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Mechanical hyperalgesia correlates with insulin deficiency in normoglycemic streptozotocin-treated rats

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The triggers and pathogenesis of peripheral diabetic neuropathy are poorly understood, and this study evaluated the role of insulinopenia in nociceptive abnormalities in the streptozotocin (STZ) rat model of diabetes to test the hypothesis that, in addition to hyperglycemia, impairment of insulin signaling may be involved in progression of neuropathy. We measured blood glucose, plasma insulin, and sciatic nerve glucose and sorbitol levels, and withdrawal thresholds for hind limb pressure pain and heat pain in STZ-injected rats that developed hyperglycemia or remained normoglycemic. The pressure pain threshold did not change in vehicle-injected controls, but during the 2 weeks after STZ, it decreased by 25–40% in STZ-hyperglycemic and STZ-normoglycemic animals ($P<0.05$). Mean heat pain threshold did not change in STZ-normoglycemic rats, but increased by about 1.5°C in STZ-hyperglycemic rats ($P<0.05$). These pain thresholds did not correlate with blood or nerve glucose or sorbitol levels, but both correlated with plasma insulin level in STZ-normoglycemic rats, and low-dose insulin replacement normalized the pressure threshold without affecting blood glucose level. Thus, at least one of early signs of diabetic neuropathy in STZ-treated rats, mechanical hyperalgesia, can be triggered by moderate insulinopenia, irrespective of glycemic status of the animals.

Keywords: Mechanical hyperalgesia; Pain; Diabetic neuropathy; Insulin; Hyperglycemia; Streptozotocin; Type I diabetes

Introduction

Chronic, systemic hyperglycemia has been historically viewed as a major trigger for the pathogenic mechanisms of neurologic complications of type I and type II diabetes (Ishii, 1995; Sugimoto et al., 2000a). For example, distal peripheral neuropathy is a frequent symptom of diabetes, and most symptoms of this neuropathy may be explained as either immediate or secondary consequences of hyperglycemia-associated activation of aldose reductase (the first enzyme in polyol pathway), which causes nerve sorbitol accumulation followed by osmotic, metabolic, oxidative and circulatory imbalance in peripheral nervous tissue (Sugimoto et al., 2000a). Further support for the hyperglycemic hypothesis comes from clinical trials showing that stringent glycemic control decreases the incidence of peripheral neuropathy by as much as 60% to 70% over several years (Boulton et al., 2004; DCCT, 1993), and from animal research demonstrating parallel development of hyperglycemia, nerve conduction slowing, and nociceptive impairments and an apparent association between hyperglycemia, sensitivity of cutaneous nociceptors, and mechanical hyperalgesia (Biessels et al., 1999; Dobretsov et al., 2001, 2003; Khan et al., 2002; Sugimoto et al., 2000a; Suzuki et al., 2002).

In spite of persuasive supporting evidence, the glucose hypothesis alone does not fully explain the variety and complexity of clinical presentations of distal peripheral neuropathy. While glucose control is an effective preventive measure, during 5 years of follow up study at least 30% of diabetic patients with apparently satisfactory control of blood glucose level still developed neuropathy (DCCT, 1993). Furthermore, there is a high incidence of painful neuropathies in pre-diabetic patients who have impaired glucose tolerance but are normoglycemic or moderately hyperglycemic (Singleton et al., 2001). Moreover, neither clinical (Chan et al., 1990; Thye-Ronn et al., 1994) nor animal (Courteix et al., 1996; Dobretsov et al., 2003; Maneuf et al., 2004; Romanovsky et al., 2004) studies have shown a correlation between hyperglycemic status and pain/hyperalgesia. Taken together, these observations suggest that factors in addition to hyperglycemia must play a role in the pathogenesis of distal peripheral neuropathy, and identification and evaluation of the causative mechanisms of peripheral neuropathy are important, not only for understanding the clinical course of the disorder, but also for the development of new strategies for its treatment and prevention.

Recent data from several independent laboratories implicate insulinopenia as a contributory factor in development of peripheral neuropathy (Brussee et al., 2004; Huang et al., 2003; Schmidt et

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al., 2004), and our initial studies suggest that mechanical hyperalgesia, a symptom of distal peripheral neuropathy, is caused by insulinopenia without accompanying hyperglycemia in a rat model using the pancreatic toxin streptozotocin (STZ) (Romanovsky et al., 2004). Establishing a neuropathic role for insufficiency of insulin signaling may help explain the apparent inconsistencies related to the glucose dependence of the origin of pain, especially in pre-diabetic patients, and the present study was, therefore, designed to examine relationships among insulinopenia, hyperglycemia, and early signs of neuropathy reflected by abnormalities of nociception in STZ-treated rats.

Materials and Methods

All experimental procedures followed "Principles of laboratory animal care" (NIH publication no. 85-23, revised 1985) and were reviewed and approved by the Institutional Animal Care and Use Committee.

