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Research Paper

Deletion of the insulin receptor in sensory neurons increases pancreatic insulin levels

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ABSTRACT

Insulin is known to have neurotrophic properties and loss of insulin support to sensory neurons may contribute to peripheral diabetic neuropathy (PDN). Here, genetically-modified mice were generated in which peripheral sensory neurons lacked the insulin receptor (SNIRKO mice) to determine whether disrupted sensory neuron insulin signaling plays a crucial role in the development of PDN and whether SNIRKO mice develop symptoms of PDN due to reduced insulin neurotrophic support. Our results revealed that SNIRKO mice were euglycemic and never displayed significant changes in a wide range of sensorimotor behaviors, nerve conduction velocity or intraepidermal nerve fiber density. However, SNIRKO mice displayed elevated serum insulin levels, glucose intolerance, and increased insulin content in the islets of Langerhans of the pancreas. These results contribute to the growing idea that sensory innervation of pancreatic islets is key to regulating islet function and that a negative feedback loop of sensory neuron insulin signaling keeps this regulation in balance. Our results suggest that a loss of insulin receptors in sensory neurons does not lead to peripheral nerve dysfunction. The SNIRKO mice will be a powerful tool to investigate sensory neuron insulin signaling and may give a unique insight into the role that sensory neurons play in modifying islet physiology.

1. Introduction

The pathogenesis of peripheral diabetic neuropathy (PDN) is complex but involves complications of hyperglycemia and reduced neurotrophic support (Singh et al., 2014). In addition to its actions on glucose, insulin acts as a neurotrophic factor for sensory neurons. Increasing evidence suggests direct neuronal insulin signaling impacts sensory neuron function and disruption of neuronal insulin signaling may contribute to PDN (Kim and Feldman, 2012). Studies of insulin action on sensory neurons in vivo face inherent difficulties due to insulin's broad systemic actions, making it difficult to identify direct actions of insulin on sensory neurons. With current PDN rodent models, neither hyperglycemia nor reductions in peripheral nervous system (PNS) insulin signaling can be isolated to establish the pathogenesis arising from either insulting factor. For example, STZ-induced diabetic mice are hyperglycemic and hypoinsulinemic and *ob/ob* mice are hyperglycemic and insulin resistant; thus, both models have elevated glucose levels and reduced insulin signaling that complicate studies direct actions of insulin on sensory neurons (Grote et al., 2013a; Grote et al., 2011).

The generation of tissue specific insulin receptor knockout mice has greatly increased our understanding of the physiological role of insulin (Kahn, 2003). The purpose of this study was to assess systemic metabolic and sensorimotor consequences generated by absent insulin signaling selectively in sensory neurons by generating sensory neuron insulin receptor knockout (SNIRKO) mice. Our prediction was that SNIRKO mice would develop neuropathy symptoms like other mouse models of PDN due to the absent neurotrophic support from insulin. Our results suggest that reductions in sensory neuron insulin signaling alone do not contribute to the symptoms of PDN. However, our results reveal a potential and surprising feedback mechanism may exist between sensory neuron insulin signaling and beta cell insulin production.

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2. Methods

2.1. Animals and genotyping

Advillin$^{Cre/+}$ mice were a generous gift of Dr. Fan Wang (Hasegawa et al., 2007) and have been previously characterized to demonstrate sensory neuron specific cre recombinase activity (da da Silva et al., 2011; Hasegawa et al., 2007; Minett et al., 2012; Zhou et al., 2010; Zurborg et al., 2011) and this was confirmed in our lab using a fluorescent reporter line *Gt(ROSA)26Sor$^{tm4(ACTB-tdTomato,-EGFP)Luo}$*/J (Tomato) (Jackson Laboratories, Bar Harbor, ME). Mice tissues were fixed via intracardial perfusion with Zamboni's fixative (4% paraformaldehyde and 15% picric acid) prior to dissection. Tissue sections were cut using a Leica CM 1950 cryostat and placed on slides in serial sections. Sections were then covered with PBS and cover slipped. Images were acquired using a Nikon Eclipse 90i microscope. Exposure times were kept constant between experimental groups. (Supplemental Fig. S1).

Mice with loxp sites flanking exon 4 of the insulin receptor gene (IR$^{lox/lox}$) were purchased from Jackson Laboratories (Bar Harbor, ME). In the presence of cre recombinase, exon 4 is deleted creating a frameshift mutation resulting in a stop codon. The resultant product would be a nonfunctional 308 amino acid truncated peptide. Male Advillin$^{Cre/+}$ mice were bred to female IR$^{lox/lox}$ to generate heterozygous Advillin$^{Cre/+}$, IR$^{lox/+}$ mice. Male Advillin$^{Cre/+}$, IR$^{lox/+}$ were bred to female IR$^{lox/lox}$ to produce SNIRKO mice with an Advillin$^{Cre/+}$, IR$^{lox/lox}$ genotype. Mice were genotyped via tail clip. Primers used for genotyping Advillin$^{Cre/+}$ were:

p1: 5′-CCCTGTTCACTGTGAGTAGG-3′,
p2: 5′-AGTATCTGGTAGGTGCTTCCAG-3′,
p3: 5′-GCGATCCCTGAACATGTCCATC-3′.

A wildtype allele produced a 500 bp fragment and a cre-expressing allele produced a 180 bp fragment. Primers used for genotyping IR$^{lox/lox}$ were:

p1: 5′-GATGTGCACCCCATGTCTG-3′,
p2: 5′-CTGAATAGCTGAGACCACAG-3′.

