Glucagon-Like Peptide 1, Insulin, Sensory Neurons, and Diabetic Neuropathy

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Abstract

Like insulin, glucagon-like peptide 1 (GLP-1) may have direct trophic actions on the nervous system, but its potential role in supporting diabetic sensory neurons is uncertain. We identified wide expression of GLP-1 receptors on dorsal root ganglia sensory neurons of diabetic and nondiabetic mice. Exendin-4, a GLP-1 agonist, increased neurite outgrowth of adult sensory neurons in vitro. To determine the effects of exendin-4 in comparison with continuous low- or high-dose insulin in vivo, we evaluated parallel cohorts of type 1 (streptozotocin-induced) and type 2 (db/db) mice of 2 months’ diabetes duration with established neuropathy during an additional month of treatment. High-dose insulin alone reversed hyperglycemia in type 1 diabetic mice, partly reversed thermal sensory loss, improved epidermal innervation but failed to reverse electrophysiological abnormalities. Exendin-4 improved both sensory electrophysiology and behavioral sensory loss. Low-dose insulin was ineffective. In type 2 diabetes, hyperglycemia was uncorrected, and neither insulin nor exendin-4 reversed sensory electrophysiology, sensory behavior, or loss of epidermal axons. However, exendin-4 alone improved motor electrophysiology. Receptor for advanced glycosylated end products and nuclear factor-κB neuronal expression were not significantly altered by diabetes or treatment. Taken together, these results suggest that although GLP-1 agonists and insulin alone are not effective, exendin-4 in combination with insulin might benefit some aspects of established diabetic neuropathy.

Key Words: Diabetes mellitus, Diabetic neuropathy, Exendin-4, Glucagon-like peptide 1, Insulin, Polyneuropathy.

INTRODUCTION

The direct neuronal impacts of the incretin hormone glucagon-like peptide 1 (GLP-1) are of interest in the development of diabetic polyneuropathy. GLP-1, like insulin, may offer direct trophic actions to neurons. For example, GLP-1 enhances nerve growth factor (NGF)-induced neurite outgrowth and cell survival in neuron-like pheochromocytoma (PC12) cells (1). The GLP-1 receptor has been identified on vagus nodose ganglia neurons (2). GLP-1 may also affect other CNS functions, including learning and memory (3), glutamate neurotoxicity (4), kainite-induced seizures, and hippocampal neuronal degeneration (3). Studies also suggest that it protects against pyridoxine sensory neuropathy (5) and some indices of diabetic neuropathy in streptozotocin (STZ) rats (6-8). GLP-1 is rapidly inactivated by dipeptidyl peptidase-4 (9), whereas the GLP-1 agonist exendin-4, isolated from Heloderma suspectum, is resistant to cleavage and has a longer half-life (10).

An untested assumption in diabetic polyneuropathy is that neuropathic deficits are fully reversible by a gold standard, insulin therapy. Human diabetic subjects are often confronted with signs and symptoms of neuropathy before deciding whether more aggressive insulin therapy may be warranted. It is uncertain whether insulin resistance also holds for neuropathic complications in type 2 diabetes. Insulin also has neurotrophic properties, and there has been a recent debate about the doses that are required to reverse neuropathic deficits.

Here, we explore the expression and function of GLP-1 receptors on adult peripheral sensory neurons and examined the role of exendin-4 in murine models of experimental types 1 and 2 diabetes. The findings indicated robust expression of GLP-1 receptors in nondiabetic and diabetic sensory neurons, increased neurite outgrowth, and impacts on experimental diabetes in response to a GLP-1 agonist; these effects differed from those of insulin.

MATERIALS AND METHODS

Mice

The protocols have been reviewed and approved by the University of Calgary Health Sciences Animal Care Committee following guidelines by the Canadian Council of Animal Care. All mice were housed in metabolic air-filtered cages in a 12-hour light cycle facility. Standard mouse diet (Pico-Vac, Brentwood, MO) and water were provided ad libitum. Swiss Webster mice, 4 weeks of age and weighing 19 to 21 g, were purchased from Charles River (Wilmington, MA). At 6 weeks of age, mice were made type 1 diabetic (n = 30) using a 3-day intraperitoneal STZ injection protocol (85, 70, and...
FIGURE 1. Glucagon-like peptide 1 (GLP-1) receptor expression in L5 dorsal root ganglia (DRG) neurons from diabetic and non-diabetic mice. (A, B) The DRG with GLP-1 receptor expression are shown in type 1 (A) and type 2 (B) diabetic mice and their respective controls. (C, D) Semiquantitative analysis of the intensity of protein expression by their luminosity in types 1 (C) and 2 (D) diabetic mice and their respective controls. In both diabetes models, there was a shift toward more intense expression of the receptor. This was significant for all categories of staining intensity in type 2 diabetes, whereas fewer negative neurons were stained in diabetic mice in type 1 diabetes (* p < 0.05 diabetic vs nondiabetics; Student t-test; n = 3/group). Scale bar = 100 μm.
Type 1 diabetes

FIGURE 2. Impact of exendin-4 on sensory neuron outgrowth. (A–D) Exposure of adult sensory neurons harvested and cultured in vitro to control conditions (A, B) or with inclusion of exendin-4 (C, D), a glucagon-like peptide 1 (GLP-1) agonist in the media for 18 hours. (E) Quantitation of neurite outgrowth showed an increase in exendin-4–exposed neurons (* p < 0.05, paired Student t-test). Scale bar = 50 μm.

