Intranasal Insulin Ameliorates Experimental Diabetic Neuropathy

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OBJECTIVE—We hypothesized that intranasal insulin (I-I) delivery targets the nervous system while avoiding potential adverse systemic effects when compared with subcutaneous insulin (S-I) for experimental streptozotocin-induced diabetic peripheral neuropathy (DPN).

RESEARCH DESIGN AND METHODS—I-I or S-I at 0.87 IU daily or placebo were delivered in separate cohorts of diabetic and nondiabetic CD1 mice during 8 months of diabetes. Radiolabeled insulin detection was used to compare delivery and biodistribution for I-I and S-I. Biweekly behavioral testing and monthly electrophysiological and quantitative studies assessed progression of DPN. At and before end point, morphometric analysis of DRG, peripheral nerve, distal epidermal innervation, and specific molecular markers were evaluated.

RESULTS—Radiolabeled I-I resulted in more rapid and concentrated delivery to the spinal cord and DRG with less systemic insulin exposure. When compared with S-I or intranasal placebo, I-I reduced overall mouse mortality and sensory loss while improving neuropathic pain and electrophysiological/morphological abnormalities in diabetic mice. I-I restored mRNA and protein levels of phosphoinositide 3-kinase/Akt, cyclic AMP response element–binding protein, and glycogen synthase kinase 3β to near normal levels within diabetic DRGs.

CONCLUSIONS—I-I slows the progression of experimental DPN in streptozotocin mice, avoids adverse effects associated with S-I treatment, and prolongs lifespan when compared with S-I. I-I may be a promising approach for the treatment of DPN. Diabetes 58:934–945, 2009

The most common form of peripheral nervous system (PNS) disease complicating diabetes mellitus is diabetic symmetric sensorimotor polyneuropathy (DPN) (1,2). Diabetic PNS is subject to behavioral, electrophysiological, and morphological changes within peripheral nerve axons, the dorsal root ganglion (DRG), and epidermal nerve fibers (2–4). Although considered an “end-stage” complication, DPN may occur early and may involve children with diabetes (5).

Clinical intervention trials in both type 1 and type 2 diabetes have demonstrated that chronic hyperglycemia has a strong association with the prevalence of complications (6,7). Beyond chronic hyperglycemia, other commonly hypothesized mechanisms relevant for pathogenesis of DPN include excessive sorbitol-aldose reductase pathway flux (8), protein kinase C isoform(s) overactivity (9), increased oxidative and nitrative stress (10), microangiopathy (11), and advanced glycation end products and their receptor (12,13). An important mechanism of DPN may also relate to impaired availability, action, or uptake of growth factors necessary to support peripheral neurons (3,4,14,15). For diabetic neuropathy, modifications in neurotrophin levels or evidence of a supportive role have been identified for many neurotrophin family members (14). An important neurotrophic factor critical in supporting peripheral neurons, and diminished in diabetes mellitus, is insulin. Both insulin and IGF-1 are important for neuronal survival and phenotypic expression in DPN mice, neuritic outgrowth through specific insulin receptor or IGF-1 receptor-mediated signaling pathways within the adult sensory neuron (4,16,17).

Insulin binds to the insulin receptor α subunit, promoting tyrosine autophosphorylation of the β subunit and subsequent phosphorylation of cellular substrates, including the insulin receptor substrate (IRS) proteins and Shc (18). Phosphorylation of IRS-1 or IRS-2 (18) creates an active signaling complex involving phosphatidylinositol 3-kinase (PI3K), Akt, and the downstream effectors cyclic AMP response element–binding protein (CREB) and glycogen synthase kinase 3β (GSK-3β), among other molecules (19).

In an experimental type 1 diabetes model of DPN, we hypothesized that a novel form of direct neuronal long-term insulin replacement therapy could slow DPN progression. Previous experiments have demonstrated that direct intrathecal insulin is capable of reversing features or preventing progression of DPN (3,4,17). We designed experiments using behavioral and electrophysiological testing to assist in delineating insulin’s trophic and antihyperglycemic effects in DPN using intranasal insulin (I-I) delivery to target insulin to the nervous system without significant alteration of blood levels of insulin or glucose (20). Intranasal delivery was first developed to bypass the blood-brain barrier and directly target growth factors and other therapeutic agents to the central nervous system (20) with travel along both olfactory and trigeminal neural conduits within extracellular pathways exclusive of axonal transport (21). Proteins as large as 27 kDa, including IGF-1, have been successfully delivered to the brain using this method (21) in rodents (22) and humans (23).
used these studies to determine complications of interventions used as secondary end points.

