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CX3CR1 contributes to streptozotocin-induced mechanical allodynia in the mouse spinal cord*

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Patients with diabetic peripheral neuropathy experience debilitating pain that significantly affects their quality of life (Abbott et al., 2011), by causing sleeping disorders, anxiety, and depression (Dermanovic Dobrota et al., 2014). The primary clinical manifestation of painful diabetic neuropathy (PDN) is mechanical hypersensitivity, also known as mechanical allodynia (MA) (Callaghan et al., 2012). MA's underlying mechanism remains poorly understood, and so far, based on symptomatic treatment, it has no effective therapy (Moore et al., 2014).

It is believed that chronic neuropathic pain is associated with the activation of glial cells, which release both pro-inflammatory as well as anti-inflammatory factors in dorsal root ganglia (DRG) and the spinal cord. Mika et al. (2013) have demonstrated that immune factors, such as cytokines and chemokines, play a crucial role in nociceptive transmission.

Fractalkine (FKN), known as CX3CL1, is the only member of the CX3C chemokine subfamily, and it is produced by neurons and astrocytes (Zhao et al., 2017), while its coupled receptor, CX3CR1, is mostly present on the surface of microglial cells, within the dorsal spinal cord. The localization of FKN and its receptor seems to guarantee an interaction between neurons and microglia, especially in neuropathic pain generation. Soluble FKN in combination with CX3CR1, activates the proliferation and migration of microglial cells surrounding the injured area (Lindia et al., 2005; Milligan et al., 2005; Cao and Zhao, 2008; Dansereau et al., 2008; Clark et al., 2009; Ji et al., 2016). However, the possible participation of the FKN/CX3CR1 axis in PDN progression has received little attention.

Thus, this study aimed at investigating alterations in MA by the FKN/CX3CR1 axis in a streptozotocin (STZ)-induced type 1 diabetes mellitus (T1DM) mouse model. STZ-induced T1DM mice showed significantly lower body weight (BW) and higher fasting blood glucose (FBG) (>11.1 mmol/L) than the saline-injected controls (Figs. 1a and 1b). Paw withdrawal thresholds (PWTs) were evaluated weekly from Weeks 1 to 10 after STZ or saline injection to monitor their progression in MA. Control diabetic mice developed MA at Week 2 after the injection of STZ and remained stable till Week 8, indicating increased sensitivity to mechanical stimulation (Figs. 1c and 1d). Spinal dorsal horn FKN-CX3CR1 protein expression was detected at different time courses during the MA period in all groups, and it increased in

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Fig. 1 Changes in body weight (BW), fasting blood glucose (FBG), mechanical paw withdrawal thresholds (PWTs), and CX3CL1/CX3CR1 expression in diabetic and control C57BL/6 mice

(a, b) Changes of BW and FBG. The diabetic group had a significantly lower BW and higher FBG. *** $P < 0.001$, vs. C57BL+saline, two-way ANOVA. (c, d) PWTs on the left and right. Compared with the control mice, the PWTs in the diabetic mice decreased significantly from Weeks 2 to 8. *** $P < 0.001$, vs. C57BL+saline, two-way ANOVA. (e, f) Changes in murine CX3CL1/CX3CR1 expression in different groups. The expression levels of CX3CL1/CX3CR1 protein in the bilateral spinal cord were determined from Weeks 1 to 6 and showed upregulation in the diabetic group. * $P < 0.05$, ** $P < 0.01$, vs. vehicle (veh, C57BL+saline), Student's t -test. (g, h) Effects of intrathecal injection of RA-CX3CR1 (TP501) on the PWTs of diabetic mice. CX3CR1's negative drug, IgG (5 $\mu\text{g/L} \times 5 \mu\text{L}$), was intrathecally injected into mice at Week 3 after STZ injection. An evident release in responses to mechanical stimuli was detected after 2 h, which was reduced after 24 h and showed higher PWTs after injection and constantly lasted for 3 d in the CXCR1 antagonist (TP501; 5 $\mu\text{g/L} \times 5 \mu\text{L}$) group. * $P < 0.05$, *** $P < 0.001$, vs. C57BL+IgG, two-way ANOVA. (i) Localization of CX3CR1 in the spinal cord of diabetic mice. Double immunofluorescence labeling showed that CX3CR1 was predominantly colocalized with Iba1. Scale bar=100 μm . (a–h) Data are expressed as mean $\pm$ standard error (SE), $n=6$ per group

