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Nicotinamide Reverses Neurological and Neurovascular Deficits in Streptozotocin Diabetic Rats

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ABSTRACT

In diabetes, activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) is an important effector of oxidative-nitrosative injury, which contributes to the development of experimental diabetic peripheral neuropathy (DPN). However, the potential toxicity of complete PARP inhibition necessitates the utilization of weaker PARP inhibitors with additional therapeutic properties. Nicotinamide (vitamin B_3) is a weak PARP inhibitor, antioxidant, and calcium modulator and can improve energy status and inhibit cell death in ischemic tissues. We report the dose-dependent effects of nicotinamide in an established model of early DPN. Control and streptozotocin-diabetic rats were treated with 200 to 400 mg/kg/day nicotinamide (i.p.) for 2 weeks after 2 weeks of untreated diabetes. Sciatic endoneurial nutritive blood flow was measured by microelectrode polarography and hydrogen clearance, and sciatic motor and hind-limb digital sensory nerve conduction velocities and thermal and mechanical algesia were measured by standard electrophysiological and behavioral tests. Malondialdehyde plus 4-hydroxyalkenal concentration in the sciatic nerve and amino acid-(4)-hydroxynonenal adduct and poly(ADP-ribosyl)ated protein expression in human Schwann cells were assessed by a colorimetric method with *N*-methyl-2-phenyl indole and Western blot analysis, respectively. Nicotinamide corrected increased sciatic nerve lipid peroxidation in concert with nerve perfusion deficits and dose-dependently attenuated nerve conduction slowing, as well as mechanical and thermal hyperalgesia. Nicotinamide (25 mM) prevented high (30 mM) glucose-induced overexpression of amino acid-(4)-hydroxynonenal adducts and poly(ADP-ribosyl)ated proteins in human Schwann cells. In conclusion, nicotinamide deserves consideration as an attractive, nontoxic therapy for the treatment of DPN.

Diabetic peripheral neuropathy (DPN) is a common complication of diabetes and can result in intractable pain or sensory loss that can ultimately predispose subjects to lower limb ulceration and eventual amputations. Preclinical studies in diabetic rodent models have shown that DPN is the result of a complex network of interrelated vascular (Stevens et al., 2000; Cameron et al., 2001), metabolic (Nakamura et al., 1999; Stevens et al., 2000; Price et al., 2004), and neurotrophic (Hounsom et al., 2001; Calcutt et al., 2004) defects, which culminate in electrophysiological deficits, abnormal sensory perception, and progressive damage and loss of unmyelinated and myelinated nerve fibers (Sima et al., 2000). Many investigators consider that glucose-induced oxidative-nitrosative stress (Stevens et al., 2000; Cameron et al., 2001; Hounsom et al., 2001; Obrosova et al., 2005b) is a critical pathogenetic mechanism that initiates a cascade of downstream metabolic and neurovascular perturbations, a construct supported by the salutary effects of antioxidants in experimental DPN (Stevens et al., 2000; Coppey et al., 2001; Schmeichel et al., 2003).

Activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) is now viewed as an important effector of oxidative-nitrosative injury (Southan and Szabo, 2003; Obrosova et al., 2004, 2005a), which results in cleavage of nicotinamide adenine dinucleotide (NAD^+) and the formation of nicotinamide and ADP-ribose residues attached to nuclear and extranuclear (e.g., mitochondrial) proteins (Southan and

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ABBREVIATIONS: DPN, diabetic peripheral neuropathy; PARP, poly(ADP-ribose) polymerase; NAD^+, nicotinamide adenine dinucleotide; NCV, nerve conduction velocity; HSC, human Schwann cells; SC, Schwann cells; HNE, hydroxynonenal; STZ, streptozotocin; STZ-D, streptozotocin-diabetic; MDA, malonyldialdehyde; 4-HA, 4-hydroxyalkenal; ROS, reactive oxygen species; DRG, dorsal root ganglion.

Szabo, 2003). PARP activation leads to the depletion of its substrate NAD^+, energy failure, inhibition of glyceraldehyde 3-phosphate dehydrogenase of glycolysis, altered gene transcription, and, in extreme cases, cell necrosis (Garcia Soriano et al., 2001; Southan and Szabo, 2003; Obrosova et al., 2004, 2005a). PARP inhibition seemed neuroprotective in pathological conditions associated with oxidative stress (Maiese and Chong, 2003; Shen et al., 2004). In animal models of DPN, PARP pharmacological inhibition (Maiese and Chong, 2003; Obrosova et al., 2004, 2005a) or gene deficiency (Obrosova et al., 2004) reversed or protected from motor and sensory nerve conduction velocity (NCV) slowing, as well as thermal and mechanical hyperalgesia. However, the potential toxicity of complete PARP inhibition because of its important role in DNA repair (Virag and Szabo, 2002) has lead to the exploration of alterative approaches utilizing PARP inhibitors at subtherapeutic doses in combination with synergistic agents (Li et al., 2005).

Nicotinamide (vitamin B_3), a water-soluble vitamin, is a weak PARP inhibitor and a biochemical precursor of NAD^+. It has been shown to improve energy status in ischemic tissues (Yang et al., 2002), exhibit antioxidant properties (Melo et al., 2000; Shen et al., 2004), regulate neuronal calcium fluxes (Shen et al., 2004), and inhibit apoptosis (Maiese and Chong, 2003; Shen et al., 2004), making it an attractive potential agent for the treatment of DPN. Moreover, nicotinamide has been used in human clinical trials with a low incidence of side effects or toxicity (Gale et al., 2004). However, the effect of nicotinamide on DPN in animal models or humans has not been reported. The data presented herein show that nicotinamide is effective at reversing early DPN in an established animal model of type 1 diabetes. It also alleviates oxidative stress in peripheral nerve of diabetic rats, as well as high glucose-exposed human Schwann cells (HSC).