**RESEARCH DESIGN AND METHODS**

We studied a total of 484 male CD1 wild-type mice with initial weight of 20 to 30 g housed in plastic sawdust covered cages with a normal light-dark cycle and free access to mouse chow and water. In all cases, mice were raised and studied in strict pathogen-free environments. All protocols were reviewed and approved by the institutional animal care and use committee at Regions Hospital (21 mice, experiment 1) and the University of Calgary Animal Care Committee using the Canadian Council of Animal Care guidelines (463 mice, experiment 2). Mice were anesthetized with pentobarbital (60 mg/kg) before all procedures. At the age of 1 month, 304 mice were injected with streptozotocin (STZ) (Sigma, St. Louis, MO) intraperitoneally once daily for each of 3 consecutive days with doses of 60 mg/kg, 50 mg/kg, and then 40 mg/kg with the remaining 180 mice injected with carrier (sodium citrate) for 3 consecutive days. Studies using harvested tissues occurred after 1 month (36 diabetic mice, 30 nondiabetic mice), 3 months (48 diabetic mice, 30 nondiabetic mice), 5 months (60 diabetic mice, 30 nondiabetic mice), and 8 months (160 diabetic mice, 90 nondiabetic mice).

Whole blood glucose measurements were performed monthly with puncture of the tail vein and a blood glucometer (OneTouch Ultra Meter; LifeScan Canada, Burnaby, BC, Canada). Hyperglycemia was verified 1 week after STZ injections with a fasting whole blood glucose level of 16 mmol/l or greater (normal range 3.8–6.8 mmol/l), a definition for experimental diabetes. All animals were weighed monthly. Mice were followed and harvested at 1, 3, 5, or 8 months of diabetes (>9 months of life). Mice that did not develop diabetes were excluded from further assessment.

Animals were inspected twice daily and examined for signs of depressed level of consciousness, ataxia, or general malaise. When such signs were identified, whole blood glucose testing was performed with a measurement of less than 3.5 mmol/l defined to represent hypoglycemia. No interventions were performed at any time with regard to additional insulin, glucose, or fluid delivery. In situations in which the mouse was obviously ill, euthanasia was performed. In circumstances in which severe hyperglycemia was found (>33 mmol/l) in an ill mouse, euthanasia was performed.

We studied cohorts with a maximum of eight mice in each group initially as a result of resource limitations. After the initial cohorts containing eight mice each were studied, a second cohort was used to obtain additional mouse data for mouse cohorts with higher levels of mortality. For any animal that experienced mortality after the 20-week point of the sensorimotor studies, the data were carried through using the last obtainable data point.

In this work, delivery of subcutaneous saline is indicated as “S-S,” subcutaneous insulin as “S-I,” intranasal insulin as “I-I,” and intranasal insulin and saline as “I-I-S.”

**Experiment 1: pharmacokinetic studies of intranasal or subcutaneous delivery.** 125I-Labeled I administration was performed to determine distribution of intranasally delivered insulin in 21 nondiabetic mice. Before experimentation, animals were acclimated for handling during awake intranasal delivery for 2 weeks. 125I-Labeled I was provided to 12 CD1 mice (male, 6–8 weeks old). The labeled I was calculated using eight mice. Each month, mice were injected with streptozotocin (STZ) (Sigma, St. Louis, MO) intraperitoneally once daily for each group, and each group underwent monthly electrophysiological testing beginning immediately after induction of diabetes and before induction of diabetes and after 1, 2, 4, 6, and 8 months of diabetes. For orthodromic sensory conduction studies, the sural nerve was used with a fixed distance of 30 mm from platinum subdermal stimulation needle electrodes (Grass Instruments, Astro-Med, West Warwick, RI) to the sciatric notch where recording electrodes were placed to measure the sensory nerve action potential (SNAP) amplitude and sensory nerve conduction velocity (SNCV). Nerve-muscle temperature was kept constant during testing at 37 ± 0.5°C using a heating lamp.

**Experiment 2a: electrophysiology during intranasal and subcutaneous insulin delivery studies.** Electrophysiological assessment of sciatric nerve function was performed as previously described (13) under halothane anesthesia. Initial baseline studies were carried out before STZ or carrier injections; no significant difference between groups was identified. Of all cohorts of mice receiving I-I, S-I, I-S, or S-S, both diabetic and nondiabetic, at least five animals were studied in each group. Whole blood glucose testing was performed at any time with regard to additional insulin, glucose, or fluid delivery. For subcutaneous delivery, cardiocentesis was performed with a measurement of less than 3.5 mmol/l defined to represent hypoglycemia. No interventions were performed at any time with regard to additional insulin, glucose, or fluid delivery. In situations in which severe hyperglycemia was found (>33 mmol/l) in an ill mouse, euthanasia was performed. In circumstances in which severe hyperglycemia was found (>33 mmol/l) in an ill mouse, euthanasia was performed.