the dorsal horn of the diabetic mice (Figs. 1e and 1f). Mice in the diabetic group were then randomly divided into two subgroups (the rheumatoid arthritis (RA)-CX3CR1-treated and the IgG-treated diabetic mouse groups). The CX3CR1 antagonist (5 $\mu\text{g}/\text{L} \times 5 \mu\text{L}$) and its negative drug IgG (5 $\mu\text{g}/\text{L} \times 5 \mu\text{L}$) were intrathecally injected into mice at Week 3 after STZ injection. An evident manifestation of responses to mechanical stimuli was detected 2 h after injection and continued up to 24 h. The CX3CR1 antagonist and the IgG were consecutively injected for 3 d. After injection, the RA-CX3CR1-treated mice showed higher PWTs compared with the control group, which lasted for 3 d (Figs. 1g and 1h). In addition, immunohistochemical analysis demonstrated the localization and expression of CX3CR1 in the spinal cord microglia (Fig. 1i). Furthermore, CX3CR1 knockout (KO) mice in the STZ-injected group showed delayed PWTs which were alleviated earlier compared with the STZ-induced wild-type mouse group (Fig. 2).

It is accepted that hyperglycemia leads to the initiation of both overactive microglia and microangiopathy. It stimulates the generation of pro-oxidants (Nishikawa et al., 2000; Guo et al., 2018), advanced glycation end products (AGEs) (Sugimoto et al., 2008), reactive nitrogen species, and the polyol pathway (Zochodne et al., 2000; Oyama et al., 2006), which induces neurovascular and mitochondrial dysfunction and finally impairs neuron and microglial functions (Inoguchi et al., 2000; Cameron et al., 2001; Xu et al., 2019). The incitement of inflammation signals induces microglia to then migrate, proliferate, synthesize, and release pro-inflammatory mediators, such as mitogen-activated protein kinase (MAPK) and nuclear factor- κB (NF- κB), among others (Svensson et al., 2005; Tsuda et al., 2005), to initiate and maintain neuropathic pain by facilitating neuron-glia interactions.

Recently, additional studies have provided evidence that supports the FKN/CX3CR1 axis as a major player in PDN. Rajchgot et al. (2019) proposed that

Fig. 2 Body weight (BW), fasting blood glucose (FBG), and mechanical paw withdrawal thresholds (PWTs) in diabetic and control C57BL/6 and CX3CR1 knockout (KO) mice

(a, b) Changes in BW and FBG in diabetic and control CX3CR1 KO mice. Significant differences in BW and FBG were observed between diabetic and control CX3CR1 KO mice. *** $P < 0.001$, vs. CX3CR1 KO+saline, two-way ANOVA. (c, d) PWTs on the left and right in diabetic and control CX3CR1 KO mice. The PWTs were significantly lower in diabetic CX3CR1 KO mice from Week 2 and were relieved by Week 8 on the left and Week 10 on the right. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, vs. CX3CR1 KO+saline, two-way ANOVA. (e, f) Changes in BW and FBG in diabetic C57BL/6 and CX3CR1 KO mice. No significant differences were observed in BW or FBG between the C57BL/6 and CX3CR1 KO diabetic groups. (g, h) PWTs on the left and right in diabetic C57BL/6 and CX3CR1 KO mice. However, the development of MA in diabetic CX3CR1 KO mice was delayed and lasted a shorter time. * $P < 0.05$, *** $P < 0.001$, vs. C57BL+STZ, two-way ANOVA. Data are expressed as mean $\pm$ standard error (SE), $n = 6$ per group

multiple molecular mechanisms are involved in the control of PDN. Using STZ-induced diabetic Sprague-Dawley rats, Yao et al. (2017) demonstrated increases in interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) in the spinal dorsal horn, indicating that these cytokines are actually involved in the PDN process. Souza et al. (2013) pointed out that FKN was a link between peripheral inflammation and satellite glial cell activation, which generates inflammatory pain. However, the role of the FKN/CX3CR1 axis in PDN, especially in STZ-induced T1DM mouse MA, remains poorly understood.

Badr et al. (2012) observed, using blood tests, that soluble FKN was significantly downregulated in STZ-induced diabetic mice during the wound healing period compared with the control group. Contrarily, Kiyomoto et al. (2013) observed that the complete Freund's adjuvant (CFA) injection-induced trapezius muscle inflammation resulted in a significant increase in FKN, but not in CX3CR1, during the MA period, indicating that FKN might directly participate in the process of trapezius muscle inflammation pain. These differences might mainly be due to its different mechanisms of action in the nervous and immune systems.

