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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, naïve 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.

Keywords

diabetes; pre-diabetes; hyperalgesia; allodynia; insulin; hyperglycemia

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1. Introduction

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; Romanovsky et al., 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 hypersensitivity 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, IENF density and length, and viscerosomatic response to colorectal distension were used to evaluate and compare different aspects of somatic and autonomic peripheral nerve function between the models.

2. 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.

2.1. 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). 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.

2.2. 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 minutes. Glucose was measured before feeding and at 30, 60, 90, and 120 minutes 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.

2.3. 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), and DCA 2000 Analyzer, reagent cartridges and control kits (Bayer Corporation, Elkhart, IN), respectively. Plasma insulin, lipids and 8-OHdG (8-hydroxydeoxyguanosine, 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), the CardioCheck PA Analyzer (Polymer Technology System Inc., Indianapolis, IN), and the highly-sensitive 8-OHdG ELISA Kit (Cosmo Bio Co. Ltd. Tokyo, Japan).

2.4. 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 four hours 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 minutes) 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 $16 \text{ g}\cdot\text{s}^{-1}$ 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 250g. 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 minutes. 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; ≈ 1.5 g stroking force) from the center of the paw to the heel served as a dynamic stimulus. Application of a 20g-bending force von Frey filament to the center of the paw for 1-2 seconds 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 minute).

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 20°C in the clear plastic chamber of the device. The test started after about 15 minutes 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 minutes intervals until three hind limb heat threshold readings were obtained for each animal. The average of these three readings was used for further analysis.

Heat withdrawal latency—Rats were allowed to acclimate in the compartments on the glass platform of a plantar test apparatus (Ugo Basile, Italy). A mobile radiant heat source located under the platform was then focused onto the center of the plantar surface of a hind-paw and switched on. The trial ended automatically, and withdrawal latency was recorded in seconds immediately upon the rat's lifting its paw or if a pre-set 30s cut-off time was achieved. Seven paw withdrawal (either hind-paw) latency readings were measured for each rat and used to calculate average withdrawal latency. For each animal, the interval between tests was 3 or more minutes.

2.5. Immunohistochemistry

Skin and pancreas samples were collected from animals that were deeply anesthetized with isoflurane. Paraffin embedding and all staining procedures followed an immunohistochemical protocol described elsewhere (Romanovsky et al., 2007). Peroxidase-DAB stained sections were observed using an upright BX51W1 microscope (Olympus, Japan) and bright-field illumination. Images were captured using a Photometrics CoolSnap camera (Roper Scientific, Ottobrunn, Germany) and analyzed with Image-Pro PLUS 5 software (Media Cybernetics, Silver Spring, MD).

Pancreas—The entire splenic lobe of the pancreas was excised, fixed in 4% formaldehyde solution for 3 days and embedded into paraffin for cross-sectioning. Four-micron thick sections were collected from three regions separated by about 250 μ m within the central part

of the sample. Serial sections representative of each region were mounted on glass slides in pairs. Of these two sections, one was labeled to identify β -cells (rabbit anti-human insulin immunoglobulins; 1:500, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and another was labeled to mark the entire islet area (rabbit antibodies raised against human PGP 9.5 (Lopez-Avalos et al., 2006); 1:400, Biogenesis, England, UK). All islets containing more than 5 β -cells and identified in parallel sections were studied. Relative β -cell area for each of these islets was measured as the ratio of the β -cell and total islet territories. Data collected from three regions of given samples were averaged.

