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Diabetes-Induced Chemogenic Hypoalgesia Is Paralleled by Attenuated Stimulus-Induced Fos Expression in the Spinal Cord of Diabetic Mice

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Abstract: **Chronic hyperglycemia in diabetes induces abnormal nerve pathologies, resulting in diabetic neuropathy (DN). Sensory symptoms of DN can manifest as positive (painful), negative (insensate), or both. Streptozotocin (STZ)-induced diabetic C57Bl/6 mice have reduced cutaneous innervation and display reduced behavioral responses to noxious stimuli, reflecting the insensate aspect of the human syndrome. Current studies were undertaken to determine whether the diabetes-induced deficits in pain responses are reflected by changes in spinal activation in this model of DN. Nocifensive responses of nondiabetic and diabetic mice to formalin injection were measured 1, 3, 5, and 7 weeks after STZ, and at each time point formalin-induced spinal Fos expression was quantified. Responses of diabetic mice were significantly reduced during the second phase of the formalin test beginning 3 weeks after STZ and during Phase 1 beginning 5 weeks after STZ. Consistent with the behavioral responses, the number of Fos-positive cells in the dorsal horn of diabetic animals was significantly reduced beginning 3 weeks after STZ and continuing 5 and 7 weeks after STZ. The deficits at 5 weeks after STZ were restored by 2-week treatments with insulin or neurotrophins. These results demonstrate that the reduced sensation occurring from progressive peripheral axon loss results in functional deficits in spinal cord activation.**

Perspective: ***The reduced expression of the immediate early gene Fos as an indicator of pain transmission supports the diabetes-induced loss of sensation in this Type 1 model of diabetes. This murine model may be better suited to understanding the insensate symptoms of diabetic patients in the absence of chronic pain.***

Key words: *Neuropathy, pain behavior, Fos, NGF, GDNF, insulin.*

Diabetic neuropathy (DN) is one of the principle chronic complications of both Type 1 and Type 2 diabetes mellitus and currently affects more than half of diabetic patients. In human patients and animal models, DN commonly manifests as a distal symmetric sensory polyneuropathy[44,47] characterized by the distal degeneration of peripheral axons.[12,18,19,26,29,34] Axonal loss can also be accompanied by segmental demyelination[22] and reduced nerve regeneration capacity.[27,38] Together, these structural changes result in reduced epidermal innervation, decreased nerve conduction velocities, and reduced amplitude of sensory nerve action potentials. However, the exact manner in which the hyperglycemic environment contributes to nerve damage is still unresolved.

In humans, diabetes-induced nerve dysfunction can produce a variable degree of motor and autonomic symptoms, but sensory deficits are the predominant feature of DN. Sensory loss develops in the majority of affected human patients, including both chronic numbness and insensitivity to pain or touch. Painful symptoms (paresthesias, hyperalgesia, tactile allodynia) are only reported in up to 32% of patients with DN, are most likely to present early in the disease progression, and have a slightly higher prevalence in Type 2 diabetes.[32,42,43,45]

Current animal models of diabetes vary in their presentation of neuropathy symptoms. Streptozotocin (STZ) is commonly used to induce diabetes in rodents because of

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its specific toxic effects on pancreatic beta cells.[28] STZ-treated rats develop mechanical, thermal, and chemogenic hyperalgesia as well as tactile and thermal allodynia.[6] These sensory abnormalities are proposed to reflect the painful aspect of human diabetic neuropathy, yet do not model the insensate symptoms suffered by the majority of human patients. In contrast, it's widely known that STZ-treated C57BL/6 mice display reduced sensitivity to mechanical, chemogenic, and, in some cases, thermal stimuli.[11,13,49] In addition, STZ-induced diabetic C57BL/6 mice display reduced dermal and epidermal innervation of the hind paw, as well as abnormalities in the central terminals of primary nociceptive neurons.[3,12] Therefore, the STZ-induced diabetic C57BL/6 mouse model may be better suited for exploring abnormal peripheral nerve function associated with insensate symptoms.[13]

