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Effect of insulin and an erythropoietin-derived peptide (ARA290) on established neuritic dystrophy and neuronopathy in Akita (*Ins2*^{Akita}) diabetic mouse sympathetic ganglia ☆

Robert E. Schmidt [a,*], Dongyan Feng [a], Qiuling Wang [a], Karen G. Green [a], Lisa L. Snipes [a], Michael Yamin [b], Michael Brines [b]

[a] Division of Neuropathology, Department of Pathology and Immunology, Washington University School of Medicine, Saint Louis, Missouri, 63110, USA
[b] Araim Pharmaceuticals, Ossining, NY, USA

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ABSTRACT

The Akita mouse is a robust model of diabetic autonomic neuropathy which develops severe diabetes following beta cell death, which occurs reproducibly at 3–4 weeks of age, and maintains the diabetic state without therapy for as long as 11 additional months. Neuritic dystrophy and neuronopathy involving prevertebral sympathetic superior mesenteric and celiac ganglia begin to develop within the first two months of onset of diabetes and are progressive with time. We have examined the effect of insulin implants resulting in normoglycemia and injections of ARA290, a small erythropoietin peptide which has no effect on glycemic parameters, on the reversal of established neuritic dystrophy and neuronopathy. We have found that 4 weeks of insulin therapy beginning at 2 months of diabetes resulted in normalization of blood glucose, body weight and HbA1c. Insulin therapy successfully reversed established neuritic dystrophy and neuronopathy to control levels. Numbers of sympathetic neurons were not significantly changed in either 3 month diabetic or insulin-treated Akita mice. Treatment with ARA290 for 7 weeks beginning at 4 months of diabetes did not result in altered metabolic severity of diabetes as measured by blood glucose, body weight or HbA1c levels. ARA290 treatment was able to decrease neuritic dystrophy by 55–74% compared to untreated diabetics or in comparison to a separate group of diabetic animals representing the 4 month treatment onset point. Surprisingly, there was no effect of ARA290 on ganglionic neuron number or ongoing neuronopathy (pale/degenerating neurons) in diabetic Akita mice during this time period. The development of neuroprotective EPO-like peptides may provide a possible future therapy for this debilitating complication of diabetes; however, it appears that discrete elements may be differentially targeted by the diabetic state and may require selective therapy.

Introduction

Autonomic neuropathy is an increasingly recognized problem in human diabetes which may result in cardiovascular, genitourinary, sudomotor and alimentary symptoms (Rundles, 1945) or remain undetected as subclinical disease. Studies of prevertebral sympathetic ganglia in autopsied diabetic human subjects demonstrate its pathologic hallmark, neuroaxonal dystrophy, which consists of terminal axonal swellings containing a distinctive admixture of subcellular elements (Duchen et al., 1980; Schmidt et al., 1993), superimposed on a mild, poorly characterized decrease in neuronal density (Schmidt et al., 1993). These defects may disconnect or misconnect ganglionic neurons and, particularly for prevertebral ganglia serving the viscera, contribute to the loss of integrated autonomic reflexes which depend on intraganglionic connections. Rodent models of diabetic sympathetic autonomic neuropathy show significant correspondence with human pathology, developing dystrophic axons in prevertebral ganglia in the presence of relative, but not absolute, preservation of sympathetic neurons (Schmidt, 2001; Schmidt et al., 2003, 2008).

We have shown that non-obese diabetic (NOD) and streptozotocin-treated NOD/severe combined immune deficient (STZ-Rx NOD/SCID) mice develop dramatic axonal as well as dendritic pathology (i.e., "neuritic dystrophy") within a few weeks of onset of diabetes (Schmidt et al., 2003, 2008). However, once diabetic, neither NOD nor STZ-Rx NOD/SCID mice survive long enough to test the ability of therapeutic agents to correct established neuropathy, clinically a more relevant

Abbreviations: CEPO, carbamylated erythropoietin; CG, celiac ganglion; EPO, erythropoietin; NOD, non-obese diabetic; STZ, streptozotocin; SMG, superior mesenteric ganglion; STZ-Rx NOD/SCID, streptozotocin-treated NOD/severe combined immune deficient.

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* Corresponding author at: Department of Pathology and Immunology (Box 8118), Washington University School of Medicine, 660 South Euclid Avenue Saint Louis, MO 63110, USA. Fax: +1 314 362 4096.
E-mail address: reschmidt@wustl.edu (R.E. Schmidt).

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paradigm than prevention of neuropathy. We have recently begun to investigate the Akita (*Ins2*^{Akita}) mouse model to address this deficiency (Schmidt et al., 2009). Akita mice are characterized by a spontaneous dominant mutation in the *insulin 2* gene on a C57BL6 mouse background which results in a tyrosine for cysteine substitution, disrupting a disulfide bridge required for proper insulin folding. This defect initiates an unfolded protein response and culminates in pancreatic β-cell apoptosis in the absence of obesity, insulitis or insulin resistance (Izumi et al., 2003; Mathews et al., 2002; Ron, 2002; Yoshioka et al., 1997). Heterozygous Akita mice are reproducibly and severely hyperglycemic (males more severely than females) and hypoinsulinemic beginning at 3–4 weeks of age but remain viable in the absence of exogenous insulin treatment for as long as 11 months, far longer than 5–8 weeks of severe hyperglycemia which can be maintained in NOD and STZ-treated NOD/SCID models over which time animals become increasingly debilitated and fragile. Within 2 months of onset of diabetes, male Akita-diabetic mice show marked neuritic dystrophy in prevertebral sympathetic ganglia identical in appearance and anatomic distribution to that which develops in other mouse models. In addition, neurons in Akita mouse prevertebral sympathetic ganglia show an unusual perikaryal alteration characterized by the accumulation of membranous aggregates and minute mitochondria and loss of rough endoplasmic reticulum culminating eventually in neuronal degeneration.

