

Acrolein Scavenger Hydralazine Prevents Streptozotocin-Induced Painful Diabetic Neuropathy and Spinal Neuroinflammation in Rats

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ABSTRACT

Diabetes-induced neuropathic pain (DNP) substantially influences people's life qualities. Hyperglycemia-induced excess free radicals have been considered as the most critical mechanisms underlying DNP. As an unsaturated aldehyde and a reactive product of lipid peroxidation, acrolein plays critical roles in diabetic nephropathy and inflammatory pain. We sought to determine whether acrolein is involved in DNP in this study. Diabetes was induced by a single intraperitoneal (*i.p.*) injection of 60 mg/kg streptozotocin (STZ). An acrolein scavenger hydralazine (5 mg/kg) was administered through a daily injection for 4 weeks, starting immediately within 30 min after STZ injection. Western blot showed that hydralazine could effectively inhibit STZ-induced upregulation of acrolein in the spinal dorsal horn on day 7–28 after STZ injection. Behavioral tests showed that STZ injection induced significant mechanical allodynia and thermal hyperalgesia, which could be alleviated by hydralazine. Immunofluorescent histochemistry and Western blot showed that STZ induced significant microglial activation. ELISA data indicated upregulation of inflammatory cytokines IL-1 β and TNF- α expression in the spinal dorsal horn. Furthermore, hydralazine effectively attenuated microglial activation and expression of inflammatory mediators. Our data indicate that acrolein might be involved in the development of neuroinflammation and behavioral consequences of DNP. *Anat Rec*, 300:1858–1864, 2017. © 2017 Wiley Periodicals, Inc.

Key words: acrolein; hydralazine; painful diabetic neuropathy; spinal cord

Neuropathic pain is one of the major complications of diabetes mellitus (Albers and Pop-Busui, 2014). Approximately 30% of diabetic patients suffer from chronic neuropathic pain (Calabek et al., 2014). Similar to other types of chronic pain, diabetes-induced neuropathic pain (DNP) is refractory to the conventional treatments and substantially influences people's life qualities (Calabek et al., 2014; Ziegler and Fonseca, 2015). Hyperglycemia-induced excess free radicals and oxidative stress has been considered as one the most critical mechanisms underlying diabetic neuropathy (Hosseini and Abdollahi, 2013). Accumulations of free radicals and reactive oxygen species would sensitize nociceptors and activate glial

cells and thus contribute to the pathogenesis of DNP. However, ideal free-radical scavengers targeting DNP are still lacking (Ziegler and Fonseca, 2015).

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Acrolein is an unsaturated aldehyde and a reactive product of lipid peroxidation (Alwis et al., 2015). Previous studies showed that acrolein could induce vicious cascades of oxidative stress that greatly amplifies its original effects (Luo and Shi, 2005; Hamann and Shi, 2009). It has been reported that acrolein contributes to many neuropathological diseases, including stroke (Saiki et al., 2009), Alzheimer's disease (Lovell and Markesbery, 2007), and spinal cord injury (Shi et al., 2011). Serum from patients with diabetic nephropathy described the utility of "aldehyde capture," suggesting future use of serum aldehyde levels in the monitoring of renal disease and treatment (Medina-Navarro et al., 2011). Acrolein levels in CSF were regarded as good biomarkers for evaluating the severity of cognitive impairment of patients with Alzheimer's disease (Mizoi et al., 2014).

Many years ago, acrolein was found to be involved in diabetic nephropathy (Suzuki and Miyata, 1999). The concentration of acrolein is significantly higher in the urine or the serum of diabetes patients (Tsukahara et al., 2003). Acrolein has also been associated with dysregulation of insulin signaling in human endothelial cells (O'Toole et al., 2014). Previous studies indicated the acrolein-derived advanced lipoxidation end-product [N (epsilon)-(3-formyl-3,4-dehydropiperidino) lysine may provide a useful risk marker for the development of proliferative diabetic retinopathy (Zhang et al., 2008). These data indicate that acrolein may play an essential role in the diabetes-related complications. However, it is still unknown whether increased serum acrolein would contribute to the development of DNP. Interestingly, acrolein could activate the transient receptor potential ankyrin 1 receptor (TRPA1) and contribute to inflammatory pain sensitivities and pain following spinal cord injury (Bai et al., 2012; Due et al., 2014; Park et al., 2015).