Animal procedures

All behavioral tests were carried out between 2 and 6 PM on male Sprague–Dawley rats (200–300 g, Harlan Inc., Indianapolis, IN); only one test per day was given to any animal. During the week before injection of STZ, rats were first acclimatized to the testing apparatus, then baseline nociceptive threshold measurements were conducted; baseline results of 3–4 pressure pain threshold or 1–2 heat pain threshold test sessions (see below) were averaged and designated as day 0 values. After acclimation and baseline tests, rats were randomly assigned to experimental or control groups. Experimental rats were injected with STZ (65 mg kg^{-1} body weight in 33 mM citrate-buffered saline given i.p.; pH 4.5) while control animals received an equivalent injection of vehicle, citrate-buffered saline. Tail blood samples were taken for glucose determination from overnight-fasted animals on the day before STZ injection and on days 3, 8 and 13 thereafter. Based on the day 3 fasting blood glucose level, the rats were categorized as normoglycemic or hyperglycemic, using a cutoff value of >6.9 mM to define hyperglycemia (American Diabetes Association, 2006). Pressure pain threshold was measured on days 3, 6, 9, 11 and 14 after STZ treatment, whereas heat pain threshold was measured on days 4 and 12. When behavioral testing followed the fasting glucose measurements, at least 6 h of free access to the food was allowed to animals before the behavioral data collection. Samples for determination of plasma insulin and sciatic nerve metabolite levels were collected within 6 h after the last pain threshold test on day 14 after STZ from animals that were deeply anesthetized with halothane.

Insulin replacement therapy was carried out in separate groups of rats that were first carried through the procedures described above. Then, starting on day 8 after STZ injection, each control and STZ-injected rat was given two daily i.p. injections of 0.2–0.4 U of insulin (human recombinant insulin, Novolin R, Novo Nordisk Pharmaceuticals Inc., Princeton, NJ) in physiological saline for 7 days. This insulin dose is about 1/10 that required to correct hyperglycemia in diabetic animals, and a lower dose was used to avoid causing hypoglycemia in the normoglycemic rats (Brussee et al., 2004). In the insulin replacement rats, PPT was measured on days 4, 6, 8, 11 and 15, and blood for insulin level determination was drawn on day 15.

Paw pressure withdrawal threshold

Pressure pain threshold was measured with an Ugo Basil Analgesy-meter (Stoelting, Wood Dale, IL, USA) as described previously (Romanovsky et al., 2004). Briefly, each test session consisted of five trials (separated by at least 15 min) on both the left and right paws of each rat. In each trial, pressure was applied to the center of the hind paw at a linearly increasing rate of 16 g s^{-1} until the animal withdrew the paw, struggled, or in rare cases vocalized; to avoid tissue damage, the pressure cutoff of the device was set at 250 g. Any trial in which pressure cutoff was reached or animal's behavioral reaction could not be unequivocally attributed to withdrawal reflex was discounted and repeated. In successful trials, nociceptive pain threshold (expressed as mass units, g) was recorded from the Analgesy-meter and the mean bilateral pressure threshold for each test session was calculated for each animal as average of ten (five per paw) threshold readings.

Hot plate withdrawal threshold

Heat pain threshold was measured using an incremental Hot/Cold Plate Analgesia Meter (IITC Life Science, Woodland Hills, CA). The animal was placed on a clean and dry aluminum plate maintained at 28°C in the clear plastic chamber of the device. The test started as soon as the rat relaxed, either standing on all four paws, sitting, or grooming its head or forepaws. During the test, the plate temperature increased at a linear rate of 10°C min^{-1} with a cutoff temperature of 55°C. Hind limb heat pain threshold was defined as a temperature at which the animal abruptly withdrew either of its hind feet from the plate surface in a sharp move, typically followed by licking of the lifted paw. Trials were discounted if the paw withdrawal appeared to associate with normal grooming behavior or repositioning rather than with reflex behavior. Testing sessions continued at 15–20 min intervals until seven hind limb heat threshold readings were obtained for each animal. The results of these determinations were averaged, and the mean value was used for further analysis.

Blood glucose and insulin measurements

Blood glucose in tail-prick samples was measured using the colorimetric Accu-Chek blood glucose monitoring system (Roche Diagnostics Corp., Indianapolis, IN, with a manufacturer's stated 97–99% accuracy and precision over the device operating range of 0.6–33.3 mM glucose). For insulin determination, blood samples were obtained by cardiac-puncture from halothane-anesthetized animals. Insulin was measured in duplicate in each sample with the Ultra Sensitive Rat Insulin ELISA Kit according to the manufacturer's protocol (Crystal Chem Inc., Downers Grove, IL; range 0.1–64 ng, sensitivity 5 pg insulin/ml).

Sciatic nerve glucose, lactate, and sorbitol measurements

About 1 cm of each sciatic nerve was exposed by blunt dissection at mid-thigh level, frozen *in situ* by Fisher's Freeze'It, excised, and stored at about −80°C until weighed at −25°C in a cryobox (Cahn microbalance). The frozen nerves were minced, thawed at −12°C in 100% ethanol, and homogenized, then serially extracted (4×) with 65% ethanol, centrifuged, lyophilized, re-dissolved in deionized water (Dienel et al., 1990), flash frozen, and stored at −80°C. Glucose and lactate levels were assayed with a YSI Biochemistry Analyzer (Yellow Springs, OH). Sorbitol levels were assayed by