Materials and Methods

Reagents. Chemicals were of reagent-grade quality and were purchased from Sigma Chemical Co. (St. Louis, MO). HSC and HSC medium were purchased from ScienCell Research Laboratories (San Diego, CA). PARP-1 and poly(ADP-ribose) antibodies were obtained from BIOMOL (Plymouth Meeting, PA), and antibody for chemically reduced amino acid-(4)-hydroxynonenal (HNE) adducts was from Calbiochem (San Diego, CA).

Animal Model. Barrier-sustained, cesarean-delivered male Wistar rats (200–300 g) were acclimatized for 1 week before being fasted overnight and rendered diabetic by an i.p. injection of streptozotocin (STZ) (45 mg/kg) (Sigma-Aldrich, St. Louis, MO) in 0.2 ml of 10 mM citrate buffer, pH 5.5. Diabetes was defined as a nonfasting plasma glucose ≥13.8 mM in tail vein blood (One Touch II, Lifescan, Inc., Milpitas, CA) 48 h after STZ injection. Animals were subsequently randomly assigned to six experimental groups: nondiabetic controls (ND), ND treated with 400 mg/kg/day nicotinamide (ND + 400), diabetic (D), diabetic treated with 200 mg/kg/day nicotinamide (D + 200), diabetic treated with 300 mg/kg/day nicotinamide (D + 300), and diabetic treated with 400 mg/kg/day nicotinamide (D + 400). Nicotinamide was administered i.p. for 2 weeks after 2 weeks without treatment, so that total duration of experiment was 4 weeks. We have previously shown that 1) PARP activation manifest by increase in both poly(ADP-ribose) immunofluorescence and poly-(ADP-ribosyl)ated protein expression is clearly present in the sciatic nerve of the STZ-diabetic (STZ-D) rat model of similar duration (Li et al., 2005b; Ilnytska et al., 2006); and 2) 2-week PARP inhibitor treatment introduced 2 weeks after STZ injections does not alleviate hyperglycemia or restore normoglycemia probably because of irreversible damage to pancreatic β cells (Obrosova et al., 2004). The blood glucose and body weight were measured every week during the study. Less than 1% of initially diabetic rats manifested restoration of normoglycemia, and those were not used for experiments.

Measurements of NCV. Hind limb sciatic motor NCV and digital sensory NCV were measured as described previously in detail (Stevens et al., 2004).

Measurement of Endoneurial Nutritive Nerve Blood Flow by H_2 Clearance. Nutritive nerve blood flow was assessed by H_2 clearance. In brief, animals were anesthetized with an i.p. injection of urethane (1g/kg), and the left carotid artery cannulated for blood pressure monitoring. Animals were artificially respired with O_2/N_2 (20:80%) via a tracheostomy. Core body temperature and hind limb muscle temperature were maintained at 37°C by radiant heat. The right sciatic nerve was exposed, and the skin around the incision was positioned to create a reservoir. A ground electrode was inserted s.c. A hydrogen-sensitive platinum electrode (tip diameter, 1–2 μm; part no. PTM23B05KT; World Precision Instruments, Sarasota, FL) was inserted into the nerve above the trifurcation. Mineral oil was used to fill the reservoir, and near nerve temperature was maintained at 37°C. The nerve was polarized with 0.25 V, and when a stable baseline was achieved, the animal received a gas mixture containing 10% hydrogen that was continued until the current change stabilized (10–30 min), at which time hydrogen flow was terminated. Current recordings were made every 30 s until baseline levels were achieved (30–60 min). Subsequently, monoexponential or biexponential clearance curves were fitted to the data (GraphPad Software Inc., San Diego, CA). Nutritive endoneurial nerve blood flow was taken as the slow component of the curve. An average of two determinations at different sites was used to determine nutritive endoneurial nerve perfusion.

Measurement of Mechanical Algesia. Mechanical algesia was determined by quantifying the withdrawal threshold of the hind paw in response to mechanical stimulation using a von Frey anesthesiometer (model 2290C; IITC Life Science, Woodland Hills, CA). The test was conducted between 9:00 and 11:30 AM each day. The rats were placed in individual Plexiglas boxes on a stainless steel mesh floor and were allowed to acclimate for at least 20 min. A 0.5-mm (diameter) polypropylene rigid tip was used to apply a force to the plantar surface of the hind paw. The force causing withdrawal was recorded by the anesthesiometer. The anesthesiometer was calibrated before each recording. The test was repeated four or five times at approximately 5-min intervals on each animal, and the mean value was then calculated.

Measurement of Thermal Sensitivity. To determine the sensitivity to noxious heat, rats were placed within a Plexiglas chamber on a transparent glass surface and allowed to acclimate for at least 20 min. A thermal stimulation meter (IITC Life Science Instruments) was used. The device was activated after placing the stimulator directly beneath the plantar surface of the hind paw. The paw withdrawal latency in response to the radiant heat was recorded. Individual measurements were repeated four or five times, and the mean value was then calculated as the thermal threshold.