55 mg/kg; dissolved in citrate buffer, pH 4.5). Control mice (n = 10) were injected with citrate buffer alone. Diabetes (defined as fasting glucose ≥16 mmol/L) was confirmed 7 to 10 days after the first injection. After diabetes confirmation, mice were left untreated for 2 months. Treatment began at 14 weeks of age and continued for 4 weeks. Behavioral and nerve conduction studies were repeated before and after treatment.

Type 2 diabetes

FIGURE 3. Hyperglycemic control in type 1 and type 2 diabetic mouse models. (A, B, D, E) Baseline blood glucose levels after 7 to 8 hours of fasting of control nondiabetic □ and diabetic animals □ before treatment in type 1 (A) and type 2 (D) mice. Fasting blood glucose levels of control □, high dose □, low dose □, untreated diabetics □, and exendin-4 □ treated type 1 (B) and type 2 (E) diabetic experimental groups from left to right during treatment 1 day before harvest. Exendin-4 had a mild impact on hyperglycemia in type 1 diabetic animals but not type 2 diabetic animals. Only titrated high-dose type 1 diabetic animals received enough insulin to lower their fasting glucose levels to normoglycemia ([A] * control nondiabetic vs diabetic, p < 0.0001; [B] ** type 1 diabetic control vs low dose, untreated, or exendin-4, p < 0.001; * low dose vs untreated diabetes or exendin-4, p < 0.05) ([D] * control nondiabetic vs diabetic, p < 0.0001; [E] * type 2 diabetic control vs high dose, low dose, untreated, and exendin-4, p < 0.0001). (C, F) Percentage of glycated hemoglobin in type 1 (C) and type 2 (F) mice at treatment end point. High-dose insulin but not exendin-4 did not significantly alter levels of glycated hemoglobin. * Type 1 diabetic control versus high dose, low dose, untreated diabetes, and exendin-4, p < 0.0001; high dose vs low dose and untreated diabetes, p < 0.001. * Type 2 diabetic control versus high dose, low dose, untreated, and exendin-4, p < 0.0001. Values are mean ± SEM. One-way analysis of variance with Tukey post hoc test and Student t-test was used in these analyses.
FIGURE 4. Electrophysiology in type 1 diabetic mice. (A) Motor nerve conduction velocity before treatment (control vs diabetes, \( p < 0.0001 \)). (B) Motor nerve conduction velocity after insulin and exendin-4 treatment. There was a nonsignificant trend toward improved motor conduction velocity with exendin-4 therapy (control vs high dose, low dose, untreated, and exendin-4 treatments, \( p < 0.0001 \)). (C) Sensory nerve conduction velocity before treatment (control vs diabetes, \( p < 0.0001 \)). (D) Sensory nerve conduction velocity after insulin and exendin-4 treatment. Note that exendin-4, but not insulin, reversed declines in conduction velocity (control vs high dose, low dose, and untreated diabetes, \( p < 0.0001 \); untreated diabetes vs exendin-4, \( p < 0.01 \)). Control nondiabetic (\( g \), A–D); type 1 diabetic animals before treatment (\( g \), A, C); high dose (\( h \), B, D), low dose (\( m \), B, D), untreated diabetic (\( b \), B, D), and exendin-4 (\( g \), B, D) treated experimental groups. Values are mean ± SEM. One-way analysis of variance with Tukey post hoc test and Student t-test as appropriate was used in these analyses.
FIGURE 5. Electrophysiology in type 2 diabetic mice. (A) Motor nerve conduction velocity before exendin-4 and insulin treatment (* control vs diabetes, \( p < 0.0001 \)). (B) Motor nerve conduction velocity after insulin and exendin-4 treatment. Note that exendin-4, but not insulin, reversed slowing of motor conduction velocity in type 2 diabetic mice (*** control vs high dose, low dose, and untreated diabetes, \( p < 0.001 \); * untreated diabetes vs exendin-4, \( p < 0.01 \)). (C) Sensory nerve conduction velocity before treatment (* control vs diabetes, \( p < 0.0001 \)). (D) Sensory nerve conduction velocity after insulin and exendin-4 treatment. There was a nonsignificant trend toward improvement in sensory conduction velocity in mice treated with exendin-4 and no improvement with insulin (* control vs high dose, low dose, untreated, and exendin-4, \( p < 0.0001 \)). Control nondiabetic (□, A-D); type 1 diabetic animals before treatment (□, A, C); high dose (■, B, D); low dose (□, B, D); untreated diabetic (□, B, D); and exendin-4 (□, B, D) treated experimental groups. Values are mean ± SEM. One-way analysis of variance with Tukey post hoc test and Student t-test as appropriate was used in these analyses.
Ten-week-old C57BKS Dock7m+/+Leprdb/J mice (n = 30) and 9-week-old control C57BLKS/J mice (n = 10) were purchased from Jackson Laboratories (Bar Harbor, ME). Diabetes (defined as fasting glucose ≥16 mmol/L) was confirmed on arrival. Treatment began at 14 weeks of age and continued for 4 weeks. Behavioral studies and electrophysiology were repeated before and after treatment. The STZ and db/db mice (n = 8) underwent a 4-week exendin-4 treatment period starting at 14 weeks of age. Mice were treated with daily subcutaneous injections of 24 nmol/kg of exendin-4 (Bachem Americas,
Torrance, CA). Mice were also divided among high-dose insulin, low-dose insulin, and no treatment groups. LinBit (LinShin Canada, Toronto, Canada) insulin implants were used to provide sustained and controlled release of insulin subcutaneously (each pellet releases 0.1 IU per day). Treatment began at 14 weeks of age and continued for 4 weeks.

