Peripheral neuropathy in mice with neuronal nitric oxide synthase gene deficiency

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Summary. Evidence for the important role of the potent oxidant peroxynitrite in peripheral diabetic neuropathy and neuropathic pain is emerging. This study evaluated the contribution of neuronal nitric oxide synthase (nNOS) to diabetes-induced nitrosative stress in peripheral nerve and dorsal root ganglia, and peripheral nerve dysfunction and degeneration. Control and nNOS-/- mice were made diabetic with streptozotocin, and maintained for 6 weeks. Peroxynitrite injury was assessed by nitrotyrosine and poly(ADP-ribose) immunoreactivities. Peripheral diabetic neuropathy was evaluated by measurements of sciatic motor and hind-limb digital sensory nerve conduction velocities, thermal algesia, tactile allodynia, and intraepidermal nerve fiber density. Control nNOS-/- mice displayed normal motor nerve conduction velocity and thermal response latency, whereas sensory nerve conduction velocity was slightly lower compared with non-diabetic wild-type mice, and tactile response threshold and intraepidermal nerve fiber density were reduced by 47 and 38%, respectively. Both diabetic wild-type and nNOS-/- mice developed diabetic neuropathy, with tactile allodynia, and intraepidermal nerve fiber degeneration. Control and nNOS-/- mice were made diabetic with streptozotocin, and maintained for 6 weeks. Peroxynitrite injury was assessed by nitrotyrosine and poly(ADP-ribose) immunoreactivities. Peripheral diabetic neuropathy was evaluated by measurements of sciatic motor and hind-limb digital sensory nerve conduction velocities, thermal algesia, tactile allodynia, and intraepidermal nerve fiber density. Control nNOS-/- mice displayed normal motor nerve conduction velocity and thermal response latency, whereas sensory nerve conduction velocity was slightly lower compared with non-diabetic wild-type mice, and tactile response threshold and intraepidermal nerve fiber density were reduced by 47 and 38%, respectively. Both diabetic wild-type and nNOS-/- mice displayed enhanced nitrosative stress in peripheral nerve. In contrast to diabetic wild-type mice, diabetic nNOS-/- mice had near normal nitrotyrosine and poly(ADP-ribose) immunofluorescence in dorsal root ganglia. Both diabetic wild-type and nNOS-/- mice developed motor and sensory nerve conduction velocity deficits and thermal hypoalgesia although nNOS gene deficiency slightly reduced severity of the three disorders. Tactile response thresholds were similarly decreased in control and diabetic nNOS-/- mice compared with non-diabetic wild-type mice. In conclusion, nNOS is required for maintaining the normal peripheral nerve function and small sensory nerve fibre innervation. nNOS gene deficiency does not protect from development of nerve conduction deficit, sensory neuropathy and intraepidermal nerve fiber loss.

Introduction

Accumulation of the potent oxidant peroxynitrite, a product of superoxide anion radical reaction with nitric oxide, is associated with multiple detrimental consequences including, but not limited by, initiation of lipid peroxidation, DNA breakage and base modifications, metalloproteinase and poly(ADP-ribose) polymerase (PARP) activations, impairment of cell signaling, changes in transcriptional regulation, inflammatory response, and, in extreme cases, cell death by necrosis and apoptosis (1-4). Evidence for the important role of peroxynitrite in diabetic complications including vascular disease (5), cardiomyopathy (2-4), peripheral (PDN; 6-11) and autonomic (12) neuropathy, and retinopathy (13) is emerging. Using a pharmacological approach with structurally different peroxynitrite decomposition catalysts and several animal models of diabetic neuropathy, we demonstrated an important role for peroxynitrite injury (nitrosative stress) in diabetes-associated nerve blood flow and nerve conduction deficits, impaired vascular reactivity of epi-neurial arterioles, thermal hyper- and hypoalgesia, mechanical hypoalgesia, tactile allodynia, and small sensory nerve fiber degeneration (6-11). Furthermore, nitrosative stress in peripheral nerve, spinal cord, and dorsal root ganglion (DRG) neurons has been identified as an important factor leading to PARP activation, another important mechanism in the pathogenesis of PDN (14-17).