Initially discovered as a mediator of erythropoiesis, for some time erythropoietin (EPO) has been recognized to have neuroprotective effects on a variety of animal models of CNS and PNS neurodegeneration. Neuroprotection by EPO (Hoke, 2006) has been described in acrylamide and cisplatin toxic neuropathies (Bianchi et al., 2007; Keswani et al., 2004a; Melli et al., 2006), HIV sensory neuropathy (Keswani et al., 2004b) and, particularly pertinent to our studies, experimental diabetic somatic neuropathy (Bianchi et al., 2004; Tam et al., 2006). Using our STZ-treated NOD/SCID mouse model, we recently showed (Schmidt et al., 2008) that EPO and carbamylated erythropoietin (CEPO) prevented the development of experimental diabetic autonomic neuropathy which is thought to reflect a poorly understood role of these agents in neuroprotection independent of a hematopoietic effect. In the experiments described here we have demonstrated the effect of insulin implants on the reversal of established neuritic dystrophy and neuronal degeneration following a course of 4 weeks of therapy. Additionally, we show that the use of a non-erythropoietic EPO-derived peptide ARA290 (Brines et al., 2008) results in a salutary effect on the frequency of neuritic dystrophy but did not prevent neuronal degeneration and loss in Akita prevertebral ganglia suggesting that these pathologic features may have a different pathogenesis.

Materials and methods

Animals

The C57BL/6J-Ins2Akita animals used were obtained from the Jackson Laboratory. All animals were housed and cared for in accordance with the guidelines of the Washington University Committee for the Humane Care of Laboratory Animals and with National Institutes of Health guidelines on laboratory animal welfare and EC Directive 86/609/EEC for animal experiments. All mice were allowed standard rat chow and water ad libitum and maintained on a 12/12 hour light/dark cycle.

Treatments

Insulin treatment

Male Akita mice became diabetic at 3–4 weeks of age and were maintained untreated for two additional months at which time they were separated into two groups. The first group received insulin containing subcutaneous implants (Linbit mouse implant, LinShin, Canada) at a dose recommended based on body weight, [2 implants for 20-gram animals with an additional implant for each 5 g of body weight (total range 2–5 implants)]. The second diabetic group received blank insulin-free implants as did C57BL6J control mice. Animals were followed by weekly measurement of body weight and blood glucose measurements for 4 weeks at which time animals were euthanized by decapitation following ketamine/xylazine treatment. HbA1c measurements were made at the time of euthanasia.

EPO-peptide (ARA290) treatment

Once male Akita mice spontaneously developed diabetes they were allowed to survive for 15–16 weeks without therapy to an age of 18–19 weeks. At that time diabetic and control animals were separated into two groups: 1) ARA290-treated mice which received daily injections of ARA290 (MW 1258, 36 μg/kg body weight /day, subcutaneously, 5 days/week, Araim Pharmaceuticals); and, 2) saline-treated mice. Animals were followed by measurement of blood glucose and body weight for 7 additional weeks at which time they were euthanized. HbA1c measurements were made at the time of euthanasia.

Tissue preparation

Male Akita and age-matched C57BL/6J control mice were anesthetized with ketamine/xylazine, the abdominal cavity opened, overlying organs displaced and immersed in cold modified Karnovsky's fixative containing 3% glutaraldehyde and 1% paraformaldehyde in sodium cacodylate buffer pH 7.4. Fixation was continued for several days at 4 °C in the same fixative. The celiac (CG) and superior mesenteric (SMG) ganglia were dissected, cleaned of extraneous tissue and rinsed in sodium cacodylate buffer. The CG were postfixed in phosphate cacodylate-buffered 2% OsO_4 for 1 h, dehydrated in graded ethanols with a final dehydration in propylene oxide and embedded in EMbed-812 (Electron Microscopy Sciences, Hatfield, PA). One-micron thick plastic sections were examined by light microscopy after staining with toluidine blue. Ultrathin sections (~90-nm thick) of individual ganglia were cut onto formvar coated slot grids, which permits visualization of entire ganglionic cross sections. Sections were post stained with uranyl acetate and Venable's lead citrate and viewed with a JEOL model 1200EX electron microscope (JEOL, Tokyo, Japan). Digital images were acquired using the AMT Advantage HR camera (Advanced Microscopy Techniques, Danvers, MA).

Morphometric studies

Quantitation of dystrophic neurites

Dystrophic elements are typically intimately related to individual neuronal perikarya and, therefore, we have routinely expressed their frequency as the ratio of numbers of lesions to numbers of nucleated neuronal cell bodies. This method, used in our previous studies (Schmidt et al., 2003, 2008, 2009), substantively reduced the variance in assessments of intraganglionic lesion frequency. In addition, its simplicity permits the quantitative ultrastructural examination of relatively large numbers of ganglia and identification of robust changes which are they infrequent enough to complicate typical non-biased counting. In the presence of neuron loss this method may overestimate the frequency of neuritic dystrophy, although loss of principal sympathetic neurons, thought to be the source of many intraganglionic dystrophic neurites, may conversely result in parallel changes in dystrophic neurites and neuron number and an unchanged ratio. In our current animal studies an entire cross section of the SMG or CG was scanned at 20,000× magnification and the number of dystrophic neurites and synapses was determined by an investigator blinded to the identity of individual animals. Dystrophic neurites consist of swollen axons, synapses, dendritic spines or dendrites containing a variety of organelles including: 1) axonal tubulovesicular aggregates; 2) pallid ribosome poor cytoplasm in dendrites; 3) axons with admixed normal and degenerating subcellular organelles and multivesicular bodies; 4) axonal