Animals were killed using isoflurane anesthesia followed by cervical dislocation. Footpad samples were fixed in paraformaldehyde/lysine/periodate and embedded in optimum cutting temperature; dorsal root ganglia (DRG) were placed in modified Zamboni fixative and embedded in optimum cutting temperature for immunohistochemistry. Sural nerves were harvested, processed, and analyzed as previously described (11).

**In Vitro Studies**

Adult male Sprague-Dawley rats (180–200 g) were anesthetized with isoflurane (Abbott Laboratories) before DRG harvesting. The DRG neurons were dissociated and maintained in vitro, as modified from Lindsay (12). Briefly, the L4 to L6 DRG were harvested from adult Sprague-Dawley rats and placed into L15 (Invitrogen, Burlington, Canada) medium, as previously described (13). Cells were placed onto poly-L-lysine (Sigma-Aldrich, St Louis, MO) and 10 μg/mL mouse laminin (Invitrogen)–coated plates. At the time of plating, a 20-nM concentration of exendin-4 (Sigma) was added to the culture medium in the treatment group. The cells were grown for 18 hours and then fixed and processed for immunocytochemistry. The sensory neurons and neurites were labeled using primary antibody against mouse NF-200 (1:800; Sigma). The secondary antibody used was anti-mouse immunoglobulin G (IgG) CY3 conjugate (1:100; Sigma) and counterstained with 4′,6-diamidino-2-phenylindole for nuclei. Total neurite outgrowth was analyzed and quantified by MetaXpress software (Molecular Devices, Sunnyvale, CA) by an observer blinded to its condition. The MetaXpress program localized cell bodies and quantified neurite outgrowth using Adobe Photoshop (San Jose, CA). The DRG neurons from the same harvesting were paired with or without exendin-4 treatment.

**Electrophysiological and Behavioral Sensory Measurements**

Multifiber motor and sensory electrophysiology were carried out under isoflurane anesthesia, as previously described (14). Motor sciatic nerve conduction recordings were carried out using stimulation at the sciatic notch and knee with recording over interosseous foot muscles. Sensory sciatic nerve conduction recordings were carried out by stimulation in the toes and recording at the knee. Near-nerve temperatures were maintained at 37°C ± 0.5°C. Examiners were blinded to the identity of the groups.

Before and after exendin-4 treatment, thermal sensitivity testing using the Hargreaves apparatus (15) was completed to assess the latency to withdraw from a thermal stimulus. In this test, the heating rate was ramped from 30°C to 58°C for 60 seconds in a consistent fashion. Mice were allowed to habituate to the testing environment 1 week before recording and were allowed to acclimate for at least 5 minutes before testing. The thermal stimulus was presented to 1 foot at a time (maximum, 30 seconds); the time for withdrawal was recorded in 3 separate trials.

Sensitivity to a mechanical stimulus was tested using a dynamic plantar aesthesiometer. The mice stood on an elevated wire mesh platform that allows for filaments to be inserted against the hind limbs through the holes of the mesh. Using a 12-mm filament, the aesthesiometer increased the force applied to the animal at a rate of 2 g per second. At the threshold force, the animal would quickly withdraw its paw from the filament. Three recordings were taken per animal, and a mean latency and threshold force were calculated. Examiners were blinded to the identity of the groups.

**Immunohistochemistry**

The L4 and L5 DRG were sectioned into 8-μm sections and immunostained for GLP-1 receptor. Secondary antibody Alexa Flour 488 goat anti-rabbit IgG (diluted 1:200) was used to identify stained sections for all primary antibodies. Sections were imaged at 20× objective magnification using fluorescent microscopy and imaged at 1,300 × 1,030 (scanned resolution). Sections were counted and quantified by their luminosity using Adobe Photoshop (San Jose, CA). The DRG sections were also stained with nuclear factor κ-light-chain enhancer of activated B cells (NF-κB), receptor for advanced glycosylation end products (RAGE), and insulin receptor β polyclonal primary antibodies. Antibody sources and dilutions were as follows: NF-κB p-50 (at 1:200; H-119, rabbit polyclonal IgG; Santa Cruz Biotechnology, Santa Cruz, CA); GLP-1 receptor (1:50; rabbit polyclonal primary antibody; Abcam, Cambridge, MA); RAGE (1:800; rabbit polyclonal antibody, ab3611; Abcam); insulin receptor β subunit (1:100; sc-711, insulin receptor-β rabbit polyclonal IgG; Santa Cruz Biotechnology). Secondary antibodies were Alexa Flour 488 and 594 (Invitrogen Biotechnology). Examiners were blinded to the identity of the groups.