**Experiment 2b: behavioral testing during intranasal and subcutaneous insulin delivery studies.** A total of 10 mice in each cohort had behavioral testing performed twice monthly to evaluate mechanical and thermal sensitivity. A 2-week training period was performed to acclimatize mice to the procedures immediately after STZ injections and during diabetes verification.

Mice were placed in a Plexiglas cage on a glass plate (for thermal testing) or on a plastic mesh floor (for tactile testing) and were allowed to acclimate for at least 5 min before recording in all cases. Thermal sensitivity was tested using the Hargreaves apparatus (24). In brief, a radiant heat source was applied individually to the middle of either hind paw for up to 60 s with the latency (seconds) to withdrawal measured. Heating rate ramped from 30 to 50°C over 60 s. In each group, 60 s and 15°C were inspected before and after thermal testing to ensure that no evidence of thermal damage was present. We were set intervals provided between a total of three trials performed during the same day. To quantify mechanical sensitivity of the foot, withdrawal in response to a stimulus consisted of sequentially smaller von Frey filaments (25). Five trials were performed at each filament size with greater number trials performed for smaller filament sizes to indicate mechanical sensitivity at the smallest positive filament size. Mechanical and thermal testing was performed on identical days with an interval of at least 1 h between the two tests.

**Other procedures and testing.** Harvesting of tissues and their subsequent morphometric processing and analysis along with quantification of epidermal fibers and all other molecular testing (Western immunoblotting, polymerase chain reactions, and electrophoretic mobility shift assays) have been described in detail in an online appendix (available at http://diabetes.diabetesjournals.org/cgi/content/full/db08-1287/DC1).

All statistical comparisons were intended between the following groups: diabetic I-I and S-I; diabetic I-I and I-S; diabetic I-I and control I-I; diabetic S-I and S-S; diabetic S-I and control S-I; control I-I and S-I; control I-I and I-S; and control S-I and S-S. Comparison testing was not performed between other groups, and Bonferroni corrections applied as appropriate for these group comparisons.

Data collected in the groups were expressed as mean ± SE in all cases. Data from each individual mouse were used to obtain means in each case. One-way matched/unmatched ANOVA and Student’s t tests were performed to compare means between diabetic and control groups. For immunohistochemistry comparisons demonstrated as low/medium/high intensity, the individual values were compared using unmatched ANOVA testing. Also, AUC statistical testing was performed for behavioral testing performed and was calculated using the trapezoidal method. Again, only the groups intended to have statistical comparisons were analyzed as such. Correlational relationships for AUC were tested using multiple linear regression analysis. For the purposes of
molecular studies and comparisons, in some cases, only one control (nondiabetic) group was used as a control value with subsequent comparisons to other diabetic groups for the molecular test studied; Bonferroni corrections were made as appropriate depending on the number of relevant comparisons.

RESULTS

Experiment 1: distribution of administered insulin.
At 1 h after I-I or S-I delivery, insulin concentrations were higher within cervical spinal cord, DRGs, and spinal dura with I-I delivery (Fig. 1). Insulin concentrations in blood were substantially higher after S-I delivery (850× greater), but insulin concentrations within kidney, liver, and lung were higher after I-I delivery.

At 6 h after I-I or S-I delivery, insulin concentrations were higher in DRGs and spinal dura after S-I delivery compared with I-I delivery (Fig. 1). After 6 h, blood concentrations of insulin were higher after I-I delivery with this peak detected at 6 h. Insulin concentrations in systemic organs were now higher after S-I delivery. When 2-h data are considered, I-I delivery led to peaks in insulin concentration within DRGs and systemic organs after 1 h and peaks in blood concentrations of insulin after 6 h. S-I delivery, in contrast, led to peaks in insulin concentration at DRG and systemic organs after 6 h, whereas blood concentrations peaked after 1 h. Blood concentrations of insulin after S-I delivery peaked at a value nearly 1,000× the peak value obtained with I-I delivery.

Mice receiving I-I treatment maintained good health throughout the 1-, 2-, and 6-h monitoring periods before...
being euthanized, whereas S-I delivery led to more frequent development of hypoglycemia-induced illness, including death, and also to reduced consciousness levels in many mice.

