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Original Contribution

PARP inhibition alleviates diabetes-induced systemic oxidative stress and neural tissue 4-hydroxynonenal adduct accumulation: Correlation with peripheral nerve function

Sergey Lupachyk, Hanna Shevalye, Yury Maksimchyk, Viktor R. Drel, Irina G. Obrosova *

Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, LA 70808, USA

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ABSTRACT

This study evaluated the role of poly(ADP-ribose) polymerase (PARP) in systemic oxidative stress and 4-hydoxynonenal adduct accumulation in diabetic peripheral neuropathy. Control and streptozotocin-diabetic rats were maintained with or without treatment with the PARP inhibitor, 1,5-isoquinolinediol, 3 mg kg^{-1} day^{-1}, for 10 weeks after an initial 2 weeks. Treatment efficacy was evaluated by poly(ADP-ribosyl)ated protein content in peripheral nerve and spinal cord (Western blot analysis) and dorsal root ganglion neurons and nonneuronal cells (fluorescence immunohistochemistry), as well as by indices of peripheral nerve function. Diabetic rats displayed increased urinary isoprostane and 8-hydroxy-2′-deoxyguanosine excretion (ELISA) and 4-hydroxynonenal adduct accumulation in endothelial and Schwann cells of the peripheral nerve, neurons, astrocytes, and oligodendrocytes of the spinal cord and neurons and glial cells of the dorsal root ganglia (double-label fluorescence immunohistochemistry), as well as motor and sensory nerve conduction velocity deficits, thermal hypoalgesia, and tactile allodynia. PARP inhibition counteracted diabetes-induced systemic oxidative stress and 4-hydroxynonenal adduct accumulation in peripheral nerve and spinal cord (Western blot analysis) and dorsal root ganglion neurons (perikarya, fluorescence immunohistochemistry), which correlated with improvement of large and small nerve fiber function. The findings reveal the important role of PARP activation in systemic oxidative stress and 4-hydroxynonenal adduct accumulation in diabetic peripheral neuropathy.

Diabetic peripheral neuropathy (DPN) affects at least 50% of diabetic subjects with both type 1 and type 2 diabetes mellitus in the United States and is the leading cause of foot amputation [1–4]. Pathogenetic mechanisms of DPN include, but are not limited to, activation of aldose reductase [5–8], the advanced glycation end-product/receptor for advanced glycation end-product axis [8–10], protein kinase C [6,11], and mitogen-activated protein kinases [12,13]; changes in arachidonic acid metabolism [14,15]; and impaired neurotrophic support [16–18]. Many of these mechanisms have been identified in both vascular endothelium and neural elements [dorsal root ganglion (DRG) neurons, Schwann cells] of the peripheral nerve as well as in the spinal cord.

Growing evidence suggests the important contribution of oxidative stress and poly(ADP-ribose) polymerase (PARP) activation to endoneurial nutritive blood flow and motor and sensory nerve conduction velocity (MNCV and SNCV) deficits, sensory neuropathy, and large and small sensory nerve fiber degeneration [19–25]. Both phenomena have been documented in the peripheral nervous system of animal models of type 1 and type 2 diabetes as well as prediabetes (reviewed in [26]). In the best-studied animal model for DPN, i.e., the streptozotocin (STZ)-diabetic rat, oxidative stress and poly(ADP-ribosyl)ation have been identified in neurons and Schwann cells of the peripheral nerve and in the vasa nervorum [21,22,25,27–29]. Furthermore, both oxidative stress and PARP activation were clearly manifest in the microvasculature of human subjects with diabetes mellitus [30]. For many years, free radical- and oxidant-induced DNA single-strand breakage was considered an obligatory step for PARP activation [31]. However, recent studies, including those related to diabetic complications, suggest that (1) PARP is also activated by metabolic factors, e.g., through phosphorylation by extracellular signal-regulated kinase [32], and (2) diabetes-induced poly(ADP-ribosyl)ation is clearly manifest in tissues in which DNA single-strand breakage is negligible or totally absent, e.g., Schwann cells of the peripheral nerve [22,33]. It has also been shown that PARP activation induced by both high glucose and nonesterified fatty acids, two major detrimental factors in the diabetic milieu, precedes and causes oxidative stress in a number of