**FIGURE 6.** Thermal withdrawal latencies for type 1 and type 2 diabetic mice. (A) Control and diabetic animals withdrawal latency before insulin and exendin-4 treatment (*p < 0.0001). Type 1 diabetic mice had a rise in thermal withdrawal latency indicating loss of thermal sensation. (B) Control, untreated type 1 diabetic animals, insulin- and exendin-4-treatment withdrawal latency after treatment. Exendin-4 treatment was associated with an improvement in thermal sensation in diabetic mice; insulin was associated with some degree of improvement in sensation, albeit incomplete (* control vs low dose and untreated diabetes, p < 0.0001; * untreated vs high dose insulin or exendin-4 treated, p < 0.05). (C) Control and type 2 diabetic animals’ withdrawal latency before insulin and exendin-4 treatment. No difference in thermal sensitivity was identified at this time point. (D) Control, untreated type 2 animals, and insulin- and exendin-4-treated withdrawal latency after treatment. In untreated diabetics, there was a nonsignificant trend toward greater sensitivity (hyperalgesia) to thermal stimulation. This was significant in exendin-4–treated mice (* control vs exendin-4 treated, p = 0.001). Control nondiabetic (CI, A–D); type 1 diabetic animals before treatment (II, A, C); high dose (II, B, D); low dose (II, B, D); untreated diabetic (II, B, D); and exendin-4 (II, B, D) treated experimental groups. Values are mean ± SEM. One-way analysis of variance with Tukey post hoc test and Student t-test as appropriate was used in these analyses.
goat anti rabbit IgG (1:200; Invitrogen). Neurons (300–500/mouse) were imaged at 20× using fluorescent microscopy and photographed at a resolution of 1,300 × 1,030 (scanned). Sections were then counted and analyzed using quantitative luminosity in Adobe Photoshop CS3. Examiners were blinded to the identity of the groups.

Footpad samples were sectioned at 25 μm. Rabbit polyclonal antibody to ubiquitin C-terminal hydrolase 1 (diluted
1:1000; primary antibody PGP 9.5; EnCor Biotechnology, Gainesville, FL) was used to label intraepidermal nerve fibers. Cy3-conjugated AffiniPure Fab Fragment Goat Anti-Rabbit IgG (H + L) secondary antibody (Jackson ImmunoResearch, West Grove, PA) (diluted 1:100) was used to identify stained sections. Sections were imaged using an Olympus laser scanning confocal microscope equipped with epifluorescence at 100× magnification; resolution at 512 × 512 and scanning step size 1 μm. Intraepidermal nerve fiber density and area were quantified as follows: vertically directed (oriented approximately 90 degrees to the surface of the skin) and total fibers (oriented in all directions) were separately counted to capture all potential epidermal branches. The approach has been internally consistent in our laboratory (16, 17) and differed from current human studies that require axons to cross the dermal-epidermal junction before counting and that yield lower numbers of axon profiles (18). All studies were carried out with the examiner blinded to the group identity of the samples.

Analysis

Values were reported as mean ± SEM. Experimental conditions were compared with a 1-way analysis of variance followed by Tukey post hoc test and Student t-test as appropriate.

RESULTS

GLP-1 Receptors Are Expressed in Sensory Neurons of Diabetic and Nondiabetic Mice and Mediate Neurite Outgrowth

A large proportion of L5 DRG neurons expressed the GLP-1 receptor. Expression was identified in both diabetic and nondiabetic mice and was mainly cytoplasmic, sparing nuclei and without prominent satellite cell staining (Figs. 1A–D). In a blinded semiquantitative analysis of neuron luminosity of both type 1 and type 2 diabetes, there was a trend in expression toward greater intensities in the diabetic mice compared with their respective controls (Figs. 1E, F). However, this trend was not confirmed in studies of whole ganglion lysates analyzed by Western immunoblot in further samples from type 1 diabetic mice (data not shown).

Exendin-4 was associated with increased neurite outgrowth, confirming the concept that its direct action through GLP-1 receptors offers trophic support to adult sensory neurons (Figs. 2A, B).

Because in vitro approaches, including those with varying culture glucose conditions, do not necessarily predict an impact on experimental neuropathy, we next tested human therapeutic doses of exendin-4 in 2 different established models of diabetes in comparison with insulin.

Exendin-4 and Insulin Effects on Hyperglycemia

Exendin-4 was associated with a borderline lowering of glucose during the 4-week treatment period and a non-significant trend toward lowering of glycated hemoglobin in type 1 diabetic mice (Figs. 3A–C). In contrast, there was no impact on hyperglycemia or glycated hemoglobin in mice with type 2 diabetes (Figs. 3D–F). Given these minor changes, it is unlikely that exendin-4 altered neuropathy through its actions on hyperglycemia.

