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Short Communication

Role of Neuroinflammation in the Pathogenesis of Painful Neuropathy in Type 2 Diabetes

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Abstract

Painful diabetic neuropathy (PDN) is an important complication of diabetes. The Zucker diabetic fatty (ZDF) rat is an established model for Type 2 diabetes (T2D). To investigate the potential role of inflammation in the pathogenesis of painful neuropathy in this model, we used a microarray to study cytokine/chemokine expression in the spinal cord dorsal horn and correlated changes in expression of TRPV1 channel which is related to thermal pain. After 6 weeks of hyperglycemia, significant changes were observed in thermal latency and in mechanical pain threshold in ZDF animals compared to their lean controls. Cytokine array revealed elevation in a number of inflammatory mediators that are associated with the emergence of pain-related behaviors. ZDF animals with PDN also exhibited increase in transient receptor potential cation channel subfamily V member 1 (TRPV1) in the DRG and the spinal cord. The increase in inflammatory markers in the spinal cord dorsal horn of Type 2 diabetic animals coincident with the onset of pain in PDN suggests that inflammation in the spinal cord may be important in the development of pain in this model.

Abbreviations

PDN : Painful Diabetic Neuropathy;
T2D : Type 2 Diabetes;
ZDF : Zucker Diabetic Fatty;
DRG : Dorsal Root Ganglia;
TRPV1 : Transient Receptor Potential Cation Channel Subfamily V member 1;
IL-1 : Interleukin-1;
TNF : Tumor Necrosis Factor;
sICAM : soluble Intercellular Adhesion Molecule;
CCL : Chemokine (C-C motif) Ligand

Introduction

Neuropathy is among one of the most common debilitating complications of diabetes. Over the course of the disease, up to 50% of the diabetic patients develop neuropathy, despite controlled blood sugar. Usually it affects the extremities, starting with feet. It begins with sensory changes such as numbness or tingling in the toes; which ultimately leads to loss of sensation. Diabetic neuropathy is the primary cause of ulcerations and infections in the feet, and in advanced cases, amputation. Up to 25% of diabetic patients with neuropathy describe abnormal peripheral sensations, including spontaneous pain, exaggerated perception of pain to mildly noxious (hyperalgesia) or innocuous (allodynia) stimuli. In some patients, impaired nociception develops into unrelenting neuropathic pain, greatly affecting quality of life [1-3].

Treatments with antidepressants, anticonvulsants and opioids are effective for pain, but are of limited efficacy, and complicated by addiction and dependency [4-6]. The available prescription medications have significant number of side effects. So, there remains a clear unmet medical need for therapies that could provide greater efficacy. The cellular mechanism(s) by which neuropathic pain develops in the diabetic patients remains poorly understood. Aberrant activation of the polyol pathway, accumulation of advanced glycation end products, microvascular insufficiency, neurotrophic factor deficiency, and dysregulation of ion channel function are strongly suggestive of a multifactorial temporal process with episodic hyperglycemia as a common initiating event [7-9].

Furthermore, neuropathic pain induces overexpression of local inflammatory cytokines released from activated macrophages, glial and Schwann cells. It has been established that proinflammatory cytokines (i.e., tumor necrosis factor [TNF]-α, interleukins [IL]-1, IL-6) cause pain [10-13]. Treatments with inhibitors of proinflammatory cytokines or anti-inflammatory cytokines may reduce pain; however, these treatments come with a number of side effects. The purpose of this study is to find a better understanding of the mechanism of pain by examining the effect of proinflammatory cytokines in the spinal cord of Type 2 diabetic animals.

Results and Methods

Study design

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. The ZDF rats were used in this study in compliance with approved institutional animal care and use protocols (IACUC, VAAAHS). Rats with greater than 300 mg/dl blood glucose level were included in the study.

Statistical analysis

The statistical significance of the difference between groups was determined by ANOVA (Systat 13) using Bonferroni's correction for the multiple post hoc analyses. All results are expressed as mean ± SEM. The animal behavior experiments, with 8-10 animals per group, were repeated twice.

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

We assessed pain 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, and mechanical nociceptive threshold using an analgesimeter (Ugo Basile, Comerio, VA, Italy) as described previously [14]. Pre-diabetic animals at 8 weeks of age showed normal responses (data not shown). After 6 weeks of diabetes, the ZDF animals showed decreased thermal latency (lean 13.05 ± 1.0 sec; ZDF 9.56 ± 1.4 sec; $P < 0.005$; n=6; Fig 1a) and decreased paw withdrawal threshold measured by Randall-Selitto test (lean 95.6 ± 7.8 gm; ZDF 56.5 ± 4.9 gm; $P < 0.001$; n=6; Figure 1b).

