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Short Communication

Increases in inflammatory mediators in DRG implicate in the pathogenesis of painful neuropathy in Type 2 diabetes

Christina Galloway, Munmun Chattopadhyay *

Department of Neurology, University of Michigan, Ann Arbor, MI, USA
VA Ann Arbor Healthcare System, Ann Arbor, MI, USA

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ABSTRACT

Background: Painful neuropathy is a common, difficult to treat complication of both Types 1 and 2 diabetes (T1D and T2D). Reports have shown that activation of inflammatory cascades play an important role in the development and persistence of neuropathic pain states, but it is not well established in painful diabetic neuropathy (PDN). Previously, studies have shown increased inflammatory cytokines in the serum of the diabetic patients with painful neuropathy. This study focuses on the changes in the levels of inflammatory mediators such as TNFα, interleukins, chemokines and cell adhesion molecules with the development of pain in the DRG of the Zucker diabetic fatty (ZDF) rat, an established model for T2D. This study also demonstrates an alteration in the levels of voltage gated sodium channel 1.7 ($Na_V1.7$) with the development of pain in DRG of the ZDF rats.

Results: Pre-diabetic ZDF animals at 8–9 weeks of age showed no thermal and mechanical hyperalgesia compared to their respective lean controls. Diabetic-ZDF animals tested for pain related behaviors showed significant thermal and mechanical hyperalgesia at 4 and 6 weeks after the onset of diabetes when compared with their age matched lean controls. These ZDF animals with PDN also showed changes in a large number of inflammatory mediators in the DRG as assessed by Western blot as well as by cytokine antibody array compared to their age matched lean controls. Further analysis by Rat cytokine antibody array of DRG of the ZDF animals with PDN at 6 weeks after diabetes when compared with ZDF animals with no pain revealed an elevation of a significant number of inflammatory mediators including, the pro-inflammatory cytokines such as TNFα, interleukin-1, 6, 13 and 17, chemokines such as MIP1 and 3, RANTES, Fractalkine and cell adhesion molecule sICAM that are associated with pain phenotype. The ZDF animals with PDN also demonstrated an increase in the protein levels of voltage gated sodium channel $Na_V1.7$ in DRG compared to lean controls with no pain.

Conclusions: The rise in inflammatory markers in the DRG of Type 2 diabetic animals and increases in voltage gated sodium channel $Na_V1.7$ in DRG with the onset of pain in PDN suggest that inflammation in the DRG may play an important role in the development of pain in this model.

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1. Introduction

Diabetes mellitus is the most common cause of neuropathy in the United States and pain is a significant complication of diabetic neuropathy occurring in 20–25% of patients with neuropathy and resulting in a significant adverse effect on quality of life measures [1]. Unfortunately, available medical treatment is relatively ineffective with limited efficacy and often complicated by side effects and dependency [2,3]. The etiology of pain in diabetic neuropathy is not well understood. Accumulating evidence suggests that the activation of inflammatory cascades in the peripheral and central nervous system may play a role in the development and persistence of neuropathic pain states induced by physical or toxic injury to peripheral nerve [4,5].

In diabetes there is evidence of systemic immune activation. Patients with painful neuropathy have increased IL-2 and TNFα mRNA and protein levels in blood [4]. Type 1 diabetes patients have increased serum TNFα [6] and studies on patients with diabetic painful neuropathy exhibit a different serum immune profile compared to patients with painless diabetic neuropathy,

Abbreviations: PDN, painful diabetic neuropathy; T2D, Type 2 diabetes; ZDF, Zucker diabetic fatty; DRG, dorsal root ganglia; $Na_V1.7$, voltage gated sodium channel isoform 1.7; IL-1, interleukin-1; TNF, tumor necrosis factor; CCL, Chemokine (C–C motif) ligand.

* Corresponding author. Address: 5248 BSRB, Department of Neurology, University of Michigan, Ann Arbor, MI, USA. Tel.: +1 734 763 5668; fax: +1 734 764 6493.

E-mail address: munmunc@umich.edu (M. Chattopadhyay).

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suggesting that immune markers in blood are associated with diabetic neuropathic pain [7]. But, the relationship of these serum inflammatory markers to nociceptive pathways in the nervous system has not been explored. We undertook these studies to evaluate the role of inflammatory mediators in DRG in T2D model of PDN.

2. Methods

2.1. Study design and behavioral test

The Zucker diabetic fatty (ZDF, Charles River, USA) rat, a substrain of the obese Zucker rat, is an established model for Type 2 diabetes in which hyperglycemia initially manifests at about 8–9 weeks of age. A blinded researcher assessed thermal hyperalgesia by measuring the latency to hind paw withdrawal from a thermal stimulus determined by exposing the plantar surface of the hind paw to radiant heat using a modified Hargreaves thermal testing device. Mechanical hyperalgesia was assessed using an analgesimeter (Ugo-Basile, Comerio, VA, Italy) by the Randall and Selitto paw pressure method as described previously [8].