We next examined whether continuous insulin therapy might reverse established hyperglycemia in type 1 and type 2 diabetic mice. In one cohort of each of the type 1 and type 2 mice, we provided 0.1 IU daily as low-dose trophic therapy. Low-dose and untreated type 1 and type 2 mice remained hyperglycemic (Figs. 3A, B, D, E). In type 1 diabetic mice, titrated high-dose treatment (~4 pellets or 0.4 IU per day) lowered fasting glucose to control levels (4–6 mmol/L) during the four-week treatment period and lowered but did not normalize glycated hemoglobin, a result of previous exposure to untreated diabetes before insulin (Fig. 3C, F). In type 2 diabetes with insulin resistance, dramatic titration of insulin up to 12 pellets daily (a physical limit to the use of implanted pellets), or 1.2 IU daily, in db/db mice failed to normalize hyperglycemia or improve glycated hemoglobin.

Exendin-4 and Insulin Effects on Electrophysiological Abnormalities

Before treatment and at 2 months of diabetes, type 1 and type 2 diabetic mice exhibited the expected slowing of both motor and sensory conduction velocities (Figs. 4A, C; 5A, C). Exendin-4 treatment in type 1 diabetic mice was associated with a borderline but non-significant trend toward improved motor conduction velocity but a significant and substantial improvement in sensory conduction slowing (Figs. 4B, D). In contrast, type 2 diabetic mice had an improvement in motor conduction velocity and a borderline, but non-significant, impact on sensory parameters.

FIGURE 7. Mechanical sensitivity in type 1 and type 2 diabetic mice. (A) Mechanical sensitivity in control and type 1 diabetic animals before insulin or exendin-4 treatment (*p < 0.05). There was loss of sensation to mechanical stimuli. (B) Withdrawal latencies to a mechanical stimulus in control, untreated streptozotocin (STZ) type 1 diabetics, and insulin- and exendin-4 treated after 1 month of treatment. Exendin-4 and high-dose insulin preserved mechanical sensation in type 1 diabetic mice. The impact was more prominent in exendin-4-treated mice (* control vs low dose and untreated diabetes, p < 0.05; * untreated type 1 vs exendin-4, p < 0.001). (C) Mechanical sensitivity in control and type 2 diabetic animals before insulin and exendin-4 treatment also demonstrated loss of sensitivity (p < 0.0001). (D) Withdrawal latencies to a mechanical stimulus in control, untreated type 2 diabetics, and insulin and exendin-4 treated after 1 month of treatment. Neither insulin nor exendin-4 had an impact on the loss of mechanical sensation in type 2 diabetic mice (* control vs untreated type 2, low-dose insulin, high-dose insulin, and exendin-4 treated, p < 0.0001). Control nondiabetic (C, A–D); type 1 diabetic animals before treatment (B, A, C); high dose (B, D); low dose (B, D); untreated diabetic (B, D); and exendin-4 (B, D) treated experimental groups. Values are mean ± SEM. One-way analysis of variance with Tukey post hoc test and Student t-test as appropriate was used in these analyses.
sensory conduction (Figs. 5B, D). Neither low-dose nor high-dose titrated insulin delivery reversed motor or sensory conduction velocity slowing in type 1 or type 2 diabetic mice (Figs. 4B, D; 5B, D, respectively).

**Exendin-4 and Insulin Effects on Behavioral Measures of Sensation**

In our model, type 1 diabetic mice had evidence of sensory loss, with a prolonged latency to withdrawal from a thermal stimulus and impaired sensitivity to a mechanical stimulus (Figs. 6A, 7A). In type 2 diabetic mice, thermal sensation was normal at 2 months while there was mechanical sensory loss (Figs. 6C, 7C). In type 1 diabetes, exendin-4 was associated with a significant improvement in thermal withdrawal latencies and fully restored mechanical sensitivity (Figs. 6B, 7B). This contrasted with no significant improvement in either modality of mice given low-dose insulin treatment (Figs. 6B, 7B). However, high-dose titrated insulin improved loss of mechanical and thermal sensitivity in type 1 diabetic mice. In type 2 diabetic mice, exendin-4 was associated with a lowering of the latency to thermal withdrawal, indicating new hyperalgesia, but no impact on loss of mechanical sensation (Figs. 6D, 7D). In type 2 animals, there was no significant impact of chronic continuous insulin at either dose on thermal or mechanical sensation (Figs. 6D, 7D).