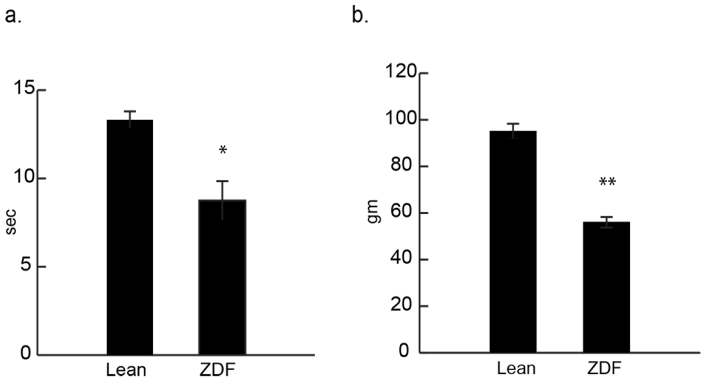

Figure1. ZDF animals presented pain-related behaviors 6 weeks after hyperglycemia.

Six weeks after diabetes, ZDF animals demonstrate thermal hyperalgesia manifested by a decrease in thermal latency compared to control lean animals (*$P < 0.005$; a) and developed mechanical hyperalgesia exhibited by decrease in hind-paw withdrawal threshold compared to compared to age-matched lean animals (**$P < 0.001$; b).

ZDF rats with PDN demonstrated activation of microglial cells with increases in TNFα, IL1-β and phospho-p38MAPK in spinal cord

By Western blot and immunohistochemistry of the spinal cord dorsal horn, we found that ZDF animals at 6 weeks of diabetes exhibited activation of microglial cells (as shown by CD11b immunohistochemistry) with significant increases in a number

of inflammatory markers including tumor necrosis factor α (TNFα), interleukin(IL)-1β along with up-regulation of phospho-p38 MAPK compared to lean control animals (Figure 2a-d). Samples of dorsal horn were prepared as described previously [16].

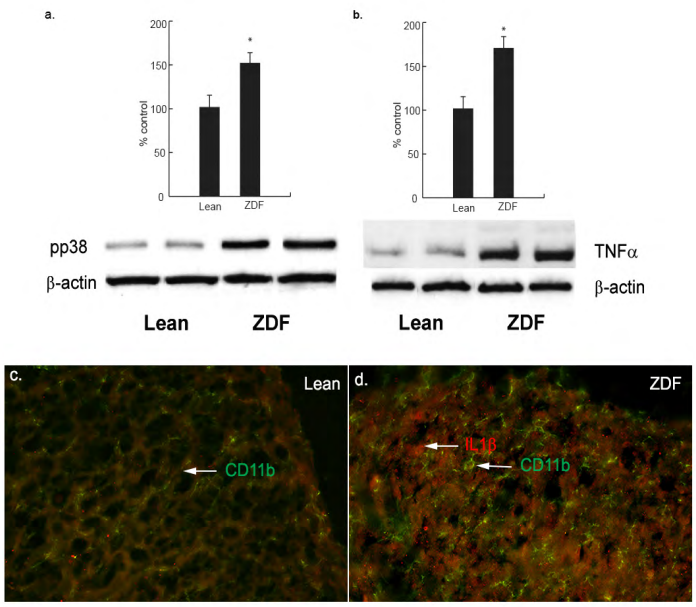

Figure 2. ZDF rats with PDN revealed increased neuroinflammation in the dorsal horn.

ZDF rats 6 weeks after diabetes show a significant increase in pp38 (a) and TNFα (b), in dorsal horn measured by Western blot. (c) Immunohistochemistry of the spinal dorsal horn demonstrated activation of microglial cells (CD11b antibody) and increased IL1β expression in ZDF animals at 6 weeks of diabetes (d).

Animals with pain-related behavior exhibited increased TRPV1 in DRG and spinal cord

In previous studies, our group and others have found that there was an increase in the amount of voltage-gated sodium channel 1.7 ($Na_V1.7$) in the DRG of the STZ-diabetic (a model of T1D) animals with PDN [14]. In this study, dorsal horn was analyzed for expression of transient receptor potential cation channel subfamily V member 1 (TRPV1) to correlate the altered TRPV1 expression with the changes in the pain-related behaviors. Western blot and immunohistochemical analyses demonstrate that TRPV1 expression is increased in the spinal cord dorsal horn of ZDF animals with pain (Figure 3a-b). ZDF rats with pain also showed a significant increase in transient receptor potential cation channel subfamily V member 1 (TRPV1) in DRG, 6 weeks after diabetes (Figure 3c).

