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ARTICLE *in* DIABETES/METABOLISM RESEARCH AND REVIEWS · NOVEMBER 2014

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Diabetes Metab Res Rev 2014; **30**: 669–678.
Published online in Wiley Online Library (wileyonlinelibrary.com) **DOI:** 10.1002/dmrr.2549

RESEARCH ARTICLE

Peroxynitrite and protein nitration in the pathogenesis of diabetic peripheral neuropathy

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Received: 17 December 2013
Revised: 4 March 2014
Accepted: 25 March 2014

Abstract

Background Peroxynitrite, a product of the reaction of superoxide with nitric oxide, causes oxidative stress with concomitant inactivation of enzymes, poly(ADP-ribosylation), mitochondrial dysfunction and impaired stress signalling, as well as protein nitration. In this study, we sought to determine the effect of preventing protein nitration or increasing peroxynitrite decomposition on diabetic neuropathy in mice after an extended period of untreated diabetes.

Methods C57Bl6/J male control and diabetic mice were treated with the peroxynitrite decomposition catalyst Fe(III) tetramesitylporphyrin octasulfonate (FeTMPS, 10 mg/kg/day) or protein nitration inhibitor (−)-epicatechin gallate (20 mg/kg/day) for 4 weeks, after an initial 28 weeks of hyperglycaemia.

Results Untreated diabetic mice developed motor and sensory nerve conduction velocity deficits, thermal and mechanical hypoalgesia, tactile allodynia and loss of intraepidermal nerve fibres. Both FeTMPS and epicatechin gallate partially corrected sensory nerve conduction slowing and small sensory nerve fibre dysfunction without alleviation of hyperglycaemia. Correction of motor nerve conduction deficit and increase in intraepidermal nerve fibre density were found with FeTMPS treatment only.

Conclusions Peroxynitrite injury and protein nitration are implicated in the development of diabetic peripheral neuropathy. The findings indicate that both structural and functional changes of chronic diabetic peripheral neuropathy can be reversed and provide rationale for the development of a new generation of antioxidants and peroxynitrite decomposition catalysts for treatment of diabetic peripheral neuropathy. Published in 2014. This article is a U.S. Government work and is in the public domain in the USA.

Keywords diabetic neuropathy; oxidative stress; nitrosative stress; antioxidants; peroxynitrite; superoxide

Introduction

Diabetic peripheral neuropathy affects a majority of patients with diabetes. Improved blood glucose control reduces the risk of diabetic neuropathy, thus implicating hyperglycaemia as a leading causative factor [1]. A number of mechanisms have been proposed linking chronic hyperglycaemia to nerve conduction slowing and other manifestations of diabetic neuropathy. The

'vascular' concept implies that endothelial dysfunction with resulting decrease in nerve blood flow and endoneurial hypoxia plays a key role in diabetic neuropathy [2–9]. The 'neurochemical' concept suggests the importance of changes in the neural elements of the peripheral nervous system, that is, the following: (1) metabolic abnormalities such as sorbitol accumulation [10,11], *myo*-inositol depletion [12,13], downregulation of Na^+/K^+ ATPase activity [13,14], (2) impaired neurotrophic support [15–17], (3) signal transduction changes [18,19], and (4) dorsal root ganglion and Schwann cell dysfunction and premature apoptosis [20]. The extent that vascular *versus* nonvascular mechanisms contribute to diabetic neuropathy remains a subject of debate.

Evidence for important role for oxidative stress in both experimental [3,9,21,22] and clinical [23,24] diabetic neuropathy is emerging. Diabetes-induced oxidative stress is a result of increased formation of reactive oxygen species and downregulation or, in some tissues [25], insufficient upregulation, of antioxidative defence provided by nonenzymatic antioxidants and antioxidative defence enzymes. Peroxynitrite, a product of superoxide anion radical reaction with nitric oxide, is a major oxidant in pathological conditions associated with oxidative stress including diabetes [26–28]. Peroxynitrite damage is stable and, in some tissues (e.g. retina) is poorly amenable to a correction with improvement of blood glucose control [29]. Peroxynitrite injury has been implicated in the 'metabolic memory' phenomenon in both experimental and clinical studies [29,30]. Peroxynitrite has multiple cytotoxic effects that include the following: (1) protein nitration and nitrosylation, (2) DNA single-strand breakage and base modification, (3) activation of poly(ADP-ribose) polymerase with resultant changes in transcriptional regulation and gene expression, (4) changes in cell signalling, (5) mitochondrial dysfunction, and (6) necrosis and apoptosis [26–28]. Accumulation of nitrotyrosine, a product of peroxynitrite-induced protein nitration, has been documented in peripheral nerve [31], *vasa nervorum* [7–9] and dorsal root ganglion [32] in streptozotocin diabetic rats, and peripheral nerve, spinal cord and dorsal root ganglion of streptozotocin diabetic and *ob/ob* mice [31,33–35] indicating that diabetes creates not just oxidative but oxidative/nitrosative stress in the peripheral nervous system. In this study, we sought to determine the effect of preventing protein nitration or increasing peroxynitrite decomposition on diabetic neuropathy in mice after an extended period of untreated diabetes.