**Experiment 2: diabetes model.** After STZ injection, mice developed diabetes within 2 weeks in greater than 85% of animals, and in each case, diabetes was maintained over the length of the study. Diabetic mice were smaller than nondiabetic mice within 1 month after STZ injection, and diabetic mice had smaller body weights throughout life (Table 1); diabetic mice receiving I-I maintained weight better than the cohort receiving S-S. Hyperglycemia was identical in mice receiving I-I or I-S, but S-I mice had more documented hyperglycemia and more episodes of illness or death associated with confirmed hyperglycemia (Table 1). Mouse glycated hemoglobin was increased in all diabetic mice after more than 9 months of life and was identical between I-I and S-I mice but was reduced in surviving S-I mice (Table 1). The mortality rate in diabetic mice was significantly higher than in nondiabetic mice, although diabetic I-I mice had improved mortality relative to diabetic I-S, diabetic S-S, and diabetic S-I mice (Table 1).

**Experiment 2: impact of intranasal insulin on somatosensory conduction velocities.** Diabetic I-I mice demonstrated less hypersensitivity in the early stages of diabetes and maintained thermal sensation better in the later stages of diabetes. Diabetic S-I mice had less thermal sensory loss than other diabetic mouse cohorts, although diabetic S-I mice had greater maintenance of sensation for tactile stimuli than diabetic mice receiving S-S or I-S (Fig. 2). During serial thermal testing, each cohort of diabetic mice also developed thermal hypersensitivity when compared with nondiabetic mice after several weeks, earlier than identified in other species or mouse strains (Fig. 2). However, diabetic I-I mice demonstrated less hypersensitivity in the early stages of diabetes and maintained thermal sensation better in the later stages of diabetes. Diabetic S-I mice had less thermal sensory loss in the later stages of diabetes (Fig. 2) when compared with diabetic mice receiving S-S and I-S. Within the first 20 weeks, AUC measurements were statistically different for diabetic I-I mice as compared with other diabetic mouse cohorts for both tactile and thermal testing, and all diabetic cohorts had thermal hypersensitivity and tactile allodynia when compared with nondiabetic mice (Fig. 2).

**Experiment 2: impact of intranasal insulin on electrophysiology.** Before induction of diabetes, there were no baseline differences in sensory behavior testing between any of the mouse cohorts identified. All diabetic mice developed tactile allodynia after 5 to 7 weeks of diabetes when compared with nondiabetic mice (Fig. 2).Diabetic I-I mice developed less allodynia in the first 3 months of diabetes (Fig. 2) as compared with the other diabetic cohorts. In the later stages of diabetes, I-I mice had less tactile sensory loss than other diabetic mouse cohorts, although diabetic S-I mice had greater maintenance of sensation for tactile stimuli than diabetic mice receiving S-S or I-S (Fig. 2). During serial thermal testing, each cohort of diabetic mice also developed thermal hypersensitivity when compared with nondiabetic mice after several weeks, earlier than identified in other species or mouse strains (Fig. 2). However, diabetic I-I mice demonstrated less hypersensitivity in the early stages of diabetes and maintained thermal sensation better in the later stages of diabetes. Diabetic S-I mice had less thermal sensory loss in the later stages of diabetes (Fig. 2) when compared with diabetic mice receiving S-S and I-S. Within the first 20 weeks, AUC measurements were statistically different for diabetic I-I mice as compared with other diabetic mouse cohorts for both tactile and thermal testing, and all diabetic cohorts had thermal hypersensitivity and tactile allodynia when compared with nondiabetic mice (Fig. 2).

**Experiment 2: impact of intranasal insulin on sensorimotor behavioral data.** Before induction of diabetes, there were no baseline differences in sensory behavior testing between any of the mouse cohorts identified. All diabetic mice developed tactile allodynia after 5 to 7 weeks of diabetes when compared with nondiabetic mice (Fig. 2). Diabetic I-I mice developed less allodynia in the first 3 months of diabetes (Fig. 2) as compared with the other diabetic cohorts. In the later stages of diabetes, I-I mice had less tactile sensory loss than other diabetic mouse cohorts, although diabetic S-I mice had greater maintenance of sensation for tactile stimuli than diabetic mice receiving S-S or I-S (Fig. 2). During serial thermal testing, each cohort of diabetic mice also developed thermal hypersensitivity when compared with nondiabetic mice after several weeks, earlier than identified in other species or mouse strains (Fig. 2). However, diabetic I-I mice demonstrated less hypersensitivity in the early stages of diabetes and maintained thermal sensation better in the later stages of diabetes. Diabetic S-I mice had less thermal sensory loss in the later stages of diabetes (Fig. 2) when compared with diabetic mice receiving S-S and I-S. Within the first 20 weeks, AUC measurements were statistically different for diabetic I-I mice as compared with other diabetic mouse cohorts for both tactile and thermal testing, and all diabetic cohorts had thermal hypersensitivity and tactile allodynia when compared with nondiabetic mice (Fig. 2).

