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Short Communication

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

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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 Zucker diabetic fatty (ZDF) rat, an established model for T2D. This study also demonstrates an alteration in 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 age matched lean controls. These ZDF animals with PDN also showed changes in 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 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.

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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 meas using a total protein assay [8]. Once the membranes were bloc 15 μL of reconstituted detection antibody cocktail were added each prepared sample. The samples with antibody cocktail we then added to the membrane and incubated at ro erature for 1 h. The membranes were washed, followed ncuba n with Streptavidin-HRP for 30 min. The intensity o a spot ter-mined by quantitative chemiluminescen ush Based age analysis system (ChemiDoc RS Sy Bio-Rad Laboratories, USA); and pixel densiti e quantitat analyzing the array image file using ima ana software (Q anti-one 4.6.1; Bio-rad Laboratories, USA). For we n blot, L4–L6 DRG from each animal considere one sample ( ) were homogenized and prepared as d bed previously [8].

2.3. DRG culture experime

For *in vit* tudi adult ra e anaesthetized with chloral hydrate (mg/kg). DRG fro these rats were collected and dissociated low agenase treatment for 1 h at 37 °C with 0.25% t , 1 mM ethylenediaminetetraacetic acid (EDTA) for 30 min at 37 ith constant shaking and then plated on Laminin, poly-D-lysine ed 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 sho y significant decrease in thermal latency (Fig. 1b ean ± 2.2 s; ZDF 10.67 ± 1.8 s; $P < 0.01$) but no signi t differenc mechanical hyperalgesia compared to the lean trols. At eeks (lean 13.16 ± 0.6 s; ZDF 10.18 ± 0.9 < 0.00 6 week nd 6 weeks after the onset of diabetes, ZDF animals significant decrease in thermal later (lean 93 ± 1.2 DF 8.47 ± 1.7 s; $P < 0.005$ at 6 weeks) an st l decrease in paw withdrawal threshold measure y Ra Selitto hod at 4 weeks (lean 78.5 ± 1.9 gm; Z 54.50 ± 2. P 005) and 6 weeks (lean 89.64 ± 8.2 gr 59.53 ± 5.7 g < 0.005; Fig. 1c and d) after the onset of abete

3.2. mals with pain ated behavior exhibited increased Na 7 in DRG

previous s es we and others have found that there is an incr in th mount of voltage-gated sodium channel 1.7 ($Na_V1.7$) of STZ-diabetic (a model of T1D) animals with N [8]. In this study, DRG were analyzed for expression of voltage ium channel isoform $Na_V1.7$ by Western blot to correlate e 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.

