Amino Acids (1998) 14: 105–112

Amino
Acids

Spinal nerve lesion induces upregulation of neuronal nitric oxide synthase in the spinal cord

An immunohistochemical investigation in the rat

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Accepted September 26, 1997

Summary. The possibility nitric oxide (NO) is involved the neurodegenrative mechanisms in the spinal cord following a chronic peripheral nerve lesion was examined using NOS immunohistochemistry. Spinal nerve lesion at L-5 and L-6 level was produced according to the Chung model, a model of neuropathic pain and rats were allowed to survive for 8 weeks. In one group of animals L-NAME was given intraperitoneally (1–2 mg/kg, i.p. daily) for 6 weeks. Sham operated rats, in which the spinal nerve was exposed but not ligated, served as controls. Ligation of spinal nerves in rats resulted in an upregulation of NOS which was most pronounced in the ipsilateral gray matter of the spinal cord compared to the contralateral side. In these rats, ultrastructural investigations showed distorted neurons, membrane disruption and myelin vesiculation. Sham operated rats did not show either NOS upregulation or structural changes in the spinal cord. Pretreatment with L-NAME significantly reduced NOS upregulation and the structural changes in the spinal cord were less pronounced. These observations strongly indicate a putative role of NOS in the pathophysiology of chronic nerve lesion. Our results may provide a new strategy to treat chronic neuropathic pain or to minimise neurodegeneration in the patients suffering from such diseases of the nervous system.

Keywords: Neuropathic pain – Spinal cord – Nitric oxide synthase – Neurodegeneration – L-NAME

Introduction

Peripheral tissue inflammation, damage, or nerve injury can lead to functional changes in the central nervous system (CNS) including an increased sensitivity to noxious stimulation or hyperalgesia (Dubner and Ruda, 1992). Peripheral nociceptors innervating the area of injury exhibit increased activity, leading to the CNS neuronal plasticity and a hyperexcitable state in the relevant spinal cord

dorsal horn (Grzybicki et al., 1996). It is these peripheral and central neuronal changes which are thought to lead the hyperalgesia (Solodkin et al., 1992). The specific mechanism(s) underlying this central or peripheral plasticity are unclear but are thought to involve changes in chemical mediators which influence synaptic transmission in the spinal cord (Yamamoto and Shimoyama, 1995).

One class of chemical mediators which has been the subject of investigation in relationship to pathologic nociceptive processing is the excitatory amino acids. Significant evidence now exist supporting a role of N-methyl-D-aspartate (NMDA) receptor activation in spinal cord plastic changes, including spinal hyperalgesia (Meller and Gebhert, 1993). Nitric oxide (NO) is a free radical gaseous molecule which is synthesised by the semiessential amino acid L-arginine hydrochloride by an enzyme nitric oxide synthase (NOS) (Dawson and Dawson, 1996). Nitric oxide (NO) is though to be the mediator of at least some of the effects of NMDA receptor activation; therefore, a role for NO production in nociceptive mechanisms including hyperalgesia has also been the subject of recent investigations (Grzybicki et al., 1996; Yamamoto and Shimoyama, 1995).

Recently involvement of nitric oxide (NO) in many physiological and pathophysiological functions of the CNS is suggested (Kimura and Steinbusch 1996; Dawson and Dawson, 1996; Sharma et al., 1997a, 1998). The synthesis on NO in the nervous system is mediated by the enzyme nitric oxide synthase (NOS) which is normally present in some neurons of the CNS (Dawson and Dawson 1996).However, its role in neurodegeneration and cell injury is still unclear. There are experimental evidences which suggest that a focal trauma to the rat spinal cord is associated with an upregulation of NOS activity in neurons of the perifocal segment (Sharma et al., 1996b). This upregulation of NOS is closely related with the pathological reaction of nerve cells indicating a putative role of NO in cell injury.

The present investigation was undertaken to find out whether a chronic spinal nerve lesion is associated with alteration in NOS activity, and if so, whether this alteration in NOS expression is related with cell injury. In addition, the influence of one NOS inhibitor, L-NAME, on the enzyme activity was examined.

Materials and methods

Animals

Experiments were carried out on 24 male Wistar rats (body weight 250–300 g) housed at controlled room temperature (21 ± 1°C) with 12 h light and 12 h dark schedule. The rat feed and tap water were supplied ad libitum.