Materials and methods

Reagents

Unless otherwise stated, all chemicals were of reagent grade quality and were purchased from Sigma Chemical Co., St. Louis, MO. Fe(III) tetramesitylporphyrin octasulfonate (FeTMPS) was prepared as previously described [36,37].

Animals

The experiments were performed in accord with regulations specified by the National Institutes of Health 'Principles of Laboratory Animal Care' and Pennington Biomedical Research Center Protocol for Animal Studies. Mature male C57Bl6/J mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). They were fed with standard mouse chow (PMI Nutrition International, Brentwood, MO, USA) and had access to water *ad libitum*. Diabetes was induced by a single injection of streptozotocin (100 mg/kg) to nonfasted animals. Blood samples for glucose measurements were taken from the tail vein 3 days after streptozotocin injection. The mice with blood glucose $\geq$13.8 m*M* were considered diabetic. The injected mice that had blood glucose concentration in nondiabetic range were given low-dose streptozotocin injections (40 mg/kg) until they developed hyperglycaemia (one to three additional injections). Experimental groups comprised of control and diabetic mice were treated with or without the peroxynitrite decomposition catalyst FeTMPS (10 mg/kg/day by oral gavage) or the protein nitration inhibitor (−)-epicatechin gallate (20 mg/kg/day i.p.). The treatments were initiated after 28 weeks of untreated diabetes and lasted 4 weeks. These doses were selected following preliminary studies using a prevention protocol and duration of diabetes of 7 weeks (data not shown). In these studies, the animals tolerated the treatments well with no apparent side effects.

Glycated haemoglobin

HbA_{1c} measurements were performed by ion-exchange HPLC in 10-μl nonfasting blood samples. The analysis was carried out by DTI Laboratories, Thomasville, GA, USA, and the blood was collected using the reagent vials and instructions provided by the company.

Behavioural 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 cut-off time of 30 s using the IITC model 336 TG combination tail-flick and paw algesia metre (IITC Life Sciences, Woodland Hills, CA) [31]. The data were reported in seconds. Tactile responses were evaluated by quantifying the withdrawal threshold of the hindpaw in response to stimulation with flexible von Frey filaments as previously described [34]. The data were reported in grammes. Tail pressure thresholds were recorded using the Paw/

DOI: 10.1002/dmrr

Tail Pressure Analgesia meter for Randall–Selitto test (Analgesy-Meter, UGO-Basile, Comerio VA, Italy) [34]. Pressure increasing at a linear rate of 10 g/s with the cut-off of 250 g to avoid tissue damage was applied to the base of the tail. The applied tail pressure that evoked biting or licking behaviour was registered by the analgesia meter and expressed in grammes. Each of these tests was repeated at least three times with a rest period of 15 min between tests. These tests were completed before the terminal procedures and on different days.

Physiological tests

On the day of terminal studies, mice were weighed and anesthetized with mixture of ketamin and xylazine (45 mg/kg and 15 mg/kg body weight, respectively, i.p.). Nonfasting blood glucose was determined. Sciatic motor nerve conduction velocity and digital sensory nerve conduction velocity were measured as previously described [38]. The motor and sensory nerve conduction velocities were reported in metres per second.

Intraepidermal nerve fibre density

Footpads were fixed in ice-cold Zamboni's fixative for 3 h, washed in 100 m*M* phosphate buffered saline (PBS) overnight and then in PBS containing increasing amounts of sucrose, that is, 10%, 15% and 20%, 3 h in each solution. After washing, the samples were snap frozen in O.C.T. (Sakura Finetek USA, Torrance, CA) 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 antiserum (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 fibre 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 reported. The length of epidermis was assessed on the microphotographs of stained sections taken a ×50 magnification with a 3I Everest imaging system (Intelligent Imaging Innovations, Inc., Denver, CO) operated with an Axioplan 2 microscope, using the NIH Image J software (National Institutes of Health, Bethesda, MD). Representative images of intraepidermal nerve fibres were obtained by confocal laser scanning microscopy at ×400 magnification, using Leica TCS SP5 confocal system (Leica Microsystems, Nussloch, Germany) [38].