Skin—Samples of plantar right or left mid-paw skin were collected (one sample per rat), fixed in picric acid-based Zamboni's fixative for 3 days and embedded into paraffin for cross-sectioning in distal-to-proximal direction. The entire sample was cut and 20 μ m-thick sections representative of four regions located at about 200-250 μ m from the center of the sample were selected for further immunohistochemical processing. Each included foot pad and inter-pad skin areas. Nerve fibers were labeled with antibodies against PGP 9.5 (1:400). Stained sections were observed with 10 $\times$ and, when required, 20 $\times$ objectives and epidermal thickness and nerve fiber density and length were measured in as many microscopic fields (10 $\times$ objective) as necessary to cover the entire length of the section (7 to 11 mm). Epidermal fibers were defined using established recommendations for the analysis of human skin biopsy samples (Lauria et al., 2005). Specifically, only nerve fibers that could be traced back to their origin at the epidermal/dermal border were counted and measured, and any fiber that branched within the epidermis was counted and measured as a single unit. Collected data were averaged first for all microscopic fields studied within the given section and then for all four sections studied for a given animal. Epidermal fiber length is expressed in μ m / fiber (all branches included) and fiber density is expressed in counts per millimeter of length of outer border of epidermis.

2.6. Electrophysiological studies

Nerve conduction—When conducted in the same animals nerve conduction and behavioral studies were done on alternate days. To further minimize possibility of interference of these measurements the NCV studies in this work were conducted on the tail, but not the limb sciatic nerve. It has been previously shown that STZ-induced diabetes causes the same degree of NCV slowing in both of these nerves (Weis et al., 1995). Animals were anesthetized with isoflurane and their body temperature was monitored by a rectal probe and maintained at 37°C by using a feed-back controlled warming pad and a radiant heat lamp (TCAT-2DF controller, Phisitemp Instruments, Clifton, NJ). For the recording, the ring silver wire electrodes were placed 8 cm and 9 cm distal to the base of the tail. Two stimulating ring electrodes were placed, one (S1) at the tail's base and another (S2) 5 cm distal to the first. A reference electrode (a common anode for stimulating electrodes) was clipped to the rat's back skin about 2 cm proximal to the S1 electrode. A recording ground clip electrode was positioned between S2 and the active recording electrodes. Rectangular constant-current stimuli (0.2 ms duration; isolated pulse stimulator 2100, A-M Systems, Carlsborg, WA) were delivered at 1 Hz, and their strength was adjusted to about 1.5x the strength required to achieve maximal amplitude of the recorded compound muscle action potentials (M-waves). Signals were recorded using a differential amplifier ISO-80 (World Precision Instruments, Sarasota, FL) and digitized and analyzed using a Micro 1401 analog-to-digital converter and Signal-4 software (Cambridge Electronic Design, Cambridge, UK). Motor conduction velocity was calculated as the measured distance between stimulating cathodes in the straight tail position divided by the difference in latencies of M-waves (time to peak of average of 10 M-waves) to stimulation at the two locations. A total of 50 frames per stimulation site were used to collect data on F-wave prevalence. The F-wave latency was measured as time between positive peaks of M- and F-waves.

Colorectal distension-induced visceromotor reflex (VMR)—measurements were conducted as described previously (Wang et al., 2008). VMR was measured in rats sedated by light isoflurane anesthesia (2%) using electromyographic (EMG) recording. The EMG electrode was inserted through a small skin incision into the external oblique muscle superior to the inguinal ligament. The signal was amplified (ISO-180), displayed on an oscilloscope and fed into a computer using CED 1401 plus and Spike 2 software. For colorectal distension, a latex balloon (glove finger, 4 cm in length) was inserted rectally into the descending colon. The balloon was distended with air four times to 20, 40, 60, and 80 mmHg (as measured with a sphygmomanometer). The interval between distention stimuli was 4 minutes and the stimulus duration was 20s. Biphasic raw EMG signals were rectified using Spike 2 software script integrated over the stimulus duration and corrected by subtraction of integral baseline activity (over 20 s preceding the stimulus). These integral EMG VMR values were expressed in area under the curve (mV/sec) units and used to determine the average stimulus-response function per rat group.

