Research report

High-mobility group box 1 contributes to mechanical allodynia and spinal astrocytic activation in a mouse model of type 2 diabetes

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A B S T R A C T

Chronic pain is one of the most common complications of diabetes. However, current treatments for diabetic pain are usually unrealistic because the underlying mechanisms are far from being clear. Immersing studies have implicated immune factors as key players in the diabetic pain. High-mobility group box 1 (HMGB1) is an important mediator of inflammatory response, but its role in diabetic pain is unclear. In the present study, we observed that db/db mice (a model of type 2 diabetes) developed persistent mechanical allodynia from postnatal 2 months. Western blot showed that in postnatal 2–5 months, HMGB1 was significantly higher than that of the heterozygous littermates (db+/+) mice. Intrathecal injection of a HMGB1 neutralizing antibody (anti-HMGB1) inhibited mechanical allodynia. Immunostaining data showed that compared with db/+ and C57 mice (postnatal 4 months), glial fibrillary acidic protein (GFAP) staining was significantly increased in the spinal cord of db/db mice. Anti-HMGB1 could effectively decrease GFAP expression. Real-time PCR showed that in postnatal 4 months, db/db mice induced significant increases of TNF-alpha, IL-1β, IL-6 and monocyte chemoattractant protein-1 (MCP-1) in the spinal dorsal horn, while anti-HMGB1 (50 µg) effectively inhibited the up-regulation of these inflammatory mediators. Our results indicate that HMGB1 is significantly up-regulated in the spinal cord of type 2 diabetes, and inhibiting HMGB1 may provide a novel treatment for diabetic pain.

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1. Introduction

Chronic pain is one of the most common chronic complications of the type 2 diabetes [2,7]. Diabetic pain is a main course of decreased life quality in patients of diabetes. However, because of the poor understanding of the underlying mechanisms, current treatments for diabetic pain are usually unrealistic [2]. Like other types of chronic pain, diabetic pain has been described simply as a course of increased neuronal activities [3]. But immersing studies have implicated immune factors as key players in the induction and maintenance of chronic pain [9,27]. We previously observed that in a mouse model of type 2 diabetes (db/db mouse), spinal IL-1β expression was significantly increased and astrocytes were obviously activated in db/db mice compared to normal mice. Intrathecal injection of an astrocytic toxin, cytokine inhibitor or interleukin-1 receptor antagonist could each significantly attenuate the allodynia [17]. These data indicate that neuroinflammation in the spinal dorsal horn may play an important role in diabetic pain. High-mobility group box 1 (HMGB1), a nonhistone DNA-binding molecule, was first discovered as a nuclear protein with rapid electrophoretic migration. However, HMGB1 is now implicated as an important mediator in inflammatory process [18]. In many disorders in the central nervous system, including brain ischemia, spinal cord injury and amyotrophic lateral sclerosis [1,15], HMGB1 has been found to play essential roles. In addition, it was reported that intrathecal administration of HMGB1 produced mechanical allodynia [5]. Very interestingly, recent studies suggest that HMGB1 plays an active role in bone cancer pain and peripheral nerve injury-induced neuropathic pain [22,25]. However, it is still unknown whether HMGB1 is involved in diabetic pain.

In the present study, based on the mouse model of type 2 diabetes (db/db mouse) we previously used [17], we firstly observed the time course of HMGB1 expression in the spinal dorsal horn. To further confirm the role of HMGB1 in diabetic pain, a
neutralizing antibody against HMGB1 was intrathecally injected into the diabetic mice and the behavioral consequences were observed. Furthermore, we detected the astrocytic activation and expression of inflammatory mediators in the spinal cord of diabetic mice after inhibiting HMGB1 expression.

