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Early Changes in Insulin Receptor Signaling and Pain Sensation in Streptozotocin-Induced Diabetic Neuropathy in Rats

Kazuhiro Sugimoto,* Irena B. Rashid,* Masaru Shoji,* Toshihiro Suda,† and Minoru Yasujima*

**Department of Laboratory Medicine, and the †Department of Endocrinology and Metabolism, Hirosaki University Graduate School of Medicine, Hirosaki, Japan.*

Abstract: The objective of the present study was to evaluate the time course of changes in peripheral nerve insulin receptor (IR) signaling and compare observed findings with behavioral responses to noxious mechanical and thermal stimuli in streptozotocin (STZ)-diabetic rats over 12 weeks of diabetes. Diabetic rats developed mechanical hyperalgesia, as indicated by decreased paw withdrawal thresholds to mechanical stimuli that were detectable after 2 weeks of diabetes; they also developed thermal hypoalgesia, as indicated by increased tail flick latencies to thermal stimuli that were detectable at 1 week of diabetes. Western blot analysis revealed decreased phosphorylated: total IR protein ratio that was detectable as early as 2 weeks of diabetes, whereas phosphorylated: total Akt protein ratio was decreased at 2 weeks and increased at 12 weeks of diabetes with unchanged PI-3K protein levels. To our knowledge, the present study is the first to demonstrate that impaired peripheral nerve IR signaling, as indicated by decreased phosphorylated:total IR protein ratio, coincides with early mechanical hyperalgesia and thermal hypoalgesia in STZ-diabetic rats. This finding may improve understanding of how altered pain sensation develops rapidly in this model.

***Perspective:** This study examined peripheral nerve IR signaling during the early course of altered nociception in STZ-diabetic rats. In diabetic rats, impaired peripheral nerve IR signaling is observed shortly after STZ injection, as is altered nociception. This finding suggests a possible role of impaired IR signaling in diabetic sensory neuropathy.*

***Key words:** Insulin signaling, Akt, sodium channel, thermal hypoalgesia, mechanical hyperalgesia, diabetic neuropathy.*

Diabetic neuropathy has been considered the most common and earliest complication of long-term hyperglycemia.[6,20] Although a number of studies have examined the mechanisms underlying hyperglycemia-induced nerve damage, the pathogenesis of diabetic neuropathy remains unclear.[56] On the other hand, evidence is accruing that insulin deficiency rather than hyperglycemia contributes to the development of diabetic neuropathy.[43,44,52,53] In the type 1 diabetic BB/W rat model, altered insulin receptor (IR) signaling in the peripheral nervous system (PNS) has been linked to neurological disorders including impaired nerve regeneration,[68] nodal/paranodal degeneration,[55] loss of sensory neurons,[32] and abnormal small fiber function and structure.[29]

The streptozotocin (STZ)-induced diabetic rat model, the most extensively studied animal model of diabetic neuropathy, exhibits a variety of types of early neurological dysfunction,[11,13,16,62] including altered pain sensation,[1,18,48] which suggest the early involvement of small nociceptive sensory neurons.[15,22] In this model, trace amounts of insulin, administered either systematically[24,47] or intrathecally,[8,64] can ameliorate mitochondrial dysfunction in sensory neurons,[24] myelinated sensory fiber atrophy,[8] epidermal unmyelinated sensory fiber loss and atrophy,[64] and altered nociceptive responses,[47] without affecting blood glucose levels. Previous studies have reported that high-affinity IRs are expressed in the PNS[58,65] and are in rats predominantly located in small- to medium-sized sensory neurons.[57] Based on these findings, we hypothesized that the insulin deficiency induced by STZ administration in rats may impair IR signaling in the PNS and result in altered nociception.

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Address reprint requests to Dr. Kazuhiro Sugimoto, Department of Laboratory Medicine, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan. E-mail: sugimoto@cc.hirosaki-u.ac.jp
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Insulin's biological functions are mediated via tyrosine phosphorylation of IRs, which is essential for activation of the receptor tyrosine kinase and its downstream signaling cascades, including the phosphatidylinositol-3 kinase (PI-3K) pathway, in which Akt is activated through phosphorylation of Thr308 and Ser473.[33,34] Interestingly, like the IRs, phosphorylated Akt has been reported to be selectively expressed in a subpopulation of small sensory neurons[42,59] involved in the processing of nociceptive information.[36,60] However, no information is available on whether peripheral nerve IR signaling and its downstream signaling molecules such as PI-3K and Akt are affected concurrently with the development of nociceptive dysfunction or whether this signaling is affected by the duration of diabetes in STZ-diabetic rats. Such information would be particularly useful for elucidating the mechanisms underlying the rapid development of altered nociception in this model.