Until now, the sources of superoxide and nitric oxide for diabetes-related peroxynitrite formation in peripheral nervous system (PNS) have been elusive. Two pharmacological studies (18,19) revealing beneficial effects of the NAD(P)H oxidase inhibitor apocynin and the xanthine oxidase inhibitor allopurinol on early PDN, suggest that these two superoxide-generating enzymes contribute to diabetes-associated superoxide production in PNS. Increased superoxide production has been documented in vasa nervorum in rat models of both Type 1 and Type 2 diabetes (16,20,21), and in high glucose-exposed human Schwann cells (22). Superoxide dismutase

Key words: nerve conduction, neuronal nitric oxide synthase, nitrosative stress, peripheral diabetic neuropathy, streptozotocin-diabetic mouse

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activity was found to be reduced in the diabetic nerve (23,24). Insufficient information is available on the role of individual isoforms of nitric oxide synthase (NOS), an enzyme producing the second component for peroxynitrite formation, i.e., nitric oxide, in nitrosative stress in PNS and PDN. Endothelial NOS (eNOS) is known to play an important role in maintenance of normal nerve blood flow, and its uncoupling in diabetes causes neurovascular dysfunction and nerve conduction deficits (25,26). Inducible NOS (iNOS) has recently been identified as a major contributor to oxidative-nitrosative stress in peripheral nerve as well as nerve conduction deficit, sensory disorders, and intraepidermal nerve fiber loss characteristic for PDN (12). The present study was aimed at evaluating the role for neuronal NOS (nNOS) in oxidative-nitrosative stress in peripheral nerve and DRG, and in development of neuropathic changes in STZ-diabetic mice, a model of Type 1 diabetic neuropathy. STZ-induced diabetic mice of all genetic strains studied so far, i.e., C57Bl6/J (6,8,9,12,27-30), 129S1/SvImJ (17), Swiss-Webster (31,32), and C57B6:129S7 (33) mice, exhibited insulin resistance at the level of peripheral tissues (28). Fasting euglycemic clamp studies demonstrate that homozygotes for the diabetic phenotype exhibit characteristic hyperglycemia (29). Non-diabetic and diabetic states. Furthermore, hyperglycemic-euglycemic clamp studies demonstrate that homozygotes exhibit hyperglycemia at the level of peripheral tissues (http://jaxmice.jax.org/strain/002986.html).

For the main experiment, a colony of nNOS−/− mice was established at Pennington Biomedical Research Center. Mature male C57Bl6/J mice were purchased from Jackson Laboratories and served as controls. All the mice were fed standard mouse chow (PMI Nutrition International, Brentwood, MO) and had access to water ad libitum. Diabetes was induced by a single injection of streptozotocin (STZ), 100 mgkg−1 d−1, i.p., to non-fasted animals. Blood samples for glucose measurements were taken from the tail vein three days after STZ injection and the day before the animals were sacrificed. The mice with blood glucose ≥13.8 mM were considered diabetic. The injected mice that had blood glucose concentration in non-diabetic range have been given low-dose STZ injections (40 mgkg−1d−1, i.p.) until they developed hyperglycemia (typically, one-three additional injections). At the end of the study (6-week duration of diabetes), the physiological and behavioral tests were performed in the following order: tactile responses to flexible von Frey filaments (first day), thermal algesia by tail-flick test (second day), thermal algesia by paw withdrawal test (third day), SNCV and MNCV (fourth day). Measurements of MNCV and SNCV were performed in mice anaesthetized with a mixture of ketamine and xylazine (45 mgkg−1 body weight and 15 mgkg−1 body weight, respectively, i.p.).

**Materials and methods**

**Reagents.** Unless otherwise stated, all chemicals were of reagent-grade quality, and were purchased from Sigma Chemical Co., St. Louis, MO. Rabbit polyclonal anti-nitrotyrosine (NT) antibody was purchased from Trevigen, Inc., 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, M.O.M. Basic Kit, Vectastain Elite ABC Kit (Standard), DAB Substrate Kit, and 3,3’-diaminobenzidine were obtained from Vector Laboratories, Burlingame, CA. Rabbit polyclonal anti-protein gene product 9.5 (ubiquitin c-terminal hydrolase) antibody was purchased from Chemicon International, Inc., Temecula, CA. Other reagents for immunohistochemistry were purchased from Dako Laboratories, Inc., Santa Barbara, CA.

**Animals and limitations of the nNOS−/− mouse model.** The experiments were performed in accordance with regulations specified by the National Institutes of Health ‘Principles of Laboratory Animal Care. 1985 Revised Version’ and the Pennington Biomedical Research Center Protocol for Animal Studies. Several breeding pairs of B6.129S4-Nos1 tm1Plh /J Pennington Biomedical Research Center Protocol for Animal Laboratory Animal Care, 1985 Revised Version and specified by the National Institutes of Health ‘Principles of experiments were performed in accordance with regulations http://jaxmice.jax.org/strain/002986.html.