2. Materials and methods

2.1. Animals

All experimental procedures received prior approval from the Animal Use and Care Committee for Research and Education of the Fourth Military Medical University (Xi’an, PR China) and also the ethical guidelines to investigate experimental pain in conscious animals [30]. All efforts were made to minimize animals’ suffering and to reduce the number of animals used. Homozygous diabetic mice (db/db) (used as a model of type 2 diabetes) and heterozygous littermates (db/+j) of C57BLKS strain (nondiabetic control) were purchased from Jackson Laboratory (stock number 000662; Bar Harbor, Maine, USA). The characterization (blood glucose and body weight) of the 2 diabetic mice was described in our previous study [17]. Age-matched C57BL6 mice were obtained from the Fourth Military Medical University (Xi’an, PR China). Mice were housed in plastic cages, three in each cage, and maintained on a 12:12 h light/dark cycle under conditions of 22–25 °C ambient temperature with food and water available.

2.2. Intrathecal injection

According to a previous report [13], we performed spinal cord puncture between L5 and L6 vertebrae with 10 μl Gastig® syringe (Hamilton, USA) and a BD Precisionglide® 30G (1/2) in. needle (Becton Dickinson, USA) under sevoflurane anesthesia. Immediately after the needle entry into subarachnoid space, a brisk tail flick could be observed. Polyclonal neutralizing antibodies against HMGB1 B box were raised in rabbits, and were affinity-purified using cyanogen bromide–activated Sepharose beads following standard procedures. Single injection of 3 μl of anti-HMGB1 (1, 10 and 50 μg; n = 6 each) or nonimmunized rabbit IgG (Sigma, St. Louis, MO, USA; n = 6, vehicle control) was performed in the end of postnatal 4 months.

2.3. Behavioral testing

Diabetic pain in mice was described as mechanical allodynia, which was evaluated by paw withdrawal frequencies (PWMs) to repeated mechanical stimuli. The procedures were described previously [29]. Mouse was placed in a Plexiglas chamber on an elevated mesh screen. Two calibrated von Frey monofilaments (0.24 and 4.33 mN; Stoelting Co., Wood Dale, IL, USA) were employed. Each filament was applied to the hind paw for approximately 1 s, and each trial was repeated 10 times to both hind paws. The data were expressed as a percentage of response frequency [(number of paw withdrawals/10 trials) × 100 % response frequency], and this percentage was used as an indication of the amount of paw withdrawal. Behavioral testing was performed in a double-blind manner by those who were blind to the animals grouping. In the time-course study, PWM was performed at postnatal 1–5 months. To evaluate the effect of the HMGB1 antibody on pain behavior, PWM was performed at 1 h, 2 h, 4 h, 8 h and 1 d after intrathecal injection.

2.4. Western blot

Mice were sacrificed and spinal cord was rapidly collected and put in liquid nitrogen. The L5 spinal cord segment was dissected on dry ice according to the termination of the L4 and L5 dorsal roots. The segment was split into the dorsal and ventral horns at the level of the central canal. The tissues were homogenized in chilled lysis buffer (50 mM Tris, 1 mM phenylmethylsulfonyl Fluoride, 1 mM EDTA, 1 μM leupeptin). The crude homogenate was centrifuged at 4 °C for 15 min at 1000 × g. The supernatant was collected and the protein concentration was measured. The electrophoresis samples were heated at 100 °C for 5 min and loaded onto 10% SDS–polyacrylamide gels with standard Laemmli solutions (Bio-Rad Laboratories, CA, USA). The proteins were electroblotted onto a polyvinylidene difluoride membrane (PVDF, Millipore, Billerica, MA, USA). The membranes were placed in a blocking solution containing Tris-buffered saline with 0.02% Tween (TBS-T) and 5% non-fat dry milk, for 1 h, and incubated overnight under gentle agitation with primary antibodies: rabbit anti-HMGB1 (1:1000; BD Pharmingen, USA); goat anti-galactosidase antibody (1:10,000; Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). All reactions were detected by the enhanced chemiluminescence (ECL) detection method (Amer- sham). The densities of protein blots were analyzed with Image G software. The densities of target proteins and GAPDH immunoreactive bands were quantified by background subtraction. The same size of square was drawn around each band to measure the density. Target protein levels were normalized against GAPDH levels and expressed as relative fold changes compared to the C57 control group.

