Original Article
Phosphorylated neuronal nitric oxide synthase in neuropathic pain in rats

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Abstract: Neuropathic pain caused by nervous system damage or system dysfunction. The pathogenesis and the mechanism underlying neuropathic pain remains unclear. The only known neurobiological component involved in the neuropathic pain is nitric oxide (NO). NO is synthesized by nitric oxide synthase (nNOS) from L-arginine and oxygen. nNOS is involved in the inflammatory pain and neuropathic pain. In this study, we aimed to identify whether KN93 reduced the pain in the rats. Sixty adult male SD rat were randomly divided into 4 groups. Sham group and model group were not received treatment. Experimental group received intrathecal injection of KN93, and negative control group received DMSO injection 30 min before pain test. After last test of pain threshold, the rats were sacrificed and lumbar spinal tissues were sampled for analysis of the expression of pnNOS and pCaMK II by quantitative PCR and Western blotting. Pain threshold was increased in the rats received KN93 treatment (P<0.01), and the expression levels of pnNOS was increased (P<0.05) in experimental group and accompanied with decrease of CaMK II expression (P<0.05). By administration of KN93, the interaction of nNOS and the adaptor protein CAPON was reduced through inhibition of CaMK II by KN93. In conclusion, this study reveals that KN93 can reduce neuropathic pain via inhibiting the activity of CaMK II, and then increase the level of phosphorylated nNOS, to reduce the interaction with CAPON.

Keywords: CAPON, phosphorylation, neuropathic pain

Introduction

Neuropathic pain is one of chronic pain disease caused by nervous system damage or system dysfunction [1, 2]. Currently, neuropathic pain resists in responding to clinical treatment and the incidence of this symptom is gradually increased [3]. It greatly affects the quality of life of patients suffering this syndrome. In some patients, the lesion or disease caused nociceptive neurons changes and lead to hyper sensitivity and activity [4]. The cause of neuropathic pain includes focal or multifocal nerve lesion of peripheral nervous system (trauma, inflammation); generalized lesion of the peripheral nervous system (alcohol, toxic, inflammation); lesions of central nervous system or complex neuropathic disorders [4]. Studies revealed that the sensitization of central and peripheral nerve plays a pivotal role in causing and maintaining neuropathic pain [4-6].

Nitric oxide (NO) regulates immune function, blood vessel dilatation as well as transmitter or modulator in the process of nociceptive stimuli [8-11]. In the biological process, NO is generated from L-arginine and oxygen with nitric oxide synthase (NOS) [12]. Peripheral nerve injury can cause the excessive expression of nNOS in the spinal dorsal horn neurons, and accompanied by hyperalgesia or even pain disorders [13-15]. nNOS plays a pivotal role in the peripheral nervous system and central nervous system. A growing number of studies have revealed that nNOS regulates multiple physiological and pathological processes, such as neuropathic pain and inflammatory pain [7, 14, 15]. After intrathecal injection of nonspecific NOS inhibitors or selective nNOS inhibitor, the mechanical pain reaction was significantly reduced in the spinal nerve ligated rats [3, 16, 17]. Moreover, mechanical pain is not observed in the nNOS gene null mice or rats with nerve injuries, which indicates that the pivotal role nNOS plays in the neuropathic pain [18-20].
It is known that CaM binds to calmodulin dependent kinase II (CaMK II), and then phosphorylates nNOS in 847th serine site to deactivate nNOS [21]. In addition, some kinase and phosphatase, such as protein kinase C (PKC) and protein kinase A (PKA), are also involved in the regulation of nNOS phosphorylation [22]. Xu and the colleagues showed that nNOS interacts with its adaptor protein CAPON in the signaling pathway [23]. However, whether phosphorylated nNOS by CaMK II reduces the interaction with CAPON and therefore increase the pain threshold is not addressed yet.