Spinal microglial activation could release proinflammatory cytokines and exaggerate the neuroinflammation, consequently enhancing diabetic pain response (Wang et al., 2014). STZ-induced upregulation of IL-1 β and TNF- α has been reported in previous studies (Pabreja et al., 2011). Whether acrolein contributed to this process was still to be studied. In this study, we tried to prove our hypothesis that acrolein might be involved in the pathophysiological process of DNP. We first observed the protein expression of acrolein in the spinal dorsal horn following induction of diabetes in rats. Then we tested the antiallo-dynic effect of an acrolein scavenger hydralazine. We also examined the expression of ionized calcium-binding adapter molecule 1 (Iba1), which has been used widely as a marker for microglia, and proinflammatory cytokines in the spinal dorsal horn after hydralazine or vehicle treatment in diabetic and sham rats.

MATERIALS AND METHODS

Animals and Treatments

Male adult *Sprague-Dawley* rats weighing 200 ± 20 g were provided by the Experimental Animal Center of Chinese PLA Army General Hospital (Beijing, China) and were housed in individual cages with free access to water and food. All experimental procedures were approved by the Animal Care Committee of Chinese PLA Army General Hospital and were performed in accordance with the guidelines on animal care of the

National Institutes of Health and the ethical guidelines (Zimmermann, 1983).

Diabetes was induced by a single intraperitoneal (*i.p.*) injection of 60 mg/kg streptozotocin (STZ, Sigma-Aldrich Co., St Louis, MO, USA). Hyperglycemia was evaluated using the Blood Glucose Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in fasting blood samples 5 days after STZ administration. To analyze the expression change of acrolein different time after induction of diabetes, rats were divided into seven groups: naive ($n = 10$), sham 7 d ($n = 10$), sham 14 d ($n = 10$), sham 28 d ($n = 10$), STZ 7 d ($n = 15$), STZ 14 d ($n = 15$), STZ 28 d ($n = 15$) (Figure 1). Rats with fast blood glucose values higher than 16.7 mmol/L were considered to be diabetic. Rats in sham control groups were injected with same volumes of vehicle rather than STZ.

To analyze the effect of acrolein scavenger hydralazine on diabetic pain, rats were divided into five groups: naive ($n = 10$), sham-HDZ ($n = 10$), sham-vehicle ($n = 10$), STZ-vehicle ($n = 15$), STZ-vehicle ($n = 15$). The hydralazine hydrochloride (Sigma, St. Louis, MO, USA) (5 mg/kg, dissolved in 0.9% saline with a concentration of 2.5 mg/mL) solution was administered (*i.p.*) through a daily injection for 4 weeks, starting immediately within 30 min after STZ injection. Previous reports suggested that 5 mg/kg hydralazine hydrochloride could effectively decrease acrolein level and has minimal influence on blood pressure (Zheng et al., 2013).

Mechanical Paw Withdrawal Threshold (PWT) Test

Behavioral tests were performed before STZ injection and at 7 d, 14 d, and 28 d after diabetes induction. For PWT test, each rat was put in a transparent plastic cage on an elevated metal mesh floor. After adaptation for 30 min period, the plantar surface of each hind paw was perpendicularly stimulated with series of von-Frey filaments (North Coast Medical, Morgan Hill, CA). The force used in the test was started from 2.0 g and the maximum force was 15.0 g. A quick withdrawal of the hind paw on stimulus was recorded as a positive response. If a positive response got, a smaller von-Frey hair was used; if a negative response was observed, a higher force was chose. The 50% mechanical PWT was determined using the Chaplan's up-down method (Chaplan et al., 1994).

Thermal Paw Withdrawal Latency (PWL) Test

PWL tests were performed before STZ injection and at 7 d, 14 d, and 28 d after diabetes induction. Each rat was individually put in chambers on an elevated glass platform, and was accommodated for 30 min in the testing environment. The radiant heat beneath the glass was focused onto the plantar surface of each hind paw. The time from the beginning of radiant heat application to the withdrawal of the rat's hind paw was recorded as the PWL. An automatic cutoff of 20 s was employed to prevent tissue damage.