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**Anesthesia, euthanasia and tissue sampling.** The animals were sedated by CO2, and immediately sacrificed by cervical dislocation. Sciatic nerves, dorsal root ganglia (DRG), and foot pads were fixed in normal buffered 4% formalin for assessment of nitrotyrosine and poly(ADP-ribose) by immunofluorescent histochemistry and intraepidermal nerve fiber density by conventional immunohistochemistry. Poly(ADP-ribose) abundance is a measure of poly(ADP-ribose) polymerase activity (14-17,34).

**Specific methods**

**Physiological tests.** Sciatic MNCV and hind-limb digital SNCV were measured as we have described elsewhere (11,14,17).

**Behavioral tests**

1. **Tactile response threshold.** Tactile responses were evaluated by quantifying the withdrawal threshold of the hindpaw in response to stimulation with flexible von Frey filaments as we have described (35).

2. **Thermal algesia.** The paw withdrawal latency in response to the radiant heat (15% intensity which produced a heating rate of ~1.3˚C per sec, cut-off time 30 sec) was determined as we have described (7-9,17,35) using the IITC model 336 TG combination tail-flick and paw algesia meter (IITC Life Science) with a floor temperature ~32-33˚C (manufacturer's set up). For assessment of tail flick response latencies, the device was set at 40% heating intensity (heating rate ~2.5˚C per sec) with a cut-off at 10 sec. In both tests, at least three readings per animal were taken at 15 min interval, and the average was calculated.

**Immunohistochemical studies.** All sections were processed by a single investigator and evaluated blindly. Low power observations of skin sections stained for PGP 9.5 were made using a Zeiss Axioskop microscope. Color images were captured with a Zeiss AxioCam HRc CCD camera at...
Table I. Initial and final body weights and blood glucose concentrations in experimental groups.

<table>
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<tr>
<th></th>
<th>Body weight (g)</th>
<th>Blood glucose (mmol/l)</th>
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<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
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<tr>
<td>Non-diabetic wild-type</td>
<td>25.8±1.0</td>
<td>30.7±0.6</td>
</tr>
<tr>
<td>Non-diabetic iNOS-/-</td>
<td>26.9±1.8</td>
<td>30.0±1.2</td>
</tr>
<tr>
<td>Diabetic wild-type</td>
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<td>24.2±0.5a</td>
</tr>
<tr>
<td>Diabetic iNOS-/-</td>
<td>26.6±0.8</td>
<td>24.8±0.5a</td>
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Data are means ± SEM, n=8-11 per group. *Significantly different from the corresponding non-diabetic groups (p<0.01).

Results

Weight gain during the 6-week study was 19% in non-diabetic wild-type and 11.5% in non-diabetic nNOS-/- mice (Table I). Both diabetic wild-type and diabetic nNOS-/- mice lost 7% of their initial body weights. Initial (after STZ treatment) body weights were 25.8±1.0 g and 26.6±0.8 g, respectively. Final body weights were 30.7±0.6 g and 24.8±0.5 g, respectively. Blood glucose concentrations at the end of the experiment were 7.1±0.4 mmol/l and 15.5±1.2 mmol/l, respectively.
Injection) blood glucose concentrations were 108 and 146% higher in diabetic wild-type and diabetic nNOS−/− mice compared with the corresponding controls. Hyperglycemia progressed with the prolongation of diabetes. Final blood glucose concentrations were similar in diabetic wild-type and diabetic nNOS−/− mice.

nNOS gene deficiency did not affect sciatic MNCV and resulted in 11% reduction in hind-limb SNCV in non-diabetic mice (Fig. 1). MNCV and SNCV were 19 and 20% lower in diabetic wild-type mice (p<0.01 for both comparisons), and 11 and 10% lower in diabetic nNOS−/− mice (p<0.05 and <0.01, respectively), compared with the corresponding non-diabetic groups.

The latency of hind paw withdrawal in response to radiant heat was increased by 100% in diabetic wild-type mice compared with the control group (p<0.01), consistent with clearly manifest thermal hypoalgesia (Fig. 2A). This is in agreement with the results of tail-flick test which also revealed increased thermal response latencies in the diabetic wild-type mice (Fig. 2B). The severity of hypoalgesia by paw withdrawal test was lower in diabetic nNOS−/− mice, compared with diabetic wild-type mice.