HSC Culture and Western Blot Analysis. HSC were cultured in commercial media according to the manufacturer's instructions. Passages 7 through 10 were used in all of the experiments. Cells were lysed in the extraction buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 50 mM NaF, and the protease/phosphatase inhibitors leupeptin (10 μg/ml), aprotinin (20 μg/ml), benzamidine (10 mM), phenylmethylsulfonyl fluoride (1 mM), and sodium orthovanadate (1 mM) and then were homogenized on ice. The homogenate was sonicated (3 × 5 s) and centrifuged at 14,000*g* for 20 min. All of the aforementioned steps were performed at 4°C. The lysates (20 μg of protein) were mixed with equal volume of 2× sample-loading buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.025% bromphenol blue and then were boiled for 5 min before loading on a gel. The lysates were fractionated in 10% SDS-polyacrylamide gel electrophoresis in an electrophoresis cell (Mini-Protean III; Bio-Rad, Her-

cules, CA). Electrophoresis was conducted at 15 mA constant current for stacking and at 25 mA for protein separation. Gel contents were electrotransferred (250 mA, 2 h) to nitrocellulose membranes using Mini Trans-Blot cell (Bio-Rad) and Western transfer buffer [25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 20% (v/v) methanol]. Free binding sites were blocked in 2% (w/v) bovine serum albumin in 20 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl and 0.05% Tween 20 for 1 h, after which membranes were probed with poly(ADP-ribose) (Trevigene, Gaithersburg, MD), PARP-1 (Biomol), or chemically reduced HNE adduct (Calbiochem) antibodies. Total content of all of the poly(ADP-ribosyl)ated proteins, HNE adducts, and PARP-1 were quantified by densitometry (Quantity One 4.5.0 software; Bio-Rad). Membranes were then stripped in 62.5 mM Tris-HCl, pH 6.7, buffer containing 2% SDS and 100 mM β-mercaptoethanol and then reprobed with β-actin antibody to confirm equal protein loading.

Biochemical Measurements. Malonyldialdehyde (MDA) plus 4-hydroxyalkenal (4-HA) concentration was measured using commercially available kits (LPO-586 assay; Oxis International, Portland, OR) as reported previously (Stevens et al., 2004).

Statistical Analysis. Data are expressed as mean ± S.E.M. Differences among experimental groups were determined by analysis of variance, and the significance of between-group differences was assessed by Student-Newman-Keuls multiple range test. Significance was defined as $p \leq 0.05$. If the variances for the variables were found to differ significantly, a logarithmic transformation was performed that corrected the unequal variances. All of the analyses were then performed on the transformed data. When between-group variance differences could not be normalized by log transformation (data sets for body weights, plasma glucose, and some metabolic parameters), the data were analyzed by the nonparametric Kruskal-Wallis one-way analysis of variance, followed by the Bonferroni/Dunn test for multiple comparisons.

All of the experiments were performed in accordance with regulations specified by the National Institutes of Health *Principles of Laboratory Animal Care* (1985 revised version) and University of Michigan Protocol for Animal Studies.

Results

Body weights and plasma glucose levels are shown in the Table 1. As expected, after 4 weeks, body weights were lower in untreated diabetic animals compared with nondiabetic controls. Body weight in animals administered nicotinamide at doses of 300 mg/kg/day and above was not significantly different from nondiabetic controls. The mechanism of the weight gain in nicotinamide-treated STZ-D rats is uncertain but may reflect increased food intake, which was not monitored.

Blood glucose concentration was increased ~4- to 5-fold in diabetic rats compared with control groups. Nicotinamide treatment of nondiabetic or diabetic rats had no significant effects on blood glucose concentration.

TABLE 1
Final body weights and blood glucose concentrations in nondiabetic and diabetic rats treated with and without nicotinamide

	n	Body Weight	Blood Glucose
		g	*mM*
ND	8	376 ± 13	4.6 ± 0.4
ND + 400	8	401 ± 14	5.1 ± 0.1
D	9	284 ± 11[a]	18.8 ± 0.8[a]
D + 200	7	310 ± 7[a]	22.2 ± 1.1[a]
D + 300	8	344 ± 4[b]	22.9 ± 0.9[a]
D + 400	10	352 ± 9[b]	19.6 ± 0.5[a]

Data are means ± S.E.M.
[a] Significantly different from ND and ND + 400 ($p < 0.01$).
[b] Significantly different from D ($p < 0.05$).

Effects of Diabetes and Nicotinamide Replacement on Motor and Sensory NCV. The effects of diabetes and nicotinamide on motor and sensory NCV are shown in Fig. 1. Sciatic motor NCV was reduced by 23% ($p < 0.001$) after 4 weeks of untreated diabetes. All of the doses of nicotinamide partially corrected this deficit, with the maximal effect (a 56% correction, $p < 0.001$) observed in the D + 400 group (Fig. 1A). In contrast, digital sensory NCV was reduced by 16% ($p < 0.001$) in diabetic rats and was 70% corrected by nicotinamide ($p < 0.01$ versus untreated diabetic group) to levels that were not significantly different from controls (Fig. 1B). Nicotinamide treatment was without effect in nondiabetic control animals.