KN93 is a synthesized methoxybenznesulfonyl derivative which competitively block calmodulin binds to CaM kinase [24]. KN93 has been shown to inhibit CaM kinase-dependent pathway in different cell types, including PC12 h cells, fibroblasts and gastric parietal cells [24-26]. In this study, we showed that the KN93 can block the activity of CaMK II, and therefore reduce the activity of nNOS and to reduce the mechanical pain in the spinal ligated rats.

Materials and methods

Experimental animals and surgical treatment

The SPF male Sprague-Dawley rats weighted 150 g-200 g were housed under specific pathogen-free conditions at the University of Jiangxi Medical College Animal House on a 12L:12D light cycle, and were administered food and water ad libitum. Experiments were followed the protocol outlined in the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication no. 85-23, revised in 1985), with approval from the University of Jiangxi Medical College Animal Care Committee.

Peripheral nerve injury was produced by spinal nerve ligation (SNL) according to the previously described method [27]. Briefly, rats were administered intraperitoneally with 300 mg/kg chloral hydrate (Sigma-Aldrich, St. Louis, MO, USA) for anesthesia; and the dorsal vertebral column was surgically exposed from L4 to L6. The paraspinal muscles were separated from the spinal processes at the L4-L6 level and the L5 transverse process was carefully removed. The right L5 spinal nerves were exposed and tightly ligated distal to the dorsal root ganglion using 6-0 silk thread. As control, sham-operated rats were also obtained by only expose L5 spinal nerves without ligations.

Intrathecal drug delivery

During surgery, the rats were implanted with a PE-10 intrathecal catheter (BD Biosciences, Bedford, MA, USA) in the lumbar enlargement (close to the L4-L5 segments) for intrathecal drug administration. After 7-day recovery, the catheter placement was verified by observing transient hind paw paralysis induced by intrathecal injection of 5 uL 2% lidocaine. Animals that failed to show any paralysis were excluded from the experiments. 5% KN93 (Sigma-Aldrich, St. Louis, MO, USA) was made up in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) and then diluted with 0.9% (w/v) saline solution. The experimental group received 50 mg/kg KN93; negative control group received 10 uL of DMSO. Model group and sham group only underwent intrathecal catheter implantation, and no injection was given. All rats received treatment 30 mins prior of pain threshold measurement.

Mechanical sensitivity measurement

Mechanical alldynia was assessed using von Frey filaments (Stoelting company, USA) by researchers who were blinded to group assignment. The ipsilateral hind paw was pressed with one of a series of von Frey filaments with gradually increasing stiffness (2, 4, 6, 8, 10, 15 and 20 g) applied to the plantar surface for 5-6 seconds for each filament. A positive paw withdrawal response was recorded if the animal briskly lifted the hind paw. The interval between trials was ≥5 min. For each trial, the same hind limb was stimulated 10 times by a single von Frey filament prior to stimulation by the next larger filament. The minimal value that resulted in ≥6 responses to 10 stimulations was recorded.

RNA extraction

The rats were deeply anesthetized with chloral hydrate (300 mg/kg) and perfused transcardially with phosphate-buffered saline (pH 7.4) followed by 4% paraformaldehyde. Total RNA from the L5 spinal segments was extracted with TRIzol™ according to manufacturer's instruction (Gibco-BRL Life Technologies Inc., Grand Island, NY, USA). Neuronal nitric oxide synthase
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(nos1), Calmodulin dependent kinase II gamma (camk2g) and nNOS-associated protein (nos1ap) were used as markers of neuropathic pain. RNA concentration and purity was determined using NanoDrop Spectrophotometer (NanoDrop 2000UV-Vis, Wilmington, DE, USA).