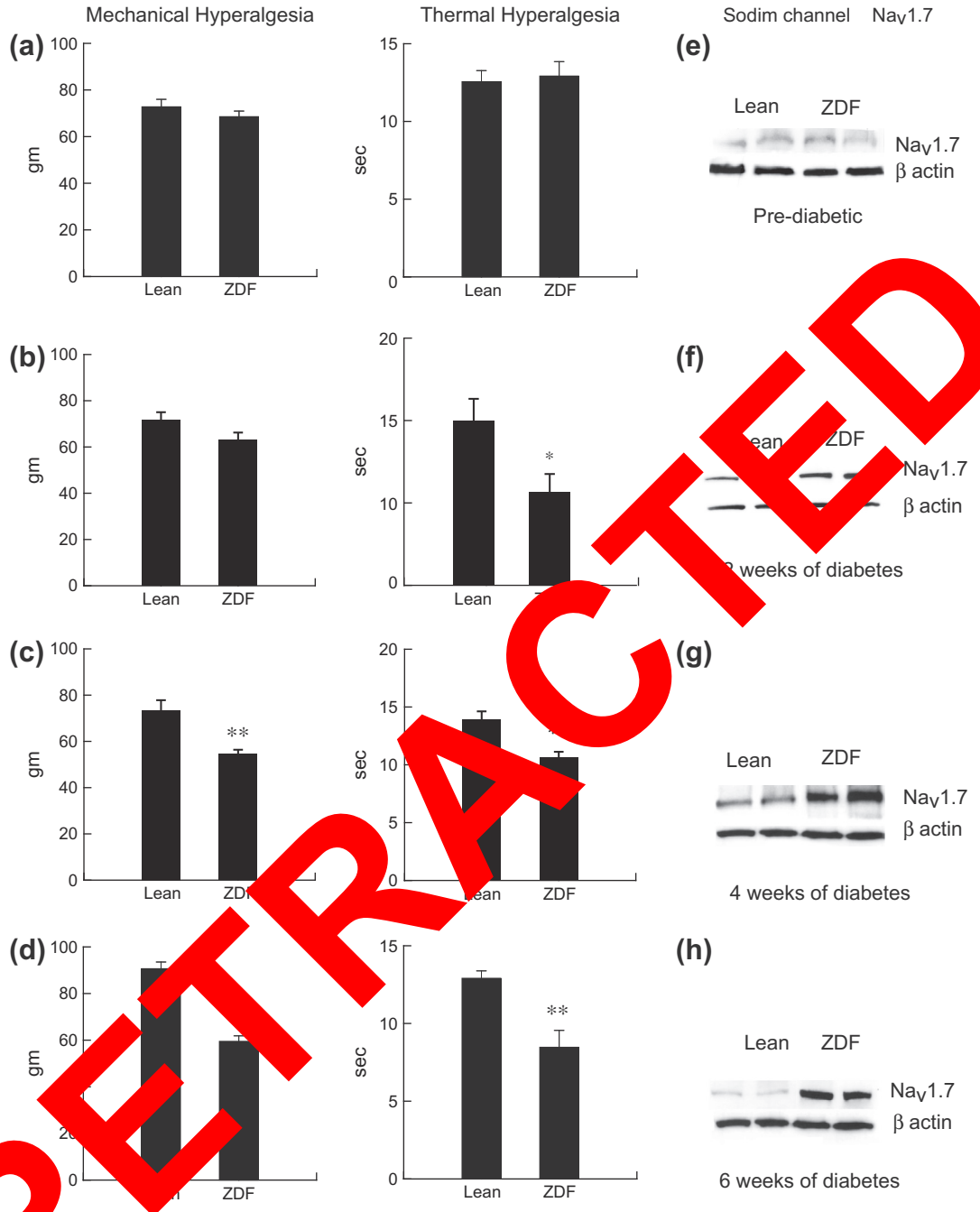

Fig. 1. ZD[illegible]n-related behaviors at 2, 4 and 6 weeks of diabetes along with changes in $Na_V1.7$ levels in DRG. (a) Pre-diabetic ZDF animals show no pain-related [illegible]rs by paw withdrawal latency to noxious thermal stimuli and mechanical nociceptive threshold by Randall-Selitto test; (b) 2 weeks after diabetes, ZDF animals demon[illegible] thermal hyperalgesia manifested by a decrease in thermal latency compared to control lean animals ($^{*}P < 0.01$; $n = 8$) and show no change in mechanical pain threshold co[illegible]d to age-matched lean animals. ZDF rats at 4 weeks (c) and 6 weeks (d) after diabetes have shown a significant decrease in their mechanical and thermal pain thresho[illegible]ompared to age-matched lean controls ($^{**}P < 0.005$; $n = 8$). (e) Pre-diabetic ZDF animals at 8 weeks of age with no pain, show no change in $Na_V1.7$ levels by Western blot analysis. (f) ZDF rats at 2 weeks after the onset of diabetes show a modest increase in $Na_V1.7$ levels in DRG compared to lean control ($n = 5$). ZDF animals with PDN, at 4 weeks (g) and 6 weeks (h) after diabetes induction exhibit a significant increase in $Na_V1.7$ levels in DRG ($n = 5$).

3.4. Levels of $Na_V1.7$ is altered by increased inflammation in cultured DRG neurons

To determine whether increase in proinflammatory cytokines would change the level of $Na_V1.7$ in the neurons, adult DRG neurons in culture incubated with rTNFα overnight showed an increase in $Na_V1.7$ protein and mRNA levels compared to untreated control cells.

4. Discussion

Inflammation plays a central role in the nervous system in response to injury which affects vasodilatation, increased vascular permeability, cell migration, and pain. Extracellular signals associated with inflammation may also lead to increased levels of pro-nociceptive chemokines/receptors that directly contribute to persistent or chronic pain behavior [5]. The pro-inflammatory