Western blot analysis of nitrated protein and 4-hydroxynonenal adducts in rat sciatic nerve and spinal cord

To assess nitrated protein expressions by western blot analysis, sciatic nerve and spinal cord segments (~20 mg) were placed on ice in 200 μl of radioimmunoprecipitation assay buffer containing 50 m*M* Tris–HCl, pH 7.2; 150 m*M* NaCl; 0.1% sodium dodecyl sulfate; 1% NP-40; 5 m*M* EDTA; 1 m*M* EGTA; 1% sodium deoxycholate and the protease/phosphatase inhibitors leupeptin (10 μg/mL), pepstatin (1 μg/mL), aprotinin (20 μg/mL), benzamidine (10 m*M*), phenylmethylsulfonyl fluoride (1 m*M*), sodium orthovanadate (1 m*M*) and homogenized on ice. The homogenates were sonicated and centrifuged at 14 000 g for 20 min. All the aforementioned steps were performed at 4 °C. The lysates (40 μg protein) were mixed with equal volumes of ×2 sample-loading buffer containing 62.5 m*M* Tris–HCl, pH 6.8; 2% sodium dodecyl sulfate; 5% β-mercaptoethanol; 10% glycerol and 0.025% bromophenol blue and fractionated in 5–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis in an electrophoresis cell (Mini-Protean III; Bio-Rad Laboratories, Richmond, CA). Electrophoresis was conducted at 15 mA constant current for stacking and at 35 mA for protein separation. Gel contents were electrotransferred (80 V, 2 h) to nitrocellulose membranes using Mini Trans-Blot cell (Bio-Rad Laboratories, Richmond, CA) and western transfer buffer (25 m*M* Tris–HCl, pH 8.3; 192 m*M* glycine; and 20% (v/v) methanol). Free binding sites were blocked in 5% (w/v) bovine serum albumin diluted in 20 m*M* Tris–HCl buffer, pH 7.5, containing 150 m*M* NaCl and 0.1% Tween 20, for 1 h. After blocking free binding sites, primary antibodies to nitrated proteins (EMD Millipore Corp., Billerica, MA, USA) or 4-hydroxynonenal adducts (EMD Millipore Corp., Billerica, MA, USA) were applied overnight, at 4 °C. Then, the antimouse or antirabbit secondary antibody was applied at room temperature for 1 h. Protein bands detected by the antibodies were visualized with Amersham ECL western blotting detection reagent (Little Chalfont, Buckinghamshire, UK). After incubation with secondary, the membranes were stripped and reprobed with β-actin antibody to confirm equal protein loading. Stripping was conducted in 25 m*M* glycine-HCl, pH 2.5 buffer containing 2% sodium dodecyl sulfate. The data were quantified by densitometry (Quantity One 4.5.0 software, Bio-Rad Laboratories, Richmond, CA,USA).

Fluorescent immunohistochemistry in dorsal root ganglia

Dorsal root ganglia (DRG) were dissected and fixed in normal buffered 4% formaldehyde for 24 h at 4 °C, dehydrated and embedded in paraffin [39]. Sections were cut at 5 μm thickness, dewaxed in xylene, hydrated in decreasing concentrations of ethanol, washed in distilled

DOI: 10.1002/dmrr

water and subjected to heat induced epitope retrieval in 10 m*M* citrate buffer (pH 6.0) with 0.05% Tween 20. To create positive control for antinitrotyrosine antibody, several deparaffinized sections from random mice were incubated with 1 m*M* peroxynitrite in 100 m*M* sodium acetate buffer, pH 5.0 for 30 min preceding the antigen retrieval step. Then, the sections were subsequently incubated with Image-iT FX signal enhancer for 30 min and blocking solution containing 2% bovine serum albumin, 5% normal goat serum and 0.3% Triton X100 in 50 m*M* Tris-buffered saline (pH 8.4) for 1 h with thorough washes between the steps. The incubation with antinitrotyrosine (1 : 200) (EMD Millipore Corporation, Billerica, MA, USA) and anti-4-hydroxynonenal adduct (1 : 500) (EMD Millipore Corporation, Billerica, MA, USA) primary antibodies were performed overnight at 4 °C. The secondary Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen, Eugene, OR, USA) conjugated goat antirabbit antibodies were applied for 1 h at room temperature at a working dilution 1 : 400. Sections were mounted in VectaShield (Vector Laboratories, Burlingame, CA, USA) mounting medium. All sections were processed by a single investigator and evaluated blindly. Colour images were captured at ×400 magnification with a 3I Everest imaging system (Intelligent Imaging Innovations, Inc., Denver, CO, USA) equipped with Axioplan 2 microscope (Zeiss). Nitrotyrosine fluorescence intensity of individual DRG neurons was quantified using the ImageJ 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 per mouse) were counted, and the average values for each animal were used to calculate group means. Fluorescence intensity was expressed as mean ± standard error of the mean for each experimental group.