**DISCUSSION**

I-I protected diabetic mice from behavioral, structural, and molecular changes associated with DPN. We propose that insulin’s neuroprotective effects on the PNS are the result of restoration of the PI3K/Akt pathway components (Fig. 4). Additionally, I-I led to less mortality than S-I delivery and provided greater protection against the effects of long-term diabetes on the PNS.

**Systemic and peripheral nervous system impact of subcutaneous and intranasal insulin.** Replacement of insulin in a type I model of diabetes through either I-I or S-I delivery led to improvements in behavioral, electrophysiological, morphological, and molecular status (Figs. 2–5 and supplemental Figs. 1–3) related to diabetes. Paradox-
**TABLE 1**
Murine weights, fasting glycemia levels, glycated hemoglobin levels, and survival numbers at induction of diabetes and at harvesting at months 1, 3, 5, and 8 of diabetes*

| Time point                  | Injection of STZ/carrier | Month 1 | Month 3 | Month 5 | Month 8 |
|-----------------------------|--------------------------|---------|---------|---------|---------|
| **Murine weight**           |                          |         |         |         |         |
| Nondiabetic S-S mice        | 25.6 ± 3.2 (n = 25)      | 32.7 ± 3.8 (n = 25) | 39.7 ± 4.0 (n = 24) | 43.4 ± 4.3† (n = 24) | 47.2 ± 4.9† (n = 23) |
| Nondiabetic S-I mice        | 25.8 ± 3.9 (n = 25)      | 30.4 ± 4.1 (n = 20) | 34.6 ± 4.7 (n = 17) | 36.2 ± 5.1† (n = 15) | 37.0 ± 5.4† (n = 12) |
| Nondiabetic I-S mice        | 25.1 ± 3.7 (n = 25)      | 32.1 ± 3.9 (n = 25) | 40.1 ± 4.2 (n = 25) | 44.7 ± 4.6 (n = 25) | 48.1 ± 5.2 (n = 24) |
| Nondiabetic I-I mice        | 25.4 ± 3.3 (n = 25)      | 31.9 ± 3.5 (n = 24) | 36.2 ± 4.9 (n = 23) | 39.1 ± 5.3 (n = 22) | 43.4 ± 4.8 (n = 21) |
| Diabetic S-S mice           | 25.2 ± 3.3 (n = 40)      | 26.9 ± 4.2 (n = 34) (3 nondiabetic) | 28.4 ± 4.3 (n = 30) | 30.6 ± 5.7 (n = 20) | 31.5 ± 5.8 (n = 16) |
| Diabetic S-I mice           | 25.4 ± 3.4 (n = 40)      | 26.4 ± 4.8 (n = 31) (2 nondiabetic) | 27.2 ± 3.8 (n = 24) | 28.2 ± 4.1 (n = 16) | 28.8 ± 4.9 (n = 12) |
| Diabetic I-S mice           | 25.2 ± 3.4 (n = 40)      | 26.2 ± 4.8 (n = 33) (3 nondiabetic) | 28.9 ± 4.2 (n = 30) | 30.2 ± 4.9 (n = 22) | 30.4 ± 5.2‡ (n = 18) |
| Diabetic I-I mice           | 25.6 ± 3.5 (n = 40)      | 27.8 ± 4.0 (n = 36) (3 nondiabetic) | 30.9 ± 4.5 (n = 35) | 34.8 ± 3.6 (n = 33) | 35.6 ± 4.9‡ (n = 30) |

**Murine glycemia and 8-month glycated Hemoglobin**

| Time point                  | Injection of STZ/carrier | Month 1 | Month 3 | Month 5 | Month 8 |
|-----------------------------|--------------------------|---------|---------|---------|---------|
| Nondiabetic S-S mice        |                          | 5.5 ± 2.3 | 5.9 ± 2.6 | 6.1 ± 2.7 | 6.2 ± 3.0 | 6.6 ± 3.2† (12.4% ± 4.8%) |
| Nondiabetic S-I mice        |                          | 5.4 ± 2.6 | 3.5 ± 2.7 | 3.9 ± 2.9 | 4.1 ± 3.0 | 4.0 ± 3.1‡ (9.2% ± 4.1%) |
| Nondiabetic I-S mice        |                          | 6.0 ± 2.8 | 5.9 ± 2.6 | 5.9 ± 2.8 | 6.1 ± 3.1 | 6.3 ± 2.7 (12.1% ± 4.7%) |
| Nondiabetic I-I mice        |                          | 5.8 ± 2.6 | 5.7 ± 2.9 | 5.6 ± 3.0 | 5.7 ± 3.0 | 5.7 ± 3.2 (12.6% ± 4.9%) |
| Diabetic S-S mice           |                          | 5.7 ± 2.7 | 3.17 ± 4.9 | 3.23 ± 6.1 | 3.22 ± 6.2 | 32.4 ± 6.0 (31.6% ± 6.2%)‡ |
| Diabetic S-I mice           |                          | 5.6 ± 2.6 | 2.47 ± 5.2 | 2.59 ± 5.8 | 2.43 ± 5.6 | 24.8 ± 6.1 (24.1% ± 6.6%)‡ |
| Diabetic I-S mice           |                          | 6.1 ± 2.9 | 3.22 ± 4.6 | 3.21 ± 5.3 | 3.21 ± 5.2 | 32.3 ± 5.8 (32.0% ± 6.0%) |
| Diabetic I-I mice           |                          | 5.8 ± 2.8 | 3.15 ± 4.5 | 3.16 ± 5.0 | 31.6 ± 5.2 | 32.0 ± 5.6 (30.2% ± 6.4%) |