Effects of Diabetes and Nicotinamide on Sciatic Endoneurial Blood Flow. Endoneurial blood flow (Fig. 2A) was reduced by 50% after 4 weeks of diabetes ($p < 0.001$). Nicotinamide treatment resulted in a dose-dependent correction of nerve blood flow deficits, with doses of 300 and 400 mg/kg/day leading to complete restoration of nerve perfusion (to 100 and 103%, respectively, $p < 0.001$). Although nicotinamide tended to increase blood flow in nondiabetic rats, this was not statistically significant. Mean systemic blood pressure (Fig. 2B) was reduced by 20% in diabetic rats ($p < 0.01$) and was increased by up to 59% ($p < 0.05$) in diabetic rats treated with 400 mg/kg/day nicotinamide to levels that did

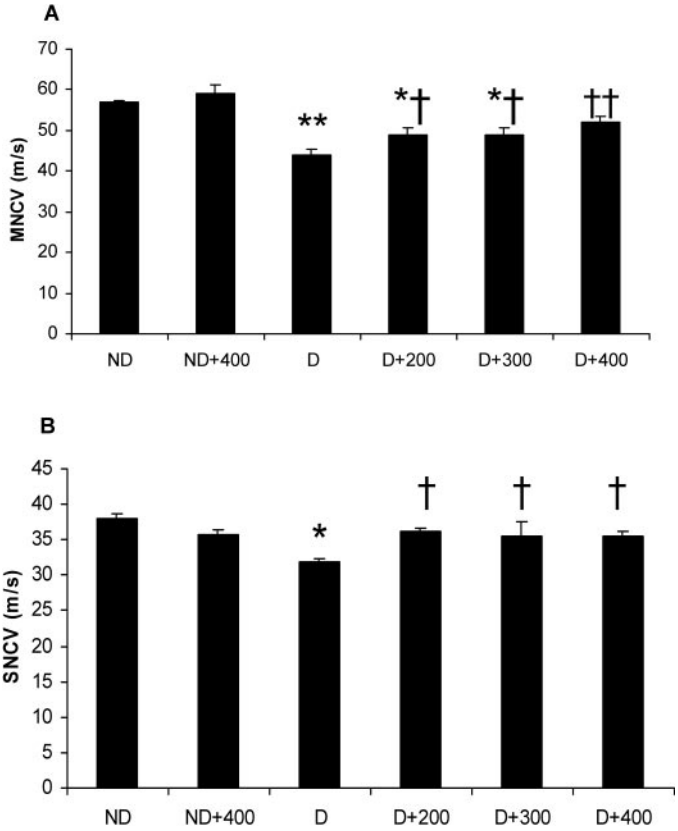

Fig. 1. Sciatic motor (A) and digital sensory (B) conduction velocity in nondiabetic and diabetic rats treated with nicotinamide. The results are expressed as mean ± S.E.M., $n = 7$ to 12 per group. ND, nondiabetic control; ND + 400, nondiabetic control rats treated with 400 mg/kg/day nicotinamide; D, untreated diabetic rats; D + 200, diabetic rats treated with 200 mg/kg/day nicotinamide; D + 300, diabetic rats treated with 300 mg/kg/day nicotinamide; and D + 400, diabetic rats treated with 400 mg/kg/day nicotinamide. *, $p < 0.05$ and **, $p < 0.01$ versus ND; †, $p < 0.05$ and ††, $p < 0.01$ versus D.

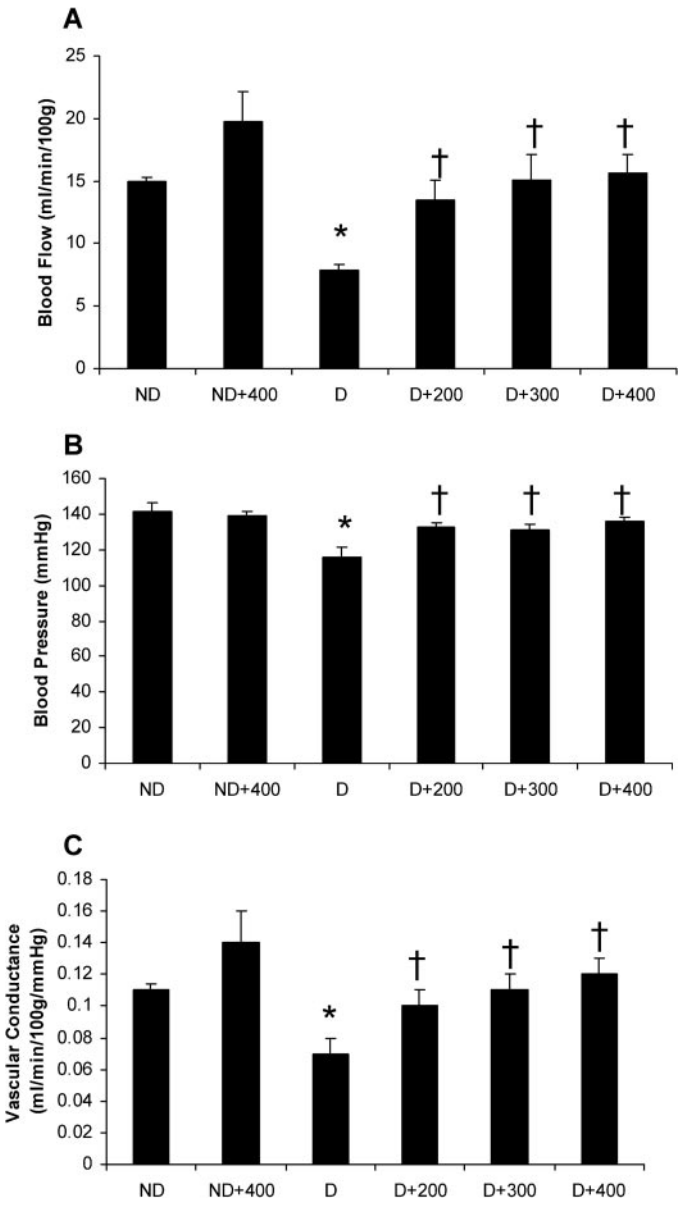

Fig. 2. Sciatic endoneurial blood flow (A), mean systemic blood pressure (B), and endoneurial vascular conductance (C) in nondiabetic and diabetic rats treated with nicotinamide. The results are expressed as mean ± S.E.M., n = 7 to 12 per group. ND, nondiabetic control; ND + 400, nondiabetic control rats treated with 400 mg/kg/day nicotinamide; D, untreated diabetic rats; D + 200, diabetic rats treated with 200 mg/kg/day nicotinamide; D + 300, diabetic rats treated with 300 mg/kg/day nicotinamide; D + 400, diabetic rats treated with 400 mg/kg/day nicotinamide. *, $p < 0.01$ versus ND; †, $p < 0.05$ versus D.