This study has some limitations that ought to be considered. Lee et al. (2013) illustrated that CX3CR1 KO mice showed reduced insulin secretion, which can gradually lead to impaired glucose tolerance. However, our experiment employed an STZ-induced T1DM mouse model to reduce continuous hyperglycemia interference, which led to some inevitable interference. In addition, CX3CR1 KO mice may have immune system dysfunction, which can weaken the mice, especially after STZ injection, and might partly reduce mechanical stimulus reflection and, unavoidably, partially affect experimental results. Furthermore, Zhang et al. (2015) illustrated that the FKN/CX3CR1 axis also participates in the development of a 2,4-dinitrofluorobenzene (DNFB)-induced itch. A number of scratches were significantly reduced at the third day after a polyclonal FKN antibody was substituted, which indicates the need to further investigate the role of FKN/CX3CR1 in diabetic pruritus.

MA in our STZ-induced T1DM mouse model was mediated by spinal FKN/CX3CR1, and CX3CR1 inhibition could significantly attenuate it. Thus, FKN/CX3CR1 plays a pivotal role in the initiation and development of MA and might be an effective cellular target for the future treatment of PDN.

Contributors

Cheng-ming NI and Bing-yu LING were responsible for drafting the manuscript, and analysis and interpretation of data. Xiang XU collected and analyzed the data. He-ping SUN and Hui JIN contributed analysis of data and manuscript preparation. Yu-qiu ZHANG helped perform the analysis with constructive discussions. Hong CAO and Lan XU contributed the conception and design of the current study. All authors have read and approved the final manuscript. Therefore, all authors have full access to all the data in the study and take responsibility for the integrity and security of the data.

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Compliance with ethics guidelines

Cheng-ming NI, Bing-yu LING, Xiang XU, He-ping SUN, Hui JIN, Yu-qiu ZHANG, Hong CAO, and Lan XU declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed. All experiments were approved by the Animal Care and Use Committee of Fudan University, Shanghai, China and followed the policies issued by the guidelines for pain research of the International Association for the Study of Pain (IASP).

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中文概要

题 目: CX3CL1 受体参与 1 型糖尿病机械痛的形成

目 的: 趋化因子 (CX3CL1) 在神经性疼痛中起重要的生理病理作用, 然而其在糖尿病神经病理痛中的

作用还有待研究。本实验主要研究了在糖尿病小鼠痛阈下调的时间窗内,脊髓背角中趋化因子CX3CL1/趋化因子受体(CX3CR1)在触诱发痛发生与发展中的作用。

创新点: 主要探讨CX3CR1在链脲佐菌素(STZ)诱导的1型糖尿病(T1DM)小鼠早期发生的机械痛性神经病变中的作用。

方法: 本实验采用健康雄性C57BL/6小鼠与CX3CR1 KO小鼠,体重20~23 g,隔夜禁食12 h(20点至次日8点),并连续三天腹腔注射100 mg/kg的STZ制备T1DM模型。以空腹血糖浓度>11.1 mmol/L且三周后小鼠机械痛阈值明显下降的情况视为T1DM模型制备成功。在小鼠机械痛阈下降的对应时间点,取腰段脊髓背角,采用蛋白质印迹法

(western blot)和免疫组化法测定CX3CL1及CX3CR1的表达情况。同时,在发生机械痛阈值下降的第三周时间窗内给予CX3CR1的中和抗体,进行机械刺激并观察其痛阈值的变化。

结论: STZ诱导的T1DM动物模型在早期表现为显著的机械诱发痛,并伴随脊髓背角CX3CL1/CX3CR1表达上调;在痛阈下降期窗内给予CX3CR1的中和抗体可抑制糖尿病小鼠的痛行为。与腹腔注射STZ形成T1DM的C57BL/6小鼠相比,CX3CR1基因敲除的糖尿病小鼠机械痛阈值下降的时间延迟,程度减轻。因此,我们推测CX3CL1/CX3CR1可能参与T1DM机械痛的形成与发展。

关键词: 趋化因子(CX3CL1);趋化因子受体(CX3CR1);机械痛;链脲佐菌素;糖尿病模型