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COMPARISON OF METABOLIC AND NEUROPATHY PROFILES OF RATS WITH STREPTOZOTOCIN-INDUCED OVERT AND MODERATE INSULINOPENIA

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Abstract—To assess the relative roles of insulinopenia, hyperglycemia and dyslipidemia in pathogenesis of diabetic neuropathy, we compared plasma insulin, glucose and lipid metabolism and peripheral nerve function in rats with streptozotocin (STZ)-induced overt and moderate insulinopenia (hyperglycemic, STZ-HG; random glucose>11 mM and normoglycemic, STZ-NG rats). While being slightly insulinopenic, STZ-NG rats are metabolically not different from control, naive animals, by having normal glucose tolerance and normal levels of plasma glucose, glycated HbA1c, cholesterol and triglycerides. Two weeks following injection of STZ, STZ-HG but not STZ-NG rats had suppressed motor nerve conduction velocity, F-wave prevalence, withdrawal responses to heat and von Frey filament stimuli. In apparent correlation with plasma insulin level, both STZ-HG and -NG rats manifested exaggerated responses in paw pressure and colorectal distension tests. These data suggest that insulinopenia may play a leading role in the diabetic impairment of deep muscle and visceral afferent pathways while hyperglycemia/dyslipidemia may represent a key requirement for the onset and progression of electrophysiological nerve impairment and loss of superficial heat and tactile perception. STZ-NG rats offer a convenient model for the investigation of the short-term effects of insulinopenia on peripheral nerve function.

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Abbreviations: CRD, colorectal distension; DPN, diffuse peripheral neuropathy; EMG, electromyographic; FG, fasting plasma glucose; FTT, food tolerance test; GTT, glucose tolerance test; HbA1c, hemoglobin A1c; IENF, intraepidermal nerve fibers; MNCV, motor nerve conduction velocity; PNS, peripheral nervous system; PPT, pressure pain threshold; RG, random plasma glucose; STZ, streptozotocin; STZ-HG, STZ-injected, hyperglycemic; STZ-NG, STZ-injected, normoglycemic; VMR, visceromotor reflex; 8-OHdG, 8-hydroxydeoxyguanosine.

Key words: diabetes, pre-diabetes, hyperalgesia, allodynia, insulin, hyperglycemia.

Diffuse peripheral neuropathy (DPN) is a frequent and severe complication of diabetes mellitus (DM). Chronic hyperglycemia represents an important precipitating neuropathy factor (Tomlinson and Gardiner, 2008). However, neuropathy may start during pre-diabetes, in people manifesting moderate impairment of glucose metabolism, but not overt chronic hyperglycemia (Smith and Singleton, 2008). Thus in addition to hyperglycemia, non-glycemic factors, such as insulinopenia and C-peptide insufficiency (type 1 diabetes) or insulin resistance and compensatory hyperinsulinemia (type 2 diabetes), or dyslipidemia and abnormalities in insulin-like growth factors production (type 1 and 2 diabetes), are likely to play an independent pro-neuropathic role (Dobretsov et al., 2007b; Zochodne, 2008). Evaluation of non-glycemic pathogenesis of DPN requires large scale longitudinal studies and detailed analysis and comparison of neuropathy, hormonal and metabolic profiles of people with diabetes and pre-diabetes as well as of asymptomatic subjects from high-risk (first-degree relatives of DM patients) populations. Such studies are expensive and difficult to organize, and research in animals appears to be an important step in understanding the function of the peripheral nervous system (PNS) under pre-diabetic conditions.

Rats with streptozotocin (STZ)-induced selective pancreatic islet β-cell injury provide a well-known STZ-hyperglycemic (STZ-HG) rat model of overt type 1 diabetes which is also widely used in pre-clinical studies of diabetic neuropathy (Sharma and Richards, 2000; Rees and Alcolado, 2005). The diabetogenic action of STZ is not absolute and with injection of low-to-intermediate doses of the toxin there are always some animals in which insulin production remains sufficient to maintain both fasting and postprandial glucose at a normal level, mimicking early stages of human type 1 pre-diabetes. This situation has been described for STZ-injected rats, baboons and minipigs (Junod et al., 1969; McCulloch et al., 1988; Kahn et al., 1992; Romanovsky et al., 2004, 2006). Recent reports have indicated that pin-prick hyperalgesia and pain on pressure that develop in STZ-diabetic rats may also be observed in STZ-normoglycemic (STZ-NG) rats. Furthermore, in experiments in STZ-NG rats the magnitude of pain on pressure correlated with plasma insulin and could be treated by a low-dose insulin replacement which did not affect plasma glucose levels. Pin-prick and deep pressure hypersensitiv-

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ity are measures of skin and deep muscle nociception, respectively (Lillioja et al., 1993; Simone et al., 1994; Graven-Nielsen et al., 2004). Thus the finding above suggested that insulinopenia may be at least as efficient as hyperglycemia in affecting these pathways during progression of diabetes. However, unlike that for overtly diabetic animals, the metabolic profile and nerve function have never been fully characterized in either model of STZ-induced moderate insulinopenia. It also remains unclear if neuronal circuitries other than those involved with pin-prick and pressure hyperalgesia may be affected by mild insulinopenia.