**Murine survival numbers**

| Time point                  | Injection of STZ/carrier | Month 1 | Month 3 | Month 5 | Month 8 |
|-----------------------------|--------------------------|---------|---------|---------|---------|
| Nondiabetic S-S mice        |                          | 25/25 (100%) | 25/25 (100%) | 24/25 (96%) | 24/25 (96%) | 23/25 (92%)† |
| Nondiabetic S-I mice        |                          | 25/25 (100%) | 20/25 (80%) | 17/25 (68%) | 15/25 (60%) | 12/25 (48%)‡ |
| Nondiabetic I-S mice        |                          | 25/25 (100%) | 25/25 (100%) | 25/25 (100%) | 25/25 (100%) | 24/25 (96%) |
| Nondiabetic I-I mice        |                          | 25/25 (100%) | 24/25 (96%) | 23/25 (92%) | 22/25 (88%) | 21/25 (84%) |
| Diabetic S-S mice           |                          | 40 (100%) | 34/37 (3 nondiabetic, 92%) | 30/37 (81%) | 20/37 (54%) | 16/37 (43%) |
| Diabetic S-I mice           |                          | 40 (100%) | 31/38 (2 nondiabetic, 82%) | 24/38 (63%) | 16/38 (42%) | 12/38 (32%) |
| Diabetic I-S mice           |                          | 40 (100%) | 33/36 (3 nondiabetic, 92%) | 30/36 (83%) | 22/36 (61%) | 18/36 (50%)‡ |
| Diabetic I-I mice           |                          | 40 (100%) | 36/39 (3 nondiabetic, 92%) | 35/39 (90%) | 33/39 (85%) | 30/39 (77%)‡ |

Data are means ± SEM and n (%). *Glycated hemoglobin values are presented in italics in the 8-month column for glycemia levels. For murine survival, Kaplan-Meier statistics were performed between cohort groups. †Significance at P < 0.05 with comparison of nondiabetic S-S and S-I mice cohort groups. ‡Significance between diabetic cohort groups receiving I-S and I-I. §Significance with comparison of diabetic I-I mice to all other nondiabetic mice using Bonferroni post hoc comparisons (α = 0.05, P < 0.0125) (nonmatched ANOVA tests, F-values range between 0.96 and 144.8 for indicated groups and time points, df = 7,4; n = 8–10). S-S = subcutaneous saline; S-I = subcutaneous insulin; I-I = intranasal insulin; I-S = intranasal saline.
ically, S-I delivery in diabetic mice led to greater mortality (Table 1), relating in part to episodes of hypoglycemia, a complication avoided by I-I. Although S-I led to improved glycated hemoglobin levels at final end point, this effect was not seen in diabetic I-I mice. These results suggest that the beneficial effects of I-I in diabetes are not primarily related to corrections in hyperglycemia (Table 1).

Role of insulin as a neuroprotective trophic factor. Insulin, a highly conserved peptide, has now emerged as a key neurotrophic factor in the nervous system, a role that is lost in type 1 diabetes. The major site of insulin’s activity, the insulin receptor, is found in high concentrations among DRG neurons and myelinated sensory root fibers and in lesser concentrations on myelinated anterior...
root fibers and in the ventral horn of the spinal cord (16,26). Intrathecal insulin prevents degeneration and promotes regeneration in injured peripheral nerve (16). Meanwhile, systemic or intrathecal insulin delivery prevents diabetes-mediated electrophysiological changes (27), whereas intrathecal insulin restores distal skin epidermal innervation (17). In vitro, insulin exerts a direct neuritic outgrowth effect through insulin receptors or perhaps through crossactivation of IGF-1 receptors (28). Insulin's greatest impact appears to be at the level of the DRG, where insulin may prevent a “dying-back” that begins in the most distal epidermal fibers (4,29).
RETRACTED

activated insulin; S-S
intranasal insulin; C
indicated groups and time points, df
(nonmatched ANOVA tests, with Bonferroni post hoc
comparison of D I-I mice to D I-S mice using multiple ANOVA testing
comparison of D I-I mice to both D S-I and D I-S mice. ‡Significance with
indicated groups and time points, df
(nondiabetic intervention counterpart (D I-I versus C I-I, D I-S versus C
S-I), D S-I versus C S-I, and D S-S versus C S-S) (