2.7. Statistical methods

Statistical analysis was conducted using Statistica Software (StatSoft, Tulsa, OK) and Origin 7.5 Graphing and Analysis Software (OriginLab, Northampton, MA). Unless stated otherwise, the data were checked for normality of distribution (Shapiro-Wilk test) and analyzed using an ANOVA or repeat measures ANOVA followed by a Tukey HSD test for post-hoc comparisons. Effects were considered as statistically significant at $p < 0.05$. Data in figures are expressed as mean $\pm$ SE.

3. Results

3.1. Study entry characteristics and diabetogenic effect of STZ

Since rats were randomly assigned to groups, entry weights and glucose levels of control and STZ-injected rats did not differ (Table 1). Similarly, there were no statistically significant baseline weight or glucose level differences between the control group and any of the sub-groups of STZ-injected rats (STZ-NG, STZ-HG and STZ-HG rats that developed ketonic coma). Interestingly however, within the STZ-injected rats, the entry weight of rats that remained normoglycemic was slightly but statistically significantly lower than that of rats that developed diabetes following the injection (Table 1).

3.2. General physical and metabolic characteristics

Multiple abnormalities evidencing severe metabolic disease were detected in diabetic STZ-HG rats in this study. These rats ceased to gain weight, maintained random and postprandial (in food tolerance test) hyperglycemia (Fig. 1, filled circles), and after 2 weeks of diabetes they had increased blood HbA1c, triglycerides and 8-OH-dG levels and decreased low density cholesterol, insulin and pancreatic β -cell area (Fig. 2, dark grey columns).

Unlike STZ-HG rats, STZ-NG rats gained weight normally and were not different from control animals in either parameters of glucose or lipid metabolism tested (Fig. 1 and 2; half-filled circles and light grey columns). However in accordance with our earlier findings (; Dobretsov and Romanovsky, 2006), these experiments show that STZ-NG rats are not absolutely normal. Compared to the control group, by the end of second week of experiments, STZ-NG rats had a decreased β -cell area (20%) and plasma insulin level (40%) (Fig. 2D and E). Furthermore, the plasma level of 8-OHdG was increased about 50% above that of control animals (Fig. 2F), suggesting systemic oxidative stress.

3.3. Nerve function profile

Pain on pressure—In support of our earlier findings, at two weeks pain pressure threshold (PPT) was decreased in both STZ-NG and STZ-HG rats (Fig. 3A). With reversed PPT measurements, the absolute pain thresholds (because of different compliance of ventral and dorsal paw surfaces) were higher than in the standard test, but still STZ-HG rats showed maximal intolerance to pressure; control rats were most tolerant and STZ-NG rats were intermediate in tolerance (Fig. 3B).

Von Frey filament and brush withdrawal tests—showed a decreased frequency of responses in STZ-HG rats to both types of stimuli (Fig. 3C and D). The difference between von Frey filament response frequencies of control and STZ-HG rats was statistically significant (Fig. 3D). A von Frey filament threshold decrease in STZ-diabetic rats to below 4 g of filament force has been previously reported (Field et al., 1999). With our experimental paradigm (20g von Frey hair) such a drop in mechanical threshold would translate to nearly 100% frequency of withdrawals, which has never been observed in any STZ-HG rats tested in our experiments.

Heat pain—Hot plate and radiant heat are the two most common tests for studies of heat nociception in rodents. Despite the fact that these tests differ in the degree of animal contact with the heated surface (localized to one paw or not localized) and outcome measures (threshold of paw withdrawal and lick vs. latency of a simple withdrawal), both produced similar results in our experiments (Fig. 4A and B). After 2 weeks, no abnormalities in heat perception were found in STZ-NG rats, but both heat withdrawal threshold and latency were increased above control values in STZ-HG rats.

Epidermal skin innervation—A loss of nociceptive heat response could result from neurodegeneration and epidermal denervation. In our studies, PGP 9.5 and peroxidase staining was used to label nerve fibers in sections of skin of experimental animals (Fig. 5A – C). With this technique we did not find any differences in the density of intraepidermal nerve fibers (IENF; Fig. 5E) between any of the studied groups of rats. Plantar foot epidermis of STZ-HG rats was thinner than that of control or NG animals (Fig. 5D). However, density of epidermal innervation expressed as IENF length per square millimeter of the epidermis did not differ between control, STZ-NG and HG rats (Fig. 5E).