not significantly differ from nondiabetic control rats ($p > 0.05$). Endoneurial vascular conductance (Fig. 2C) was reduced by 30% in diabetic rats compared with controls. Nicotinamide completely reversed this deficit at all of the doses (to 100, 110, and 120% of control values at 200, 300, and 400 mg/kg/day, respectively).

Effect of Diabetes and Nicotinamide on Mechanical and Thermal Algesia. The effects of diabetes and nicotinamide at 400 mg/kg/day on paw withdrawal thresholds in response to noxious stimuli with rigid von Frey filaments and paw withdrawal latencies in response to thermal noxious stimuli are shown in Fig. 3, A and B. The paw withdrawal threshold to noxious mechanical stimuli was reduced by 37%

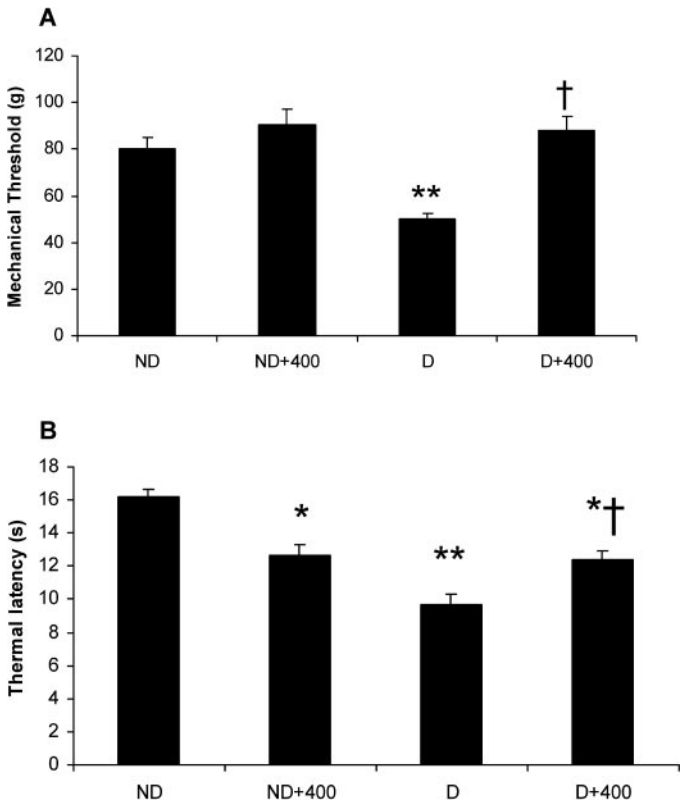

Fig. 3. Hind limb thermal withdrawal latency (A) and mechanical noxious threshold (B) in nondiabetic and diabetic rats treated with nicotinamide. The results are expressed as mean ± S.E.M., n = 6 to 8 per group. ND, nondiabetic control; ND + 400, nondiabetic control rats treated with 400 mg/kg/day nicotinamide; D, untreated diabetic rats; D + 400, diabetic rats treated with 400 mg/kg/day nicotinamide. *, $p < 0.05$; **, $p < 0.01$ versus ND; †, $p < 0.05$ versus D.

in untreated diabetic rats compared with the nondiabetic control group ($p < 0.01$; Fig. 3A), consistent with diabetes-induced mechanical hyperalgesia. Nicotinamide resulted in complete ($p < 0.01$ versus untreated diabetic animals) correction of this variable to levels that were indistinguishable from the nondiabetic control group (110% of nondiabetic control). Although the mechanical withdrawal threshold of nondiabetic control animals treated with nicotinamide was 14% greater than in the untreated nondiabetic group, this difference did not achieve statistical significance.

The paw withdrawal latency in response to the radiant heat stimulus was reduced by 40% ($p < 0.01$) in untreated diabetic rats compared with controls ($p < 0.01$; Fig. 3B). Nicotinamide treatment of diabetic animals resulted in a 42% correction of this deficit ($p < 0.05$). In nicotinamide-treated nondiabetic rats, thermal response latency was reduced by 21% ($p < 0.05$).

Sciatic nerve MDA + 4-HA concentration was increased 1.8-fold ($p < 0.001$) in diabetic rats compared with nondiabetic controls. This accumulation of lipid peroxidation products was entirely prevented by 400 mg/kg/day nicotinamide (Fig. 4).

HNE adduct abundance was increased 1.25-fold in HSC cultured in 30 mM glucose compared with those in 5.5 mM glucose (Fig. 5), and this increase was completely prevented by nicotinamide (Fig. 5). Nicotinamide did not affect HNE adduct expression in HSC cultured in 5.5 mM glucose.