Abbreviations: DPN, diabetic peripheral neuropathy; DRG, dorsal root ganglion; 4-HNE, 4-hydroxynonenal; ISO, 1,5-isoquinolinediol; MDA, malondialdehyde; MNCV, motor nerve conduction velocity; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PNS, peripheral nervous system; ROS, reactive oxygen species; SNCV, sensory nerve conduction velocity; STZ, streptozotocin.

* Corresponding author. Fax: +1 225 763 0274.
E-mail address: obrosoig@pbrc.edu (I.G. Obrosova).

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cell targets for diabetic complications, including Schwann cells of the peripheral nerve [34], retinal pericytes and endothelial cells [35], and renal podocytes [36]. The aforementioned studies have predominantly been performed in in vitro cultured cells and used cell-permeable agents generating fluorescent compounds in reactions with intracellular reactive oxygen species (2′,7′-dichlorodihydrofluorescein diacetate, hydroethidine) for oxidative stress evaluation. Note that the inter-experiment reproducibility of such measurements is quite low.

To further explore the relations between oxidative stress and PARP activation in diabetes, this in vivo study evaluated the effects of the potent and specific PARP inhibitor 1,5-isoquinolinediol on systemic oxidative stress and 4-hydroxynonenal protein adduct accumulation in tissue targets for DPN, i.e., peripheral nerve, spinal cord, and DRG neurons. We employed a well-studied STZ-diabetic rat model, which displays clearly manifest systemic and neural oxidative stress [21,23,25,27–29,37–39], and reliable quantitative techniques for evaluation of oxidative injury.

Methods

Reagents

Unless otherwise stated, all chemicals were of reagent-grade quality and were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Mouse monoclonal anti-poly(ADP-ribose) was purchased from Trevigen, Inc. (Gaithersburg, MD, USA), and rabbit polyclonal anti-4-hydroxynonenal–Michael adduct (4-HNE adduct) antibody was purchased from Calbiochem (San Diego, CA, USA), mouse monoclonal anti-S-100 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal anti-glutamine synthetase, anti-NeuN, and anti-glial fibrillary acidic protein antibodies were obtained from Chemicon (Billerica, MA, USA) and mouse monoclonal anti-APC-Ab7 from Calbiochem. Isolectin GS-IB4 from *Griffonia simplicifolia,* Alexa Fluor 594 conjugate, goat anti-rabbit Alexa Fluor 488, goat anti-mouse Alexa Fluor 594, and Prolong Gold antifade reagent were purchased from Molecular Probes (Eugene, OR, USA). Avidin/biotin blocking kit, Vectastain Elite ABC kit (Standard*), DAB substrate kit, and 3,3′-diaminobenzidine were obtained from Vector Laboratories (Burlingame, CA, USA). Other reagents for immunohistochemistry were purchased from Dako Laboratories (Santa Barbara, CA, USA).

Animals

The experiments were performed in accordance with regulations specified by the National Institutes of Health *Principles of Laboratory Animal Care,* 1985 revised version, and the Pennington Biomedical Research Center *Protocol for Animal Studies.* Male Wistar rats (Charles River, Wilmington, MA, USA), body weight 250–300 g, were fed a standard rat chow (PMI Nutrition Int., Brentwood, MO, USA) and had access to water ad libitum. STZ diabetes was induced as described [22]. Blood samples for glucose measurements were taken from the tail vein ~48 h after the STZ injection and the day before the study termination. All rats with blood glucose levels ≥13.8 mM were considered diabetic. Diabetic rats were maintained on suboptimal doses of insulin (~1–2 U every second day) to prevent ketoacidosis and weight loss. The experimental groups comprised control and diabetic rats treated with or without the PARP inhibitor 1,5-isoquinolinediol (ISO), 3 mg kg^{-1} day^{-1} ip, for 10 weeks after the first 2 weeks without treatment. An initial 2-week period without treatment was introduced to avoid β-cell regeneration and alleviation of hyperglycemia, which is known to occur when PARP inhibitors are administered together with streptozotocin or shortly after induction of diabetes. At the end of the study, the rats were placed in individual metabolic cages (Lab Products, Inc., Seaford, DE, USA) and urine was collected for 24 h. Urine specimens were centrifuged at 12,000 *g* (4 °C, 10 min) and frozen for subsequent assessment of 8-isoprostane and 8-hydroxy-2′-deoxyguanosine by ELISA.

