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Activation of spinal $GABA_B$ receptors normalizes N-methyl-D-aspartate receptor in diabetic neuropathy

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ABSTRACT

N-methyl-D-aspartate receptor (NMDAR) activity is increased, while $GABA_B$ receptor is downregulated in the spinal cord dorsal horn in diabetic neuropathy. In this study, we determined the interaction of NMDARs and $GABA_B$ receptors in streptozotocin (STZ)-induced diabetic neuropathy. The paw withdrawal threshold (PWT) was significantly lower in STZ-treated rats than in vehicle-treated rats. Intrathecal injection of baclofen, a $GABA_B$ receptor agonist, significantly increased the PWT in STZ-treated rats, an effect that was abolished by pre-administration of the $GABA_B$ receptor specific antagonist CGP55845. Spinal NR2B, an NMDA receptor subunit, protein and mRNA expression levels were significantly higher in STZ-treated rats than in vehicle-treated rats. Intrathecal baclofen significantly reduced the NR2B protein and mRNA expression levels in STZ-treated rats. Intrathecal administration of CGP55845 eliminated baclofen-induced reduction of NR2B protein and mRNA levels in STZ-treated rats. In addition, the phosphorylated cAMP response element-binding (CREB) protein level was significantly higher in the spinal cord dorsal horn in STZ-treated rats compared with vehicle-treated rats. Intrathecal injection of baclofen significantly decreased phosphorylated CREB protein level in STZ-treated rats; an effect was blocked by CGP55845. These data suggest that activation of $GABA_B$ receptors in the spinal cord dorsal horn normalizes NMDAR expression level in diabetic neuropathic pain.

1. Introduction

Hyperalgesia or allodynia are two major complains in patients with diabetic neuropathy [1,2]. The spinal cord dorsal horn is importantly involved in the perception of nociceptive signals in diabetic neuropathy [3]. Increased primary afferents may lead to neuroplasticity in the spinal cord and contribute to the hyperactivity of dorsal horn neurons in the spinal cord (central sensitization) in diabetic neuropathic pain [4–7]. Although extensive studies have been performed to determine the signaling mechanisms underlying hyperactivity of spinal cord dorsal horn neuron in diabetic neuropathy, the precise mechanism is not clear.

N-methyl-D-aspartate receptors (NMDARs) are heteromeric protein complexes consisting of 3 subunits: NR1, NR2 and NR3. NR2A and NR2B are the predominant subunits in the spinal cord and the distribution of NR2B subunits is restricted to the superficial dorsal horn of the spinal cord [8,9]. It has been shown that activation of spinal NMDAR activity contributes to hyperalgesia in neuropathic pain condition. For example, application of NMDA induces a greater increase in the calcium influx in spinal lamina II neurons in nerve-ligated rats than in control rats [10]. Consistently, NMDAR antagonists ketamine, memantine or MK-801 potently reduce evoked responses of dorsal horn neurons in spinal nerve-ligated rats [11]. Furthermore, the NR2B subunit-specific antagonist ifenprodil reduces the amplitude of NMDAR currents in nerve-ligated mice [12]. Although, diabetic neuropathy is associated with increased glutamate release from primary afferent terminals in the spinal cord [13,14], an increase in spinal NMDAR activity in diabetic neuropathy has not yet been specifically documented. Previous studies have shown that phosphorylation of cAMP response element-binding protein (CREB), a transcription factor, is increased in the spinal cord in pain sensation including neuropathic [15], inflammatory [16], and diabetic neuropathy [17]. Also, NMDAR function is regulated by CREB signaling [18,19].

$GABA_B$ receptors are densely expressed in the spinal cord dorsal horn [20,21]. Activation of spinal $GABA_B$ receptors produces antinociception in both acute and chronic pain [22,23]. Furthermore, intrathecal administration of baclofen has been used as an adjuvant analgesic to treat patients with neuropathic pain [24]. It has been shown that activation of presynaptic $GABA_B$ receptors decreases synaptic GABA, glycine, and glutamate release in the spinal dorsal

Abbreviations: NMDA, N-methyl-D-aspartate; NMDAR, N-methyl-D-aspartate receptor; PWT, paw withdrawal threshold; STZ, streptozotocin; CREB, cAMP response element-binding; p-CREB, phosphorylated CREB.

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horn [25–27]. In addition to inhibiting presynaptic neurotransmitter release, activation of postsynaptic $GABA_B$ receptors suppresses Ca^{2+} permeability of NMDARs to decrease Ca^{2+} signals in postsynaptic spines [28]. The reduction of NMDAR activity may contribute to antinociception induced by baclofen. On the basis of previous findings, we hypothesize that activation of GABAB receptor dowregulates NMDARs in diabetic neuropathy. In this study, we also studied the possible signaling pathways involved in the downregulation of NMDARs by activation of $GABA_B$ receptors.

2. Materials and methods

2.1. Diabetic neuropathic pain and behavioral test

Male Sprague–Dawley rats (6–8 weeks old, 200–300 g) were used in this study according to the guidelines with the Animal Care and Use Committee of the Hebei Medical University. All efforts were made to minimize the suffering and number of rats used. Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ, 50 mg/kg, Sigma Chemical, St. Louis, MO, USA) freshly dissolved in 0.9% sterile saline [6]. After STZ injection, rats developed persistent mechanical allodynia and hyperalgesia within 1 week and the hyperalgesia reached to maximum 2 weeks after STZ injection [5,29]. We started to perform all the tests and measurements at least 3 weeks after STZ injection. Tactile allodynia was assessed using a series of calibrated von Frey filaments (Stoelting Co., Wood Dale, IL, USA) applied perpendicularly to the plantar surface of both hindpaws with sufficient force to bend the filament for 6 s. Brisk withdrawal or paw flinching was considered to be a positive response. In the absence of a response, the filament of the next greater force was applied. Following a response, the filament of the next lower force was applied. The tactile stimulus producing a 50% likelihood of withdrawal response was calculated by using the 'up–down' method [5,7].