To address these questions, measurements of random glucose, glucose in food tolerance test, random plasma cholesterol and triglycerides, islet β-cell area, plasma insulin and oxidative stress DNA damage marker 8-OHdG (8-hydroxydeoxyguanosine) were utilized to characterize the state of the disease. Measurements of pain on pressure, motor nerve conduction velocity and F-wave characteristics, withdrawal responses to dynamic and punctate tactile and heat stimuli, intraepidermal nerve fibers (IENF) density and length, and viscero–somatic response to colorectal distension were used to evaluate and compare different aspects of somatic and autonomic peripheral nerve function between the models.

EXPERIMENTAL PROCEDURES

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

Animals and induction of diabetes

This report is based on the results of 10 independent experiments conducted between 2007 and 2009 using male Sprague–Dawley rats (200–350 g, Harlan Inc., Indianapolis, IN, USA). STZ was dissolved in a citrate buffer (pH=4.5) immediately before injection and given intraperitoneally at the 65 mg/kg dose to a rat fed *ad libitum*. On day 3 after injection of STZ random blood glucose was measured and rats that developed hyperglycemia (>11 mM) or remained normoglycemic were designated as STZ-HG and STZ-NG animals, respectively. Each experiment included 12 to 22 animals with four controls (i.p. injection of citrate buffer) per group. Not all tests were conducted in all studied rats. However, except for measurements of oxidative stress markers, the results of every test conducted by the end of second week after STZ-injection were collected in at least two independent experiments.

Food tolerance test

In the food tolerance test, overnight fasted rats were placed in individual cages with access to a standard (3 g/kg of rat weight) piece of Teklad Global #2016 diet; an amount that was completely consumed by a rat within 5–10 min. Glucose was measured before feeding and at 30, 60, 90, and 120 min thereafter. A food tolerance test instead of a glucose tolerance test (FTT vs. GTT) was chosen because while GTT amplifies glucose intolerance, FTT is less stressful and it is more relevant as a measure of postprandial hyperglycemia—the state that is frequently implicated as an alternative to chronic hyperglycemia trigger of DPN.

Colorimetric and ELISA determinations

Glucose and glycated hemoglobin (HbA1c) were measured in blood samples obtained by tail-prick technique using the colorimetric Accu-Chek blood glucose monitoring system (Roche Diagnostics Corp., Indianapolis, IN, USA), and DCA 2000 Analyzer, reagent cartridges and control kits (Bayer Corporation, Elkhart, IN, USA), respectively. Plasma insulin, lipids and 8-OHdG (a marker of oxidative DNA damage) were measured in samples of ventricular blood collected from isoflurane-anesthetized animals and processed according to the manufacturer's protocols using, respectively, the Ultra Sensitive Rat Insulin ELISA Kit (Crystal Chem Inc., Downers Grove, IL, USA), the CardioCheck PA Analyzer (Polymer Technology System Inc., Indianapolis, IN, USA), and the highly-sensitive 8-OHdG ELISA Kit (Cosmo Bio Co. Ltd. Tokyo, Japan).

Behavioral tests

All behavioral tests were carried out between 2 and 6 PM on rats that had free access to food and water for at least 4 h prior the test; only one test per day was given to any animal. During the week before injection of STZ, rats were acclimatized to the given testing apparatus; the results of those acclimating sessions were discarded.

Paw pressure withdrawal threshold. Pressure pain threshold (PPT) was measured with an Ugo Basil analgesy-meter (Stoelting, Wood Dale, IL, USA) as described previously. 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 $g \cdot s^{-1}$ until the animal struggled to withdraw or succeeded in withdrawing the paw. To avoid tissue damage the pressure cutoff of the device was set at 250 g. The nociceptive pain threshold (expressed as mass units, g) was recorded from the analgesy-meter and the bilateral pressure threshold for each test session was calculated for each animal as the average of ten (five per paw) threshold readings. The analgesy-meter is designed to apply the stimulus via a conical rounded stylus to the dorsal surface of the paw supported on a flat plinth. In the experiments with "reversed" PPT measurements, the apparatus was modified by switching the positions of the plinth and stylus, so that the stylus was now facing the ventral surface of the paw. The remaining settings and protocol of "reversed" PPT measurements were the same as described for the standard procedure.

Von Frey filament and camel brush tests. Rats were placed in an elevated testing cage with a wire mesh bottom and allowed to acclimate for 30 min. During the test session withdrawal reactions to tactile dynamic and then to static stimuli were measured. Stroking the plantar surface of the foot with a camel hair brush (#2; $\approx$1.5 g stroking force) from the center of the paw to the heel served as a dynamic stimulus. Application of a 20 g-bending force von Frey filament to the center of the paw for 1–2 s was used as a static stimulus. For each rat, the percentage of brisk lifting withdrawals in 10 trials with the brush and 10 trials with the von Frey filaments was determined and documented (the interval between sequential trials in the same paw of the same rat was not less than 1 min).

Hot plate withdrawal threshold. Heat pain threshold was measured using an incremental hot/cold plate analgesia meter (IITC Life Science, Woodland Hills, CA, USA). The animal was placed on a clean and dry aluminum plate maintained at 20 °C in the clear plastic chamber of the device. The test started after about 15 min of acclimation time, when the rat was relaxed, standing on all four paws, sitting, or lying down. During the test, the plate temperature increased at a linear rate of 5 °C min^{-1} with a cutoff temperature of 50 °C. Hind limb heat pain threshold was defined as the temperature at which the animal abruptly withdrew its paw from the plate and licked either of its hind feet. Testing sessions continued at 15–20 min intervals until three hind limb