The relationship between an animal's behavior and pain status is inherently subjective, and it is not absolute that rodent nocifensive withdrawal behaviors are directly related to the perceived stimulus intensity. Fos is an immediate-early transcription factor expressed in second-order spinal neurons in response to a noxious peripheral stimulus and has been used as a surrogate for peripheral nerve activation. In response to formalin injection into the hind paw, Fos is expressed in a temporal and spatial pattern consistent with the magnitude of nociceptive input from the hind paw.[1,4,39] To test the hypothesis that diabetes-induced peripheral nerve damage results in suppressed spinal activation, we compared spinal Fos expression in response to formalin injection in nondiabetic and diabetic mice during the progression of neuropathy. In addition, we tested whether insulin or neurotrophin treatments restored Fos expression in a manner consistent with improved behavioral responses. Our results suggest that STZ-induced sensory loss in C57Bl/6 mice reduces Fos expression in the dorsal horn in a manner consistent with peripheral nerve damage and reduced primary afferent input. Moreover, treatments that improve aspects of the neuropathy can similarly improve stimulus-induced Fos expression in the spinal cord.

Materials and methods

Animals

All animal use was in accordance with NIH guidelines and conformed to the principles specified by the University of Kansas Medical Center Animal Care and Use Protocol. In all studies, 8-week-old male C57BL/6 mice (Charles River, Wilmington, MA) were housed 2 to 4 mice per cage on a 12:12-hour light/dark cycle under pathogen-free conditions with free access to mouse chow and water.

Experimental Design

To assess whether progressive behavioral deficits in diabetic mice represent true hypoalgesia, nondiabetic and diabetic mice were injected with STZ or vehicle on day 0 and killed 1, 3, 5, or 7 weeks later. Formalin testing was performed on the day of death for each separate end point, and formalin-induced Fos expression was evaluated. Based on these results, further experiments were performed to determine whether the concomitant deficits observed in behavioral responses and Fos expression could be restored. At 5 weeks after STZ, nondiabetic and diabetic mice treated with insulin, neurotrophins, or CSF were tested for formalin responses, killed, and evaluated for Fos expression.

Diabetes Induction

Diabetes was induced by a single intraperitoneal injection of streptozotocin (180 mg/kg body weight; Sigma, St. Louis, MO) dissolved in 10 mmol/L sodium citrate buffer, pH 4.5.[50] Nondiabetic mice were injected with sodium citrate buffer alone. Animal weight and tail vein blood glucose levels using glucose diagnostic reagents (Sigma) were measured 1 week after STZ and every other week thereafter to assess diabetes. Only STZ-injected mice with blood glucose levels greater than 16.0 mmol/L (288 mg/dL) were included in the diabetic groups; STZ-injected mice with blood glucose levels below that standard were not included in the study.[50] Blood collection for the final time point was taken subsequent to formalin testing so that behavioral measurements would not be influenced by the blood draw. Weight and blood glucose levels were compared between nondiabetic and diabetic untreated animals using 2-way analysis of variance (ANOVA) followed by post hoc analysis using the Fisher's PLSD test. Repeated measures (RM) AVOVA was used to analyze weight and blood glucose of treated nondiabetic and diabetic animals before and after treatment.

Behavioral Analysis

The formalin test was performed before the animals were killed 1, 3, 5, and 7 weeks after STZ on nondiabetic and diabetic mice by an experienced experimenter blinded to the condition of the mice. After a 1 hour-habituation to individual observation chambers, mice were injected subcutaneously with 20 μL of formalin (5% formaldehyde) into the dorsal surface of the right hind paw, using a 1-mL insulin syringe and 28-gauge needle. The amount of time devoted to the injected foot (licking and biting) was recorded in two 10-minute windows during the acute (Phase 1; 0 to 10 minutes after injection) and inflammatory (Phase 2; 40 to 50 minutes after injection) phases of the formalin test. Differences in the attentive time spent to the injected foot during each phase were compared between nondiabetic and diabetic mice at each time point using unpaired *t* tests.

Fos Immunocytochemistry

The expression of Fos protein was examined in the spinal cords of nondiabetic and diabetic mice. Two hours after formalin injection, when Fos protein is expressed at maximal levels,[4] mice were anesthetized with Avertin (1.25% vol/vol tribromoethanol, 2.5% *tert*-amyl alcohol, 200 μL/10 g body weight; Sigma) and transcardially per-