2.2. Implantation of intrathecal catheter and administration of drugs

Intrathecal catheters were inserted in diabetic and age-matched normal rats anesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg). One end of polyethylene-10 tubing was inserted intrathecally through an incision in the cisternal membrane and advanced 7–9 cm caudal until the tip of the catheter was positioned at the lumbar spinal level (L5 to L6). The other end of the intrathecal catheter was sutured to the musculature and skin at the incision site and externalized to the back of the rat [30,31]. The rats were allowed to recover for 5–7 days following the procedure. Only animals with no evidence of neurological deficits after catheter implantation were used for this study. Intrathecal injections of vehicle and drugs (such as baclofen and CGP55845 or a combination of baclofen and CGP55845) were performed at least 3 weeks after STZ injection. These injections were performed once a day for 4 consecutive days. For rats administered a combination of drugs, the interval between each injection was 15 min. The PWT value was determined 30 min after each intrathecal injection of drugs. The spinal cord dorsal horn tissues were obtained from each group of rats after measuring PWT in the 4th day administration of drugs for Western and PCR analysis, as described below.

Drugs in a volume of 10 μl were injected via this catheter, followed by 10 μl of saline for flushing. R(+)-baclofen hydrochloride and STZ were purchased from Sigma Aldrich and dissolved in saline in the designated concentration. CGP55845 was obtained from Tocris Bioscience and dissolved in DMSO (0.05% in saline).

2.3. Western blot analysis

With the rats under deep anesthesia by intraperitoneal injection of pentobarbital (60 mg/kg), the spinal cord lumbar enlargement was removed and the tissue was homogenized in Radio Immuno Precipitation Assay (RIPA) lysis buffer containing F-proteinase. An equal amount of protein (60 μg) was loaded into each lane, separated electrophoretically by 10% sodium dodecyl sulfate polyacrylamide gel electrophores and electroblotted onto polyvinylidene difluoride (PVDF) membranes. After being blocked in 5% nonfat dry milk for 1 h, the membranes were incubated with anti-NR2B and anti-p-CREB (Santa Cruz, 1:500 dilution), anti-NR2B (Abcam, 1:150) and anti-β-actin (Santa Cruz, 1:200 dilution) at 4 °C overnight. The membrane was rinsed and incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody at 1:4000 dilution for 3 h at 37 °C. The membrane was developed using an enhanced chemiluminescence kit (Bio-RAD, Hercules, CA) according to the manufacturer's instructions. The intensity of the bands was captured digitally and analyzed quantitatively by a Kodak EDAS120 system and Quantity One 4.6 software and the integrated optical density of the bands was normalized to background values. Whole-brain lysates were used as a positive control in each immunoblotting experiment.

2.4. Real-time RT-PCR

Total RNA was extracted from the spinal cord dorsal horn tissues using TRIzol (Invitrogen Technology). Then 5 μg of total RNA extraction was reverse-transcribed to synthesized cDNA. The primer sequences for each PCR were: GADPH (496 bp): Forward: 5′-GATGGGTGTGAACCAC GAGAAAT-3′; reverse: 5′-ACGGATACATTGGGGGTAGGAA-3′; NR2B (475 bp): Forward: 5′-AGGAAGCCACCTACATTTTTGA-3′; reverse: 5′-CGAGGCCACACATAATACTTCA-3′.

Reaction conditions followed the manufacturer's protocol with optimization of primers, $MgCl_2$ concentrations, and annealing temperature. The PCR program included an initial denaturation step at 95 °C for 5 min, 31 cycles of 30 s at 94 °C, 30 s at 58 °C, and 5 min at 72 °C for extension. Then, PCR products were separated by 1% agarose gel electrophoresis and visualized with ethidium bromide. Gel images were acquired by a Kodak EDAS120 system and Quantity One 4.6 software.

2.5. Data analysis

All the data are presented as means ± SEM. The PWT values in each group of rats were compared by using two-way ANOVA with Bonferroni's post hoc test. The band density values in Western blot and PCR were compared by using un-paired t-test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect of intrathecal baclofen on PWT in diabetic rats

Rats that received STZ injection developed a robust reduction of PWT 3 weeks after the injection of STZ [14,32]. We did not include rats that did not show a reduction of PWT in test with tactile stimuli after STZ treatment. The PWT value (3.25 ± 0.53 g) in STZ-treated rats was significantly lower than the value (11.3 ± 2.0 g, n = 36) in vehicle-treated rats (n = 12, Fig. 1). Intrathecal injection of baclofen (0.5 μg, 10 μl) in STZ-treated rats increased PWT value (9.86 ± 3.21 g, n = 12) to the level in vehicle-treated rats. Furthermore, intrathecal injection of $GABA_B$ receptor antagonist CGP55845 (10 μg, 10 μl) completely abolished baclofen-induced increase in PWT in STZ-treated rats (n = 12, Fig. 1). These data suggest that stimulation of spinal $GABA_B$ receptors reduces hyperalgesia in diabetic neuropathy.

3.2. Stimulation of $GABA_B$ receptor decreased spinal NMDAR expression

Because activation of NMDARs is critically involved in hyperactivity of spinal cord neuron in diabetic neuropathy, we determined NR2B subunit expression in the spinal cord in STZ-treated rats (3 weeks after STZ injection) before and after administration of baclofen. We first measured NR2B protein levels in the lumbar portion of the spinal