Nerve conduction velocity and F-wave characteristics—An abnormal result in nerve conduction tests is one of the most consistent signs of neuropathy in human patients and in animal models of diabetes. In our studies, STZ-rats manifested a decline in tail motor nerve conduction velocity (MNCV), F-wave prevalence, and lengthening of minimal F-wave latency. However, none of these characteristics has been significantly ($p < 0.05$) changed in STZ-NG rats (Fig. 6A-C; panel D shows an example of the recording).

Autonomic nervous system—Autonomic neuropathy frequently accompanies somatic neuropathy in diabetes (Schmidt et al., 1981). The function of the autonomic nervous system in this work was assessed using repeated colorectal distension (CRD) technique for measurements of pressure-induced visceromotor reflex. The results of these studies are shown in Fig. 6E. Indicative of visceral hypersensitivity the average EMG responses to CRD were higher in STZ-HG rats than in control at every tested pressure. Albeit to a lesser degree than STZ-HG rats, STZ-NG animals also demonstrated statistically significant exaggerated visceral sensitivity.

3.4. Insulinopenia and pain on pressure

Our previous studies have shown that two weeks following injection with STZ, pain on pressure threshold in STZ-NG rats relates closely to the degree of insulinopenia developing in these animals. It has also been shown that the defect in insulin production observed in a baboon model of STZ-induced moderate insulinopenia spontaneously reverses with time. Some of these experiments, therefore, were expanded to monitor changes in PPT beyond our standard 2 week-long period. Fig. 7A summarizes all of our data on PPT studied over a total of four weeks of experiment. A slow but statistically significant continuous increase in PPT of control rats was observed with time (114 ± 4 g at the study entry vs. 130 ± 6 g after 4 weeks of experiment; $p < 0.05$; RM-ANOVA). Over the same time STZ-HG rats became pressure-intolerant by the second week of experiment, and their PPT remained unchanged thereafter. Unlike that in control or STZ-HG rats, changes in PPT of STZ-NG rats were transient. These animals were maximally sensitive to pressure during the first and second weeks of experiment but recovered by the fourth week of study to the PPT level that was not different from PPT of control rats (Fig. 7A; $p > 0.05$; RM-ANOVA and post-hoc Tukey HSD test).

Evaluation of baseline data showed a weak but statistically significant correlation between a rat's weight and its PPT, with a slope of regression line of 0.18 g increase in PPT per each gram of weight gain ($R = 0.31$; $p < 0.01$; 121 rat). Correction for this weightdependence completely eliminated the upward drift of PPT in control rats (data not shown). Weight changes however, could not account for changes in PPT of STZ-injected rats. Therefore, plasma insulin was measured and compared to PPT and random plasma glucose (RG) of animals from 2- and 4-week-long experiments (Fig. 7B and C). The major finding of this analysis was that while for control or STZ-HG rats insulin did not differ significantly between 2 week and 4 weeks (open and closed circles in Fig. 7B and C), the level of insulin was statistically significantly higher in 4-week STZ-NG rats as compared to their 2-week counterparts (Fig. 7, symbols connected with arrows pointing toward 4 week data points). This “recovery of insulin production” in STZ-NG rats did not result in changes in RG, but it was associated with correction of PPT to a level not different from either control group of rats. Overall, relationships between plasma insulin, RG and PPT matched closely those described previously, suggesting that, in rat, different degrees of insulinopenia are required to manifest abnormalities in nerve function and glucose metabolism, with the former being more sensitive to failure in insulin production.