The abundance of PARP-1 protein in HSC was not affected by 30 mM glucose or presence of nicotinamide (Fig. 6, A and

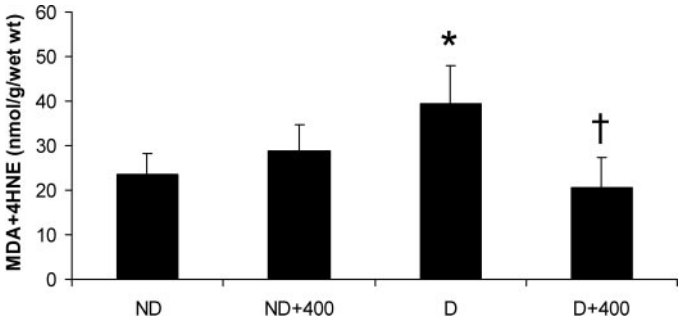

Fig. 4. Sciatic nerve MDA plus 4-HA content in nondiabetic and diabetic rats treated with nicotinamide. The results are expressed as mean ± S.E.M., $n = 6$ to 8 per group. ND, nondiabetic control; ND + 400, nondiabetic control rats treated with 400 mg/kg/day nicotinamide; D, untreated diabetic rats; D + 400, diabetic rats treated with 400 mg/kg/day nicotinamide. *, $p < 0.05$ versus ND; †, $p < 0.05$ versus D.

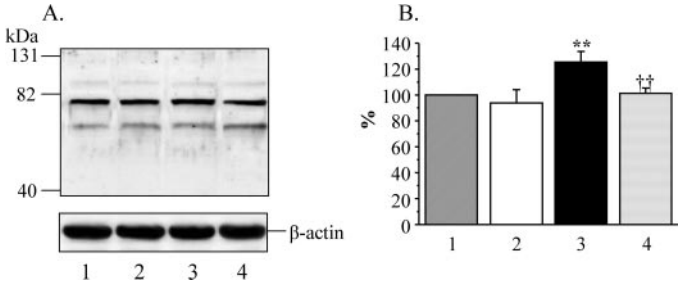

Fig. 5. A, representative Western blot analysis of HSC chemically reduced HNE adducts. Equal protein loading was confirmed with β-actin antibody. Lane 1, 5.5 mM glucose; lane 2, 5.5 mM glucose + 25 mM nicotinamide; lane 3, 30 mM glucose; lane 4, 30 mM glucose + 25 mM nicotinamide. B, chemically reduced HNE adduct content in HSC cultured in 5.5 mM glucose (1), 5.5 mM glucose + 25 mM nicotinamide (2), 30 mM glucose (3), and 30 mM glucose plus 25 mM nicotinamide (4). Chemically reduced HNE adduct content in cells cultured in 5.5 mM glucose is taken as 100%. Mean ± S.E.M., $n = 5$ per group. **, $p < 0.01$ versus cells cultured in 5.5 mM glucose; ††, $p < 0.01$ versus cells cultured in 30 mM glucose without nicotinamide.

B). In contrast, poly(ADP-ribosyl)ated protein abundance was increased 1.7-fold in HSC cultured in high glucose (Fig. 6, C and D), and this increase was reduced by nicotinamide.

Discussion

Growing evidence implicates activation of the nuclear enzyme PARP in the development of DPN (Southan and Szabo, 2003; Obrosova et al., 2004, 2005a). Therefore, a nontoxic pharmacological inhibitor of PARP would seem desirable. We explored the efficacy of various doses of nicotinamide in STZ-D rats. Nicotinamide corrected diabetes-induced sciatic nerve lipid peroxidation and nerve perfusion deficit, and dose-dependently attenuated nerve conduction slowing, as well as mechanical and thermal hyperalgesia. Furthermore, nicotinamide prevented high glucose-induced formation of HNE adducts and poly(ADP-ribosyl)ated proteins in HSC. As a drug with multiple actions that can be administered at high dose to human subjects (Gale et al., 2004), nicotinamide has advantages over other more potent PARP inhibitors, e.g., 3-aminobenzamide (Obrosova et al., 2005a), 1,5-isoquinolinediol (Ilnytska et al., 2006), and PJ34 (Li et al., 2004). Most important is that nicotinamide not only counteracts NAD^+ cleavage by PARP but also acts as physiological NAD^+ precursor that activates glucose utilization in glycolysis and pentose phosphate pathway and counteracts dyslipidemia and gluconeogenesis (Obrosova et al., 1988). Thus, nicotinamide deserves consideration as an attractive, nontoxic therapy for the treatment of diabetic neuropathy.

The beneficial effects of nicotinamide on nerve perfusion are consistent with a key role for PARP activation and oxidative stress in endothelial dysfunction (Garcia Soriano et al., 2001). To evaluate the effect of nicotinamide on sensory hyperalgesia, we utilized an acute 4-week STZ-D rat model. Both thermal and mechanical hyperalgesia typically develop in short-term (4–6 weeks) STZ-induced diabetic rodents (Cameron et al., 2001; Li et al., 2005a) before hypoalgesia subsequently develops at longer time points (Calcutt et al., 2004). This temporal change in thermal sensitivity parallels the pattern of sensory change that has been observed in subjects with diabetes, albeit over a much longer time course (Dyck et al., 2000). This transient thermal hyperalgesia has been postulated to reflect alterations in neuropeptide metabolism (Calcutt et al., 2004) and/or neuronal calcium signaling (Li et al., 2005a), which would provide a mechanistic explanation for the salutary effects of nicotinamide (Shen et al., 2004). The subsequent development of thermal hypoalgesia may reflect impairment of C fiber function secondary to impaired neurotrophic support. In nondiabetic rats, thermal response latency was selectively reduced, and in diabetic rats, there was complete correction of mechanical algesia compared with partial correction of thermal hyperalgesia in response to nicotinamide. Although the mechanism(s) of this effect is unknown, it may reflect differential sensitivity of large myelinated versus small unmyelinated fibers in this animal model.