Statistical analysis

The results are presented as mean ± standard error of the mean. 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 (datasets for body weights and plasma glucose), the data were analysed by the nonparametric Kruskal–Wallis one-way analysis of variance, followed by the Bonferroni/Dunn test for multiple comparisons. Individual pair-wise comparisons in Table 1 and Figure 1 were made using the unpaired two-tailed Student's *t*-test. Significance was defined at $p < 0.05$.

Results

Table 1 provides baseline data for a representative group of control and streptozotocin diabetic mice entered into this study. At baseline (prior to induction of streptozotocin diabetes), the endpoints examined were indistinguishable from those observed in control mice after additional 28 or 32 weeks (Tables 1 and 3). All endpoints examined motor and sensory nerve conduction velocities, thermal response latency and mechanical and tactile response thresholds were alike for the mice at baseline and 28 and 32 weeks later. Table 1 and Figure 1 also provide data on the effect of untreated diabetes of 28 weeks duration on neuropathic endpoints used in this study. Diabetic mice displayed significant motor and sensory nerve conduction velocity deficits and impaired thermal response, mechanical response threshold and tactile response threshold as well as reduction in intraepidermal nerve fibre density (Figure 1A and B).

Data in Table 2 demonstrate that weight of control and streptozotocin diabetic mice with or without treatment was not significantly different. Diabetic mice were significantly hyperglycaemic as indicated by elevated nonfasting blood glucose level at the time of the study and increased haemoglobin A_{1c}. Treatment of 4 weeks with FeTMPS or epicatechin gallate did not alter the hyperglycaemic status of the mice.

Table 1. Motor and sensory nerve conduction velocity, thermal and mechanical algesia and tactile sensitivity in control mice at baseline and after 28 weeks of untreated diabetes

Determination	Baseline (20)	28 weeks control (30)	28 weeks diabetes (40)
MNCV (m/s)	52.3 ± 0.8	51.7 ± 1.1	45.1 ± 1.0*
SNCV (m/s)	40.6 ± 0.4	41.4 ± 0.7	33.2 ± 2.0*
Thermal response latency (s)	10.8 ± 0.7	10.9 ± 0.8	14.6 ± 0.5*
Mechanical withdrawal thresholds (g)	115 ± 4	110 ± 4	139 ± 5*
Tactile response thresholds (g)	1.85 ± 0.17	1.72 ± 0.13	0.85 ± 0.09*

A subpopulation of control mice at baseline, before injection with streptozotocin, was examined. After 28 weeks, control mice and untreated diabetic mice were reexamined in order to determine the effect of diabetes on endpoints prior to beginning treatments. Data are presented as the mean ± standard error of the mean.
The number of mice in each group is indicated in parentheses.
MNCV, motor nerve conduction velocity; SNCV, sensory nerve conduction velocity.
*$p < 0.01$ compared with 28 week control mice.

DOI: 10.1002/dmrr

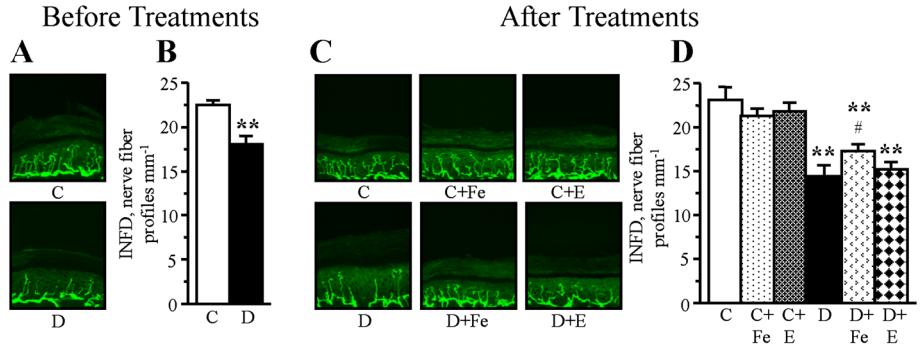

Figure 1. Representative images of intraepidermal nerve fibre profiles, magnification ×400 (A, C), and intraepidermal nerve fibre densities (B, D), in experimental groups at 28 weeks (before treatments with FeTMPS or epicatechin gallate) (A and B) and after 4 weeks of treatment (C and D). Mean ± standard error of the mean, the number of mice for each study group for (C) and (D) are the same as described in Table 2. Along the *x*-axis, C represents control mice; D, diabetic mice; E, epicatechin gallate treated mice; and Fe, FeTMPS treated mice. $^{**}p < 0.01$ *versus* nondiabetic mice; $^{\#}p < 0.05$ *versus* untreated diabetic mice