2.5. Immunohistochemistry

Mice were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) through the ascending aorta after deep anesthesia by pentobarbital (60 mg/kg, i.p.). The L5 spinal cords were collected, post-fixed in the same fixative solution for 2 h, cryoprotected for 24 h at 4 °C in 0.1 M phosphate buffer containing 30% sucrose and frozen-sectioned at 10 μm. After being blocked in 0.01 M phosphate-buffered saline containing 0.3% Triton X-100 and 10% goat serum for 1 h at room temperature, the sections were incubated with primary mouse anti-GAPDH (1:2000; Millipore) overnight at 4 °C and then with FITC-conjugated horse anti-mouse IgG (1:200; Vect- tor, Burlington, CA, USA) for 1 h at room temperature. Images were obtained using a confocal laser microscope (FV1000; Olympus, Tokyo, Japan) and digital images were captured with Fluoview 1000 (Olympus). Five nonadjacent sections from the L5 segments were selected randomly. Images were evaluated by a computer-assisted image analysis program (MetaMorph 6.1). To measure the area of GFP* immunopositive somata, we used the Threshold Image function in Measure of MetaMorph 6.1 to set the low and high thresholds for the immunofluorescent intensity which was determined to be a signal. Our image data were collected using the same region and the same size of field within same lamina to avoid any variance and difference in staining between laminae. A standardized field area was sampled arbitrarily from regions within randomly selected dorsal horn sections. Then the immunoreactivi- ties for GFP within the superficial dorsal horn were averaged across the five spinal sections for each experimental group.

2.6. Real-time reverse transcription polymerase chain reaction (RT-PCR)

After deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.), mice were sacrificed. L5 spinal dorsal horn was rapidly harvested and total RNA was extracted with Trizol (GIBCO/BRL Life Technologies Inc., Grand Island, NY, USA). Complementary DNA (cDNA) was synthesized with oligo (dT)12–18 using Superscript™ III Reverse Transcriptase for RT-PCR (Invitrogen, Carlsbad, CA, USA). The primers used were presented in Table 1. Equal amounts of RNA (1 μg) were used to prepare cDNA using the SYBR® Premix Ex Taq™ (Takara, Tokyo, Japan) and analyzed by real-time PCR in a detection system (Applied Biosystems, Foster City, CA, USA). The amplification protocol was: 3 min at 95 °C, followed by 42 cycles of 10 s at 95 °C for denaturation and 45 s at 60 °C for annealing and extension. Target cDNA quantities were estimated from the threshold amplification cycle number (Ct) using Sequence Detection System software (Applied Biosystems). Data were analyzed by those who

Table 1
Primer sequence for the rat genes characterized in this experiment.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
<th>Sequences</th>
<th>Accession number</th>
</tr>
</thead>
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<tr>
<td>TNF-alpha</td>
<td>Forward Primer</td>
<td>5′-TGTGCTGCCCACCAAGAGA-3′</td>
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</tr>
<tr>
<td></td>
<td>Reverse Primer</td>
<td>5′-TGGTGCTGGTGGTGCGTCA-3′</td>
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<tr>
<td>IL-4</td>
<td>Forward Primer</td>
<td>5′-TGCTGATGACCTGGGG-3′</td>
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<tr>
<td></td>
<td>Reverse Primer</td>
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<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>Forward Primer</td>
<td>5′-CCCCCTGAGAAGCAGTATG-3′</td>
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</tr>
<tr>
<td></td>
<td>Reverse Primer</td>
<td>5′-CAAAGTGTCCAGGCGACAC-3′</td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
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<td></td>
<td>Reverse Primer</td>
<td>5′-TACCCGCTAGGCTTCTGTT-3′</td>
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were blind with respect to group assignment. A ΔCt value was calculated for each sample by subtracting their Ct value from the Ct value for the corresponding GAPDH to normalize the differences in cDNA aliquots. Each cDNA quantity was then calculated with the following formula: $2^{-\Delta\Delta Ct}$. GAPDH was served as an internal standard control for variations in RT-PCR efficiency. In each experiment, PCRs were done in triplicate and all experiments were repeated twice.

