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Increases in PKC gamma expression in trigeminal spinal nucleus is associated with orofacial thermal hyperalgesia in streptozotocin-induced diabetic mice

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ABSTRACT

Painful diabetic polyneuropathy (PDN) at the early phrase of diabetes frequently exhibits increased responsiveness to nociception. In diabetic patients and animal models, alterations in the transmission of orofacial sensory information have been demonstrated in trigeminal system. Herein, we examined the changes of protein kinase Cγ subunit (PKCγ) in trigeminal spinal nucleus (Sp5C) and observed the development of orofacial thermal sensitivity in streptozotocin (STZ)-induced type 1 diabetic mice. With hyperglycemia and body weight loss, STZ mice exhibited orofacial thermal hyperalgesia, along with increased PKCγ expression in Sp5C. Insulin treatment at the early stage of diabetes could alleviate the orofacial thermal hyperalgesia and impaired increased PKCγ in Sp5C in diabetic mice. In summary, our results demonstrate that PKCγ might be involved in orofacial thermal hyperalgesia of diabetes, and early insulin treatment might be effective way to treat orofacial PDN.

Introduction

In diabetes mellitus (DM), approximately 50% of patients experience diabetic peripheral neuropathy (DPN) (Hong and Wiley, 2005; Rossi, 2010; Ziegler, 2008). At the early to intermediate stages of DPN, painful diabetic neuropathy (PDN) is one of the most common complications and frequently manifested by thermal and mechanical hyperalgesia (Baron et al., 2009; Courteix et al., 1996; Sugimoto et al., 2000). Because PDN is affected by complicated multifactors, the underlying mechanism is still unclear. Pharmacological management is considered to be the most important therapeutic option for treating DPN, but pain control is still inadequate and unsatisfactory in patients (Finnerup et al., 2010; Tesfaye et al., 2010).

It has been established that streptozotocin (STZ)-induced type 1 diabetic rodents exhibit hyperalgesia to thermal, mechanical and chemical noxious stimulations in hind paws, indicating the involvement of spinal circuitry might be essential for the development and maintenance of PDN in lower limbs (Courteix et al., 1993; Malcangio and Tomlinson, 1998). However, the trigeminal circuitry may also be affected in PDN (Arap et al., 2010; Nones et al., 2013; Urban et al., 1999). In diabetic patients, there are reports of an increased frequency of orofacial pain (Arap et al., 2010). In addition, individuals with diabetes experience greater adverse effects from orofacial pain than non-diabetes (Arap et al., 2010). In animal models, the orofacial heat nociceptive thresholds were reduced in STZ-induced experimental diabetes in rats (Ziegler, 2008). In comparison with a large amount of experimental work in spinal circuitry, the mechanism of orofacial sensory changes in trigeminal circuitry has rarely been reported in diabetes.

It is well known that the trigeminal spinal nucleus (Sp5C) locates at brain stem and is highly selective for orofacial

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nociceptive transmissions in trigeminal circuitry (Luccarini et al., 1998; Martin et al., 2010; Pajot et al., 2000). Sp5C has also been regarded as a morphological and functional organization comparable to the spinal dorsal horn (Davies and North, 2009; Gauriau and Bernard, 2004). Similar to the substantia gelatinosa of the spinal cord, the Sp5C receives primary afferents of the trigeminal cranial nerves that carry nociceptive information from the orofacial area (Gauriau and Bernard, 2004). Although Sp5C has been demonstrated that plays an important role in orofacial neuropathic pain, there is little evidence in PDN of diabetes.

Protein kinase C (PKC) are a group of enzymes that play important roles in intracellular signal transductions. Among at least 10 isoenzymes, the gamma subunit of PKC (PKCγ) is an important contributor to the increased pain sensitivity through spinal cord circuit that occurs after injury (Malmberg et al., 1997; Mori et al., 1990). In contrast to the widely distributed α, βI and βII, PKCγ is restrict distributed in the superficial dorsal horn the spinal and trigeminal systems (Polgar et al., 1999; Tanaka and Saito, 1992). Moreover, it is well defined that injury-induced nociceptive processing occurs within PKCγ-containing neurons in spinal dorsal horn (Martin et al., 2001). However, little is known about the possible role of the PKCγ signaling pathway in the Sp5C, particularly in PDN.

Thus, based on these considerations, the current study is plan to examine: (1) the development of the orofacial heat sensitivity in STZ-induced diabetic mice; (2) the changes of PKCγ expression at different time points after STZ-induced experimental diabetes; (3) the effects of early insulin intervention in diabetic mice on orofacial thermal pain and PKCγ expression in the Sp5C.

Materials and methods

Experimental animals

Male C57BL/6 mice weighing 20–25 g at the ages of 6–8 weeks from Laboratory Animal Resources of the PLA general hospital (Beijing, PR China) were utilized. All animal studies were conducted using approved protocols and carried out in accordance with the Principles of Laboratory Animal Care (NIH Publication no. 85-23, revised 1985). Mice were fasted for 20 h before diabetes was induced with a single injection of streptozotocin (STZ, Sigma, St. Louis, MO, USA) at 100 mg/kg body weight, which was freshly dissolved in ice-cold sodium citrate (pH 4.5). On the 7th day after STZ administration, whole blood was obtained from the mice tail vein and glucose levels were measured using the blood glucose monitoring meter (Active; Roche Diagnostics, Mannheim, Germany). Only mice with blood glucose concentration > 20 mM were further used. Citrate buffer-treated mice were used as a normoglycemic control (blood glucose < 12 mM). All animals were housed in standard conditions (12 h light/dark cycles) with free access to food and water.