Spinal nerve lesion

Spinal nerve lesion at L-5 and L-6 was produced (n = 5) according to the Chung model. In brief, under Halothane anaesthesia, left spinal nerve corresponding to L-5 and L-6 segments was dissected out and ligated according to the neuropathic pain model of Chung described earlier (Kim and Chung, 1992; Sharma et al., 1996b). The spinal nerves contain both sensory and motor fibres. After the ligation, the muscles were sutured and the skin was closed. These rats were allowed to survive 8 weeks after the operation. This experimental condition is approved by the Ethical Committee of Uppsala University, Uppsala, Sweden.

Control group

In a separate group of rats in which the spinal nerve was exposed but not ligated were used as sham operated controls (n = 5). For comparison normal rats (n = 5) were used as intact controls. Survival time was 8 weeks for both the groups.

Drug treatment

In a separate group of nerve ligated rats (n = 5) or sham operated (n = 4) rats L-nitro-arginine-methyl-ester (L-NAME) was given intraperitoneally as repeated injections 1 day before the experiment (2 mg/ kg, i.p., twice daily) until 3 days after the lesion and was then continued daily (1 mg/kg, i.p., once daily) for a period of 6 weeks. These rats were allowed to survive 8 weeks after the experiment.

Parameters measured

The following parameters were measured in control, sham operated and nerve liagted rats simultaneously in a blind fashion.

NOS immunohistochemistry. The NOS immunohistochemistry was done on L-5 segment of the spinal cord using free floating Vibratome sections (60 µm thick) using monoclonal antibody raised against constitutive isoform of neuronal nitric oxide synthase (for details see Sharma et al., 1996b, 1997a, 1998).

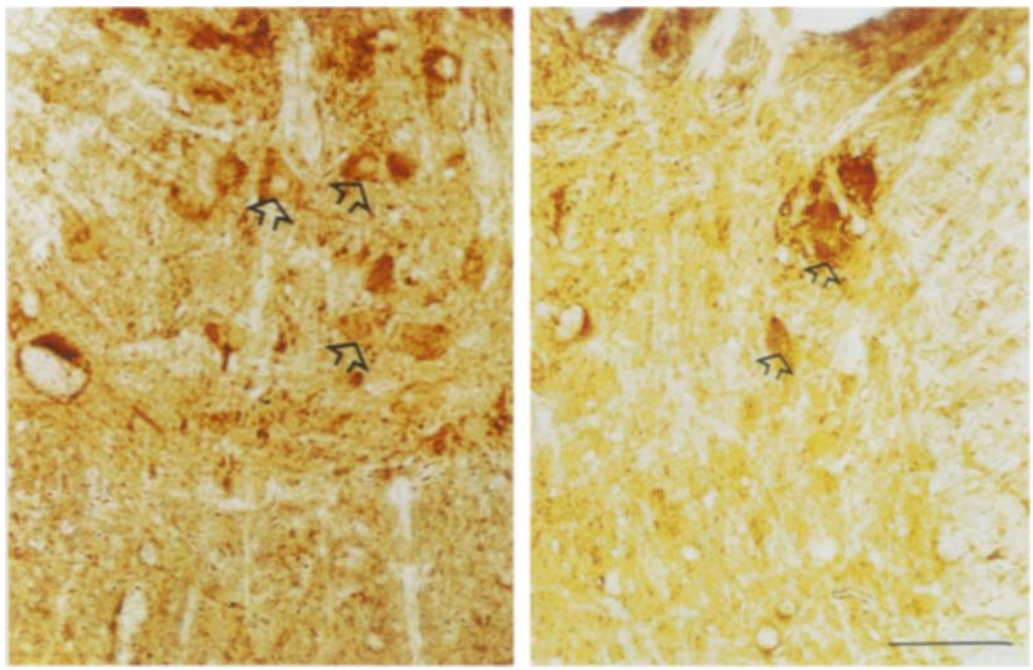

Fig. 1. A representative example of NOS immunoreactivity (bold arrow heads) in the ipsilateral ventral horn of L-5 segment 8 weeks after peripheral nerve lesion (left) and its modification by L-NAME treatment (right). Pretreatment with chronic L-NAME significantly thwarted the NOS immunostaining in the cord. Thus only a few NOS positive cells are visible in the treated rat (thin arrow heads) compared to untreated rat

Morphological study. Some tissue pieces from the L-5 segment of the cord were embedded in epon for routine light and electron microscopy for structural investigation as described earlier (Sharma et al., 1997a, 1998). In brief, for high resolution light microscopy, about 1 μm thick high sections were cut and stained with toluidine blue and examined under light microscope for gross pathology. For semiquantitative analysis, the number of distorted nerve cells were counted in dorsal and ventral horn in both ipsilateral and contralateral side of the L-5 spinal cord segment (Sharma et al., 1996a).