Table 2. Body weights, blood glucose levels and haemoglobin A_{1c} in control and streptozotocin-induced diabetic mice treated with or without FeTMPS or epicatechin gallate

Determination	Control (10)	Control + FeTMPS (10)	Control + epicatechin (10)	Diabetic (10)	Diabetic + FeTMPS (16)	Diabetic + epicatechin (14)
Body weight (g)	31.0 ± 0.7	30.8 ± 1.1	29.3 ± 0.8	30.1 ± 0.8	29.9 ± 0.5	28.1 ± 0.6
Blood glucose (m*M*)	7.9 ± 0.4	6.9 ± 0.2	6.9 ± 0.3	19.2 ± 3.6*	22.7 ± 2.7*	21.8 ± 2.8*
Hb A_{1c} (%)	3.30 ± 0.04	3.29 ± 0.04	3.25 ± 0.04	4.98 ± 0.28*	4.64 ± 0.26*	5.01 ± 0.20*

Control and diabetic mice treated with or without FeTMPS or epicatechin gallate for 4 weeks after 28 weeks of untreated diabetes. At baseline, the mice weighed 24.0 ± 0.2 g. Data are presented as the mean ± standard error of the mean.
The number of mice in each group is indicated in parentheses.
*$p < 0.01$ compared with control mice.

Both FeTMPS and epicatechin gallate treatment of diabetic mice for 4 weeks after 28 weeks of untreated diabetes significantly improved sensory nerve conduction velocity deficit, thermal hypoalgesia (treatment with epicatechin gallate was significantly better than treatment with FeTMPS for this endpoint), mechanical withdrawal threshold and tactile response threshold, but only FeTMPS treatment significantly improved motor nerve conduction velocity and improved intraepidermal nerve fibre density compared with untreated diabetic mice (Table 3 and Figure 1C and D). However, for most endpoints, treatment with FeTMPS or epicatechin gallate did not fully restore diabetic neuropathy endpoints to control values.

Our findings implicate both peroxynitrite injury *in toto* and its component, protein nitration, in the development of advanced diabetic peripheral neuropathy; they also suggest that protein nitration does not account for all detrimental effects of peroxynitrite, and that peroxynitrite, the most potent oxidant in biological systems, contributes to the development of diabetes-associated neuropathic changes through both protein nitration and oxidative stress. To examine these, further studies were performed of the effect of treatment with FeTMPS or epicatechin gallate on the level of nitrated proteins in dorsal root ganglion neurons, sciatic nerve and spinal cord. Treating diabetic mice with FeTMPS or epicatechin gallate for 4 weeks after 28 weeks of untreated diabetes significantly reduced levels of nitrated proteins in dorsal root ganglion neurons, sciatic nerve and spinal cord (Figure 2A–C, respectively). In all cases, nitrated proteins levels were returned to near control values. In contrast, only treatment with FeTMPS was effective in reducing 4-hydroxynonenal adducts, a marker of oxidative stress, in dorsal root ganglion neurons, sciatic nerve and spinal cord (Figure 3A–C, respectively).

Discussion

The emerging role of peroxynitrite in the pathogenesis of diabetic neuropathy suggests that interventions aimed at preventing peroxynitrite formation could be an effective target for the treatment of diabetic neuropathy. Accumulation of nitrotyrosine, a biological marker of peroxynitrite formation, has been documented in the vascular endothelium, heart, retina and kidney of streptozotocin diabetic

DOI: 10.1002/dmrr

Table 3. Motor and sensory nerve conduction velocity, thermal and mechanical algesia and tactile sensitivity in control and streptozotocin-induced diabetic mice treated with or without FeTMPS or epicatechin gallate

Determination	Control (10)	Control + FeTMPS (10)	Control + epicatechin (10)	Diabetic (10)	Diabetic + FeTMPS (16)	Diabetic + epicatechin (14)
MNCV (m/s)	52.2 ± 0.6	50.6 ± 1.1	48.8 ± 0.6*	42.6 ± 1.3‡	48.0 ± 1.2$^{*,\#}$	44.4 ± 1.7*
SNCV (m/s)	40.3 ± 0.6	39.3 ± 0.3	39.8 ± 0.5	32.8 ± 3.8‡	36.2 ± 0.7$^{‡,\#}$	36.3 ± 0.8$^{‡,\#}$
Thermal response latency (s)	11.2 ± 0.4	10.7 ± 0.2	11.3 ± 0.1	14.8 ± 0.2‡	13.2 ± 0.4$^{‡,\#}$	12.3 ± 0.4$^{*,\#,\neq}$
Mechanical withdrawal thresholds (g)	109 ± 5	111 ± 2	117 ± 3*	141 ± 2‡	122 ± 1$^{‡,\#}$	119 ± 2$^{*,\#}$
Tactile response thresholds (g)	1.67 ± 0.05	1.69 ± 0.05	1.72 ± 0.05	0.91 ± 0.03‡	1.17 ± 0.07$^{‡,\#}$	1.19 ± 0.07$^{‡,\#}$

