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Full Length Research Paper

Efficiency of intrathecal glial cell line-derived neurotrophic factor on nitric oxide and nitric oxide synthase activity in rat with neuropathic pain

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The aim of this study was to investigate the efficiency of intrathecal glial cell line-derived neurotrophic factor (GDNF) on nitric oxide (NO) and nitric oxide synthase (NOS) activity following a spinal nerve ligation (SNL) of male Sprague Dawley rats. The rats were randomly divided into four groups: normal (control), sham-operated, SNL (SNL followed by a physiological saline injection into the subarachnoid space), and GDNF (SNL followed by a GDNF injection into the subarachnoid space). Each group was divided into three subgroups (n = 10). The rats in each subgroup were euthanised 3, 7, and 14 days after the operation. Rat behaviour was evaluated before euthanising, and the ipsilateral spinal cords were harvested after euthanising to determine the NO content and NOS activity. Compared with the control and sham-operated groups, the NO content and NOS activity in the SNL group increased significantly 3 days after the operation; this increase was maintained until 14 days after the operation (*P* < 0.01 or 0.05). A significant decrease was observed in the NO content and NOS activity in the GDNF group compared with the SNL group. The decrease continued until 14 days after the operation (*P* < 0.01 or 0.05). The results indicated that the NO and NOS activity in the rat spinal cord are associated with SNL-induced neuropathic pain. The decreased neuropathic pain from the intrathecal GDNF is correlated with the decrease in NO content and NOS activity in the spinal cord.

Key words: Glial cell line-derived neurotrophic factor, neuralgia, nitric oxide, nitric oxide synthase.

INTRODUCTION

Glial cell line-derived neurotrophic factor (GDNF) is a small protein that was isolated and purified from the mouse glial cell line B49 in 1993 (Lin et al., 1993). GNDF has been proven to be closely associated with neuropathic pain owing to its nutritional and improved effect on the primary afferent neuron regeneration, as well as its role in the damage sensory formation of the spinal dorsal horn (Boucher et al., 2000; Wieseler et al., 2004; Airaksinen et al., 2006; Ricart et al., 2006). A number of studies demonstrate that in the nociceptive information delivery process, nitric oxide (NO), as a messenger, is involved in the pain regulation of the peripheral and central nervous system at different levels, particularly in the pain regulation of the spinal cord.

Increasing evidence shows that nitric oxide synthase (NOS) inhibitors have a significant anti-nociceptive effect, and that spinal cord plasticity based on the NO synthesis system plays an important role in the maintenance and occurrence of pain following a nerve injury (Meller et al., 1992, 1994; Yaksh, 1999). Previous study findings show that a subarachnoid injection of GDNF can significantly reduce the neuropathic pain, mechanical hyperalgesia, and cold-induced persistent pain in rats (Jia et al., 2009). The mechanism of NO and NOS effects in the spinal cord is unclear. The aim of this study was to induce a rat neuropathic pain model through spinal nerve ligation (SNL) and to observe the changes in NO content and NOS activity to investigate the GDNF mechanism on

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neuropathic pain.

MATERIALS AND METHODS

Animal selection and grouping

Healthy male Sprague Dawley rats aged six weeks and weighing 180 to 200 g were provided by the Vital River Laboratory Animal Technology [Beijing, China; No. SCXK (Jing) 2007 to 2008]. All procedures were approved by the Animal care committee (Ningbo University, Ningbo, China) and were in accordance with the Chinese law on animal experiment. The rats were housed in sawdust-lined plastic boxes, with five rats per box, natural illumination, and free access to food and water. The rats were randomly assigned to four groups: normal control, sham-operated, SNL (SNL followed by a physiological saline injection into the subarachnoid space), and GDNF (PeproTech, Inc., Rocky Hill, NJ, USA) (SNL followed by a GDNF injection into the subarachnoid space). Each group was further divided to three subgroups (n = 10) according to the time at which the rats were euthanised: 3, 7, and 14 days post-surgery. After euthanising, the spinal cord tissue from the affected side of the rats was harvested to measure the NO content and NOS activity.