**Epidermal Innervation Is Not Corrected by Insulin or Exendin-4 in Type 2 Diabetes**

We analyzed mean axon densities oriented vertically to the surface of the skin and total axon profiles (all orientations) in 30 sections per mouse footpad to calculate a mean value for each mouse and, subsequently, a mean value for each group. In untreated type 1 diabetic mice, there was only a mild reduction in total epidermal axon density. A nonsignificant trend for vertical axon epidermal density was evident at 3 months when control and untreated diabetic mice were compared (Fig. 8A, B). In type 2 diabetic mice, there were significant reductions in vertical axon density in control versus untreated diabetic mice (Fig. 8C). Exendin-4 did not significantly alter vertical or total epidermal profile densities in type 1 diabetic mice. In contrast to untreated type 1 diabetic mice, continuous high-dose insulin treatment was associated with normal total axon density and nonsignificant trends toward improved vertical axon density measurements (Fig. 8B). Low-dose insulin was associated with a borderline but nonsignificant trend toward improvement in epidermal density. In type 2 diabetic mice, neither exendin-4 nor the insulin regimens improved epidermal innervation (Fig. 8C, D).

**Proximal Sural Axons Are Not Targeted by Diabetes**

To evaluate peripheral nerve proximal to the epidermal terminals, we analyzed sural myelinated axon densities and caliber. In both type 1 and 2 diabetic models, there were no changes in axon density and only mild but nonsignificant trends toward axonal atrophy (Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A328; Figure, Supplemental Digital Content 2, http://links.lww.com/NEN/A329; Figure, Supplemental Digital Content 3, http://links.lww.com/NEN/A330, Figure, Supplemental Digital Content 4, http://links.lww.com/NEN/A331). None of the regimens had an impact on these measurements.

**RAGE and NF-kB Pathways Are Not Activated After 3 Months of Diabetes**

In type 1 diabetes, there were only mild nonsignificant trends toward greater expression of RAGE immunofluorescence in untreated diabetics. This trend was reversed in high-dose insulin–treated mice, and there was no impact from low-dose insulin or exendin-4 (Fig. 9). Similar trends were noted in NF-kB expression, specifically of nuclear profiles. In type 2 diabetic mice, there were no significant changes or trends toward changes in either RAGE or NF-kB nuclear expression between diabetic mice and controls. Insulin infusion did not alter the expression. Additional expression studies of insulin receptor-β, the subunit of the insulin receptor, did not identify differences among diabetic mice, controls, or insulin-treated mice in either type 1 or 2 diabetics (Figure, Supplemental Digital Content 5, http://links.lww.com/NEN/A332).

**DISCUSSION**

In this study, we evaluated the potential impact of GLP-1 agonism on the development of neuropathy in types 1 and 2 experimental diabetes. To understand its potential actions, we compared its impact to both low- and high-dose insulin therapy, the latter previously considered to be a therapeutic gold standard. We identified the expression of GLP-1 receptors on adult sensory neurons, providing an avenue for direct signaling. We also show that exendin-4, a GLP-1 agonist, either acts as a direct trophic molecule on adult sensory neurons or increases their sensitivity to endogenous growth factors. The effects of GLP-1 are similar to those recently reported by Himeno et al (7) who noted dose-dependent increases in neurite outgrowth from

**FIGURE 8.** Footpad intraepidermal nerve fibers. (A–D) Quantitative data for intraepidermal axon density are shown for type 1 diabetic mice (A, B) and type 2 diabetic mice (C, D) and for vertically oriented axons (A, C) or total axon profiles (B, D). In type 1 diabetes, there was only a borderline reduction in innervation identified in total epidermal axon density in untreated diabetic mice versus controls (B) *p* = 0.03. There were nonsignificant trends toward ongoing reductions in low dose and exendin-4–treated mice but not high-dose insulin–treated mice. In type 2 diabetes, there was a decline in vertical axon density (C) *p* < 0.05 with no impact of insulin or exendin-4. Control nondiabetic ○ high dose □; low dose □; untreated diabetic □; and exendin-4 □ treated experimental groups, respectively, from left to right. Values are mean ± SEM. One-way analysis of variance with Tukey post hoc test and 1- or 2-tailed Student t-test was used. (E–N) Examples of epidermal footpads from type 1 diabetics (E–I) and type 2 diabetics (J–N). Panels are from control nondiabetics (E, J), high-dose insulin (F, K), low-dose insulin (G, L), untreated diabetics (H, M), and exendin-4–treated diabetics (I, N). Scale bar = 20 μm.
juvenile mouse DRG sensory neurons. Despite these promising findings, however, we show mixed actions in reversing experimental neuropathy in vivo with improvements in some indices but not others.

Our protocol for evaluating not only exendin-4 but also insulin was demanding, requiring reversal of established indices of neuropathy in concurrent and well-established models of type 1 and type 2 diabetes. We used well-defined diabetic polyneuropathy time points and end points that are likely more illustrative of a clinically relevant impact than a preventive or evolutionary study. The effects of exendin-4 differed from those of insulin, arguing against the possibility that its actions could be attributed to endogenous insulin release. Nonetheless, that possibility cannot be excluded; insulin levels in this study were below detectable levels (not shown) and of insufficient sensitivity to detect small changes.

Low potentially neurotrophic actions of insulin did not reverse established features of neuropathy in either type 1 or 2 models. High-dose titrated continuous insulin reversed hyperglycemia and improved mechanical sensory loss but only partially improved thermal sensory loss and did not reverse electrophysiologic abnormalities. The present studies also confirm that it is particularly challenging to control hyperglycemia in a type 2 diabetic model, illustrating a dramatic insulin resistance. Practical considerations precluded implantation of our db/db mice with larger numbers of pellets. Type 2 diabetic mice had associated refractory neuropathic abnormalities that did not improve.

