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STZ causes depletion of immune cells in sciatic nerve and dorsal root ganglion in experimental diabetes

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ABSTRACT

Streptozotocin (STZ) treatment, a common model for inducing diabetes in rodent models, induces thermal hyperalgesia and neuronal toxicity independently of hyperglycemia by oxidizing and activating TRPA1 and TRPV1. Following treatment with STZ, $CD45^{+}$ immune cells were found to be depleted in sciatic nerve (SN) and DRG in mice, prior to hyperglycemia. Macrophages were also lost in DRG and NFκB-p65-activation was increased in SN macrophages. Immune cells were significantly reduced in both SN and DRG up to three weeks, post-treatment. Loss of PNS-resident macrophages in response to STZ-mediated toxicity may affect the regenerative capacity of the nerve in response to further injury caused by diabetes.

1. Introduction

Pain and the progressive loss of sensation in the hands and feet are the characteristic symptoms of diabetic neuropathy (DN), which affects 30–50% of patients suffering from diabetes. DN is one of the most difficult late complications to treat, with only ca. 40–60% of patients achieving partial relief via pharmacological intervention. The lack of effective treatment opinions, other than glucose lowering, may be due to the difficulties in translating findings in animal models of DN to treatments in a clinical setting. Administration of streptozotocin (STZ) is an accepted pharmacological method for the induction of type 1 diabetes in rodent models. STZ, a nitrosourea derivative, is taken up by cells via glucose transporter-2 (GLUT2), which is highly expressed by insulin-producing beta cells (Wang and Gleichmann, 1995). Once inside the cell, the alkalizing properties of STZ causes DNA damage in a variety of different mechanisms, ultimately leading to cell death (Bolzán and Bianchi, 2002). However, STZ can also affect the peripheral nervous system (PNS), in which there is no documented expression of GLUT2. In rats, mechanical and thermal hyperalgesia was observed within the first two weeks following administration of STZ, independently of blood glucose levels (Bishnoi et al., 2011; Romanovsky et al., 2004). Transient Receptor Potential Vanilloid 1 channel (TRPV1), a receptor for various noxious stimulate and heat, was shown to be required for early glucose-independent thermal hyperalgesia induced by STZ in mice and rats. Resiniferatoxin, a TRPV1 antagonist, attenuated the early hyperalgesic response to heat in rats which had received STZ (Bishnoi et al., 2011). Furthermore, mice lacking TRPV1 did not show hyperalgesia to heat in response to STZ treatment (Pabbidi et al., 2008b). Pabiddi et al. showed that the response of Dorsal Root Ganglia (DRG) neurons to capsaicin was increased in the presence of STZ, and that STZ-treatment in vitro caused a ROS-mediated toxic effect (Pabbidi et al., 2008a). Persistent stimulation of TRPV1 in the context of diabetes leads to denervation (Koivisto et al., 2012), explaining the hypoalgesia found after treatment with high doses of STZ.

Recently Andersson et al. showed that STZ yields peroxynitrite in neurons upon decomposition (Andersson et al., 2015). The peroxynitrite oxidizes the cysteine residues in the intracellular domain of TRPA1, a receptor expressed on TRPV1 positive neurons (Kwan et al., 2006). Oxidization of TRPA1 cysteine residues and modification of Lysine710 by glucose, oxidized by the STZ-derived peroxynitrite, could together explain the STZ-mediated activation of TRPA1. Peroxynitrite mediates neuronal toxicity during inflammation (Mander and Brown, 2005) and nitroxidative species can also induce neuroinflammatory signaling via danger-associated molecular patterns (DAMPs), which contribute to pain sensations after injury (Grace et al., 2016).

Despite the evidence for neurotoxic effects of STZ, early measurements and/or interventions for DN after STZ treatment are still common. These treatments, whilst effective at preventing a potential ROS-mediated injury caused by STZ, may not necessary be treating the

Abbreviations: DN, diabetic neuropathy; DRG, dorsal root ganglion; HbA1c, haemoglobin A1c; MFI, mean fluorescence intensity; PCL, peritoneal cavity lavage; SN, sciatic nerve; STZ, streptozotocin; DAMPs, danger-associated molecular patterns.

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hyperglycemia-dependent damage that is occurring in diabetes. Understanding to what extent and how STZ-treatment affects the nerve *in vivo* is therefore important for interpretation of the many published animal studies that have investigated treatment and development of DN.

STZ has previously been shown to cause increase expression of pro-inflammatory cytokines in the spinal cord (Bishnoi et al., 2011). It is therefore reasonable to hypothesize that if STZ-treatment causes an injury to the PNS, either directly or mediated by hyperglycemia, this would result in an immune activation in the sciatic nerve (SN) or DRGs. Mechanic nerve injury in the PNS causes recruitment, accumulation, proliferation and activation of macrophages (Barrette et al., 2008; Mietto et al., 2015) to clear debris and pathogens and to orchestrate tissue reconstruction. Increased infiltration of $CD45^+$ immune cells, which enter the nerve from circulation, is an indicator of inflammation (DeFrancesco-Lisowitz et al., 2014). CD11b is present on infiltrating $CD45^{hi}$ monocytes and macrophages, as well as on $CD45^{low}$ microglia in the central nervous system (Guillemin and Brew, 2004), or satellite glial cells (SGCs) in the DRGs (van Velzen et al., 2009). MHC class II is present on inflammatory M1 macrophages and is up-regulated as macrophages and microglia become increasingly pro-inflammatory (Kigerl et al., 2009). Anti-inflammatory M2 macrophages, identified by their expression of the mannose receptor CD206, provide neurotropic factors and are required for tissue regeneration in the PNS (Martinez et al., 2013). The role for macrophages in the different stages of nerve regeneration in the PNS has recently been reviewed elsewhere (DeFrancesco-Lisowitz et al., 2014). In particularly anti-inflammatory $CD206^+$ M2 macrophages have been shown to promote axon outgrowth in the PNS (Mokarram et al., 2012) and M2 macrophages have also been shown to secrete endogenous opioids, decreasing pain from injury to the PNS (Pannell et al., 2016). The aim of this study was to investigate the consequences of STZ-treatment on the innate immune system, primarily the macrophage, in the PNS, and identify a time point in the course of diabetes, at which this effect has been resolved.