Preparation of the neuropathic pain rat models

The SNL rat models were established according to the methods reported by Kim and Chung (1992). Following an intraperitoneal injection of 1% sodium pentobarbital (40 mg/kg), the rats were placed in a ventral position such that the Ping iliac spine (L6) was in a horizontal position. A 1.5 cm incision in the skin was made above and below the midline of the back. The left paravertebral muscles were bluntly dissected between L4 and S2 to expose the L6 transverse process and sacral cornu. Part of the L6 transverse process was removed to expose the L4 to 5 spinal nerve. The L5 spinal nerve was isolated and tightly ligated using a 6 to 0 silk suture and the L6 spinal nerve was dissociated from the sacral cornu and ligated by performing a hemostasis and incision suture. The rats from the sham-operated group underwent a spinal nerve exposure without ligation. The same person performed all of the experimental procedures to maintain consistency.

Subarachnoid catheter

All the operations were carried out under sterile conditions according to previously reported methods (Storkson et al., 1996). Following an intraperitoneal injection of 1% sodium pentobarbital (40 mg/kg), the rats were placed in a prone position and a longitudinal incision was made from L5 to 6. A self-made guided needle was inserted vertically, positioned to the subarachnoid space with a clear sense of breakthrough and movement in the rat's tail, and the needle was inclined to the rat's head. A polyethylene catheter (PE-10) was introduced into the subarachnoid space through the self-made guided needle, and the catheter tip was positioned 3 to 3.5 cm near the lumbar enlargement of the spinal cord.

The distal end of the catheter was tunnelled subcutaneously to emerge at the neck. After implanting the intrathecal catheter, the rats displaying evidence of motor dysfunction were sacrificed. The location of the catheter tip was confirmed by pumping an intrathecal infusion of 20 µL 2% lidocaine to induce motor paralysis of the hind limbs within 30 min. The eligible rats were housed in sawdust-lined plastic boxes, with five rats per box, natural illumination, and free access to food and water.

Determination of the NO content in the spinal cord tissues

After the sacrifice, the vertebral lamina was removed and a 0.2 g spinal cord tissue from the lumbar intumescent segment was rinsed with cold physiological saline (0 to -4°C) and then placed into a tube containing cold physiological saline to prepare a 10% spinal cord tissue homogenate (pH 7.5, 0.025 mol/L sucrose, 0.05 mol/L Tris-HCl, 0.1 mmol/L ethylenediaminetetraacetic acid). The homogenate was centrifuged for 10 min at 6,000 r/min, and 500 µL supernatant was harvested. All procedures were performed at 0 to 4°C. All kits used in these procedures were supplied by the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Considering its active chemical nature, NO was converted into NO_3^- and NO_2^- *in vivo*. We determined the NO content by measuring the amount of NO_3^- and NO_2^-, expressed as µmol/g protein. The Lowry protein assay was used.

Determination of total NOS (TNOS) and inducible NOS (iNOS) activities

NO is biosynthesised endogenously from L-arginine and oxygen by NOS enzymes, and NO reacts with nucleophilic substances to produce coloured compounds. Total NOS activity was determined based on this principle. The constitutive NOS and iNOS activities were measured based on their different sensitivities to calcium. The NOS activity (U/mg protein) was calculated using the following equation: [($Absorbance_{550}$ test - $Absorbance_{550}$ blank) / Molar absorption coefficient × Total volume of reaction solution / sample volume (µL) × 1 / (Coloured optical path × Reaction time)] / Protein content (mg/L).

Statistical analysis

All statistical analyses were processed using the SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) and expressed as Mean ± standard deviation (SD). The normality test was conducted using the Kolmogorov–Smirnov test, one-way analysis of variance was employed for an intergroup comparison, and paired *t* test was used for intragroup comparisons. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

Assessment of the NO content

Compared with the control and sham-operated groups, NO content was significantly increased in the SNL group on day 3 and remained so until 14 days after the operation ($P < 0.01$ or 0.05). In the GDNF group, NO content was significantly lower than in the SNL group, staying at this level until 14 days after the operation ($P < 0.01$ or 0.05). No difference was observed in the NO content between the sham-operated and GDNF groups (Table 1).