2.7. Statistical analysis

All data were collected by experimenters blinded to the surgery and reagents and statistical analyses were done by using SPSS® version 16.0 software (SPSS Inc, Chicago, IL, USA). Data were expressed as mean ± standard error mean (mean ± SEM). Two-way analysis of variance (ANOVA) (Group × time) was used for analysis of behavioral data as well as morphological analysis. Dunnett’s C post hoc test, assuming the variances were not equal, was employed whenever appropriate and significance was set at 0.05 for all statistical tests.

3. Results

3.1. Mechanical allodynia in the type 2 diabetic mice

In our previous study, we reported that compared with db/+ and C57 mice, the body weight and blood glucose of db/db mice was significantly higher throughout the period tested, suggesting that db/db mice developed diabetic features [17]. Behavioral study showed that from postnatal 2 months, the PWF of db/db mice was significantly increased, compared with db/+ and C57 mice (P < 0.05; Fig. 1), suggesting that diabetic mice significantly developed mechanical allodynia. Then the mechanical allodynia continued thereafter throughout the period tested. Db/+ and C57 mice did not display mechanical pain hypersensitivity at any observed time points. In a recent study [21], locomotor function of the db/db mice was evaluated and these mice did not exhibit gross motor coordination defects, which confirmed that the performance in pain behavior tests was not caused by the locomotor functions.

3.2. HMGB1 is increased in the spinal dorsal horn of diabetic mice and contributes to diabetic pain

To explore the potential roles of HMGB1 in diabetic pain, we examined HMGB1 expression in different postnatal time points with Western blot. HMGB1 expression in the C57 and db/+ mice was not significantly different at any observed postnatal time points (data not shown). Spinal expression of HMGB1 in diabetic mice was expressed as fold changes compared with that of C57 mice at postnatal 5 months. The protein level of HMGB1 was not increased until postnatal 2 months and gradually up-regulated till the end of the test. In postnatal 2–5 months, HMGB1 was 2.33 ± 0.42 folds, 2.41 ± 0.35 folds, 2.87 ± 0.42 folds, and 3.13 ± 0.64 folds of C57 mice, which were significantly higher than that of the C57 and db/+ mice (Fig. 1C, P < 0.05).

In order to further confirm the involvement of HMGB1 in diabetic pain, we injected a neutralizing antibody of HMGB1 (anti-HMGB1) and observed the behavioral consequences. Treatment with anti-HMGB1 significantly reversed mechanical allodynia induced by diabetes. Intrathecally administering 1 μg of anti-HMGB1 could decrease PWF slightly, but there were no statistical differences compared to that of vehicle group at any time points (Fig. 2A, B, P > 0.05). However, 10 μg of anti-HMGB1 could down-regulate PWF significantly and this anti-allodynic effect lasted to 1 d after administration and 50 μg of anti-HMGB1 produced a more significant effect (Fig. 2A, B, P < 0.05). To evaluate the efficacy of neutralizing antibody, at 4 h after administration, rats were sacrificed and Western blot analysis was performed to evaluate the expression of HMGB1. Injection of 1 μg of anti-HMGB1 could inhibit HMGB1 slightly, but not significantly compared to that of vehicle group. However, 10 μg or 50 μg of anti-HMGB1 significantly blocked the expression of HMGB1. These results indicated that anti-HMGB1 treatment showed an effective and reliable anti-allodynic effect in a dose-dependent manner and this effect was through inhibiting HMGB1 expression.
induction of a number of inflammatory mediators in astrocytes [20]. In the present study, we tried to demonstrate whether up-regulated HMGB1 contributed to astrocytic activation and mechanical allodynia. Immunohistochemistry indicated that compared with db/+ and C57 mice (postnatal 4 months), GFAP staining was significantly increased in the spinal cord of db/db mice at the same postnatal period. Intrathecally administering 1 µg of anti-HMGB1 could decrease GFAP expression slightly, although not significantly (Fig. 3A–G, P > 0.05). However, 10 µg and 50 µg of anti-HMGB1 could inhibit GFAP immunodensity significantly (Fig. 3A–G, P < 0.05). We checked the expression of GFAP at different time following anti-HMGB1 (50 µg) injection by Western blot analysis. The intensity of GFAP was slightly but not significantly decreased at 1 h but was remarkably down-regulated at 2–8 h. However, GFAP level returned to normal level at 1 d after anti-HMGB1 (50 µg) injection (Fig. 3H, P > 0.05). These results showed that anti–HMGB1 treatment could inhibit astrocytic activation in the diabetic mice.