Statistical analysis

For quantitative data obtained, Student's unpaired t-test was used to evaluate the statistical significance of the data obtained. A p-value less than 0.05 was considered to be significant.

Results

Spinal nerve lesion and NOS immunohistochemistry

Ligation of spinal nerves in rats resulted in an upregulation of NOS which was most pronounced in the ipsilateral gray matter of the spinal cord compared to the contralateral side (Fig. 1). Sham operated rats did not show NOS upregulation (Fig. 2). The immunostained material was mainly confined within the neuronal cytoplasm, however in few cases the cell nucleus is also stained (Fig. 2).

Spinal nerve lesion and spinal dorsal horn morphology

In these rats, morphological investigations showed marked neurodegenrative changes in the spinal cord. These changes were most pronounced in the ispilateral dorsal horn and also seen in some parts of the ventral horn (Fig. 2). These changes include vacuolation of neuronal cytoplasm, degenration of myelin and distorted neurons.

Effect of L-NAME pretreatment

Pretreatment with L-NAME resulted in a slight but significant reduction in NOS upregulation (Fig. 1). In these drug treated rats, structural changes were also considerably reduced (Fig. 2).

Discussion

Our results show that an experimental model of chronic neuropathic pain produced by peripheral nerve lesion, is associated with neurodegenerative changes in the spinal cord. Our observation is limited to the survival period of 8 weeks. Thus it is not clear from this study whether these neurodegenerative changes seen in the spinal cord following nerve lesion are maximal. There are reports that 2 weeks after nerve lesion changes in dorsal root ganglia can be seen using morphological approach (Steel et al., 1994). Our observation further added the information that spinal cord itself show marked cell changes corresponding to the nerve lesion segment. Since we did not analyse morphological changes in the spinal cord above or below the nerve lesioned segment it is not certain whether morphological changes seen

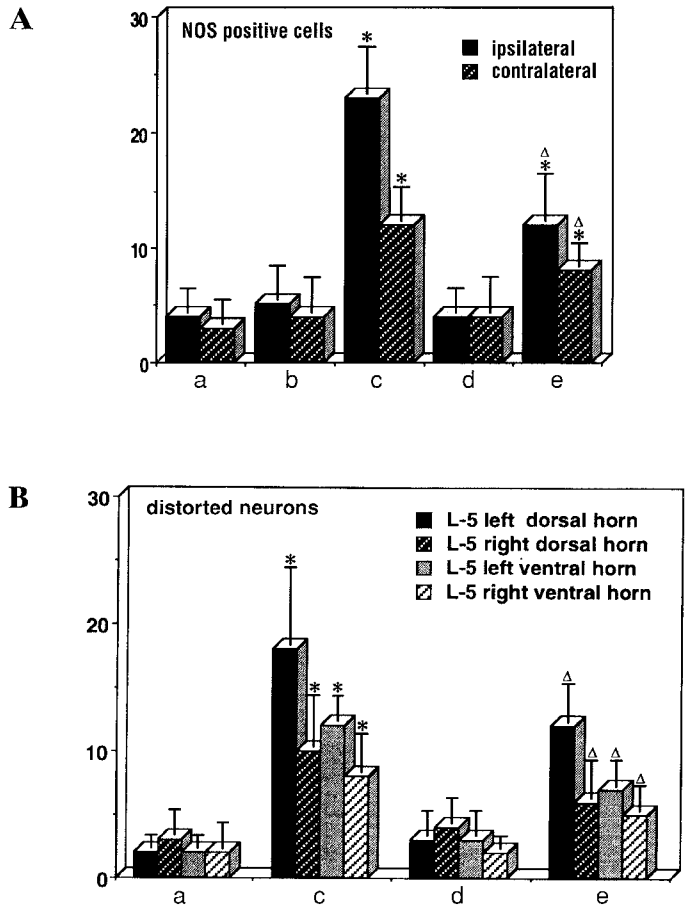

Fig. 2. **A** Semiquantitative analysis of NOS upregulation in the L-5 segment of the spinal cord 8 weeks following peripheral nerve lesion and its influence by L-NAME treatment. **B** Semiquantitative analysis of distorted nerve cells in the L-5 segment of the spinal cord 8 weeks following peripheral nerve lesion and its influence by L-NAME treatment (*$p < 0.05$, Student's unpaired t-test compared from control group; $\Delta p < 0.05$, compared from spinal nerve lesioned group). *a* control; *b* sham; *c* nerve lesion; *d* L-NAME + sham; *e* L-NAME + lesion

in the spinal cord would represent a general phenomena or it is limited to a focal changes in the cord. To further elaborate this finding additional investigation of various level of the neuraxis in this model are needed, which however require further studies.