Assessment of the TNOS activity

Compared with the control and sham-operated groups, TNOS activity in the SNL group was significantly increased on day 3 and was maintained until 14 days after the

operation ($P < 0.01$). The TNOS activity was significantly lower in the GDNF group compared with the SNL group; this behaviour was also maintained until 14 days after the operation ($P < 0.01$ or 0.05). A significant difference in TNOS activity was observed between the sham-operated and GDNF groups ($P < 0.01$ or 0.05) (Table 2).

Assessment of the iNOS activity

Compared with the control and sham-operated groups, iNOS activity in the SNL group was dramatically increased on day 3 and continued to increase until 14 days after the operation ($P < 0.01$). Seven days after the operation, iNOS activity was significantly lower in the GDNF group compared with the SNL group, remaining at this level until 14 days after the operation ($P < 0.01$ or 0.05). No significant difference was observed in iNOS activities of the sham-operated and GDNF groups until 14 days after the operation ($P < 0.01$) (Table 3).

DISCUSSION

The present study demonstrated that NO content and NOS activity significantly increased in the SNL group on post-operation days, and that the intrathecal administration of GDNF significantly reduced the SNL-induced NO production and NOS activity in the spinal cord. GDNF, a member of the transforming growth factor beta superfamily, is the most active growth factor for motor neurons (Watabe et al., 2001; Malcangio, 2003; Paratcha et al., 2003; Dong et al., 2006; Honq et al., 2008). Jia et al. (2009) found that SNL rats experienced a 50% reduction in paw withdrawal threshold on the operated side and an increased number of paw lifts on a 5°C cold plate one day after the operation. These effects were still evident until 14 days after the operation. This behaviour indicated that SNL rats exhibit cold hyperalgesia. In our previous study, a subarachnoid injection of GDNF reduced the mechanical and cold hyperalgesia expression in the SNL rats at 3 and 5 days after the operation, respectively (Jia et al., 2009).

NO is a new type of non-classical neurotransmitter and messenger molecule that participates in the pain regulation of the peripheral and central nervous system in various ways. Results showed that the NO content increased significantly in the rats with SNL-induced hyperalgesia until 14 days after the surgery. These results are in accordance with the results of Mabuehi et al. (2003). These findings further prove that the NO produced in the spinal cord plays an important role in pain information transmission and hyperalgesia occurrence (Wang et al., 2001), although the mechanism through which this occurs is complex.

When the peripheral nerve impulse generated by a noxious stimulation is inserted into the spinal cord through the Aδ and C fibres, excitatory amino acids from the spinal cord dorsal horn neurons are precipitated to bind with N-Methyl-D-Aspartate (NMDA) and non-NMDA receptors to realise a continued polarisation of the spinal cord dorsal horn neurons. After the excitatory post-synaptic potential (EPSP) is formed, the Ca^{2+} channels are opened and the Ca^{2+} influx binds with calmodulin to activate the NOS. L-arginine reacts with Nicotinamide adenine dinucleotide phosphate (NADPH) and O_2 to produce NO+NADP+NADPH in the NOS presence. The NO produced rapidly diffuses in and out of the cells to activate soluble guanylate cyclase and generate monophosphate (cGMP), activating the protein kinase to increase the release of neurotransmitters, accumulate prostaglandin, and enhance the response of the NMDA receptors in postsynaptic neurons. Consequently, the spinal cord dorsal horn nociceptive neurons are excited, which, in turn induces pain (Li and Clark, 2001; Tao and Johns, 2002; Yoon et al., 2005). A large amount of NO harvested from persistent noxious stimuli can activate guanylate cyclase to produce more cGMP. The generated cGMP can penetrate into the deep dorsal horn to enhance the response of mass dynamic neurons to external stimuli (Lin et al., 1997).

This study confirmed that the intrathecal administration of GDNF significantly reduces the SNL-induced NO production in the spinal cord, thereby controlling the formation and development of the central nervous system sensitisation.