Astrocytic activation in diabetic mice is likely to enhance gene transcription and consequently to modulate synthesis of inflammatory mediators such as IL-1β, TNF-alpha, monocyte chemotactic protein (MCP-1), IL-6, which positively contribute to pain sensitization [8]. With real-time RT-PCR, we observed that in postnatal 4 months, db/db but not db/+ or C57 mice induced significant increases of TNF-alpha (2.16 ± 0.25 folds of C57, P < 0.05), IL-1β (3.28 ± 0.21 folds of C57, P < 0.05), IL-6 (2.23 ± 0.11 folds of C57, P < 0.05) and MCP-1 (1.83 ± 0.21 folds of C57, P < 0.05) in the spinal dorsal horn (Fig. 4A). Treatments with HMGB1 antibody (50 µg) but not vehicle control, significant inhibited TNF-alpha (1.56 ± 0.14 folds of C57, P < 0.05), IL-1β (2.18 ± 0.15 folds of C57, P < 0.05), IL-6 (1.33 ± 0.18 folds of C57, P < 0.05) and MCP-1 (1.37 ± 0.22 folds of C57, P < 0.05). We also examined the mRNA levels of these inflammatory mediators at different time intervals following anti-HMGB1 (50 µg) injection. TNF-alpha was significantly lower than the baseline value throughout the observing period (1 h to 1 d), while IL-1β was not obviously lower at 1 h. IL-6 and MCP-1 expressions were only remarkably decreased from 2 h to 8 h after anti-HMGB1 (50 µg) injection (Fig. 4B). These results suggested that spinal HMGB1 up-regulation in diabetic mice contributed to synthesis of inflammatory mediators.

4. Discussion

In the present study, we provide evidence of the novel contribution of spinal HMGB1 in diabetic pain. We observed that HMGB1 is significantly increased in the spinal dorsal horn of type 2 diabetic mice and HMGB1 expression correlates with mechanical allodynia. In addition, blocking HMGB1 could reverse diabetic pain with decreased astrocytic activation and down-regulated expression of inflammatory mediators.

Cumulating data suggest that HMGB1 is not only a nuclear protein, but also an important mediator in inflammatory response. After stimulation, HMGB1 can be released into the extracellular space either actively or passively. Recent studies suggest that in conditions such as oxidative stress, HMGB1 could be released [24]. In diabetes, hyperglycemia was reported to not only directly induce oxidative stress [23], but also contribute to increased aldose reductase activity, nerve hypoxia/ischemia, activation of protein kinase C, and insulin-like growth factor deficiency [6]. These pathological pathways all in turn converge in producing dramatic oxidative stress [10]. Thus we hypothesize that hyperglycemia-induced oxidative stress may be a key mechanism for the initial increase of HMGB1.

Released HMGB1 binds to its receptors, including the receptor for advanced glycation end products (RAGE), Toll-like receptor2 (TLR2), and TLR4, which are highly expressed on immune