Anesthesia, euthanasia, and tissue sampling

The animals were sedated with CO_2 and immediately sacrificed by cervical dislocation. Sciatic nerves and spinal cords were rapidly frozen in liquid nitrogen and stored at −80 °C before assessment of poly(ADP-ribosyl)ated proteins and 4-HNE adducts by Western blot analyses. A portion of the sciatic nerves and spinal cords as well as DRG from the untreated diabetic group was fixed in 4% formalin and later used for 4-HNE-adduct localization to individual cell types in the peripheral nervous system (PNS), using double-label fluorescence immunohistochemistry with specific cell markers, as well as extra-nuclear and intranuclear poly(ADP-ribose) localization (sciatic nerve only). The rest of the 4% formalin-fixed DRG was used for assessment of 4-HNE-adduct and poly(ADP-ribose) abundance by fluorescence immunohistochemistry.

Specific methods

Motor and sensory nerve conduction velocities

Sciatic motor and hind-limb digital sensory nerve conduction velocities (MNCV and SNCV) were assessed at three time points, i.e., at the beginning of the study (before induction of diabetes), at 2 weeks (before interventions), and at 12 weeks (final measurements), as we described previously [22].

Behavioral tests

Assessment of thermal algesia (by paw-withdrawal latencies), mechanical algesia (rigid von Frey filament test), and tactile allodynia (flexible von Frey filament test) was performed at three time points, i.e., at the beginning of the study (before induction of diabetes), at 2 weeks (before interventions), and at 12 weeks (final measurements), as we described previously [22–24].

Western blot analysis of 4-HNE adducts and poly(ADP-ribosyl)ated proteins in sciatic nerve and spinal cord

Western blot analysis of 4-HNE adducts and poly(ADP-ribosyl)ated proteins in sciatic nerve and spinal cord was performed as we described in detail previously [15,25]. We employed 5–17% sodium dodecyl sulfate–polyacrylamide gels, and the electrophoresis was conducted for 2 h. Protein bands were visualized with the BM chemiluminescence blotting substrate (POD; Roche, Indianapolis, IN, USA). Membranes were then stripped and reprobed with β-actin antibody to verify equal protein loading. The data were quantified by densitometry (Quantity One 4.5.0 software; Bio-Rad Laboratories, Richmond, CA, USA).

Fluorescence immunohistochemistry of 4-HNE adducts and poly(ADP-ribose) in DRG

All sections were processed by a single investigator and evaluated blindly. 4-HNE-adduct and poly(ADP-ribose) abundance was assessed by fluorescence immunohistochemistry as described [25]. Low-power observations of DRG sections stained for 4-HNE adducts and poly (ADP-ribose) were made using a Zeiss Axioplan 2 imaging microscope. Color images were captured with a Photometric CoolSNAPTM HQ CCD camera at 1392×1040 resolution. Low-power images were generated with a 40× Acroplan objective using the RS Image 1.9.2 software. 4-HNE-adduct and poly(ADP-ribose) fluorescence intensity of individual DRG neurons was quantified using the ImageJ 1.43q software (National Institutes of Health, Bethesda, MD, USA) and normalized per neuronal area. Because of highly variable size of individual nuclei and low nuclear HNE-adduct fluorescence, the latter was subtracted from total neuronal HNE-adduct fluorescence. For assessment of 4-HNE-adduct and poly(ADP-ribose) fluorescence, 43–