Diabetes-induced PARP activation has been shown to be both a consequence of increased reactive oxygen species (ROS) and nitrogen species production secondary to DNA single-strand breakage, as well as a contributor to oxidative-nitrosative damage, thereby resulting in a self-perpetuating cycle (Obrosova et al., 2004, 2005a; Southan and Szabo, 2003). Extracellular signal-regulated kinase and p38 mitogen-activated protein kinase (Purves et al., 2001; Agthong and Tomlinson, 2002) function as transducers for the detrimental effects of hyperglycemia in DPN by promoting neurodegeneration (Agthong and Tomlinson, 2002). These signal transducers can be activated by glucose and oxidative stress (Wang et al., 1998; Purves et al., 2001) by a mechanism involving PARP (Veres at al., 2004). Therefore, the properties of nicotinamide that can function as both a superoxide anion radical scavenger and PARP inhibitor would make it an attractive agent to combat these pathogenetic pathways. However, nicotinamide does not directly alter the activity of either p38 or c-Jun N-terminal kinase (Chong et al., 2002), which suggests that its effects may be more distal, such as preventing the activation of caspase-1 and caspase-3. Similar therapeutic effects have been reported for PARP inhibitors used at doses that are unlikely to have direct antioxidant effects (Li et al., 2005b), for potent antioxidants that do not inhibit PARP (Stevens et al., 2000; Coppey et al., 2001), and for weaker antioxidants such as taurine, which also possess calcium-regulating properties in the dorsal root ganglion (DRG) neurons (Li et al., 2005a).

Several lines of evidence suggest that, in addition to axonal atrophy and myelinated fiber loss characteristic for advanced DPN, Schwann cells (SC) contribute to the early axonopathy and painful sensory neuropathy that develop in the absence of demyelination. First, SC contain abundantly expressed aldose reductase playing an important role in both axonopa-

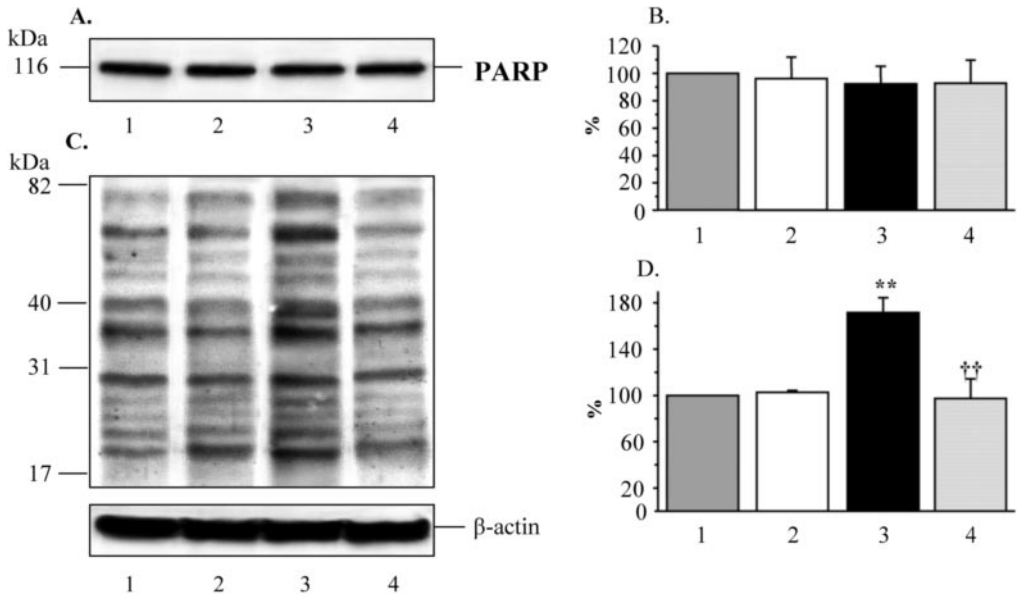

Fig. 6. A, representative Western blot analysis of HSC PARP-1 protein. Equal protein loading was confirmed with β-actin antibody. Lane 1, 5.5 mM glucose; lane 2, 5.5 mM glucose + 25 mM nicotinamide; lane 3, 30 mM glucose; and lane 4, 30 mM glucose + 25 mM nicotinamide. B, total PARP-1 protein content in HSC cultured in 5.5 mM glucose (1), 5.5 mM glucose + 25 mM nicotinamide (2), 30 mM glucose (3), and 30 mM glucose + 25 mM nicotinamide (4). Total PARP-1 protein content in cells cultured in 5.5 mM glucose is taken as 100%. C, representative Western blot of HSC poly(ADP-ribosyl)ated proteins. Equal protein loading was confirmed with β-actin antibody. Lane 1, 5.5 mM glucose; lane 2, 5.5 mM glucose + 25 mM nicotinamide; lane 3, 30 mM glucose; and lane 4, 30 mM glucose + 25 mM nicotinamide. D, total poly(ADP-ribosyl)ated protein content in HSC cultured in 5.5 mM glucose (1), 5.5 mM glucose + 25 mM nicotinamide (2), 30 mM glucose (3), and 30 mM glucose plus 25 mM nicotinamide (4). Total poly(ADP-ribosyl)ated protein content in cells cultured in 5.5 mM glucose is taken as 100%. In B and D, the results are expressed as mean ± S.E.M., $n = 5$ per group. **, $p < 0.01$ versus cells cultured in 5.5 mM glucose; ††, $p < 0.01$ versus cells cultured in 30 mM glucose.