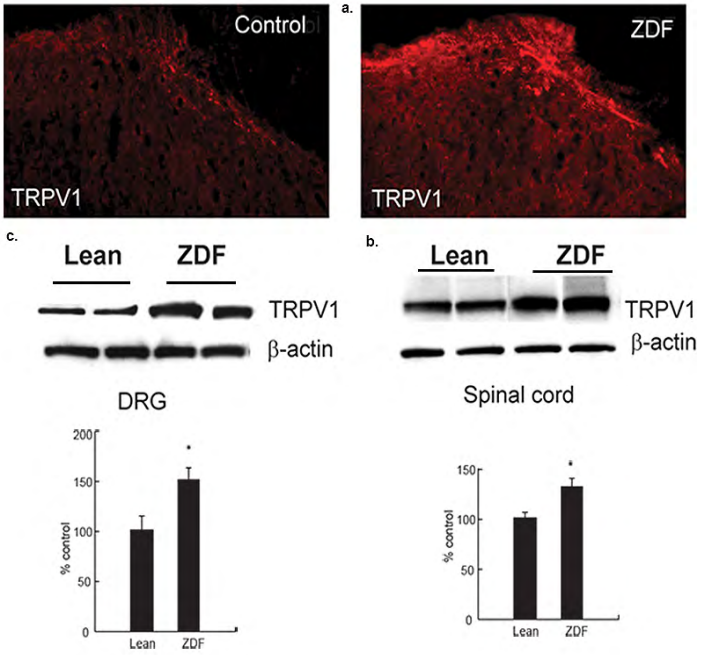

Figure 3. ZDF rats with PDN demonstrated increased TRPV1 in DRG and spinal cord.

(a) Immunohistochemical analysis exhibited an increase in TRPV1 levels in spinal cord dorsal horn of ZDF animals with PDN, 6 weeks after diabetes. Western blot analysis showed a significant increase in TRPV1 in spinal cord (b) and DRG (c) of ZDF rats with PDN 6 weeks after diabetes compared to lean control.

ZDF rats with PDN revealed increases in a number of inflammatory mediators in spinal cord dorsal horn

To detect the relative expression of 29 cytokines, chemokines and cell adhesion molecules in the spinal dorsal horn, Rat Cytokine Array (ARY008; R&D Systems, USA) was used. The total protein was isolated and quantified; array was performed according to manufacturer's protocol [15]. 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 was quantitated by analyzing the array image file using image analysis software (Quanti-one 4.6.1; Bio-rad Laboratories, USA). Analysis of the array (Figure 4) reveals a significant increase in 27 out of 29 cytokines/chemokines/cell adhesion molecules in the spinal cord dorsal horn of ZDF animals with painful neuropathy 6 weeks after diabetes compared to their respective age-matched lean controls. Interleukins, IL-10 and IL-4 expression were not changed in these animals. These two cytokines are anti-inflammatory in their effects.