4. Discussion

The purpose of this work is to provide an as complete as possible characterization and comparison of metabolic and nerve function profiles of STZ-NG and HG rats

4.1. STZ-rats and type 1 diabetes

The STZ-HG rat is a well known model of type 1 diabetes. Both in humans and STZ-HG rats, the disease is characterized by chronic hyperglycemia, dyslipidemia, weight loss, systemic oxidative stress and inflammation, all resulting from a nearly 90% depletion of pancreatic insulin-producing β -cells and insulinopenia. The major limitation of the STZ-HG rat model is that diabetes develops within days while the human disease can take years to progress (see (Vardi et al., 1991; Devendra et al., 2004)). Considering this problem, studies in STZ-NG rats are of obvious interest. STZ-NG rats are moderately insulinopenic, but nonetheless maintain normal fasting and random glucose, normal HbA1c and normal glucose in glucose- and food-tolerance tests (Fig. 1 and 2, and). STZ-NG rats also have normal lipid metabolism but a detectable accumulation of the blood markers of oxidative stress (Fig. 2). Thus the STZ-NG rat appears as a simple model mimicking at least two

important features of early stages of human type 1 pre-diabetes: moderate insulinopenia and oxidative stress. As in STZ-NG baboons, insulinopenia in STZ-NG rats is reversible, and yet this model provides us with approximately a 2 week-long window for studies of immediate direct (not complicated by hyperglycemia or dyslipidemia) pathogenic effects of a decline in insulin production.

The question regarding variation in the sensitivity of individual rats to the same dose of STZ remains unresolved. Within-strain differences in the diabetogenic effects of STZ could result from between-animals differences in the abundance of β -cell-specific Glut-2 isoform of glucose transporter (responsible also for β -cell-specific uptake of STZ) or differences in activation of islet cell poly(ADP-ribose)polymerase at the final stages of STZ-induced cytotoxic cascade (Cardinal et al., 1999; Yang and Wright, 2002)). Furthermore, glucose and STZ compete for cell entry. Therefore, the time elapsed between when the rat had its last meal and the STZ-injection constitutes another factor of variability. In our experiments rats had free access to food before STZ, and no attempts were made to monitor the amount and timing of food consumption during that period. In addition, the size of the pancreas, the major target of STZ, does not linearly relate to the rat body weight (Schneeman et al., 1983). As a result, the same dose of STZ (per kg body weight) may be expected to be more diabetogenic in larger animals. This factor could account for the observation in this study of a 10 g difference of pre-STZ weights of STZ-NG and STZ-HG rats (Table 1).

4.2. Neuropathy in STZ-HG rats

Hyperglycemia and diabetes have been studied for many years in STZ-treated rats. Our data reported here are largely consistent with that previous work with a few exceptions. First, a novel finding of this work is the observation of visceral hypersensitivity in STZ-HG animals. Visceral hypersensitivity is a frequent sign of human diabetic gastroparesis (Kumar et al., 2008). To our knowledge, our study is the first to describe this phenomenon in a rat model of diabetes. Second, our findings suggest a loss of perception of superficial touch in STZ-HG rat, in contrast to previous results suggesting an enhanced sensitivity, including those using the same rat strain (Harlan, Indianapolis; (Calcutt et al., 1996; Chen and Pan, 2002; Hong et al., 2004)), STZ concentration (65 mg/kg; (Hoybergs and Meert, 2007)), duration of experiment (1 -4 weeks) and site and type of stimulus (plantar foot and standard von Frey filaments), which reported a 30-50% decrease in STZ-HG rat tactile withdrawal thresholds. The underlying cause of these discrepancies remains to be determined; however, it is possible that the mechanisms of withdrawal responses to noxious (sharp tip) and innocuous (flat tip) stimulation with von Frey filaments are different and, therefore, may be differentially affected by diabetes. Our current finding of a loss of perception to superficial touch in STZ-HG rats appears to also disagree with our previous work showing early development of pin-prick hyperalgesia (a decrease in withdrawal threshold to sharp von Frey filaments) in STZ-NG rats. Central integration of A β - and C-fiber activity in the standard von Frey filament test is very sensitive to duration and frequency of the filament application (Bove, 2006), and this could be one of the reasons for the discrepancy in the results of this and previously published reports. In this study, the duration of the stimulus was 1 sec, while 5-6 seconds-long stimuli were used by others in reporting tactile allodynia in studies of STZ-HG rats.