3.3. HMGB1 contributes to spinal astrocytic activation and inflammatory response in diabetic mice

We previously observed that astrocytic activation contributed to mechanical allodynia in the db/db mouse [17] and an in vitro study suggested that HMGB1 could induce transcriptional/translational...
cells [19]. Receptor activation leads to the consequent release of cytokines/chemokines, including TNF-alpha, IL-1, IL-6, and MCP-1 [18,28]. In the present study, we also observed that inhibiting HMGB1 expression with the specific neutralizing antibody could significantly block the expression of these cytokines/chemokines. Previous studies have confirmed the positive contribution of these cytokines/chemokines to chronic pain. For example, it was previously reported that through binding with its receptor on the neurons, IL-1β increases calcium conductivity of neuronal NMDA receptor, possibly via intracellular signaling pathways leading to the phosphorylation of NMDA receptor NR1 subunit [12]. Activation of NMDA receptor in the spinal dorsal horn plays a pivotal role in central sensitization and thus chronic pain [16]. In addition to triggering release of proinflammatory cytokines in spinal dorsal horn, HMGB1 could also be released after cytokines stimulation [22]. So there is a positive feedback circuit between HMGB1 and proinflammatory cytokines. This positive feedback circuit enlarges the effect of HMGB1 on diabetic pain and makes it more difficult to be treated by clinical therapy.

In the present study, we also observed a remarkable astrocytic activation in diabetic mice. Interestingly, inhibiting HMGB1 could significantly block astrocytic activation. As we have discussed above, HMGB1 could binds to TLR2 and TLR4, which are also expressed on spinal astrocytes. We thus assume that HMGB1 may activate astrocytes through these two TLRs. Great deals of studies have been performed targeting TLRs as critical mediators of chronic pain [11]. It has been reported that after nerve injury, TLR2 is required for proinflammatory cytokines expression and pain hypersensitivity [14] and up-regulated TLR4 in the spinal cord are required for glial activation and pain response [4]. Another important receptor of HMGB1, RAGE, has not been reported to be expressed on spinal astrocytes. However, a recent study showed that HMGB1 is expressed on GFAP-positive satellite cells in dorsal root ganglia [15]. Previous studies have reported that RAGE is significantly increased in diabetes [26]. So RAGE may also mediate the pronociceptive effect of HMGB1 in diabetic pain.

In summary, we observed that type 2 diabetes induced a significant increase of HMGB1, which could activate spinal astrocytes and expression of inflammatory cytokines and chemokines, which could modulate NMDA-mediated synaptic transmission and pain response. In addition, cytokines and/or chemokines further increase release of HMGB1 in the spinal cord, and enhance nociceptive transmission in a manner of positive feedback. Our data thus indicate that HMGB1 may play an important role in diabetic pain and inhibiting HMGB1 expression may provide a novel treatment for diabetic pain.

Fig. 3. Diabetes-induced astrocytic activation in the spinal dorsal horn is reversed by intrathecal anti-HMGB1 (Ab) treatment. (A–C) Compared to the C57 (postnatal 4 months) or db/+ (postnatal 4 months) mice, expression of glial fibrillary acidic protein (GFAP) in the spinal dorsal horn are significantly higher at the same postnatal period. Enhanced staining appeared in all areas of the spinal grey matter, with the most prominent increase in superficial dorsal horn laminae I-III. Diabetes-induced up-regulation of GFAP is significantly attenuated by the anti-HMGB1 (Ab) treatment (D–F), compared with the vehicle control. (G) Statistical results of the immunostaining data. *P<0.05 vs vehicle control group; #P<0.05 vs C57 group. (H) The expression of GFAP at different time points following anti-HMGB1 (10 μg) injection indicated by Western blot analysis. *P<0.05 vs control value (before injection).
**Fig. 4.** Effects of anti-HMGB1 (Ab) treatment on diabetes-induced cytokines/chemokine expression revealed by real-time RT-PCR. Data are normalized against GAPDH levels and expressed as relative fold changes compared to the C57 group. (A) Compare with C57 (postnatal 4 months) and db/db (postnatal 4 months) mice, expression of TNF-alpha, IL-1β, IL-6, and monoocyte chemotactic protein (MCP-1) in db/db mice is significantly higher at the same postnatal period. **P** < 0.05 vs C57 group; #P < 0.05 vs vehicle control group. (B) The expression of cytokines/chemokine at different time points following anti-HMGB1 (10 µg) injection. **P** > 0.05 vs control value (before injection).

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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