Western Blotting

Six rats in each group were sacrificed by decapitation under deep anesthesia with isofurane. The lumbar

segment of spinal cord was dissected. The sample was further divided into dorsal and ventral parts, and then the dorsal horns were homogenized with a hand-held pestle in homogenization buffer (50 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, 0.1 mM EGTA, and 1 μ M leupeptin). Protein concentration was measured by the bicinchoninic acid (BCA) Protein Assay kit (Pierce, WI, USA). Proteins (30 μ g) were denatured in Laemmli sample loading buffer (Bio-Rad, Hercules, CA) at 99°C for 5 min and were then separated on 10% SDS-polyacrylamide gels and then electroblotted on nitrocellulose membranes. After blocking with 5% nonfat milk for 1 h, the membrane was then incubated overnight at 4°C with primary antibodies: mouse antiacrolein (1:200, Abcam, Cambridge, UK), rabbit anti-Iba-1 (1:1000; Millipore, Billerica, MA, USA), and mouse anti- β -actin (1:2000, Santa-Cruz Biotechnology, Santa Cruz, California, USA). The proteins were detected by horseradish peroxidase-conjugated secondary antibodies and visualized with chemiluminescence reagents provided with the ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA), following exposure to hyperfilm (Kodak, Rochester, New York, USA). The intensity of the blots was quantified using densitometry analysis with Image J software. The relative protein levels of acrolein and Iba-1 were normalized to the β -actin densities of the same sample. The data were expressed as relative fold changes relative to the naive groups.

Immunofluorescent Histochemistry

Under anesthesia with chloral hydrate (*i.p.*, 350 mg/kg), rats were perfused with 0.1 M phosphate buffer (PB, pH 7.3) containing 4% paraformaldehyde. The lumbar segments of spinal cord were then removed and post-fixed and then cryoprotected with 30% sucrose. Coronal sections (30 μ m) were cut using a Leica cryostat (Leica CM1800, Heidelberg, Germany). Five sections from each animal were selected randomly for immunofluorescent staining. After being rinsed in 0.01 M PBS (pH 7.3) and blocked with 2% goat serum, the sections were incubated overnight at 4°C with rabbit anti-Iba-1 IgG (1:1000, Millipore). The sections were then incubated for 2 h with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200, Abcam) after washed with 0.01 M PBS (10 min \times 3 times). Images were taken with a confocal laser microscope (FV1000; Olympus, Japan).

ELISA Analysis

The spinal dorsal horns were collected and homogenized in PBS with protease inhibitors. Then the samples were thawed and centrifuged at 10,000g for 5 min to remove cellular debris. The total protein concentration of the supernatant was quantified by the BCA Protein Assay kit (Pierce). Cytokine protein levels were detected with rat ELISA kits according to manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The average level of TNF- α and IL-1 β was calculated based on a standard curve provided by the kit. All assays were performed in duplicates using recommended buffers, diluents, and substrates. The tissue cytokine concentrations were expressed as pg protein/mg. The detection limits for TNF- α and IL-1 β were both 5 pg/mg.

Data Analysis

For behavioral studies, the data of paw withdrawal frequency and latency were suitable for parametric statistics. Analysis of variance (ANOVA) for repeated measures was used to determine the time course of STZ treatment and drug effects. One-way ANOVA was used to identify the source of significant interactions at each time point, followed by Tukey *post hoc* tests. All the data were expressed as mean \pm SEM, and $P < 0.05$ was considered as statistical significance.

RESULTS

STZ-Induced Persistent Elevation of Acrolein Protein Adducts in Rat Spinal Dorsal Horn

The expressions of acrolein following injection of STZ were examined by Western blot analysis. In the rats receiving vehicle injections (sham groups), acrolein expressions were not increased compared with naive rats at any observing period ($P > 0.05$). However, in the STZ treatment group, a significant increase of acrolein could be observed at day 7 ($P < 0.05$ *vs.* naive and sham groups) after diabetes induction. In addition, the expression of acrolein was further increased on day 14 ($P < 0.05$ *vs.* naive and sham groups) and 28 ($P < 0.05$ *vs.* naive and sham groups) after STZ injection.