Quantitative reverse transcription PCR

Total cellular RNA was reserve transcribed from each rat with Oligo(dT) 12-18 utilizing SuperScript® III Reverse Transcriptase for qRT-PCR following instructions from manufacturer (Invitrogen Life Technologies, Carlsbad, CA, USA). The sequences of primers used in this study are shown in Table 1. qPCR was conducted by using 1 µg of RNA from each sample in presence of 0.1 uM to 0.5 uM primers as well as SYBR® Premix Ex Taq™ according to manufacturer’s instruction (Takara Bio, Inc., Tokyo, Japan) and analysed with a qRT-PCR detection system (Applied Biosystems, Foster City, CA, USA). The reaction conditions were 95°C for 10 mins, then 40 cycles of 95°C for 15 sec and 60°C for 1 min. All the expression levels were normalized to GAPDH expression.

Western blot

The tissue of spinal cord were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Na-deoxycholate, 1% NP-40) supplemented with 1% phosphatase and 1% protease inhibitor cocktails, 5 mM NaF and 1 mM PMSF. Gel electrophoresis was performed according to standard protocols [28]. Antibodies and working dilutions for western blot: AR (1:100, Genetex, Irvine, CA, USA), GAPDH (1:100,000, Millipore, Billerica, MA, USA), AMPK and p-AMPK-Thr172 (1:1000, Cell Signalling, Danvers, MA, USA), MID1 (1:400, Sigma-Aldrich), α4 (1:500, Abcam, Cambridge, UK), N-flag (1:1000, Sigma-Aldrich), PP2A (1:1000, Millipore). Immoblot bands were scanned and quantified using a scanning densitometer (Odyssey; Li-Cor Biosciences, Lincoln, NE, USA). The housekeeping protein GAPDH served as loading control.

Co-immunoprecipitation

For analysis of nNOS-CAPON interaction, co-immunoprecipitation (CoIP) was performed as described previously [29] with few modification. Briefly, cells were washed with PBS pH 7.4 twice and lysed with NP40 buffer (50 mM Tris-Cl pH 8.0, 150 mM NaCl, 1% NP-40). Lyssates were precleared by the addition of 50 µl of agarose beads for 30 min. Total protein (600 µg) and 4 µg of antibody were used for each IP and rotated overnight in 4°C. Beads (30 µg) were added to each IP and rotated for 2 h, followed by centrifugation at 1000 rpm for 3 min. Supernatants were removed, and pellets were washed four times with NP40 buffer. Complexes were eluted in SDS lysis buffer. Western blotting was performed with specific antibodies to visualize proteins interacting with CAPON.

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**Table 1. Primers used for PCR**

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Gene symbol</th>
<th>5’ primer</th>
<th>3’ primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_133605.1</td>
<td>camk2g</td>
<td>5’-GCTCTTGAGAATGGGCAA-3’</td>
<td>5’-CCTCGAGATCGTGTAGT-3’</td>
</tr>
<tr>
<td>NM_138922.1</td>
<td>nos1ap</td>
<td>5’-ATAGAGCGAGTCGCGTGATG-3’</td>
<td>5’-CCTCGATCTTCCTGGCATCT-3’</td>
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<tr>
<td>NM_052799.1</td>
<td>nos1</td>
<td>5’-TGACACCACTAGCATTACC-3’</td>
<td>5’-GCAGTCAGGGCATGAA-3’</td>
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<tr>
<td>NM_017008.4</td>
<td>gapdh</td>
<td>5’-CCCGAATGATCCGTTGTG-3’</td>
<td>5’-TAGGCCAGGATGCCCTTATTAG-3’</td>
</tr>
</tbody>
</table>