Control and diabetic mice treated with or without FeTMPS or epicatechin gallate for 4 weeks after 28 weeks of untreated diabetes. Data are presented as the mean ± standard error of the mean.
MNCV, motor nerve conduction velocity; SNCV, sensory nerve conduction velocity.
*, $^{‡}p < 0.05$ and $p < 0.01$ compared with control mice, respectively.
$^{\#}p < 0.01$ compared with untreated diabetic mice.
$^{\neq}p < 0.05$ compared with diabetic mice treated with FeTMPS. The number of mice in each group is indicated in parentheses.

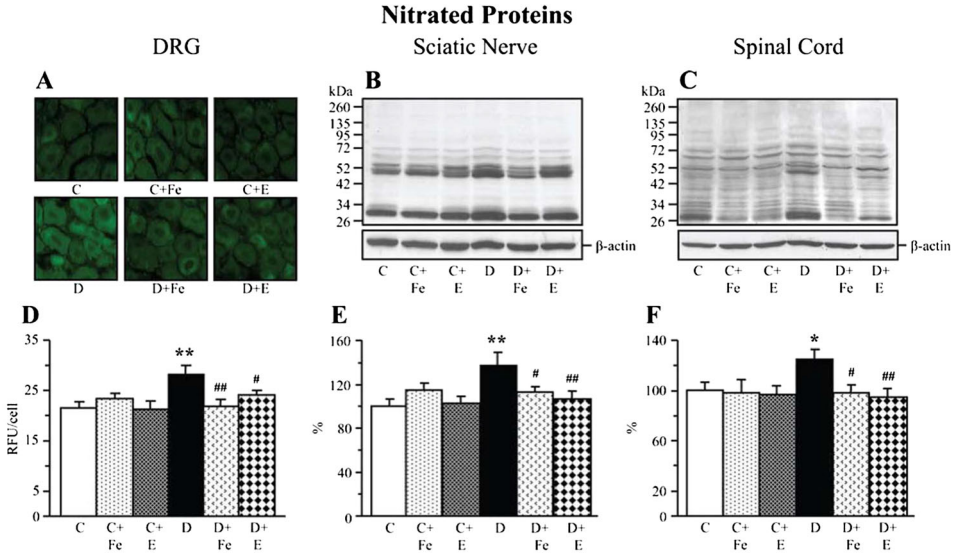

Figure 2. Nitrated proteins in dorsal root ganglion neurons (DRG), sciatic nerve and spinal cord isolated from control (C) and diabetic (D) mice treated with or without FeTMPS (Fe) or epicatechin gallate (E) for 4 weeks after 28 weeks of untreated diabetes. Mean ± standard error of the mean, the number of mice for each study group is the same as described in Table 2. Along the *x*-axis, C represents control mice; D, diabetic mice; E, epicatechin gallate treated mice; and Fe, FeTMPS treated mice. *$p < 0.05$ compared with control; **$p < 0.01$ compared with control; $^{\#}p < 0.05$ compared with untreated diabetic; $^{\#\#}p < 0.01$ compared with untreated diabetic

rodents as well as in the circulation, vasculature, myocardium and kidney of human subjects with diabetes [26–28,30,39–49]. However, one concern of past studies of interventions to disrupt oxidative/nitrosative stress in diabetic rodent models is that most of them have been performed using short-term diabetic durations. There is a need for studies of chronic disease in animal models to determine whether interfering with oxidative/nitrosative stress can improve diabetic neuropathy endpoints after long-term neuropathic conditions. To address this issue, we studied the effect of treating streptozotocin-induced diabetic mice with a peroxynitrite decomposition catalyst or protein nitration inhibitor for 4 weeks following 28 weeks of untreated diabetes on diabetic neuropathy related endpoints.