Acrolein Scavenger Hydralazine-Alleviated Diabetic Pain Behavior

As acrolein expressions were significantly elevated in the spinal dorsal horn after STZ injection, we then observed whether acrolein correlated with STZ-induced painful diabetic neuropathy. Hydralazine (5 mg/kg) was administered (*i.p.*) through a daily injection for 4 weeks, starting immediately within 30 min after STZ injection. PWTs to mechanical stimuli and thermal PWLs were tested on days 0, 7, 14, and 28 after STZ injection. Behavioral tests showed that PWTs of diabetic rats (STZ-vehicle group) were significantly decreased on day 14 ($P < 0.05$ *vs.* naive) and 28 ($P < 0.05$ *vs.* naive). The inhibitory effect of hydralazine on PWTs was not significant on day 14 ($P > 0.05$ *vs.* STZ-vehicle), but was significant on day 28 ($P < 0.05$ *vs.* STZ-vehicle) after STZ injection (Figure 2A). STZ-induced thermal hyperalgesia could be observed on day 7 after induction. The PWLs in STZ-hydralazine were not significantly elevated on day 7 ($P > 0.05$ *vs.* STZ-vehicle), but were significantly increased on day 14 ($P < 0.05$ *vs.* STZ-vehicle) and day 28 ($P < 0.05$ *vs.* STZ-vehicle) after STZ injection (Figure 2B). It should also be noticed that hydralazine could not completely block STZ-induced mechanical allodynia or thermal hyperalgesia. PWTs and PWLs in the STZ-hydralazine group were still lower than that of the sham or naive group, on day 14 and day 28 ($P < 0.05$). These results showed that systemic injection of acrolein scavenger hydralazine could successfully alleviate diabetic pain behavior. To further confirm the scavenging effect of hydralazine on acrolein, we sacrificed the rats on day 28 after behavioral tests and checked the expressions of acrolein. Western blotting analysis showed that compared to the vehicle, hydralazine could effectively inhibit STZ-induced upregulation of acrolein in the spinal dorsal horn ($P < 0.05$). Acrolein expression in

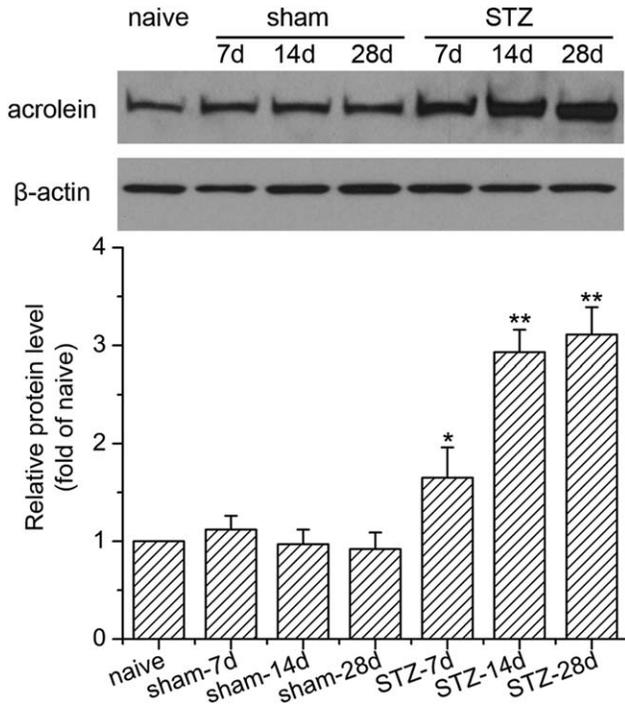


Fig. 1. Streptozotocin (STZ)-induced persistent elevation of acrolein protein adducts in rat spinal dorsal horn shown by Western blot analysis. Rats were divided into seven groups: naïve ($n=6$), sham 7 d ($n=6$), sham 14 d ($n=6$), sham 28 d ($n=6$), STZ 7 d ($n=12$), STZ 14 d ($n=12$), STZ 28 d ($n=12$). In the rats receiving vehicle injections (sham groups), acrolein expressions were not increased compared with naïve rats at any observing period. In the STZ treatment group, a significant increase of acrolein could be observed at day 7 and was further increased on day 14 and 28 after STZ injection. Data are normalized against β -actin levels and expressed as fold change of the naïve group. *, **: $P < 0.05$, $P < 0.01$ versus naïve group.

sham-hydralazine group was also lower than that of sham-vehicle group, although the difference was not significant (Figure 3).