4.3. Neuropathy in STZ-NG rats

The complex variety of individual presentations of diabetic diffuse peripheral neuropathy (DPN) in humans implies that different symptoms of DPN have different pathogeneses. In support of this statement, our previous and current and more complete comparison of metabolic and neuropathy profiles of STZ-HG and STZ-NG rats firmly suggest that diabetes-associated disturbance of autonomic function and function of the nociceptors

responsible for deep pressure and superficial pin-prick pain sensations may start early in STZ-treated animals and without involvement of glycemic factors or dyslipidemia. Conversely, impairment of glucose and/or lipid metabolism may indeed be critical contributors to the progression of motor nerve and heat and light touch skin afferent dysfunction observed here in STZ-HG rats only. As in our work, motor nerve conduction velocity (MNCV) was found to be decreased in the tail and sciatic nerves of STZ- or alloxan-injected rats, but only in those animals that developed overt hyperglycemia (Miyoshi and Goto, 1973; Greene et al., 1975).

Recently, it was argued that heat hyperalgesia developing in a STZ-HG mouse model could result from a direct neurotoxic effect of STZ leading to sensitization of skin capsaicin-sensitive neurons (Pabbidi et al., 2008). No heat hyperalgesia was detected in our experiments in STZ-NG rats (see above). However, the time course (peak at first week and spontaneous recovery by third week) of heat withdrawal latency changes described for STZ-mouse was very similar to that observed in this study for PPT (Fig. 7A), raising the question that pressure hyperalgesia, pin-prick and visceral hypersensitivity measured in STZ-NG animals are merely artifacts of neurotoxicity of STZ. We do not have sufficient data to argue this possibility with regard to visceral or pin-prick pain. Nonetheless, there are at least several lines of evidence indicating that it is insulinopenia, not direct STZ-toxicity responsible for pressure hyperalgesia in STZ—treated animals. First, in STZ-HG rats both metabolic disease and pain on pressure develop within the first weeks following toxin injection and persist thereafter over the following months without much change. STZ-induced death of β -cells explains the persistence of metabolic disease; there is, however, no equivalent structural injury in the nervous system that could explain the persistence of pressure hyperalgesia independent of metabolic causes. Second, in STZ-NG rats PPT correlates with plasma insulin with a linear regression coefficient of 0.94. Such strong correlation leaves small room for confounding factors. Third, PPT is fully corrected in STZ-NG rats by low-dose insulin replacement and in STZ-HG rats by high-dose insulin replacement (Courteix et al, 1996). Insulin replacement is efficient even after months of untreated STZ-induced diabetes. Finally, recovery of original PPT level occurs in STZ-NG rats at the fourth week of experiment (Fig. 7). Again, this occurs as an apparent consequence of the spontaneous recovery of insulin production in these animals. Direct STZ-toxicity is unlikely to explain all these observations. Insulin and C-peptide deficiency parallel each other in type 1 diabetes. The role of the latter in pressure hyperalgesia is ruled out by recovery of PPT in insulin-replacement experiments.