In this study, 28 weeks of untreated diabetes in streptozotocin-treated C57Bl6/J mice resulted in slowing of motor and sensory nerve conduction velocity, thermal hypoalgesia accompanied by a decrease in intraepidermal nerve fibre density, tactile allodynia and mechanical hypoalgesia. Similar results have been reported by our group in mice after a much shorter duration of streptozotocin

DOI: 10.1002/dmrr

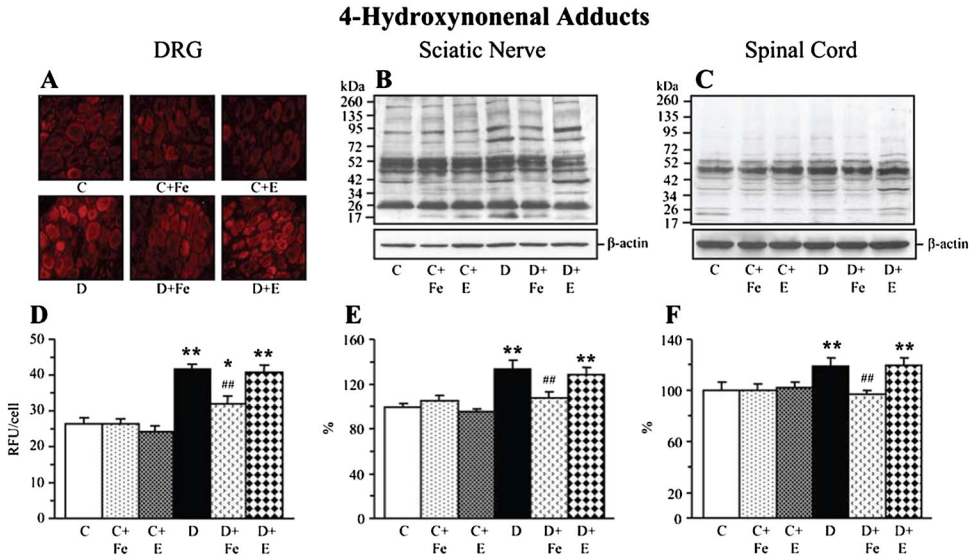

Figure 3. 4-Hydroxynonenal adducts in dorsal root ganglion neurons (DRG), sciatic nerve and spinal cord isolated from control (C) and diabetic (D) mice treated with or without FeTMPS (Fe) or epicatechin gallate (E) for 4 weeks after 28 weeks of untreated diabetes. Mean ± standard error of the mean, the number of mice for each study group is the same as described in Table 2. Along the *x*-axis, C represents control mice; D, diabetic mice; E, epicatechin gallate treated mice; and Fe, FeTMPS treated mice. $^{*}p < 0.05$ compared with control; $^{**}p < 0.01$ compared with control; $^{\#\#}p < 0.01$ compared with untreated diabetic

diabetes (6–12 weeks) and in leptin-deficient *ob/ob* mouse [34,35,38,50]. This raises the issue of what are the correct conditions to use regarding duration of untreated diabetes and treatment that would best represent the patient should the compound being evaluated preclinically advance to clinical trials. Studies from our group using the same C57Bl6/J mouse model of type 1 diabetes show that the extent of nerve conduction deficits and impaired behavioural response to a painful stimuli are about the same in mice after 6 or 24 weeks of untreated diabetes [34,38,50]. It is unknown whether this means that the degree of severity of these deficits is the same for the acute *versus* chronic diabetic mice. Even though the effect of acute and chronic diabetes on the endpoints being measured was similar, we do not know what mechanism(s) may be contributing to the deficits after acute or chronic hyperglycaemia. It is unlikely that mechanism(s) contributing to diabetic neuropathy develop at the same rate. Therefore, a given treatment applied after acute *versus* chronic hyperglycaemia could have different affects depending on the mechanism(s) responsible for the pathogenesis at the time treatment is initiated. Likewise, it is unknown what the correct treatment period should be. In our studies, a short treatment phase of 3 or 4 weeks did not result in complete recovery of diabetic neuropathy endpoints [34,38,50]. Whether this means that multiple mechanism(s) may be contributing to the deficits and the monotherapy approach was not sufficient to fully restore function or that the selected treatment was not given for long enough periods to enact full recovery is unknown. The only way to answer these questions would be to perform a longitudinal study that incorporates different durations of untreated diabetes and treatment. The downside of this approach is that it is time consuming and expensive. Thus, investigators are left with selecting duration of diabetes and a treatment period that they hope at the end of their experiment will indicate whether the mechanism being targeted has a role in diabetic complications.

The major findings in this study were that both a peroxynitrite decomposition catalyst (FeTMPS) and epicatechin gallate, a protein nitration inhibitor, improved diabetic neuropathy endpoints after treating chronic streptozotocin diabetic mice for only 4 weeks. Both FeTMPS and epicatechin gallate reduced the level of nitrated proteins in the dorsal root ganglion neurons, sciatic nerve and spinal cord; whereas only FeTMPS improved oxidative stress as demonstrated by reduction of 4-hydroxynonenal adducts. This implies that nitrosative stress plays a significant role in the development/progression of diabetic neuropathy attributed to oxidative stress. It is important to stress that treatment of chronic streptozotocin diabetic mice with epicatechin gallate for 4 weeks did not improve intraepidermal nerve fibre density. In contrast, FeTMPS treatment did increase the number of nerve fibres in the epidermis of the hindpaw compared with untreated diabetic mice. Both treatments improved thermal nociception. This suggests that recovery of thermal sensitivity does not necessarily require recovery of nerve fibres. Others have reported in studies with diabetic mice and rats that changes in thermal sensitivity and intraepidermal nerve fibre density may not be paralleling factors [51,52].