Hydralazine-Ameliorated Neuroinflammation in the Spinal Dorsal Horn of Diabetic Rats

We sacrificed the rats on day 28 and tested the neuroinflammation in the rat spinal dorsal horn. Immunofluorescent histochemistry showed that STZ induced obvious microglial activation in the spinal dorsal horn. In the sham-treated rats, hydralazine has no detectable effect on microglial morphology; however, microglial activation was significantly suppressed by hydralazine (Figure 4A–E). Western blotting showed that Iba-1 expression in STZ-hydralazine group was significantly lower than that of STZ-vehicle group, indicating the effect of hydralazine on microglial activation after STZ injection (Figure 4F,G). However, in the sham groups, hydralazine had no effect on Iba-1 expression ($P > 0.05$), indicating that hydralazine inhibited activated but not inactivated microglia. Similar to the behavioral data, hydralazine could not completely inhibit STZ-induced microglial activation. Iba-1 in the STZ-hydralazine group was still higher than that of the sham or naïve group. In

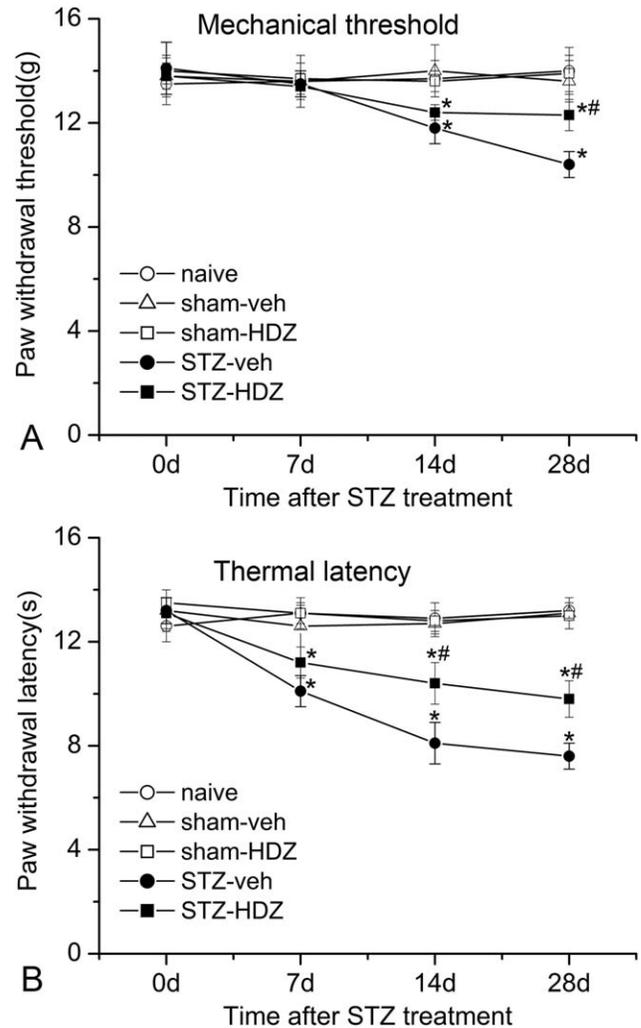


Fig. 2. Hydralazine (HDZ)-alleviated streptozotocin (STZ) injection-induced mechanical allodynia (A) and thermal hyperalgesia (B). Rats were divided into five groups: naïve ($n=6$), sham-HDZ ($n=6$), sham-vehicle ($n=6$), STZ-vehicle ($n=12$), STZ-vehicle ($n=12$). Hydralazine (5 mg/kg) was administered (i.p.) through a daily injection for 4 weeks, starting immediately within 30 min after STZ injection. Mechanical paw withdrawal thresholds to mechanical stimuli and thermal paw withdrawal latencies were tested on days 0, 7, 14, and 28 after STZ injection. *, #: $P < 0.05$ versus naïve or STZ-vehicle (veh) group, respectively.

addition, we also checked the expression of inflammatory cytokines in the spinal dorsal horn after hydralazine treatment. ELISA data showed that IL-1 β and TNF- α were both robustly increased in the diabetic spinal cord ($P < 0.05$ vs. naïve group, Figure 4H,I). Hydralazine effectively attenuated the expression of these inflammatory mediators: IL-1 β ($P < 0.05$ vs. STZ-vehicle, Figure 4H) and TNF- α ($P < 0.05$ vs. STZ-vehicle, Figure 4I). However, IL-1 β and TNF- α expressions in STZ-hydralazine group were still higher than that of the naïve or sham groups. These data indicate that systemic hydralazine treatment could decrease the production of inflammatory cytokines in the spinal dorsal horn of diabetic rats.