**Figure 1.** Sciatic motor nerve conduction velocities (A) and hind-limb digital sensory nerve conduction velocities (B) in control and diabetic wild-type and nNOS−/− mice. Mean ± SEM, n=8-10 per group. C, control mice; D, diabetic mice. *p<0.05 and **p<0.01 vs corresponding non-diabetic groups.

**Figure 2.** Paw withdrawal latencies in response to radiant heat (A) and tail-flick test response latencies (B) in control and diabetic wild-type and nNOS−/− mice. Mean ± SEM, n=8-11 per group. C, control mice; D, diabetic mice. *p<0.01 vs corresponding non-diabetic groups; **p<0.01 vs diabetic wild-type mice.

**Figure 3.** Tactile response thresholds in response to stimulation with flexible von Frey filaments in control and diabetic wild-type and nNOS−/− mice. Mean ± SEM, n=8-11 per group. C, control mice; D, diabetic mice. *p<0.01 vs non-diabetic control mice.
Non-diabetic nNOS−/− mice displayed 47% reduction in tactile response thresholds, compared with non-diabetic wild-type mice (Fig. 3). Diabetic wild-type mice developed clearly manifest tactile allodynia, whereas tactile response threshold were similarly reduced in non-diabetic and diabetic nNOS−/− mice, compared with wild-type controls.

INFD was reduced by 38% in diabetic wild-type mice and by 27% in diabetic nNOS−/− mice compared with the corresponding controls (p<0.05 for both comparisons, Fig. 4). Note, however, that nNOS gene deficiency was associated with a 32% decrease in INFD in non-diabetic mice.

NT immunofluorescence was 31% higher in non-diabetic nNOS−/− mice compared with non-diabetic wild-type mice, but the difference between two non-diabetic groups did not achieve statistical significance. NT immunofluorescence increased by 69 and 37% in the sciatic nerves of diabetic wild-type and nNOS−/− mice, respectively, compared with the corresponding non-diabetic controls (p<0.01, Fig. 5). DRG NT immunofluorescence was comparable in non-diabetic wild-type and nNOS−/− mice (Fig. 6). It was increased by 56% in diabetic wild-type mice compared with non-diabetic controls (p<0.01). In contrast, diabetic nNOS−/− mice were protected against peroxynitrite injury in DRG and maintained nitrotyrosine immunoreactivity at the level detected in non-diabetic mice.

PAR fluorescence was similar in DRG of non-diabetic wild-type and nNOS−/− mice (Fig. 7). It was 23% higher in DRG of diabetic wild-type mice compared with non-diabetic controls (p<0.01). In contrast, diabetic nNOS−/− mice displayed normal DRG PAR immunofluorescence (Fig. 8). The
percentage of DRG neurons with weak PAR immunofluorescence was lower, and of those with moderate and intense immunofluorescence higher in diabetic wild-type mice compared with the corresponding control group. nNOS gene deficiency reduced the percentage of neurons with moderate and intense PAR fluorescence and increased the percentage of neurons with weak PAR fluorescence in diabetic mice.

Discussion

nNOS is expressed in many cell types in peripheral nervous system including Schwann cells of peripheral nerve (36), DRG neurons (37,38), and motoneurons, oligodendrocytes, and astrocytes of spinal cord (39,40). One can not exclude that it is also expressed in vasa nervorum considering recent report of the presence of nNOS in vascular endothelium (41). Note, that nNOS is constitutively expressed in some types of neurons, and can be induced in others (42). Sciatic nerve (43) or pelvic nerve (44) transection induced nitric oxide synthase expression in small to medium sized DRG neurons suggesting that this change may be limited to, or most prominent in, C-fiber afferents.

The findings of the present study suggest that nNOS is required for maintaining the normal peripheral nerve function and small sensory nerve fibre innervation. The latter is not surprising because nNOS is the main producer of nitric oxide which serves as a neurotransmitter in PNS (45). nNOS gene deficiency in non-diabetic mice affected both large and small sensory nerve fibers and was associated with reduction in sensory nerve conduction velocity, tactile response thresholds,
and loss of intraepidermal innervation. Other report suggests that nNOS plays a critical role in Schwann cells proliferation during peripheral nerve regeneration (46). On the other hand, nNOS overexpression was implicated in neuropathic pain resulting from nerve injury or peripheral inflammation (47,48). nNOS expression was reduced in sciatic nerve of C57BLKS/J-\(^{m+/+}\)Leprdb homozygous (db/db) mice compared with non-diabetic controls, and in high glucose-exposed cultured mouse Schwann cells compared with the cells cultured at normal glucose concentration (36). The 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor rosuvastatin reversed reduction of nNOS expression in sciatic nerves of diabetic mice, and prevented high glucose-induced nNOS down-regulation in mouse Schwann cells, by mechanisms that involved phosphoinositide 3-kinase and phosphorylation of Akt (36). In the same study, co-treatment of diabetic mice with rosuvastatin and the selective nNOS inhibitor 1,2-trifluoromethylphenyl imidazole, blunted beneficial effects of rosuvastatin on MNCV and SNCV deficits, thermal sensitivity, and nerve vascularity which suggests an important role for nNOS-derived NO in peripheral nerve function. Of interest, NOS enzymatic activity in DRG was increased rather than reduced in rats with STZ-diabetes of 12-month duration (37). This activation, however, was not due to an increase in nNOS mRNA expression or immunoreactivity.