Treatment of streptozotocin diabetic mice with FeTMPS or epicatechin gallate did not fully restore nerve conduction

DOI: 10.1002/dmrr

velocity deficits or behavioural endpoints with tactile response threshold being the least responsive to treatment. Tactile allodynia, a condition where light touch is perceived as painful, has been observed in streptozotocin diabetic rats and mice [34,53,54]. The mechanism(s) of this response is not understood, although it has been prevented or reversed by insulin therapy the inhibitor of catechol-*O*-methyltransferase, antioxidant nitecapone and the poly (ADP-ribose)polymerase inhibitor 1,5-isoquinolinediol [53–56]. As discussed earlier, there could be several reasons why FeTMPS or epicatechin gallate treatment was minimally successful in improving tactile allodynia. Treatment for 4 weeks after chronic hyperglycaemia may have been too short of an intervention period to achieve full recovery for a diabetes complication that is progressive. Kowluru *et al*. (2007) demonstrated that improvement of glycemic control in diabetic rats after 6 months of poor control failed to reverse peroxynitrite accumulation in retina capillaries (29). This result is consistent with the metabolic memory phenomenon and could help explain the slow recovery of peripheral nerve function in these studies. Moreover, it could explain why FeTMPS was found to generally be more beneficial than epicatechin gallate treatment. FeTMPS treatment would enhance decomposition of nitrosative stress thereby speeding recovery, whereas epicatechin gallate treatment would prevent or reduce formation of new nitrosylated proteins and not necessarily enhance turnover or decomposition of existing nitrosylated proteins; thus, the effects of the preformed nitrosylated proteins would persist. Therefore, after extended periods of hyperglycaemia followed by improvement in glycemic control, reversal of complications may be slow and only improved with appropriate interventions. It is also well known that diabetic neuropathy is multifactorial, and it is likely that diabetes-induced nitrosative stress may be only one mechanism contributing to diabetic neuropathy as defined by the many different endpoints that are often used in rodent studies of diabetes complications and that tactile allodynia as an endpoint may be less responsive to prevention of nitrosative stress.

Peroxynitrite decomposition catalysts or inhibition of protein nitration may alleviate diabetic neuropathy by preventing the direct effects of protein nitration and nitrosylation, DNA breakage and base modification, lipid peroxidation and indirectly by reducing poly(ADP-ribose) polymerase activation [27,28,35,57]. Furthermore, we have shown that peroxynitrite decomposition catalysts can improve diabetes-induced decrease in endoneurial blood flow and vascular dysfunction of not only epineurial arterioles, blood vessels that provide circulation to the sciatic nerve, but also coronary and mesenteric arteries [58]. Improvement in vascular function was likely due to preventing the quenching of nitric oxide an important vasodilator. Future studies will focus on longer treatment periods and perhaps combination therapies to maximize recovery.

Our findings indicate that both peroxynitrite injury *in toto* and its component, protein nitration, play an important role in the development of chronic diabetic peripheral neuropathy. We found that both functional and structural neuropathic changes in chronic diabetes are amenable to treatment even after an extended period of nontreatment. Neither inhibition of peroxynitrite injury *in toto* nor inhibition of protein nitration resulted in complete correction or alleviation of diabetic neuropathy endpoints. Treatment with a peroxynitrite decomposition catalyst alleviated peripheral nerve dysfunction and increased intraepidermal nerve fibre density, whereas a protein nitration inhibitor resulted in a significant improvement of functional indices only. Our findings suggest that peroxynitrite decomposition catalysts and protein nitration inhibitors may find use in management of diabetic peripheral neuropathy and justify further efforts aimed at developing both classes of agents that would be suitable for human application.

Acknowledgements

This material is based upon work supported in part by the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development, Biomedical Laboratory Research and Development (BX001680-01, MAY), the National Institute of Diabetes and Digestive and Kidney Diseases grant DK073990 (MAY), DK081147 (IGO and MAY) and the National Institute of General Medicine Sciences grant GM036298 (JTG) from NIH. The content of this manuscript are new and solely the responsibility of the authors and do not necessarily represent the official views of the granting agencies.

Conflicts of interest

None declared.

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