catalyst FP15 treatment, magnification ×40. Nitrotyrosine fluorescence in sciatic nerves (B) and DRG (D) of C and D rats with and without the peroxynitrite decomposition catalyst FP15 treatment. Data are means ± SE; n = 6–8/group. D + 3, D + 5, and D + 10, D rats treated with 3, 5, and 10 mg·kg⁻¹·day⁻¹, respectively, of the peroxynitrite decomposition catalyst FP15. **P < 0.01 vs. C; ###P < 0.01 vs. untreated D group.
important player in PDN (9, 37), in type 1 diabetic subjects (11, 10, 34).

Perxynitrite damage can lead to PDN via multiple mechanisms including, but not limited, by PARP activation, an important player in both motor and sensory neuropathy (35, 39, 49). Of particular interest is the absence of diabetes-induced PARP activation in DRG, consistent with our previous observations in rats with 4-wk duration of STZ-diabetes (Obrosova IG, unpublished observations). Note that structurally diverse PARP inhibitors alleviated motor and sensory nerve conduction velocity deficits, neurovascular dysfunction, and peripheral nerve energy failure as well as manifestations of sensory neuropathy in the rats with 4-wk duration of diabetes (35, 39, 49). Therefore, PARP activation in the peripheral nerve, but not in DRG, plays an important role in the pathogenesis of early PDN. In the present study, both diabetes-induced PARP activation in the peripheral nerve and manifestations of PDN were dose-dependently corrected by a peroxynitrite decomposition catalyst treatment.

In addition to a key role in PDN, our findings dissect an important contribution of peroxynitrite to diabetes-induced impairment of endothelium-dependent vascular relaxation in vasa nervorum as well as coronary and mesentery arteries. Nitric oxide and endothelium-derived hyperpolarizing factor are the two major factors controlling acetylcholine- and bradykinin-induced relaxation in these vascular beds (20, 55). Diabetes-induced superoxide formation in vascular endothelium leads to generation of peroxynitrite (16, 18), thus creating

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**Fig. 6.** Representative microphotographs of immunofluorescent staining of poly(ADP-ribose) in sciatic nerves (A) and DRG (C) of C and D rats with and without the peroxynitrite decomposition catalyst FP15 treatment, magnification ×40. Poly(ADP-ribose) fluorescence in sciatic nerves (B) and DRG (D) of C and D rats with and without the peroxynitrite decomposition catalyst FP15 treatment. Data are means ± SE; n = 6–8/group. **P < 0.01 vs. C; ##P < 0.01 vs. untreated D group.
nitric oxide deficiency and reducing the nitric oxide-dependent component of vascular responses to acetylcholine and bradykinin. The observed beneficial effects of FP15 treatment on both nitrotyrosine immunofluorescence and acetylcholine-induced relaxation of epineurial arterioles are indicative of an important role of peroxynitrite in the mechanisms underlying impaired reactivity of vasa nervorum in experimental PDN. Interestingly, an improvement of epineurial arteriole reactivity occurred despite the absence of any effect of FP15 on diabetes-induced superoxide production. Apparently, peroxynitrite, but not superoxide itself, causes impairment of endothelium-dependent reactivity of vasa nervorum in STZ-diabetic rats. The latter is probably true for other vascular beds considering that diabetes-induced impairments of endothelium-dependent vascular reac-

Fig. 7. Representative microphotographs of superoxide immunofluorescent staining in epineurial arterioles (A) and aorta (C) of C and D rats with and without the peroxynitrite decomposition catalyst FP15 treatment, magnification ×40. Superoxide fluorescence in epineurial arterioles (B) and aorta (D) of C and D rats with and without the peroxynitrite decomposition catalyst FP15 treatment. Data are means ± SE; n = 6–8/group. *P < 0.05 vs. C. RFU, relative fluorescence units.

Fig. 8. Representative microphotographs of nitrotyrosine immunofluorescent staining in epineurial arterioles (A) and aorta (C) of C and D rats with and without the peroxynitrite decomposition catalyst FP15 treatment, magnification ×40. Nitrotyrosine fluorescence in epineurial arterioles (B) and aorta (D) of control and diabetic rats with and without the peroxynitrite decomposition catalyst FP15 treatment. Data are means ± SE; n = 6–8/group. *P < 0.05 vs. C; #P < 0.01 vs. untreated D group.
tivity of coronary and mesentery arteries (the present study) and corpus cavernosum (46) were corrected by FP15 and another peroxynitrite decomposition catalyst, 5,10,15,20-tetraakis(N-methyl-4'-pyridyl) porphyrinato iron III, respectively. Peroxynitrite has also been reported to reduce endothelium-dependent relaxation of pulmonary arteries (3). Note that, in addition to reducing nitric oxide availability, peroxynitrite formation has been reported to reduce the endothelium-derived hyperpolarizing factor-mediated component of vascular relaxation (40) and leads to production of the potent vasoconstrictors such as endothelin-1 and cyclooxygenase-2-derived prostanoids (31, 43). The vascular relaxation in response to sodium nitroprusside was not affected by diabetes or FP15 treatment. The latter is consistent with previous findings from our group (20, 55) suggesting that, in contrast to other pathological conditions, i.e., hypertension (42), short-term diabetes does not affect the function of the smooth muscle layer and endothelium-independent relaxation of large arteries.

In conclusion, peroxynitrite plays an important role in neovascular dysfunction, MNCV and SNCV deficits, sensory neuropathy, and impaired vascular reactivity of epineurial arterioles as well as coronary and mesentery arteries in STZ-diabetic rats. A peroxynitrite decomposition catalyst corrected nerve blood flow and conduction slowing and alleviated small sensory nerve dysfunction and impairment of endothelium-dependent relaxation. The effective dose of a peroxynitrite decomposition catalyst (5 mg kg⁻¹ day⁻¹) was ~100- to 500-fold lower than the effective doses of such well-studied antioxidants as vitamin E, butylated hydroxytoluene, N-acetyl-l-cysteine, and α-lipoic acid. The results provide the rationale for development of peroxynitrite decomposition catalysts as a novel class of therapeutics for prevention and treatment of diabetic neuropathy and vascular dysfunction.

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