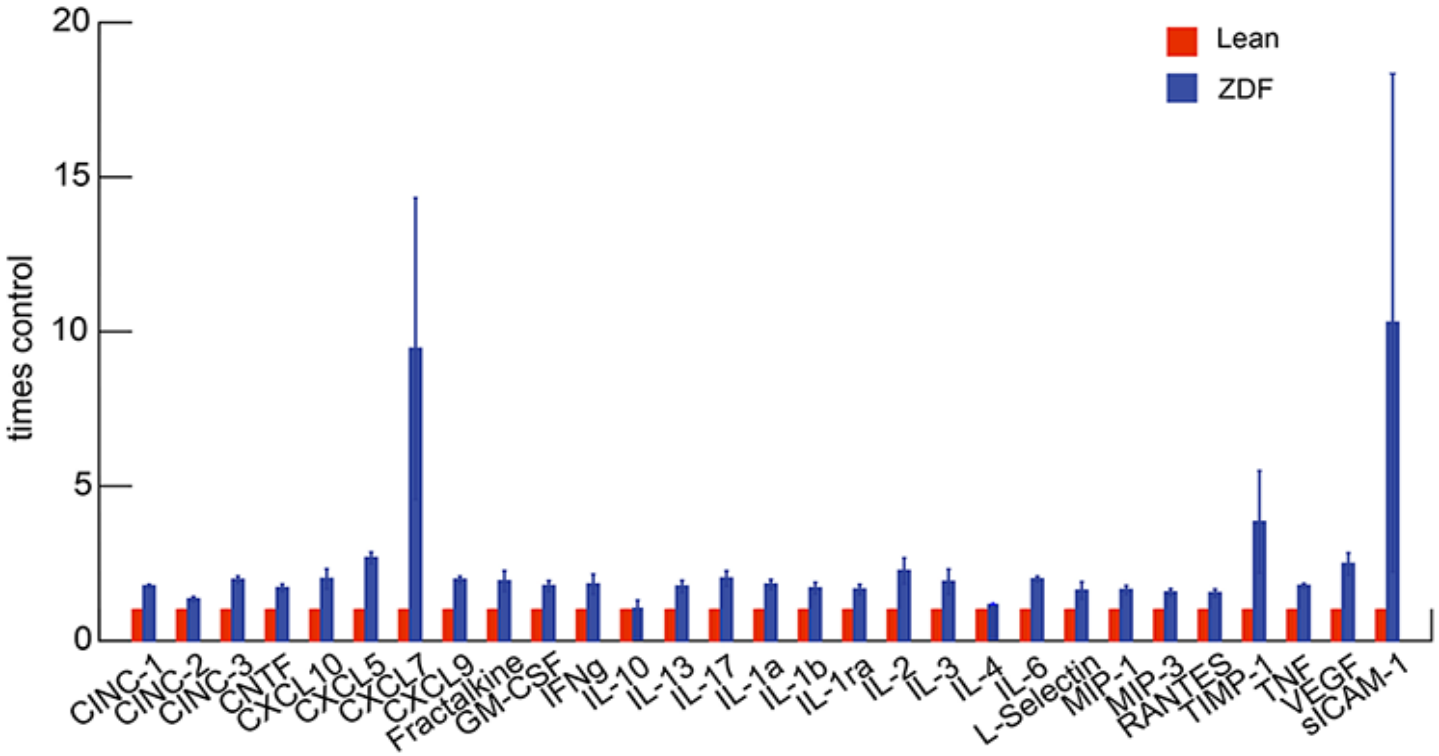

Figure 4. Analysis of inflammatory markers in dorsal horn of Type 2 diabetic rats.

ZDF animals with PDN show an increase in significant number of inflammatory markers that are associated with development and maintenance of pain by cytokine array of dorsal horn of ZDF rats 6 weeks after diabetes compared to lean animals. Out of 29 cytokines, chemokines and adhesion molecules, 27 are increased significantly (P<0.05) compared to lean controls. The 2 anti-inflammatory cytokines IL-10 and IL-4 did not show any difference compared to controls.

Discussion

Nervous system inflammation plays an essential role in pain and injury [16] which leads to increased vascular permeability, plasma extravasation and cell migration. Inflammation may also cause elevated levels of pro-nociceptive chemokines/receptors which directly contribute to pain behavior [17]. So far, the research focused on improving the treatment of chronic pain has largely ignored the role of neuro-inflammation in diabetic neuropathy. In this study the inflammatory mediators that are altered in the animals with PDN, fall into the following major categories: cytokines, chemokines, cell adhesion molecules and trophic factors. (1) Cytokines have been implicated in the development of hypersensitivity in other models of neuropathic pain caused by physical injury, and are important for the regulation of immune responses and nociception. The important pro-inflammatory cytokines that are altered in this study are TNFα and interleukins, particularly IL1α, IL1β, IL-6, IL13 and IL17 [17]. (2) Members of the CC and CXC family of chemokines are implicated in behavioral hypersensitivity and vascular inflammation and are increased after nerve injury [18-22]. The chemokines that are changed in this study are CCL3 (MIP-1α) and CCL20 (MIP-3α), CCL5 and CXCL10 (RANTES and IP-10), CXCL7 (thymus cytokine) and CX3CL1 (fractalkine). (3) Cell adhesion molecules exemplify essential biomarker for inflammatory processes [23]. There is a significant increase in cell adhesion molecule, sICAM-1 in the spinal cord of diabetic animals. (4) Trophic factors have been linked with pain in patients. This study reveals alterations in expression of ciliary neurotrophic factor (CNTF) and vascular endothelial growth factor (VEGF) in spinal cord; previous studies have associated the changes in CNTF with hyperalgesia in ALS patients in a phase I trial, and changes in VEGF with increased pain in patients with bladder pain. [24, 25].

In this study, we found that a number of cytokines, chemokines and cell adhesion molecules are altered with the development of pain in T2D. The study also revealed an increase in transient receptor potential cation channel subfamily V member 1 (TRPV1) in the DRG and the spinal cord of the animals. Recent studies have shown that elevated levels of TNF-α are responsible for the up-regulation of voltage gated sodium channel activity [26, 27] and TRPV1 receptors [28]. Therefore, the results from this study suggest that the substantiation of these inflammatory mediators in spinal cord may be responsible for the development of painful neuropathy in Type 2 diabetes con-

comitant with increases in the voltage gated ion channels. We have now established that ZDF animals at 6 weeks after hyperglycemia with PDN have increased levels of TNFα, TRPV1 and increased phosphorylation of p-38 in spinal cord along with changes in a number of other proinflammatory cytokines and chemokines. These results suggest that painful diabetic neuropathy is, at least in part, an 'inflammatory' condition. Therefore, this study may help understand the possible role of these pro-nociceptive markers to identify novel analgesic targets.

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Authors' contributions

HJY contributed experimentally; carried out the behavior test and cytokine array; VT contributed in immunohistochemistry and western blot. MC contributed to the design and analysis of the study, wrote the manuscript.

Competing interests

The authors declare that they have no competing interests.

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