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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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ABSTRACT

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. Immerging 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.

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 immerging 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

Abbreviations: DRG, dorsal root ganglion; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; HMGB1, high-mobility group box 1; MCP-1, monocyte chemoattractant protein-1; PWF, paw withdrawal frequency; RT-PCR, reverse transcription polymerase chain reaction; TLR, toll like receptor.

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

Genes	Primers	Sequences	Accession number
TNF-alpha	Forward primer Reverse primer	5′-TGATCGGTCCCAACAAGG A-3′ 5′-TGCTTG GTG GTTTGCTACGA-3′	AY427675
IL-1β	Forward primer Reverse primer	5′-TGCTGATGTACCAGTTGGGG-3′ 5′-CTCCATGAGCTTTGTACAAG-3′	NM031512
IL-6	Forward primer Reverse primer	5′-GCCCTTCAGGAACAGCTATG-3′ 5′-CAGAATTGCCATTGCACAAC-3′	NM012589
MCP-1	Forward primer Reverse primer	5′-CAGATCTCTCTTCCTCCACCACTAT-3' 5′-CAGGCAGCAACTGTGAACAAC-3′	M57441
GAPDH	Forward primer Reverse primer	5′-CCCCCAATGTATCCGTTGTG-3′ 5′-TAGCCCAGGATGCCCTTTAGT-3′	NM01008

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/+*) 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 Gastight® syringe (Hamilton, USA) and a BD Precisionglide® 30G (1/2) in. needle (Becton Dickinson, USA) under sevofluorane 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 nonimmuned 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 (PWFs) 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, PWF was performed at postnatal 1–5 months. To evaluate the effect of the HMGB1 antibody on pain behavior, PWF 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), mouse anti-glial fibrillary acidic protein (GFAP; 1:2000; Millipore) or goat anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1: 1000; Santa-Cruz Biotechnology, Santa Cruz, CA, USA). Bound primary antibodies were detected with the anti-rabbit, anti-mouse or anti-goat horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000; Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). All reactions were detected by the enhanced chemiluminescence (ECL) detection method (Amersham). The densities of protein blots were analyzed with Image G software. The densities of target proteins and GAPDH immunoreactive bands were quantified with 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-GFAP (1:2000; Millipore) overnight at 4 °C and then with FITC-conjugated horse anti-mouse IgG (1:200; Vector, Burlingame, 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 GFAP 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 immunoreactivities for GFAP 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