Moreover, tetrodotoxin-resistant (TTX-R) voltage-gated sodium channel $Na_v1.8$ has also been reported to be distributed predominantly in nociceptive sensory neurons[2,49] and to be involved in neuropathic and inflammatory pain states.[3,19,26,69] Previous studies have indicated that the altered nociception in STZ-diabetic rats is associated with changes in the expression and activity of $Na_v1.8$ in sensory neurons.[15,21] On the other hand, in rat models of neuropathic pain due to partial nerve injury and inflammatory pain, massive increase in $Na_v1.8$ protein expression in peripheral axons has been suggested to contribute to peripheral sensitization.[12,19] However, no studies have examined peripheral nerve $Na_v1.8$ protein expression and its association with altered nociception as well as with peripheral nerve IR signaling in STZ-diabetic rats.

In the present study, we therefore assessed the protein expression and phosphorylation of IRs as well as downstream signaling molecules such as PI-3K and Akt in peripheral nerves from STZ-diabetic rats with various durations of diabetes and examined the relationships between the findings obtained and behavioral responses to mechanical and thermal stimuli as well as the protein expression of $Na_v1.8$ in diabetic rats.

Methods

Experimental Animals

Male Wistar rats were housed on sawdust in plastic cages, maintained on a 12 hour-12 hour light-dark cycle at 22° ± 2°C and 55% ± 5% relative humidity, and allowed free access to tap water and standard laboratory chow. All animal protocols were approved by the Animal Research Committee and followed the Guidelines for Animal Experimentation of Hirosaki University.

Induction of Diabetes by STZ

After an overnight fast, diabetes was induced in 10-week-old rats by a single intravenous injection of STZ (45 mg/kg; Sigma, St. Louis, MO) into the tail vein. STZ solution was prepared freshly by dissolving it in 0.05 mol/L citrate buffer (pH 4.0). Age-matched nondiabetic rats received an injection of citrate buffer alone and served as controls. Diabetic condition was assessed by serial measurement of nonfasting tail vein blood glucose level using an Accu-Chek Compact Plus blood glucose meter (Roche Diagnostics K.K., Tokyo, Japan) up to 12 weeks after STZ injection, and diabetic rats with constant hyperglycemia (blood glucose levels of more than 300 mg/dL) were included in the study. To evaluate the degree of chronic hyperglycemia in diabetic rats, hemoglobin A1c levels were measured in whole blood drawn from the left cardiac ventricle using the Dimension clinical chemistry system (Dade Behring Inc., Newark, DE) in a subset of diabetic and control rats at the indicated time points.

Behavioral Tests of Nociception

It was not possible for the investigator (K.S.) to be blinded to animal status because of the obvious emaciation of diabetic animals. Ninety-five diabetic and 49 age-matched control rats were used to evaluate changes in mechanical and thermal perception at the indicated time points after STZ injection. Before starting the experiments, the animals were allowed to acclimatize to handling-related stress for at least 1 week. The experimental procedures described below were then carried out. The mechanical nociceptive threshold was assessed with the Randall-Selitto test using an Analgesymeter (Ugo-Basile, Varese, Italy). A constantly increasing pressure stimulus (with increase at a rate of 16 g/s) was applied to the dorsal surface of the rat hind paw while the animal was gently restrained under a soft towel; to avoid tissue damage, a cutoff of 250 g was used. The pressure was increased until the animal withdrew the paw, squeaked, or struggled. One measurement per paw was performed with an interval of longer than 15 minutes between measurements; for each animal, the results for the 2 paws were averaged for use in statistical analysis. Thermal sensitivity was assessed using a Tail Flick Analgesymeter (MK-330B; Muromachi Kikai Co, Ltd., Tokyo, Japan). The animal was gently wrapped in a towel and placed on the top of the instrument with the tail in the sensing groove. The tail flick latency was determined by exposing the animal's tail to a radiant heat source and recording the time taken to remove the tail from the noxious thermal stimulus. The radiation intensity was chosen on the basis of the intensity required to elicit a basal tail flick response of 4 to 8 seconds in control rats. For each animal, 2 to 3 recordings were made at an interval of longer than 15 minutes; the mean value was used for statistical analysis.

Tissue Sampling

A subset of diabetic rats with constant hyperglycemia and age-matched control rats were killed by exsanguination from the left cardiac ventricle under deep anesthesia with pentobarbital (~100 mg/kg), and the anterior tibial muscle, brain, and bilateral sciatic nerves were collected at the indicated time points. In the case of the sciatic nerves, we carefully peeled off the epineurial and perineurial tissues to ensure that the epineurial connec-