2. Materials and methods

2.1. Mice

Wild-type C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). All procedures were approved by the Animal Care and Use Committee at the Regierungspräsidium Karlsruhe, Germany (G216/10).

2.2. STZ-treatment

STZ was administered by intraperitoneal injection (50 mg/kg body weight in 50 mM Sodium citrate; pH 4.5) for 3 or 5 consecutive days in eight week old male mice, whilst age-matched controls received sodium citrate. Blood glucose levels were determined in blood samples from the tail vein using ACCU-CHEK glucose sticks (Bierhaus et al., 2004; Bierhaus et al., 2012). Mice were sacrificed using carbon dioxide, and peritoneal cavity lavage was performed post-mortem. Following perfusion with ice-cold PBS, both SN and DRGs (L1-L6) were dissected out and processed for analysis.

2.3. Thermal sensitivity assays

Sensitivity to heat-induced pain was measured with an electronically controlled hot-plate analgesia meter (*Columbus Instruments, Columbus, Ohio, USA*) at 50 °C, as previously described (Bierhaus et al., 2004; Menéndez et al., 2002). Each mouse was removed from the hot plate when a jumping escape response occurred or hind paws were licked, or after a maximal cut-off time of 60 s. The latency until mice showed the first signs of discomfort (hind paw lifting, licking, or shaking, and jumping) were recorded. Three measurements were taken per animal, with a time interval of ca. 30 min between each measurement to minimize a habituation effect. The foot withdrawal latency of the mice was also measured using the Hargreaves apparatus (*Ugo Basile, Comerio, Italy*) (Hargreaves et al., 1988). The cut-off time of the equipment was preset at 33 s with an intensity of 60. Briefly, each mouse was placed into the testing enclosure and allowed time (ca. 30 min) to acclimatize before measurements were taken. Three measurements of foot withdrawal latency were made on each hind paws. Each trial was separated by an interval of ca. 30 min to decrease the possibly of skin injury and alterations of sensitivity of cutaneous nociceptors. The measurements from all mice within a group were averaged and the difference in latency times between the control and STZ-treated mice was calculated as a measure of thermal sensitivity, as previously described (Bierhaus et al., 2004; Bierhaus et al., 2012).

2.4. Flow cytometry

The protocol for preparing nerves for flow cytometry was adapted from (Barrette et al., 2008) with the modification that the digested material was pass first through a 140 μm metal mesh (*Sigma Aldrich Co., St. Louis, USA*) and then through a 70 μm metal mesh on ice and stained with the appropriate antibodies (Supplementary Table 1). The cells were analyzed using Becton Dickinson LSR II flow cytometer (*Heidelberg, Germany*) and FlowJo version xV0.7 (*OR, USA*). The gating for CD45 and CD11b were based upon parallel staining of the blood (Supplementary Fig. 1) and the gates for CD68 and CD206 were based upon stainings of the macrophage-rich peritoneal lavages (Supplementary Fig. 2).

2.5. Statistical analysis

GraphPad Prism version 6.05 and Microsoft Excel were used for analysis. Outliers were excluded based on interquartile range × 1.5 and did not alter the conclusions of the experiments. Data represents mean ± SD unless otherwise stated. Statistical significance was tested using two-tailed Student's *t*-test: $^{***}p < 0.0001$, $^{**}p < 0.001$, $^{*}p < 0.05$.

3. Results

3.1. Loss of leukocyte from PNS, prior to hyperglycemia, is associated with onset of thermal hyperalgesia

Following treatment with STZ for three consecutive days, healthy C57BL/6 mice were found to have significantly increased thermal sensitivity, responding 1.30 ± 0.29 s faster than the control mice, indicating hyperalgesia (Fig. 1A). Blood glucose was not significantly elevated in STZ-treated mice, as compared to control group (Fig. 1B). However, the mice had significantly reduced body weight (Fig. 1C). Flow cytometry analysis of single cell suspensions ($DAPI^+$; Fig. 2A) of SN and DRG showed that the proportions of cells in PNS expressing CD45 were reduced in the STZ-treated mice (Fig. 2B–C). Furthermore, $CD45^+CD11b^+$ cells, a general marker of myeloid cells, were reduced in the DRG following STZ-treatment (Fig. 2B–D). The expression of ICAM-1, a molecule required for leukocyte recruitment, in the DRGs was significantly decreased after STZ treatment (Fig. 2E). The loss $CD45^+CD11b^+$ cells in PNS was not the result of a reduction of $CD11b^+$ cells from blood as the proportion of $CD45^+$ leukocytes expressing CD11b was increased in the blood (Fig. 3A). The proportion of $CD11b^+$ cells, of the remaining leukocytes in the DRGs, was also increased (Fig. 3B). In order to investigate if the relative proportions of monocytes or neutrophils were changed in blood or DRG after STZ treatment, the cells were stained with CD45, CD11b, Ly6G and Ly6C (Fig. 3C–D). As a proportion of all leukocytes, the percentage of monocytes ($CD11b^+Ly6C^{+/++}Ly6G^-$) (Fig. 3E–F), neutrophils ($CD11b^+Ly6C^+Ly6G^+$) (Fig. 3G–H) or inflammatory monocytes