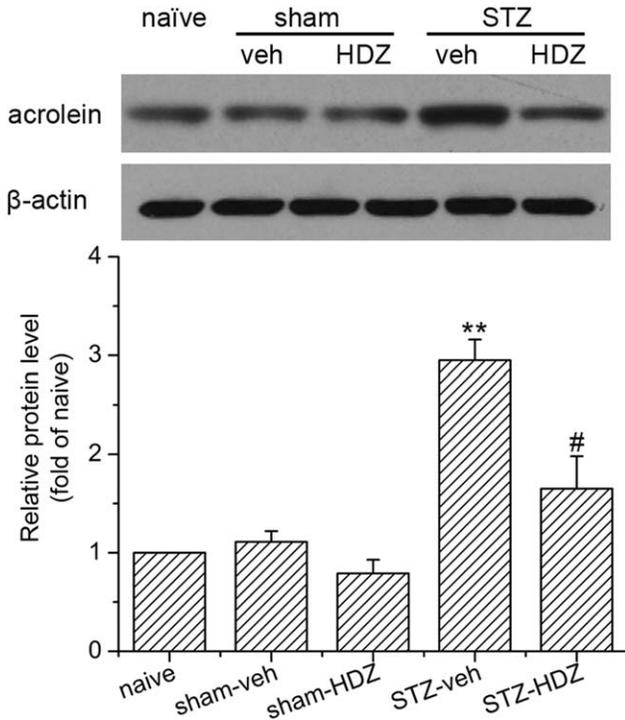


Fig. 3. Hydralazine (HDZ)-inhibited acrolein expression in spinal dorsal horn of diabetic rats. Rats were sacrificed on day 28 after behavioral tests. Western blotting analysis showed that hydralazine could effectively inhibit STZ-induced upregulation of acrolein in the spinal dorsal horn. Acrolein expression in sham-hydralazine group was also lower than that of the sham-vehicle group. Data are normalized against β -actin levels and expressed as fold change of the naïve group. **: $P < 0.01$ versus naïve, #: $P < 0.05$ versus STZ-vehicle (veh) group, respectively.

DISCUSSION

In this study, we found a persistent elevation of acrolein in the spinal dorsal horn following induction of diabetes in rats. An acrolein scavenger hydralazine could partially inhibit DNP. Furthermore, neuroinflammatory response in the spinal dorsal horn of diabetic rats could be ameliorated by hydralazine treatment. Our results indicated that acrolein could be an effective target for the treatment of chronic pain induced by diabetes.

Acrolein was reported to be increased in urine and serum of diabetic patients (Zhang et al., 2008; Hosseini and Abdollahi, 2013; Albers and Pop-Busui, 2014). To our knowledge, this is the first time elevated acrolein has been found in spinal cord of rat diabetes model. Acrolein has exogenous and endogenous sources (Watzek et al., 2012). In this study, the increased acrolein may have the following sources: (a) neutrophil-derived myeloperoxidase induced threonine degradation; (b) polyamines catabolism; and (c) lipid peroxidation of polyunsaturated fatty acids (Stevens and Maier, 2008). However, due to the diffusive nature of acrolein, the cell source of acrolein is difficult to be determined. Acrolein could be released from neural and nonneural cells and quickly diffused and caused cell injuries. Together with our results, increased expression of acrolein in the spinal cord could be a common factor of pathological pain. And