2.2. Cytokine array and Western blot

Rat Cytokine Array (ARY008; R&D Systems, USA) was used to simultaneously detect the relative expression of 29 cytokines, chemokines and cell adhesion molecules. This antibody array detects multiple analytes in tissue lysates. L4–L6 DRG from rats ($n = 5$) were homogenized and prepared according to manufacturer's instructions. The sample protein concentrations were measured using a total protein assay [8]. Once the membranes were blocked, 15 μL of reconstituted detection antibody cocktail were added to each prepared sample. The samples with antibody cocktail were then added to the membrane and incubated at room temperature for 1 h. The membranes were washed, followed by incubation with Streptavidin-HRP for 30 min. The intensity of each spot was determined by quantitative chemiluminescence, using a PC-based image analysis system (ChemiDoc XRS System, Bio-Rad Laboratories, USA); and pixel densities were quantitated by analyzing the array image file using image analysis software (Quanti-one 4.6.1; Bio-rad Laboratories, USA). For western blot, L4–L6 DRG from each animal considered as one sample ($n = 5$) were homogenized and prepared as described previously [8].

2.3. DRG culture experiment

For *in vitro* studies, adult rats were anaesthetized with chloral hydrate (400 mg/kg i.p.). DRG from these rats were collected and dissociated following 0.25% collagenase treatment for 1 h at 37 °C with 0.25% trypsin, 1 mM ethylenediaminetetraacetic acid (EDTA) for 30 min at 37 °C with constant shaking and then plated on Laminin, poly-D-lysine-coated coverslips at 10^5 cells per well in a 24-well plate in 500 μl of defined neurobasal media containing B27, Glutamax I, Albumax II and penicillin/streptomycin (Gibco-BRL, Carlsbad, CA, USA), supplemented with 100 ng/ml of 7.0S NGF per ml (Sigma, St. Louis, MO, USA). 4 day old DRG neurons in culture were incubated with 15 ng/ml of recombinant TNFα (rTNFα, Sigma) for overnight and collected for qRT-PCR and western blot analysis for $Na_V1.7$ level in DRG (Supplementary Fig. 3).

2.4. Statistical analysis

The statistical significance of the difference between groups was determined by ANOVA (Systat 11) using Bonferroni's correction for the multiple post hoc analyses. All results are expressed as mean ± SEM.

3. Results

3.1. ZDF rats with Type 2 model of diabetes showed thermal hyperalgesia, mechanical hyperalgesia 6 weeks after diabetes

Pre-diabetic animals at 8–9 weeks of age showed normal responses similar to their age-matched lean controls (Fig. 1a). At 2 weeks after diabetes, ZDF animals showed only significant decrease in thermal latency (Fig. 1b; lean 14.93 ± 2.2 s; ZDF 10.67 ± 1.8 s; $P < 0.01$) but no significant difference in mechanical hyperalgesia compared to the lean controls. At 4 weeks (lean 13.16 ± 0.6 s; ZDF 10.18 ± 0.9 s; $P < 0.005$ at 6 weeks) and 6 weeks after the onset of diabetes, the ZDF animals showed significant decrease in thermal latency (lean 12.93 ± 1.2 s; ZDF 8.47 ± 1.7 s; $P < 0.005$ at 6 weeks) and substantial decrease in paw withdrawal threshold measured by Randall-Selitto method at 4 weeks (lean 78.5 ± 1.9 gm; ZDF 54.50 ± 2.5 gm; $P < 0.005$) and 6 weeks (lean 89.64 ± 8.2 gm; ZDF 59.53 ± 5.7 gm; $P < 0.005$; Fig. 1c and d) after the onset of diabetes.

3.2. ZDF animals with pain-related behavior exhibited increased $Na_V1.7$ in DRG

In previous studies we and others have found that there is an increase in the amount of voltage-gated sodium channel 1.7 ($Na_V1.7$) in DRG of STZ-diabetic (a model of T1D) animals with PDN [8]. In this study, DRG were analyzed for expression of voltage gated sodium channel isoform $Na_V1.7$ by Western blot to correlate the changes in $Na_V1.7$ with the changes in pain-related behaviors. We did not find any increase in the level of $Na_V1.7$ in 8 weeks old pre-diabetic ZDF animals without PDN (Fig. 1a and e). 2 weeks after diabetes, ZDF animals showed significant increase in thermal hyperalgesia ($P<0.01$) but not mechanical hyperalgesia and a moderate increase in $Na_V1.7$ (Fig. 1b and f). At 4 and 6 weeks after diabetes, ZDF animals showed significant thermal and mechanical hyperalgesia along with a substantial increase in $Na_V1.7$ levels in DRG (Fig. 1c, d and g, h).

3.3. ZDF rats with painful diabetic neuropathy demonstrated increased neuroinflammation in DRG

Pre-diabetic ZDF animals showed no significant change in the expression of any of the 29 pro-inflammatory cytokines or chemokines in DRG at 8 weeks of age (Supplementary Fig. 2) compared to their age-matched lean controls. ZDF animals with PDN 6 weeks after diabetes when compared with their respective age-matched lean control animals showed a significant increase in 27 out of 29 cytokine/chemokines/cell adhesion molecules; only 2 cytokines, IL-10 and IL-4, those have anti-inflammatory properties, did not change in these animals (Supplementary Table 1).

By Western blot of DRG, we found that ZDF animals at 6 weeks of diabetes exhibited significant increases in a number of inflammatory markers, including tumor necrosis factor α (TNFα), interleukin-1 β (IL1β) and phospho-p38 MAPK protein compared to lean control animals (Fig. 2a–c). ZDF animals with PDN at 6 weeks of diabetes when compared with pre-diabetic ZDF animals without PDN showed significant increases in 19 inflammatory mediators (Fig. 2d), including the pro-inflammatory cytokines such as TNFα, interleukin (IL)-1α and β, IL-6, IL-13 and IL-17, chemokines such as MIP1 and 3, RANTES, Fractalkine and cell adhesion molecule sICAM in DRG of ZDF with PDN.