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Physical property

Axonal fiber density

(per mm²)

Nondiabetic I-I mice 4–6 18,024 ± 136 17,522 ± 124
Nondiabetic I-S mice 4–6 18,122 ± 142 17,113 ± 132
Nondiabetic S-I mice 4–6 18,098 ± 138 17,222 ± 128
Nondiabetic S-S mice 4–6 18,055 ± 151 17,151 ± 147
Diabetic I-I mice 4–5 18,104 ± 157 16,377 ± 168*
Diabetic I-S mice 4 18,085 ± 164 14,982 ± 175*
Diabetic S-I mice 4–5 18,063 ± 161 15,522 ± 115*
Diabetic S-S mice 4–5 18,002 ± 168 14,916 ± 172*

Axonal area (µm²)

Nondiabetic I-I mice 4–6 33.6 ± 0.8 28.7 ± 0.7
Nondiabetic I-S mice 4–6 32.9 ± 0.7 27.4 ± 0.8
Nondiabetic S-I mice 4–6 32.9 ± 0.8 27.6 ± 0.7
Nondiabetic S-S mice 4–6 33.4 ± 0.7 27.3 ± 0.5
Diabetic I-I mice 4–5 33.8 ± 0.7 26.8 ± 0.6**‡
Diabetic I-S mice 4 32.7 ± 0.7 23.2 ± 0.8*
Diabetic S-I mice 4–5 33.1 ± 0.8 24.3 ± 0.7*
Diabetic S-S mice 4–5 32.6 ± 0.8 23.6 ± 0.7*

Axonal diameter (µm)

Nondiabetic I-I mice 4–6 5.34 ± 0.20 4.92 ± 0.15
Nondiabetic I-S mice 4–6 5.12 ± 0.21 4.77 ± 0.14
Nondiabetic S-I mice 4–6 5.28 ± 0.20 4.98 ± 0.14
Nondiabetic S-S mice 4–6 5.19 ± 0.19 4.83 ± 0.13
Diabetic I-I mice 4–5 5.18 ± 0.16 4.34 ± 0.11**‡
Diabetic I-S mice 4 5.08 ± 0.15 3.80 ± 0.12
Diabetic S-I mice 4–5 5.22 ± 0.12 4.02 ± 0.11
Diabetic S-S mice 4–5 5.11 ± 0.19 3.78 ± 0.10*

Myelination thickness

(µm)

Nondiabetic I-I mice 4–6 1.02 ± 0.05 0.96 ± 0.04
Nondiabetic I-S mice 4–6 1.03 ± 0.05 0.93 ± 0.05
Nondiabetic S-I mice 4–6 1.02 ± 0.04 0.95 ± 0.05
Nondiabetic S-S mice 4–6 1.04 ± 0.05 0.94 ± 0.04
Diabetic I-I mice 4–5 1.03 ± 0.05 0.85 ± 0.04†‡
Diabetic I-S mice 4 1.02 ± 0.04 0.79 ± 0.03*
Diabetic S-I mice 4–5 1.02 ± 0.04 0.82 ± 0.04*
Diabetic S-S mice 4–5 1.03 ± 0.05 0.76 ± 0.04*

Data are means ± SEM. *Significance between diabetic mice and their
nondiabetic intervention counterpart (D I-I versus C I-I, D I-S versus C
I-S, D S-I versus C S-I, and D S-S versus C S-S) (α = 0.05, P < 0.016)
(nomatched ANOVA tests, F-values range between 0.88 and
8.76 for indicated groups and time points, DF = 4,3, n = 4–6). ‡Significance with comparison of D I-I mice to both D S-I and D I-S
mice. †Significance with comparison of D I-I mice to D I-S mice using
multiple ANOVA testing with Bonferroni post hoc t test comparisons
(α = 0.05, P < 0.016) (nomatched ANOVA tests, F-values range between 0.98 and 3.55 for indicated groups and time points, df = 5,4,
n = 4–6). D = diabetic; I-I = intranasal insulin; C = control; I-S =
intranasal saline; S-I = subcutaneous insulin; S-S = subcutaneous saline.