**Table 2. The mechanical pain threshold in rats (g)**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sample No.</th>
<th>1 day prior to surgery (g)</th>
<th>1 day after surgery (g)</th>
<th>3 day after surgery (g)</th>
<th>5 day after surgery (g)</th>
<th>1 h</th>
<th>2 h</th>
<th>4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>11</td>
<td>13.7±0.6</td>
<td>7.5±0.8a</td>
<td>4.8±0.4a</td>
<td>2.5±0.5a</td>
<td>2.4±0.1</td>
<td>2.5±0.1</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td>N</td>
<td>11</td>
<td>13.2±0.4</td>
<td>6.7±0.5a</td>
<td>4.5±0.3a</td>
<td>2.7±0.5a</td>
<td>2.5±0.1c</td>
<td>2.8±0.2</td>
<td>3.0±0.1c</td>
</tr>
<tr>
<td>E</td>
<td>8</td>
<td>13.4±0.6</td>
<td>7.0±0.4a</td>
<td>3.7±0.3a</td>
<td>2.4±0.5a</td>
<td>55.4±3.3b</td>
<td>48.9±2.3</td>
<td>47.0±2.0b</td>
</tr>
<tr>
<td>Sham</td>
<td>15</td>
<td>12.7±0.7</td>
<td>12.2±0.4</td>
<td>12.1±0.5</td>
<td>12.6±0.7</td>
<td>12.7±0.5</td>
<td>12.6±0.5</td>
<td>12.7±0.6</td>
</tr>
</tbody>
</table>

a=P<0.05, compared with corresponding data 1 day prior to surgery; b=P<0.01, compared with sham group; c=P<0.01, compared with model group.
Immunohistochemistry

Immunofluorescence (IF) was used for analysis of nNOS-CAPON interaction in the rat spinal cord tissues based on methods been published in the past with minor modifications [30]. Briefly, after incubation with primary antibody, in either CAPON (1:1000, ab190686) or nNOS (1:800, ab76067) (Abcam, Cambridge, Massachusetts, US) for overnight, tissues were incubated with secondary antibody conjugated with either Alexa 488 (CAPON) or PE (nNOS) in 1:700 dilution (Abcam, Cambridge, Massachusetts, US) for 2 h. Then tissues were washed with PBS and mounted with DAPI mounting media for analysis.

Statistics

Data are presented as means ± SEM, and analyzed using SPSS 19.0 (SPSS Inc, USA). For behavioral data, comparisons were performed...
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Using repeated measures of analysis of variance (ANOVA). For others, comparisons were performed using ANOVA followed by Bonferroni tests. Data were considered as significant when $P<0.05$.

Results

Mechanical pain sensitivity decreased after intrathecal KN93 delivery

Firstly, we defined the pain sensitivity of these rats before and after KN93 treatment. It is very clear that 1 day before SNL surgery, rats had very similar pain threshold (Table 2). After surgery, the pain threshold was reduced ($P<0.05$) in the rats in model group, experimental group as well as negative control group, but not in the sham group. It clearly illustrate that the surgery was successfully reduced the pain threshold of rats underwent complete SNL surgery. After given KN93 or DMSO, the rats in the experimental group showed a great elevation ($P<0.01$) in the pain threshold, when compared with model group (Table 2). However, the negative control group showed a reduced pain threshold ($P<0.01$), when compared with sham group (Table 2).

KN93 inhibiting pCaMK II expression and promoting pnNOS expression

Next we examine the role of KN93 in the phosphorylation of nNOS in the rats suffering neuropathic pain. We determined the gene expression levels of phosphorylated CaMK II (pCaMK II), nNOS and phosphorylated nNOS after KN93 administration. The expression level of pCaMK II was reduced ($P<0.05$) in the experimental group and the sham-operated group when compared with the model group (Figure 1A). No change was observed in nNOS expression levels (Figure 1B). Moreover, the expression of pnNOS is significantly increased ($P<0.05$) in both experimental group and sham-operated group (Figure 1C).

Knowing that gene expression levels of pCaMK II and pnNOS were changed after KN93 treatment, we examined the protein expression levels. It is very clear that as pCaMK II gene expression level decreased in experimental group and sham-operated group, the abundance of pCaMK II protein reduced as well (Figure 1D). No obvious change can be seen from nNOS protein (Figure 1D), and pnNOS appeared to be more in the experimental group and sham-operated group (Figure 1D).