4.4 Insulinopenia and neuropathy

These data add to a growing pool of literature emphasizing the importance of insulin in controlling the normal function of peripheral nervous system (PNS) (; Singhal et al., 1997; Brussee et al., 2004; Toth et al., 2006). Furthermore, the function of some neuronal pathways, such as those involved with perception of nociceptive pressure and possibly with superficial pin-prick and visceral mechanosensitivity, appears to be substantially more demanding to the level of circulating insulin than the mechanisms of glucose homeostasis. The critical concentrations of plasma insulin required for maintenance of PPT and glucose are in the ranges of 1.5 – 2 ng/ml and 0.5 - 1 ng/ml, respectively (see Fig. 7 for random and (Dobretsov et al., 2007a) for fasting glucose data). The higher tolerance of glucose metabolism to insulinopenia than that of the PNS may derive from a combination of factors. Liver, skeletal muscle and fat cells express high-affinity insulin receptors (IR) at a higher density than do neurons, and the liver has a small fraction of hybrid insulin/insulin-like growth factor 1 receptors binding insulin with low affinity (Karagiannis et al., 1997; Bailyes et al., 1997; Sugimoto et al., 2000). Another factor that could play a role in vulnerability of the PNS is its relatively weak anti-oxidant defenses (Romero et al., 1990). Hyperglycemia is

a powerful pro-oxidative stress condition. Insulinopenia, however, appears on its own to be a factor that may shift the balance between production and elimination of free oxygen species toward the former (Bravi et al., 2006; Yu et al., 2006). In support of this, we note that 8-OHdG levels were higher in the plasma of STZ-NG than those of control animals (Fig. 2F).

It is not clear why a specific group of afferents, among many different types of neurons present in the PNS, appears to be the most sensitive to mild insulinopenia and systemic oxidative stress. Insulin controls expression of NO-synthase and, thus, *via* availability of NO, controls skin and skeletal muscle blood flow and relaxation of the distal colonic smooth muscle (Middleton et al., 1993; Clark et al., 2003). Therefore, moderate insulinopenia could result in increased excitability of skin, gastric and muscle nociceptors by modifying local tissue conditions. Another mechanism could be sensitization of skin-viscero-muscular nociceptive convergence neurons (either because of a direct effect of insulinopenia or because of weakening segmental or descending inhibition) resulting in a parallel progression of pin-prick, visceral and muscle mechano-hyperalgesia. As discussed above, however, the large scale sensitization of second order neurons within the skin nociceptive pathway would likely manifest itself in allodynic phenomena. No such phenomena were detected in this study.

4.5. Conclusion

These data suggest that deterioration of peripheral nerve function may start early in the course of diabetes, either being driven directly by insulinopenia or with participation of the effects of oxidative stress. In rat, pain on pressure appears as a uniquely sensitive indicator of moderate insulinopenia. While pain on pressure is present in as many as 70% of people with type 2 diabetes and painful DPN (Otto et al., 2003), PPT was not assessed in type 1 pre-diabetes patients. Therefore, the clinical relevance of these findings is still to be determined. Nonetheless, STZ-NG rats appear to be a good model for evaluation of the role of insulin in peripheral nerve functions in the short-term (2-3 weeks). Our data do not exclude the possibility that abnormalities in MNCV or in superficial heat or tactile stimuli perception that were observed in STZ-HG rats only, still have an insulin-dependent component. Data showing that near-nerve or intrathecally-supplied insulin can correct nerve conduction in overtly diabetic rats support this suggestion. A greater degree of insulinopenia or longer periods of mild insulinopenia might be needed to reveal this component.

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Abbreviations

8-OHdG	8-hydroxydeoxyguanosine
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
PPT	pressure pain threshold
RG	random plasma glucose
STZ	streptozotocin
STZ-HG	STZ-injected, hyperglycemic
STZ-NG	STZ-injected, normoglycemic
VMR	visceromotor reflex

FIGURE 1.

Weight (A), random (B) and peak in FTT (C) glucose of control and STZ-injected rats. Number of rats studied indicated by a number next to the symbol. In B and C, horizontal dotted and dashed lines show 7.8 mM and 11 mM random glucose thresholds of clinical pre-diabetes and diabetes, respectively.

FIGURE 2.