A wildtype allele produced a 279 bp fragment and an allele with loxp insertion produced a 313 bp fragment. All primers were added to a supermix to genotype SNIRKO mice and the PCR product was amplified for 35 cycles (94 °C for 15 s, 62 °C for 15 s, 72 °C for 90 s) in a 40 μL reaction (Supplemental Fig. S2). IR$^{lox/lox}$ were used as controls for all experiments. All experiments were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee.

2.2. Insulin receptor quantification

2.2.1. RT-PCR

Total RNA was isolated from control and SNIRKO DRG to assess Cre/loxP recombination as described previously (Guerra et al., 2001). A reverse primer specific for exon 6 of the insulin receptor was used for reverse transcription: 5′-GTGATGGTGAGGTTGTGTTTGCTC-3′. The reaction was carried out using an iScript select kit (Bio-Rad) at 42° for 30 min, followed by 85° for 5 min. The generated cDNA was then used for PCR template. Primers to exon 3, 5′-GCTGCACAGCTGAAGGCC TGT-3′, and exon 5, 5′-CTCCTCGAATCAGATGTAGCT-3′ were used to amplify the region corresponding to exon 4. PCR conditions were 94° for 30 s followed by 35 cycles of 94° for 30 s, 58° for 30 s, 72 °for 1 min and a final extension at 72° for 7 min. A 585 bp fragment indicates an intact insulin receptor and a 435 bp fragment indicates cre/loxp recombination and deletion of the 150 bp exon 4.

2.2.2. Western blots

Insulin receptor protein expression was quantified in gastrocnemius muscle and DRG using Western blot analysis. Samples were homogenized in Cell Extraction Buffer (Invitrogen, Carlsbad, CA) containing 55.55 μL/mL protease inhibitor cocktail, 200 mM Na_3VO_4, and 200 mM NaF. Protein concentration of the supernatant was measured with a Bradford assay (Bio-Rad, Hercules, CA). Samples were boiled with Lane Marker Reducing Sample Buffer (Thermo Scientific, Waltham, MA) for 3 min. Thirty μg of protein was loaded per lane and samples were separated on a 4–15% gradient tris-glycine gel (Bio-Rad). After gel electrophoresis, samples were transferred to a nitrocellulose membrane and blocked in 5% milk. Following incubation with primary (insulin receptor β subunit (Santa Cruz), actin (Millipore) and secondary antibodies, bands were visualized with film and analyzed with ImageJ (NIH).

Akt activation was assessed using western blots in muscle and DRG following an intraperitoneal injection of insulin at 10.0 U/Kg or vehicle control as previously described (Grote et al., 2013b). Mice were fasted 3 h prior to insulin injection. Thirty minutes after insulin stimulation mice were sacrificed and tissues were harvested. Western samples were then prepared and blots were probed with total Akt and p-(Ser473)Akt (Cell Signaling, Danvers, MA).

2.3. Metabolic parameters

Metabolic parameters were monitored throughout the course of SNIRKO development and testing. Mice weights were recorded at 3, 5, 6, 7, 8, 16, 22, and 28 weeks of age.

2.3.1. Glucose and hemoglobin A1C

Blood glucose levels were determined using a glucose diagnostic assay (Sigma-Aldrich, St. Louis, MO). Mice were fasted 3 h prior to glucose measurement and blood was collected via tail snip at 6, 10, 16, 22, and 29 weeks of age. In addition, long term glucose levels were assessed by determining hemoglobin A1C levels immediately prior to sacrifice at 29 weeks of age using A1CNow + Meter (Bayer, Leverkusen, Germany).

2.3.2. Insulin and IGF-1

After a 3-h fast, whole blood was collected via tail snip and allowed to clot on ice for 30 min. Samples were then centrifuged at 3000*g* for 15 min. The resultant serum supernatant was used for analysis. Serum insulin and IGF-1 levels were measured with ELISAs from ALPCO (Salem, NH). Serum insulin levels were measured alongside glucose at 6, 10, 16, 22, and 29 weeks of age and IGF-1 levels were determined at sacrifice.

2.3.3. Intraperitoneal glucose tolerance test (IPGTT)

At 28 weeks of age, both IR$^{lox/lox}$ and SNIRKO glucose tolerance was analyzed with an IPGTT. After a 6-h fast, mice were administered a glucose bolus of 2 g/kg body weight via IP injection. Blood glucose measurements were taken immediately prior to glucose stimulation and at 15, 30, 60 and 120 min thereafter.

2.4. Sensorimotor behavior and neuron analysis

2.4.1. Thermal sensitivity

Mice were acclimated to the behavior facility and equipment for a minimum of 2 days. On test days, mice were acclimated to the behavior facility for 30 min and subsequently to the Hargreaves table for 30 min prior to data collection. The table surface was maintained at 30 °C and mice were housed in individual clear plastic cages. A 4.0 V radiant heat source was applied to the mid plantar surface of the hind paw, and time to withdrawal was measured (Hargreaves et al., 1988). Four trials were recorded for each hind paw, alternating paws between trials. Data was presented as the average latency to withdrawal across both paws.

2.4.2. Mechanical sensitivity

Mice were acclimated to the mesh grid in clear individual plastic cages. The up-down method was used to test paw mechanical sensitivity (Dixon, 1980). A set von Frey monofilaments (Stoelting, Wood Dale, IL) capable of exerting forces of 0.0045, 0.02, 0.068, 0.158, 0.178, 1.2,