acrolein could be a new target for developing strategies for pain treatment. We observed that the acrolein scavenger hydralazine could effectively hamper microglial activation and synthesis of inflammatory cytokines. Acrolein toxicity includes induction of oxidative stress and neuroinflammation (Moghe et al., 2015). Acrolein may very well serve as inflammation-associated compound that contributes to the chronic nature of diabetes-related neuropathic pain. In our study, we observed a strong activation of neuroinflammation after STZ injection, which is in consistent with previous reports (Wang et al., 2014). Hyperglycemia in diabetes increases oxidative stress in nerve (Catanzaro et al., 2013). The damaged nerve induces inflammation of Schwann cells and axons and lead to the release of proinflammatory cytokines, including IL-1 β and TNF- α , which disturb homeostasis of glia in the spinal cord and cause microglial (but not astrocytic) activation (Wang et al., 2014). Inhibition of microglia with the selective inhibitor minocycline inhibits the elevated IL-1 β and TNF- α , and diabetes-related mechanical allodynia and thermal hyperalgesia (Pabreja et al., 2011). In our study, we found that microglial activation could be suppressed through decreasing acrolein levels. This provides some evidence for the involvement of lipid peroxidation in glial cell activation in the pathogenesis of DNP. Neuroimmune activation could be suppressed by inhibition of oxidative stress, suggesting acrolein could be the upstream event of neuroimmune response in the state of diabetes.

The detailed mechanism underlying acrolein-induced microglial activation is unknown. It has been reported that mice exposed to acrolein for 4 days exhibited an enhanced inflammatory reaction with activated macrophages and neutrophils, and higher level of cytokines in the bronchial fluid. Similarly, carbonyl scavenger bisulfite could inhibit the inflammatory response (Ong et al., 2012). However, other studies showed that acrolein may inhibit proinflammatory response in human T lymphocyte (Lambert et al., 2007) and could inhibit macrophage responses (Kirkham et al., 2004). These results suggest that acrolein may have contradictory effects on inflammatory response. Some believe that duration of acrolein exposure could determine its final outcomes: acute acrolein exposure might suppress innate immunity while chronic exposures enhance inflammation (Moghe et al., 2015).

It should be noticed that in diabetic patients, successful treatment of hyperglycemia does not always attenuate established DNP (Tomlinson and Gardiner, 2008), indicating that DNP is not always dependent on hyperglycemia. Hyperglycemia is critical for inducing metabolic alterations and producing acrolein and other products of lipid peroxidation (Catanzaro et al., 2013). After these products are generated, they can operate independently of blood glucose to induce neuroinflammation and cause persistent pain. So, acrolein and other products but not blood glucose itself could be effective targets for treatment of DNP.

Hydralazine has been used for a long time as an anti-hypertensive drug (Hamann and Shi, 2009). Nucleophilic nitrogen of the hydralazine could form a hydrazone when react with acrolein. Among all the aldehyde-trapping agents, the effect of hydralazine is the most potent (Burcham et al., 2000). It has shown an effect in preventing acrolein-mediated cell death *in vitro* and spinal cord injury *in vivo* (Lambert et al., 2007; Shi et al.,