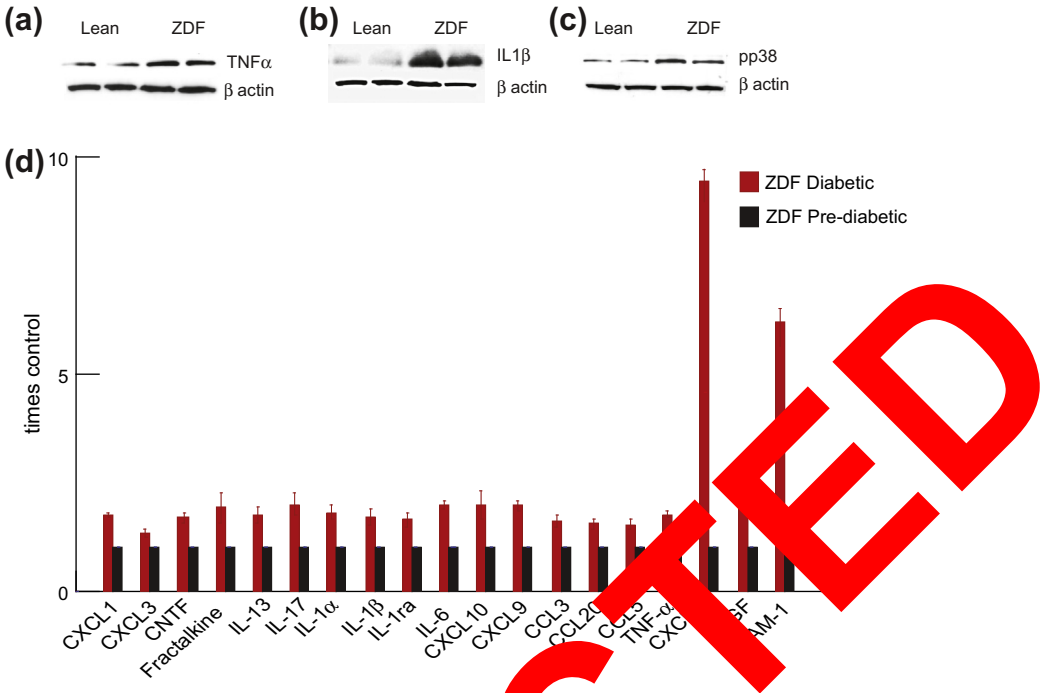

Fig. 2. ZDF rats with painful neuropathy have increased inflammatory markers in DRG com[pared] to ZDF anim[als] with no pain. ZDF animals at 6 weeks of diabetes exhibited significant increases in inflammatory markers tumor necrosis factor α (TNFα; a), interleukin[-1β] (IL1β; b) and [phos]pho-p38 MAPK (c) protein in the DRG of the animals with painful neuropathy compared to lean control animals without painful neuropathy measure[d by] Western blo[t] analysis ($n = 5$). (d) ZDF animals with painful neuropathy at 6 weeks after the onset of diabetes have shown an increase in a significant number of inflamma[tory] markers in the DRG including pro-inflammatory cytokines such as TNFα, interleukin-1, 6, 13 and 17, chemokines such as MIP1 and 3, RANTES, Fractalk[ine] and cell adhesion [molecule] sICAM by rat cytokine antibody array compared to pre-diabetic ZDF animals without any pain phenotype ($n = 5$).

cytokines elevated in the DRG of ZDF animals with[out] weeks compared to pre-diabetic ZDF without PDN in t[he] study[,] TNFα and interleukins, particularly IL1α, IL1β, IL-6[,] [IL-]3 and [IL-]7. These cytokines have also been implicated in th[e] dev[elopment] sensitivity or pain in other models of [neu]ropathic [pain] caused by physical injury which are important [in] the regulati[on] of immune responses and nociception [5]. M[ost] of the CC and [CXC] family of chemokines that are significantly incr[eased] compared to pre-diabetic ZDF animals with[out] pain include [CCL]3 (MIP-1α) and CCL20 (MIP-3α) [9], CCL[5] and CXCL10 (RANTES and IP-10) [10], CXCL7 (thymus cytok[ine] and [CX3]CL1 (Fractalkine), which are increased in other mode[ls] of [neu]ropathic pain resulting from physical injury to nerve and are [also] indicated [in] behavioral hypersensitivity and vasc[ular] inflamma[tion] [6]. Cell adhesion molecule sICAM-1 th[at] represents [an] important biomarker for inflammatory processes [that] was [also] increased in ZDF animals with PDN. In recent past [studies] have been implicated with pain in patients. Trophic [fac]tors, including ciliary neurotrophic factor (CNTF) which has been [associ]ated with hyperalgesia in ALS patients in a phase I trial, and i[ncr]eased levels of vascular endothelial growth factor (VEGF) which was linked with severity of pain in patients with bladder pain [12,13], were also elevated in ZDF animals with PDN in this study.

To date, research focused on improving the treatment of chronic pain has largely ignored the role of inflammation-associated factors in nociceptive pathways in the peripheral nervous system in diabetic subjects. In these studies, we found that a number of cytokines, chemokines and cell adhesion molecules are altered in T2D animals with PDN. Similar to our previous STZ-diabetic studies, these studies also documented that ZDF animals with pain demonstrate increased $Na_V1.7$ levels in DRG. In recent studies it has been shown that elevated levels of TNF-α are responsible for the up-regulation of voltage gated sodium channel activity [14]. We found that the adult DRG neurons in culture when pre-incubated with 15 ng/ml of recombinant TNFα (rTNFα) for overnight, to determine whether the increase in proinflammatory cytokines would change the level of $Na_V1.7$ in the neurons, showed an increase in $Na_V1.7$ protein and mRNA levels compared to control cells. This preliminary study suggests that TNFα plays an essential role in modulation of voltage gated sodium channel (Supplementary Fig. 3). We have shown that ZDF animals with PDN starting at 2 weeks had a small increase in the levels of $Na_V1.7$ compared to no change in the pre-diabetic ZDF animals without any pain. At 4 and 6 weeks after the onset of diabetes, DRG of the animals had significant increase in the levels of $Na_V1.7$. These animals at 6 weeks of diabetes also showed an increased phosphorylation of p-38 in DRG along with simultaneous changes in a number of proinflammatory cytokines and chemokines as stated above. Hence, the results from this study may suggest that the elevation of these inflammatory mediators in DRG may be responsible for the development of painful neuropathy in Type 2 diabetes, which may be associated with increases in the voltage gated sodium channel $Na_V1.7$. Therefore, the painful diabetic neuropathy may possibly be considered, at least in part, a 'neuro-inflammatory' condition. Taken together, a better understanding of the role of these pro-nociceptive markers may lead to the development of novel analgesic targets.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2013.04.009.

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