In the present study, diabetic wild-type mice displayed clearly manifest nitrosative stress in both peripheral nerve and DRG. nNOS gene deficiency prevented diabetes-induced peroxynitrite injury manifest by nitrotyrosine accumulation, in DRG neurons, but not in peripheral nerve which is indica-
tive of different roles of nNOS-derived NO in peroxynitrite formation in these two tissue-sites of PDN. Despite the lack of neuronal nitrosative stress, diabetic nNOS<sup>−/−</sup> mice displayed reduction in MNCV and SNCV, sensory disorders, and intraepidermal nerve fiber loss, although nerve conduction deficits and thermal hypoaesthesia were less severe in diabetic nNOS<sup>−/−</sup> mice, than in diabetic wild-type mice. Note, that in our previous study (11), diabetic iNOS<sup>−/−</sup> mice which were protected from nitrosative stress in peripheral nerve, but not DRG neurons, preserved normal nerve conduction velocities and thermal sensitivity. These findings suggest that nitrosative stress in peripheral nerve, rather than DRG neurons, contributes to the development of early PDN. The roles iNOS and nNOS in advanced PDN still remain to be established.

The importance of oxidative-nitrosative stress in peripheral nerve, but not DRG, in early PDN is further supported by the pattern of changes in PARP activity. Evidence for the important role for PARP activation in diabetic complications including endothelial dysfunction (49), cardiomyopathy (50), peripheral (14-17) and autonomic (51) neuropathy, nephropathy (52), and retinopathy (53,54) is emerging. Accumulation of poly(ADP-ribosyl)ated proteins, a sign of PARP activation, has been documented in peripheral nerve and DRG neurons of animal models of Type 1 and Type 2 diabetes and high glucose-exposed cultured human Schwann cells (6-11,14-17,22,35,55) PARP activation has been implicated in neurovascular dysfunction, motor and sensory nerve conduction velocity deficits, peripheral nerve energy failure, thermal hyper- and hypoaesthesia, mechanical hypoaesthesia, tactile alldynia, and intraepidermal nerve fiber loss characteristic for PDN (14-17,56). In the present study, PARP activation was clearly manifest in both sciatic nerve and DRG neurons of animal models of Type 1 and Type 2 diabetes and high glucose-exposed cultured human Schwann cells (6-11,14-17,22,35,55) PARP activation has been implicated in neurovascular dysfunction, motor and sensory nerve conduction velocity deficits, peripheral nerve energy failure, thermal hyper- and hypoaesthesia, mechanical hypoaesthesia, tactile alldynia, and intraepidermal nerve fiber loss characteristic for PDN (14-17,56). In the present study, PARP activation was clearly manifest in both sciatic nerve and DRG neurons of animal models of Type 1 and Type 2 diabetes and high glucose-exposed cultured human Schwann cells (6-11,14-17,22,35,55) PARP activation has been implicated in neurovascular dysfunction, motor and sensory nerve conduction velocity deficits, peripheral nerve energy failure, thermal hyper- and hypoaesthesia, mechanical hypoaesthesia, tactile alldynia, and intraepidermal nerve fiber loss characteristic for PDN (14-17,56).

In conclusion, nNOS is required for maintaining the normal peripheral nerve function and small sensory nerve fibre innervation and appears to be the main source of nitric oxide for diabetes-associated peroxynitrite formation in DRG neurons. In contrast, this isoform does not seem to contribute to peroxynitrite formation in diabetic peripheral nerve. nNOS gene deficiency does not protect from development of nerve conduction deficit, sensory neuropathy, and intraepidermal nerve fiber loss. These findings, together with our previous studies, suggest that oxidative-nitrosative stress in axons and Schwann cells, rather than DRG neurons, plays a major role in, at least, early PDN.

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References