Insulin’s downstream signaling pathways. Insulin stimulation upregulates protein-tyrosine phosphorylation (30) through downstream activation of IRS-2 (18). Insulin also modulates the inner mitochondrial membrane potential through activation of the PI3K pathway (31), stimulating phosphorylation of Akt and Akt substrates such as CREB (32–35). PI3K promotes translocation of voltage-dependent calcium channel currents to the neurolemma in an Akt-dependent manner (36). Activated Akt is important for sensory neurite extension and branching (37), and the PI3K–Akt pathway has a positive regulatory effect on myelin-associated glycoprotein (MAG) expression in Schwann cells, Schwann cell differentiation (38), and promotion of myelination (39) through Schwann cell biosynthesis of fatty acids (40). Similar to insulin, IGF-I also activates the PI3K/Akt pathway (41), leading to phosphorylation of Akt effectors, including CREB and GSK-3β (19). IGF-I also leads to accumulation of pAkt within DRG neuronal nuclei and increases CREB-mediated transcription (19). In our studies, prevention of downregulation of PI3K/Akt (Fig. 4) in murine DRG was associated with amelioration of behavioral, electrophysiological, and morphological changes pertaining to diabetes.

CREB is also a neuroprotective molecule; CREB phosphorylation inhibits apoptosis in embryonic neurons (42), whereas the loss of CREB results in impaired axonal growth (43). Meanwhile, GSK-3β, downstream of PI3K/Akt, is a neuron-specific promoter of apoptosis in DRG neurons when it is active (nonphosphorylated...
Phosphorylation of GSK-3 by Akt renders it inactive, leading to antiapoptotic properties (44,45). GSK-3 also regulates the transcriptional activities of CREB (46,47) and may regulate gene expression and activity of transcriptional factor binding to the MAG promoter region (38). GSK-3β also promotes Schwann cell differentiation, suggesting that the PI3K/Akt/GSK-3β pathway is crucial for initiation and possibly mainte-
nance of myelination through promotion of MAG expression (38). Thus, insulin may be important in maintaining conduction velocities (Fig. 3) by direct effects on Schwann cells. In our studies, I-I delivery was associated with elevation of pCREB and pGSK-3β levels and reversal of diabetes-associated suppression of CREB-DNA binding within diabetic mouse DRGs (Fig. 4 and supplemental Fig. 3).
Usefulness of intranasal delivery in diabetic neuropathy. Intranasal administration allows insulin to bypass the blood-brain barrier and enter the brain and spinal cord parenchyma, as well as CSF, within 1 h. Its entry into the nervous system and CSF likely occurs through extracellular bulk flow transport along both olfactory and trigeminal neural pathways and may use perivascular channels of blood vessels entering the CNS (21). This method of insulin delivery permitted us to study the impact of insulin without affecting glycemia levels such as occurs with systemic insulin delivery, easing difficulty in dissecting the relative contributions of hyperglycemia and insulin’s trophic properties (48). Prior studies using intranasal delivery of insulin-like molecules such as IGF-I have demonstrated safety and efficacy in experimental stroke (22). I-I delivery in humans has led to improvements in memory (23) within minimal impact on plasma glucose levels, which remain in the euglycemic range (20,23). Although the use of I-I for the management of systemic diabetes has been limited, in our mouse cohorts, I-I was also associated with better maintenance of body weight and improved mortality (Table 1).

Limitations of our results using I-I delivery in diabetic mice must be acknowledged. Our results must be considered under the limitations of working with a murine model, and the inability to achieve a long-term model of murine type 1 diabetes with optimal glycemic management as a suitable control group. The mouse cohorts were subjected to intensive testing throughout their lifetime, which may have led to stress impacting on behavioral testing results. Diabetic CD1 mice developed sensory behavioral changes earlier than has been observed in other rodent models of diabetes, which may limit portability of these findings to other models. It is also possible that hypoglycemia may have impacted on sensorimotor testing: the impact of hypoglycemia on the diabetic I-S and control I-S cohort groups was anticipated but unavoidable. Distribution of insulin within the diabetic nervous system may differ from results obtained in nondiabetic mice examined in our radiolabeling studies. In addition, although I-I–obtained concentrations were higher in nervous system tissues, tissue concentrations at later time points were not different between I-I and S-I delivery, suggesting that differences in systemic exposure and possibly different metabolic rates for insulin may also play a role in our results. Based on our studies, it is difficult to develop a more appropriate control group of diabetic mice with long-term glycemic control based on the STZ-induced diabetic model. However, our results also provide evidence for potentially robust benefits of insulin independent of its actions on hyperglycemia. These results support its role as an important neurotrophic factor in the management of diabetic neuropathy. Our results support the development of human I-I clinical trials for the prevention and slowing of the development of DPN.

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