The possible mechanisms of cell reaction in the spinal cord following nerve lesion is not clear from this study. In order to find out a potential role of substances involved in neurodegeneration, we examined NOS immunoreactivity in the cord after the peripheral nerve lesion. Our results point out a marked upregulation of NOS immunoreactivity in the spinal cord following 8 weeks after the lesion. This upregulation was closely correlated with the lesion site. However the NOS positive neurons can be found widely distributed in both sensory and motor areas of the cord. This observation suggest that NO

production from activated NOS is some how involved in the probable mechanisms of nerve lesion induced neurodegeneration.

NO is a free radical gaseous molecule which is synthesised by the enzyme nitric oxide synthase (NOS) from the L-Arginine (Dawson and Dawson, 1996). Thus an upregulation of NOS is considered to be mainly responsible for NO production (Kimura and Steinbusch, 1996). There are reports that activated NOS thus produce NO which is mainly contributing to the cell injury (Dawson and Dawson, 1996; Sharma et al., 1996b). This is further evident with the fact that ischemia, infarction, stroke and trauma are able to induce upregulation of NOS in areas showing cell injury or necrosis (for details see Dawson and Dawson, 1996).

Further evidence of NOS induced cell injury came from the observations that inhibitors of NOS are mainly neuroprotective in nature. However, not all NOS inhibitors are neuroprotective (Sharma et al., 1998). It appears that the dose and drug type plays important role. Thus L-NAME can induce neuroprotection in some cases of ischemia and stroke depending on doses. However in many other cases this compound is not effective in achieving neuroprotection.

This discrepancy in dose and effect relationship is not yet clear. Few reports suggest that at high doses of L-NAME, it is possible that endothelial isoform of NOS can also be inhibited which lead to aggravation of cell injury in animal models of ischemia (Dawson and Dawson, 1996). Thus use of low dose sometimes is associated with neuroprotection in animal models of cell injury.

To further confirm in our model that upregulation of NOS is some how involved in the neurodegeneration in the spinal cord, we used chronic L-NAME pretreatment. Since chronic L-NAME treatment was not done before in literature, we decided to initiate the treatment with a low dose of the compound (2 mg/kg, i.p. twice daily) commencing from 1 day before the nerve lesion until 3 days after the lesion and then reduced the dosage to 1 mg/kg once daily until 6 weeks after the nerve ligation.

In order to testify the efficacy of the dose of L-NAME we used NOS immunohistochemistry and examined the morphology of the spinal cord. Our results suggest that L-NAME in this dose did not however completely abolished the NOS immunohistochemistry. Thus NOS positive neurons can still be found in the spinal cord of nerve lesioned rats indicating that this dose of L-NAME was not sufficient enough to inhibit NOS production completely in these animals. Interestingly, in these animals the changes in spinal cord were significantly less compared to untreated animals. This observation strongly suggest that NOS upregulation is somehow related with the neurodegeneration obtained in this model of chronic neuropathic pain caused by spinal nerve lesion and further support the idea that the dose of L-NAME is important determinant of neuroprotection which depends on the ability of NOS inhibition.

Alternatively use of specific and prolonged inhibitors of NOS such 7-nitroindazole is needed ion this model to further characterise the relationship between NOS upregulation and neurodegeneration. Further studies are in progress to find out a role of other isoforms of NOS such as iNOS and eNOS in the mechanism of neurodegeneration.

In conclusion, our observations strongly indicate a putative role of NO as evident with NOS upregulation in the pathophysiology of chronic nerve lesion. Further studies using other neuroactive drugs and growth factors in this model may provide a new strategy to treat chronic neuropathic pain or to minimise neurodegeneration in the patients suffering from such diseases of the nervous system.From our study, it appears that NOS inhibtiors may be of some use in the treatment of chronic nerve lesion or pain mechaniasm as well as in neurodegenration. Furthermore, the Chung model of neuropathic pain using a peripheral nerve lesion seems to be a new promising model to study spinal cord neurodegeration.

Acknowledgements

This investigation was supported by grants from Swedish Medical Research council nr. 2710, 9077 and 11205. The skillful technical assistance is provided by Inga Hörte, Kärstin Flink and Ingmarie Olsson. The secretarial assistance of Aruna Misra is greatly appreciated.

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Received August 25, 1997