Pain behavioral test

Thermal heat hyperalgesia on the orofacial area was measured as previously reports (Nones et al., 2013; Zhang et al., 2012). In order to reduce the influence of constraint-induced stress on behavioral results, each animal was habituated to being held by the experimenter several times (but without application of the heat stimulus) for 2 days. To establish the time-course of thermal hyperalgesia in the STZ-induced diabetic mice, the heat stimulus was applied on the day preceding STZ injection to determine basal responsiveness and days 7, 14 and 28 after STZ injection. Mouse was removed from its home cage and gently held by the experimenter. Mice ($n = 6$ in each group) were habituated to the testing environment for 30 min before tests. A radiant heat stimulus (50 °C) was positioned 1 cm from the surface applied to the maxillary whisker pad skin by radiant heat (IITC Life Science, Woodland Hills, CA, USA). The heat latency time to display either head withdrawal or vigorous flicking of the snout was recorded. The radiant heat intensity was adjusted so that basal latency time is between 10 and 13 s, with a cut-off of 20 s to prevent tissue damage. All behavioral studies were performed under blind conditions.

Immunohistochemistry

Mice ($n = 6$ in each group) were anesthetized by intraperitoneal injection of pentobarbital sodium (5 mg/100 g). Mice were perfused with 50 ml of 4% (w/v) formaldehyde and post-fixed at 4 °C for 2 h. The brainstems were harvested and then stored in 30% sucrose solution overnight at 4 °C. Then, tissues were cut into transversal 20 μm thick serial sections in a cryostate (Leica CM1800, Germany). The sections were blocked within 10% normal goat serum for 1 h and incubated with rabbit antisera against PKCγ (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. After PBS washing for three times, Alexa488-conjugated donkey anti-rabbit IgG (1:500 dilution; Invitrogen, Carlsbad, CA) was applied with incubation for 2 h at room temperature, followed by PBS washing and slide mounting. All sections were visualized under a confocal laser scanning microscope (FV-1000, Olympus, Japan). PKCγ positive cells were determined by counting the number of profiles (cell bodies) and analyzed under fluorescence microscopy. For calculation of the number of immunofluorescent profiles in Sp5C, five sections from a series of every fifth serial section were selected randomly from each mouse and were evaluated by using ImageJ software (National Institutes of Health, www.rsb.info.nih.gov/ij).

Western blotting

Mice ($n = 6$ in each group) were transcardially perfused with saline and the tissue samples were dissected. Samples were homogenized in SDS sample buffer containing a mixture of protease inhibitors (Sigma, St. Louis, MO, USA). Protein concentrations were determined by BCA Protein Assay (Pierce, Rockford, IL, USA). Fifty micrograms of protein were loaded for each lane and separated on 10% SDS–PAGE gel. After the transfer, the blots were locked with a blocking buffer (5% nonfat dry milk in TBS-T) for 2 h at room temperature and then incubated with PKCγ antibody (rabbit, 1:1000, Santa Cruz) overnight at 4 °C. For loading control, the blots were probed with β-actin antibody (1:10,000 dilution; Sigma, St. Louis, MO, USA). The blots were further incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature; the reaction products were visualized with enhanced chemiluminescence (Bio-Rad, Hercules, CA, USA).

Drug treatments

The dosage of insulin was chosen on the basis of previous study and calculated by body weight in mice (Hoybergs and Meert, 2007). Mice ($n = 6$ in each group) were scheduled to receive an intraperitoneal (i.p.) injection of insulin (0.2 IU) or same amount of saline daily from day 7 for 7 days. Thermal latency time were evaluated 2 h prior to i.p. treatment (to determine baseline hyperalgesia) and then at 2 h intervals for up to a maximum of 4 h after drug administrations. The changes in PKCγ expression in Sp5C were examined after 7-day-treatment.

Statistical analyses

In Sp5C, the number of PKCγ-positive products was calculated and analyzed. Briefly, five sections from a series of every fifth serial section were selected randomly from each mouse ($n = 6$ in each group for statistical analyses). The relative optical densities (ROD) of the immunostainings were analyzed with ImageJ software. The ROD was calculated by subtracting the background from the integrated optical density (IOD) of the positive staining. The values for ROD were statistically analyzed among groups. Data from immunofluorescent products were presented as fold change *vs.* the control group. In behavioral tests, analysis of the time-course of behavioral data among saline- and drug-treated groups was performed by two factors (group and times) repeated measures analysis of variance (ANOVA). Data were expressed as mean ± SD and were analyzed by SPSS 16.0 (SPSS Inc., Chicago, IL, USA). The differences between groups were considered as statistically significant at a value of $p < 0.05$.

Results

Changes in body weights, blood glucose levels and orofacial thermal hyperalgesia

STZ is widely used to induce diabetes in these investigations about diabetes and related PDN. In the present study, STZ (100 mg/kg, i.p.) was administered to mice to induce diabetes (Hayashi et al., 2006). There was no difference in basal blood glucose concentration and body weight between STZ-treated mice and control mice at onset of the study (Fig. 1A). Compared with controls, mice treated with STZ presented a significant increase in the blood glucose at day 7 (23.25 ± 2.35 mM *vs.* 5.85 ± 0.50 mM, $p < 0.05$). There were also significant changes in blood glucose levels along the 4 weeks of observation between STZ and control mice (25.72 ± 0.93 mM *vs.* 5.57 ± 0.47 mM at day 28, $p < 0.05$).

Decreased body weights were found in STZ mice along with hyperglycemia (Fig. 1B). Fourteen days after STZ injection, body weights in the diabetic mice were significantly reduced compared with the control group (17.83 ± 1.83 g *vs.* 27.33 ± 1.21 g, $p < 0.05$)