NOS is an important factor in limiting NO synthesis, and the *in vivo* biological role of NO mainly depends on NOS activity. Thus, measuring NOS is a key link in studying the biological effects of NO. The iNOS system has been described as calcium-insensitive, and is mainly found in macrophages and astrocytes. Under pain stimulation, the astrocytes in the spinal dorsal horn are vitalised by the iNOS, and the NO generation is significantly promoted. In this study, we found that the iNOS activity in the spinal cord was enhanced in the SNL-induced neurotrophic pain rats, accounting for 20% of the TNOS. Considering this calcium-insensitive characteristic, no difference was observed in the *in vitro* and *in vivo* measurement of iNOS activity. iNOS is expressed in activated microglia, and massive amounts of NO can activate astrocytes to produce prostaglandin, enhancing the excitability of the pain transmission neurons. As a result, an increased iNOS activity effectively promotes pain occurrence (Lui and Lee, 2004; Naik et al., 2006).

In this study, the neuronal presynaptic membrane was depolarised after the peripheral nerve injury; therefore, glutamate or NMDA was released into the synaptic cleft to bind with the NMDA receptor or other excitatory amino acid receptors. As the receptor channel opened, the Ca^{2+} influx coupled with calmodulin protein activated the NOS with the assistance of the NADPH. The L-arginine then reacted with NADPH and O_2 to produce NO, activating

Table 1. Effects of GDNF on nitric oxide contents in the spinal cord of SNL rats (μmol/g protein, Mean ± SD).

Group	3 days	7 days	14 days
Control (n=6)	3.26±0.14	3.22±0.12	3.30±0.17
Sham-operated (n=10)	3.11±0.38	3.37±0.38	3.32±0.19
SNL (n=10)	3.63±0.34**	3.71±0.30*	3.80±0.22**
GDNF (n=10)	3.28±0.22$^{\#}$	3.36±0.25$^{\#}$	3.48±0.28$^{\#\#}$

GDNF: glial cell line-derived neurotrophic factor; SNL: spinal nerve ligation; *P < 0.05, **P < 0.01 *vs.* sham-operated group; *P < 0.05, **P < 0.01 versus sham-operated group; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01 versus SNL group.

Table 2. Effects of GDNF on total nitric oxide synthase activity in the spinal cord of SNL rats (U/mg protein, Mean ± SD).

Group	3 days	7 days	14 days
Control (n=6)	11.77±2.22	11.73±2.12	11.74±2.05
Sham-operated (n=10)	11.60±1.61	11.64±1.95	11.79±1.99
SNL (n=10)	15.95±1.85**	17.60±2.02**	17.90±1.79**
GDNF (n=10)	14.08±1.81**$^{\#}$	13.61±2.33*$^{\#\#}$	13.79±2.03**$^{\#}$

GDNF: Glial cell line-derived neurotrophic factor; SNL: spinal nerve ligation; *P < 0.05, **P < 0.01 versus sham-operated group; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01 versus SNL group.

Table 3. Effects of GDNF on inducible nitric oxide synthase activity in the spinal cord of SNL rats (U/mg protein, Mean ± SD).

Group	3 days	7 days	14 days
Control (n=6)	2.25±0.53	2.21±0.54	2.15±0.59
Sham-operated (n=10)	2.52±0.60	2.52±0.66	2.41±0.44
SNL (n=10)	3.79±0.57**	3.84±0.69**	3.63±0.53**
GDNF(n=10)	3.16±0.96	2.84±0.55$^{\#\#}$	2.83±0.48**$^{\#\#}$

GDNF: glial cell line-derived neurotrophic factor; SNL: spinal nerve ligation; *P < 0.05, **P < 0.01 versus sham-operated group; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01 versus SNL group.

the soluble guanylate cyclase to generate cGMP. The generated cGMP, as a secondary messenger, was involved in the protein phosphorylation, induced the c-Fos expression and switched on the related genes to participate in the pain or nociceptive transmission. The intrathecal administration of GDNF can reduce the SNL-induced NOS activity, thereby reducing the NO synthesis to alleviate the sensitisation of the spinal cord neurons and reduce the nociceptive information transmission to the central nervous system (Myung, 2006; Naik et al., 2006).

Conclusion

The intrathecal administration of GDNF can effectively relieve SNL-induced neuropathic pain. The mechanisms of this effect correlate to the decrease of NO content and NOS activity in the spinal cord.

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