In this context, the differing effects of exendin-4 and insulin, albeit also incomplete, are interesting. We deliberately selected a dose of exendin-4 that was equivalent to therapeutic doses used in patients with type 2 diabetes. The GLP-1 receptors are coupled to a variety of downstream pathways such as phosphoinositide 3 kinase and cyclic adenosine monophosphate, both of which impact peripheral neuron function and are altered in diabetes (13, 19–22). In diabetic sciatic nerve and DRG, GLP-1 agonists increased pERK1/2 levels, an effect that might be occurring in either local Schwann cells or axons (8). In the present study, the effects of exendin-4 on hyperglycemia were minor and, therefore, were unlikely to have altered neuropathic findings. Type 1 diabetic mice exhibited loss of thermal and mechanical sensation after 2 months of untreated diabetes, confirming work by others (23).

Exendin-4 treatment was associated with an improvement in thermal sensation and fully reversed mechanical sensory loss. Several previous studies have reported mechanical hypersensitivity in diabetic neuropathy, including the db/db type 2 model (22, 24–29); this contrasts with the loss of mechanical sensitivity that we observed. Our studies began at 14 weeks of age (when allodynia might be expected to decline) and continued until 18 weeks of age. As diabetes progresses, loss of epidermal innervation and a shift to hyposensitivity likely occur. Exendin-4 exacerbated thermal hyperalgesia in type 2 diabetic mice and had no impact on mechanical sensory loss. We did not examine downstream pathways of which phosphoinositide 3 kinase–Akt might have particular interest and significance in this regard.

Our results also differ to some degree from additional recent studies published during completion of the present work. Jolivalt et al (8) studied STZ-induced diabetes in mice, a type 1 model treated with exendin-4 for 8 weeks without an insulin treatment arm in a preventive paradigm. They noted preservation of motor conduction velocity and epidermal axons. There was no impact on thermal sensation. Sensory conduction and mechanical sensitivity were not tested. The GLP-1 receptor protein was not altered by diabetes in whole ganglion lysates. GLP-1 agonism was associated with rises of pERK1/2 in diabetic nerves. Overall, those results indicate that in a less demanding preventive paradigm, the benefits of exendin-4 were incomplete, as in the present study. Here, a longer reversal paradigm model had a partial impact on several indices of neuropathy that differed from those of insulin. In an additional reversal paradigm also recently published, Himeno et al (7) offered exendin-4 to type 1 diabetic mice after 12 weeks of diabetes. As in the present study, they noted improvements in motor and sensory conduction. Their findings were not compared with effects of insulin. Although physiological sensory modalities such as thermal and mechanical sensation were not evaluated, exendin-4 improved perception of an electrical current, as well as epidermal innervation. There was no impact on nerve blood flow measured by laser Doppler flowmetry, although this measurement in mice is of uncertain importance in experimental diabetes (30). As above, no changes in mRNA or total protein levels of GLP-1 were identified, but neuron-specific expression also was not examined. In our analysis, although there were semiquantiative trends toward greater neuron-specific intensity in both models of diabetes (performed by blinded analysis), we could not confirm the changes by Western immunoblots of ganglion lysates.

Previous studies have reported that loss of epidermal axons, or small-fiber neuropathy, occurs in both type 1 and type 2 diabetes in human and experimental models, but it may not be evident at early time points when functional abnormalities are present (31–37). Exendin-4 did not appear

**FIGURE 9.** Immunohistochemistry of dorsal root ganglia (DRG) neurons. Images and protein expression analysis of receptor for advanced glycosylated end products (RAGE), nuclear factor-kB (NF-kB) expression in DRG of diabetic and nondiabetic control mice. (A–F) Sections of L5 DRG from type 1 nondiabetic littermate controls (A, B) and untreated type 1 streptozotocin (STZ) mice (C, D) or type 2 nondiabetic littermate controls (E, F) and untreated type 2 db/db mice (G, H). (I–K) Semiquantitative protein expression luminosity analysis results in type 1 STZ mice, including controls, low-dose insulin, high-dose insulin, untreated, and exendin-4 treatment groups. (L–N) Semiquantitative protein expression luminosity analysis in type 2 db/db mice, including controls, low-dose insulin, high-dose insulin, untreated, and exendin-4 treatment groups. There were trends toward greater RAGE and NF-kB nuclear expression in type 1 diabetics (fewer negative neurons, more highly expressing neurons) and a reversal of this trend with high-dose insulin therapy, but the differences were not statistically significant (analysis of variance, NS). Scale bar = 100 μm.
to improve innervation in either model. These results differ from previous reports with the dipeptidyl peptidase-4 inhibitor vildagliptin which prevents the degradation of GLP-1. Use of this agent, beginning at 4 weeks of STZ-induced type 1 diabetes, prevented epidermal axon loss at 16 and 32 weeks of diabetes (38). The differences in our results could be attributed to time points, model, and drug differences. Jin et al (38) began treatment only after 4 weeks of diabetes in rats, whereas we waited 8 weeks in an accelerated mouse model and did not treat the mice as long. Taken together, it appears that the strict anatomical axon investment of the epidermis does not necessarily correlate with function.