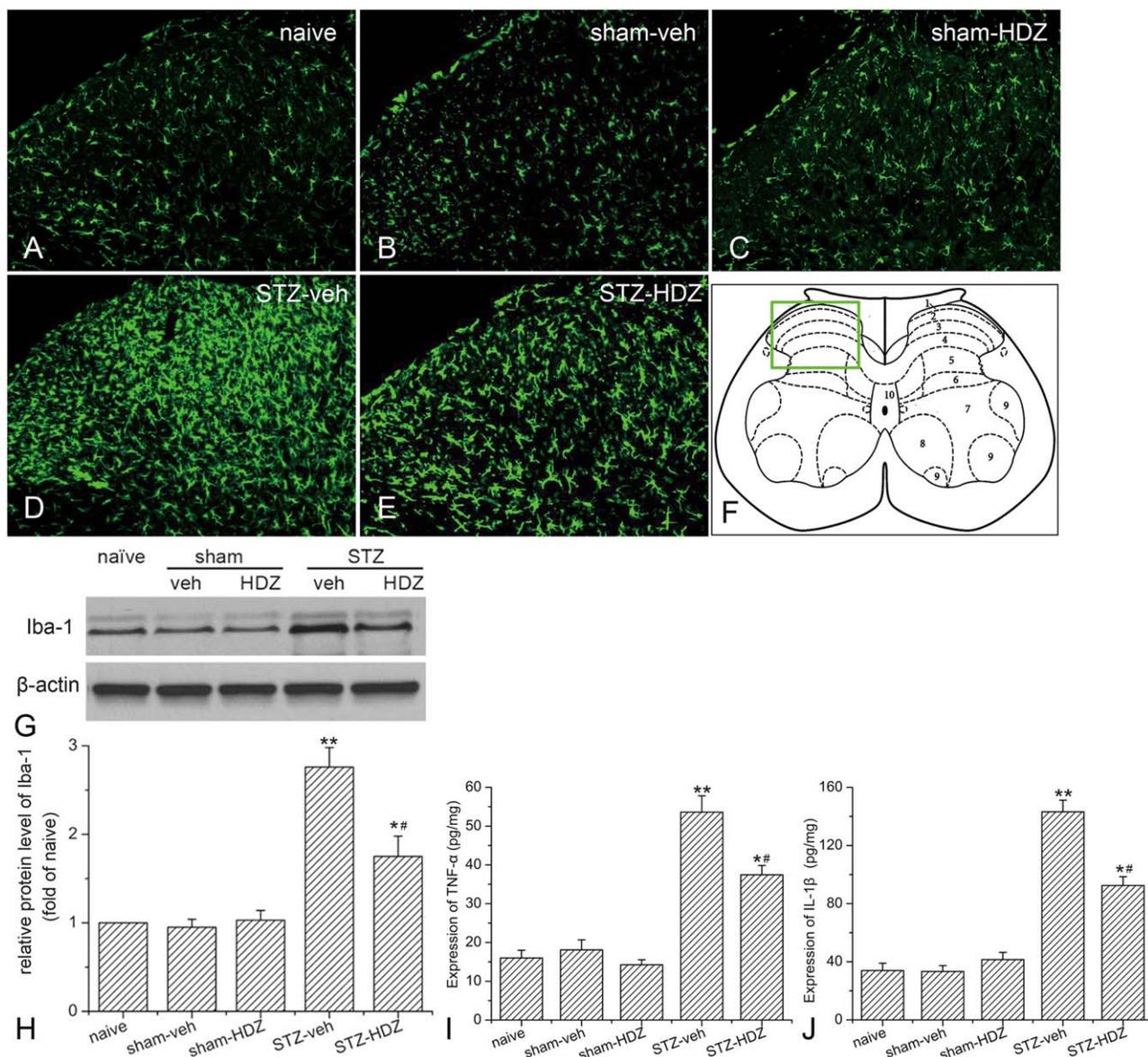


Fig. 4. Hydralazine-ameliorated neuroinflammation in the spinal dorsal horn of diabetic rats. Immunohistochemistry showed that streptozotocin (STZ) induced a significant microglial activation in the rat spinal dorsal horn (D) in comparison to naive (A) and vehicle treated rats (sham-veh; B). Hydralazine did not alter microglial density of sham rats (sham-HDZ; C) but effectively decreased STZ-induced microglial activation (STZ-HDZ; E). (F) shows the scheme presenting an overview over the detected region. Western blot data are expressed as fold change of the naive group. STZ induced a significant increase of microglial marker Iba-1 expression in the spinal dorsal horn, which was significantly inhibited by hydralazine (G, H). ELISA data showed that IL-1 β (I) and TNF- α (J) were both robustly increased in the diabetic spinal cord. Hydralazine effectively attenuated the expression of these inflammatory mediators. Scale bar = 100 μ m in (E) (applied in A-E). *, **: $P < 0.05$ or 0.01 versus naive, #: $P < 0.05$ versus STZ-vehicle (veh) group, respectively.

2011). To our knowledge, this study is the first demonstration that hydralazine has the acrolein-scavenging effect in diabetes and inhibited DNP. We observed that persistent administration of 5 mg/kg hydralazine could effectively inhibit DNP. This dose has been chosen in accordance to previous reports and would not affect the blood pressure (Hamann and Shi, 2009; Due et al., 2014). We have also observed in this study that hydralazine has no effect on baseline pain response in sham rats, indicating that this drug does not influence

nociceptive sensitivity in normal rats. Long-term treatment with hydralazine brings a concern about the side effect. The potential side effects of intraperitoneal hydralazine (0.1–10 mg/kg) treatment on locomotion, anxiety/depression, and motor coordination were tested in previous studies (Bai et al., 2012). Hydralazine has no effect on locomotion or motor coordination, but could alleviate pain-related anxiety-like behaviors.

Taken together, our data provide some evidence of the active participation of acrolein in the pathogenesis of

DNP. The antiallodynic effect of hydralazine on DNP would make it serve as a therapeutic option for pain related to diabetes.

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