thy (Price et al., 2004) and sensory neuropathy (Calcutt et al., 2004) in diabetes. Second, aldose reductase-containing SC are the main source of ROS and reactive nitrogen species in diabetic peripheral nerve (Obrosova et al., 2005b). Third, the SC is a major site of diabetes-induced PARP activation in the peripheral nervous system (Obrosova et al., 2004). PARP activation contributes to overexpression of inflammatory cytokines [e.g., tumor necrosis factor-α (Ha et al., 2002) implicated in DPN (Gonzales-Clemente et al., 2005)]. Finally, SC play an important role in neurotrophic support by synthesizing nerve growth factor, ciliary neurotrophic factor, as well as desert hedgehog protein (Matsuoka et al., 1991; Johann et al., 1993; Mirsky et al., 1999). Diabetes-associated deficits of these factors have been implicated in axonopathy and/or sensory neuropathy and neuropathic pain (Calcutt, 2002; Calcutt et al., 2003). All of these findings provide the rationale for exploring metabolic disturbances in SC, for a better insight into pathogenetic mechanisms of axonopathy and sensory neuropathy associated with diabetes.

In our previous studies, HSC exposed to 30 mM glucose displayed increased superoxide production, accumulation of poly(ADP-ribosyl)ated and nitrosylated proteins, and a PARP inhibitor sensitive induction of inducible nitric oxide synthase expression (Obrosova et al., 2005a). Although HSC exposure to 30 mM glucose does not precisely mimic mild hyperglycemia in human diabetes mellitus, it is highly unlikely that interrelationships among different pathogenetic mechanisms identified at 30 mM glucose concentrations disappear or become opposite at more physiological 7 to 12 mM glucose concentrations. It would also take much longer for any metabolic abnormality to develop in such "mild" conditions, considering that even diabetic rodents with blood glucose concentrations of 20 to 30 mM do not develop clearly manifest biochemical changes in tissue sites for diabetic complications immediately after development of hyperglycemia. For example, first signs of oxidative stress in the peripheral nerve develop approximately 10 days after achievement of hyperglycemia in STZ-D rats (I. G. Obrosova, unpublished observations). Therefore, relatively mild hyperglycemia is probably responsible for the slow development of diabetic neuropathy and other complications in human subjects over a number of years compared with diabetic rodents. For these reasons, and because of time limitations associated with any cell culture experiment, in vitro studies of mechanisms underlying diabetic complications are usually performed in cells, i.e., SC (Minea et al., 2002) and sensory neurons (Purves et al., 2001) and cultured in 25 to 30 mM glucose.

High glucose-induced accumulation of poly(ADP-ribosyl)ated proteins and HNE adducts in HSC was alleviated by nicotinamide, which is consistent with reports of its neuroprotective effect in neural cell lines exposed to high glucose concentrations or hypoxia/reoxygenation (Shen et al., 2004; Obrosova et al., 2005a). In rat primary cortical neurons, nicotinamide reduced lactate dehydrogenase release, ROS production, calcium influx, caspase-3 activation, and cell injury after oxygen-glucose deprivation and reoxygenation (Shen et al., 2004). In DRG neurons exposed to high (45 mM) glucose, nicotinamide reduced superoxide and hydrogen peroxide production and dose-dependently counteracted glucose-mediated (or hydrogen peroxide-induced) cell death (Vincent et al., 2005). Interestingly, recent evidence suggests that the cytoprotection afforded by nicotinamide may be concentration-dependent in both animal and cell culture models (Yang et al., 2002; Veres et al., 2004). In high glucose-exposed DRG neurons, 12.5 mM nicotinamide was required to prevent increased superoxide production (Vincent et al., 2005), and the

neuroprotective concentrations ranged from 10 to 25 mM. The present studies in high glucose-exposed HSC showed inhibition of lipid peroxidation by 25 mM nicotinamide, consistent with the construct that various concentrations of this agent are required to inhibit oxidative stress originating from different sources and in different cell types. Therefore, our data suggest that, in diabetes, nicotinamide could exert a direct neuroprotective effect in both neurons and glial cells, which complements its ability to prevent neurovascular dysfunction, thereby providing an effective treatment for DPN.

In conclusion, nicotinamide attenuated nerve lipid peroxidation in STZ-D rats in vivo and in HSC in vitro and was effective in combating neurovascular dysfunction, nerve conduction deficits, and abnormal sensory responses in early DPN. The relatively low toxicity of nicotinamide compared with other PARP inhibitors suggests that it deserves consideration as a candidate drug for treatment of DPN. However, the ability of considerably more potent antioxidant therapy to reverse clinical diabetic neuropathy has proved disappointing, highlighting the potential importance of the additional metabolic actions of nicotinamide. Those probably include, but may not be limited to, improvement of energy metabolism as a result of increased NAD biosynthesis, partial inhibition of PARP, and associated decrease in neuronal Ca^{2+} accumulation and glutamate excitotoxicity. The potential of the combination therapy including nicotinamide, the xanthine oxidase inhibitor allopurinol, and the potent antioxidant and metabolic enhancer DL-α-lipoic acid to reverse established neuropathy complicating diabetes is currently being evaluated in a 2-year clinical trial.

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