Increased pain threshold in rats is associated with decreased interactions of phosphorylated nNOS with CAPON

After detection of pCAMK II and pnNOS up-regualtion in the dorsal root ganglion of rats, next we identified whether increased express of pnNOS is associated with the adaptor CAPON. Currently it has been revealed that the interaction of nNOS and its adaptor protein CAPON is involved in the process of neuropathic pain and maintaining the pain. It is obvious that both nNOS and pnNOS interact with CAPON, however, after phosphorylation of nNOS, the interaction is reduced (Figure 2). With the presence of KN93, the interaction between pnNOS and CAPON is further reduced (Figure 2).

Then we visualized both CAPON and nNOS by Immunofluorescent. It is very clear that the Immunofluorescent intensity is reduced after given KN93 (Figure 3A-D), when compared to the rats injected with DMSO (Figure 4A-D). This evidence further verified the role of KN93 in the process of neuropathic pain.

Discussion

The pathogenesis of neuropathic pain is very complicated, it can be induced in multiple sites of the neuronal system, and the symptoms vary in the clinical conditions. It is commonly seen in the patients experiencing central or peripheral
never damage, including diabetes, Multiple sclerosis, neuropathy post herpes infection, trauma caused spinal damage etc. On the other words, neuropathic pain may be caused by central or peripheral neuronal damage [31-35]. This study revealed nNOS phosphorylation plays a pivotal role in the pathogenesis of neuropathic pain, and also showed by inhibiting CaMK II; pain threshold is increased in rats. The coIP and immunofluorescent results showed interactions between nNOS and CAPON, which can be reduced through phosphorylation of nNOS by CaMK II, and then lead to the improvement of mechanical pain threshold of rats.

Our study utilized rat SNL model, using qPCR and Western blotting to determine nNOS expression levels in the spinal dorsal horn and dorsal root ganglion. Firstly we identified that the pain threshold is significantly increased after administration of KN93 in rats. Then we observed as the expression level of pCaMK II decreased, the expression level of pnNOS increased. By utilizing coIP and Immunofluorescent, we identified nNOS interacted with CAP-

Figure 3. The co-expression sites of nNOS and CAPON of the experimental group. A. DAPI staining; B. nNOS staining; C. CAPON staining; D. Merged nNOS and CAPON staining. Arrows (→) indicates the co-expression sites of nNOS and CAPON.
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ON, and the interaction was reduced when nNOS phosphorylated.

Early study revealed chronic neuropathic pain can be reversed by KN93 [36], however, the mechanisms of how KN93 reverse the chronic pain induced by SNL was not addressed. Other study showed the interaction of nNOS with adaptor protein CAPON are involved in the process of increasing the pain threshold and therefore reduce the pain [37]. This study evaluated that the effect of KN93 is associated with pCaMK II expression in the spinal cord tissues of rats. Previous study demonstrated that pCaMK II can be activated when low dose of KN93 was injected [38]. Our study further demonstrated that neuropathic pain can be reversed by pCaMK II inhibitor. Our data suggests pCaMK II is involved in pathogenesis of neuropathic pain, as well as maintain the chronic pain.

In conclusion, we identified the role of phosphorylation of nNOS in the process of neuropathic pain in rats. As nNOS gets phosphorylated, the interaction with the adaptor protein...

Figure 4. The co-expression sites of nNOS and CAPON of the negative control group. A. DAPI staining; B. nNOS staining; C. CAPON staining; D. Merged nNOS and CAPON staining. Arrows (→) indicates the co-expression sites of nNOS and CAPON.
CAPON is reduces, increase the pain threshold, and therefore relief the neuropathic pain. Intracathal injection of KN93 may be potential novel treatment for SNL caused abnormal pain and other neuropathic pain. KN93 may also be the target for new therapy for pain control or management.

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Disclosure of conflict of interest

None.

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