Tests characterizing glucose and lipid metabolism, pancreatic injury and oxidative stress in STZ-NG and -HG rats at two weeks of experiment

In panels A - F, open, light grey and dark grey columns represent respectively, control, STZ-NG and STZ-HG groups of rats; number above the column indicates the number of animals studied. Asterisks and double asterisks indicate statistical difference of given group mean from control or from control and from remaining group of animals ($p < 0.05$; two-sample t test). Panels G and H show examples of immunoperoxidase detection of insulin-producing pancreatic islet cells. Adjacent 5 μ m thick sections were stained with PGP 9.5 antibodies to characterize the total area of identified islets (G) and with antibodies to insulin to characterize the area of these islets occupied by β -cells (H). Calibration bar is 25 μ m

FIGURE 3.

Pressure pain threshold (A,B) and frequency of withdrawal to brush (C) and 20-g von Frey filament (D) measured in STZ-NG and HG rats at two weeks of experiment.

In all panels, number above the column indicates number of animals studied. Open, light and dark grey columns are, respectively control, STZ-NG and STZ-HG rats. Asterisks and double asterisks indicate statistical difference of given group mean from control.

FIGURE 4.

Heat pain threshold (A) and latency (B) measured after 2 weeks of experiments in control and STZ-injected rats.

Opened, light grey and dark grey columns represent respectively, control, STZ-NG and STZ-HG groups of rats; number above the column indicates the number of animals studied. (*, $p < 0.05$; ANOVA)

FIGURE 5.

Epidermal thickness and innervation in control and STZ-injected rats (2 weeks of experiment)

Panels A, B and C (control, STZ-NG and STZ-HG rats, respectively) show a view of skin sections with nerve fibers entering and branching in the epidermis (arrows; PGP 9.5 label). White dotted lines in images mark outer and inner borders of epidermis. Calibration bar is 50 μm . Panels D, E and F, show the results of analysis of mean epidermal thickness (D), IENF (number of fibers per mm of epidermis) and IENF length, expressed as total length of IENF per mm^2 of epidermal area (F). Epithelium of STZ-NG rats is thinner than that of control or STZ-NG rats (**). No other between-group statistical significance of studied parameters was detected by ANOVA and Tukey HSD test.

FIGURE 6.

Nerve conduction studies and assessment of visceral hypersensitivity in STZ-NG and STZ-HG rats.

Open, light grey and dark grey columns represent respectively, control, STZ-NG and STZ-HG groups of rats; number above the column indicates a number of animals studied.

Asterisks and double asterisks indicate statistical difference of given group mean from control or from control and from remaining group of animals ($p < 0.05$; two-sample t test).

Between group difference for data on visceral sensitivity was also supported by repeated measures-ANOVA: $F=32.9$ (d.f. = 45; $p < 0.01$). Panel D shows example of rat tail M and F-wave recordings induced by the nerve stimulation at the base of the tail (proximal) and more distally along the tail (distal). In this particular example time between positive peaks of M – waves is 1.8 ms, distance between electrodes 5.7 cm and resulting MNCV is 31.7 m/s

FIGURE 7.

Changes in PPT over time and role of insulinopenia.

In A, open, half-filled and filled circles represent control, STZ-NG and STZ-HG rats, respectively. In B and C, open and closed circles represent averages of the results from 2-week-long and 4-week-long experiments, respectively. In all panels, the size of experimental group is given by a number next to the symbol (see text for other details).

Table 1

Study entry characteristic of control and STZ-injected rats and their sub-groups

Group	Weight, g	RG, mM	# animals
Control	270 ± 5	5.7 ± 0.2	36
Injected with STZ	269 ± 3	5.6 ± 0.1	85
STZ-NG	264 ± 4	5.7 ± 0.1	43
All STZ-HG	274 ± 4 *	5.5 ± 0.1	42
STZ-HG, ketonic **	269 ± 8	5.3 ± 0.2	15

* -denotes difference from STZ-NG rats at P=0.047 (two-sample t-test)

** -ketonic coma developed during first week of injection with STZ and resulted in animal loss. No data collected in these animals at any time before or after STZ were used in this study.