In view of the known neurotrophic properties of insulin (39), the effects of continuous low-dose systemic infusions that are insufficient to alter hyperglycemia are of interest (40–45). In 3 previous paradigms, we encountered reversal of experimental neuropathy using direct neuronal applications of low doses of insulin, that is, near-nerve, intrathecal, and intranasal (45, 46). In these studies, hyperglycemia was unaltered despite correction of neuropathy. Short-term largely preventive studies in rats have identified partial benefits from low-dose continuous insulin without improved hyperglycemia (47, 48). In the present work, however, we found no role for continuous low-dose systemic insulin in reversing neuropathy. We believe that insulin must have direct access to neurons to provide trophic actions; systemic therapy is, therefore, insufficient.

Sima and colleagues (49) have hypothesized that axoglial dysjunction allows the migration of sodium channels into neighboring interneural regions, leading to decreased sodium channel nodal density and persistent changes in axon conduction velocity (50). Several studies have shown that insulin can prevent electrophysiological abnormalities in experimental diabetes (51). Indeed, Biessels et al (52) reported partial but incomplete reversal of sensory and motor conduction velocity abnormalities in STZ rats. Our mice were exposed to untreated diabetes for a longer period, had diabetes for a larger proportion of their shorter lifespan, and develop distal sural axonal degeneration sooner than rats (53). An interesting possibility not studied here is that exendin-4 may have improved conduction slowing in type 1 diabetic animals through stimulation of residual endogenous release of C-peptide, a potential insulin sensitizer that has a direct impact on neuropathy (54, 55).

In type 2 diabetic mice, hyperglycemia and conduction slowing were both refractory to high doses of insulin (1.2 IU per day, 23 IU/kg per day; corrected for their obesity) without an impact on glycemia, in contrast to effects of 0.4 IU per day (12 IU/kg per day) in type 1 diabetic animals. Robertson and Sima (56) studied db/db diabetic mice at 13 weeks of age (≈8 weeks of diabetes) and administered titrated (glucose, ≈8 mmol/L) subcutaneous late evening Lente insulin (2–12 IU) for 17 days. There was improvement in motor conduction velocity, but a second trial at 18 weeks of diabetes for 21 days had no impact. In our experiments, db/db mice began insulin treatment at 3 to 4 months of age (14 weeks of diabetes), but the physical limitations of implantation did not allow us to titrate the dose as high as Robertson and Sima (56) did to correct hyperglycemia. Obese ob/ob mice given insulin therapy for 4 weeks (from 8 to 12 weeks of age) had a reversal of hyperglycemia and thermal and mechanical hypersensitivity (28). Very high doses of injected insulin (10–40 NPH IU per day in 2 divided doses) were used, and insulin was given for prevention rather than reversal of neuropathy. It is possible that earlier or more intense therapeutic interventions requiring injection strategies rather than continuous-release insulin pellets may facilitate rescue before the development of insulin resistance. As also discussed by Sima et al (49), several differing features of type 1 and 2 diabetes in concurrent models may account for their phenotypic differences: circulating insulin levels, loss of C-peptide, features of insulin resistance now known to also develop directly in neurons (39), or other alterations.

A final observation was that protein upregulation of the RAGE–NF-κB system was modest to absent in these models. Although chronic continuous insulin did eliminate the mild trends toward their activation in DRG, expression was unimpressive. Here, we studied earlier models of experimental diabetes compared with 5-month investigations of RAGE deletion in separately published work (14). Although the extent of protein expression does not discount a role for this system earlier in diabetes, other approaches to evaluate that potential early role may be required.

Overall, our findings identify remarkable and unexpected variations in how several indices of neuropathy respond to a GLP-1 agonist, in contrast to the effects of insulin. Taken together, there are several important implications in our present findings. First, electrophysiological abnormalities, behavioral changes, and epidermal innervation each represent independent “hits” on neurological function that are only roughly correlated. Second, we do not understand how specific interventions may independently target each of these alterations. Their correlation breaks down in rigorous reversal paradigms, in parallel type 1 and 2 model comparisons, and in the impact of specific agents.

In conclusion, the GLP-1 agonist exendin-4 directly impacts sensory neuron behavior. However, its effects on established models of type 1 and 2 diabetes are complex and incomplete, a result also encountered with continuous insulin therapy. Although more investigation is required, including whether insulin and GLP-1 might have synergistic actions, it is possible that GLP-1 agonists may be of interest in both forms of human diabetes. Overall, exploitation of the GLP-1-pathway may offer adjunct approaches to treat intractable neuropathy.

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REFERENCES


42. Sima AA, Lattimer SA, Yagihashi S, Greene DA. Axo-glial dysjunction: A novel structural lesion that accounts for poorly reversible slowing of