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Spinal nerve lesion induces upregulation of constitutive isoform of heme oxygenase in the spinal cord

An immunohistochemical investigation in the rat

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Summary. The influence of carbon monoxide (CO) on chronic spinal nerve lesion induced spinal cord neurodegeneration was examined using immunohistochemical expression of the constitutive isoform of its synthesising enzyme, hemeoxygenase-2 (HO-2) in a rat model. Spinal nerve lesion at L-5 and L-6 level was produced according to the Chung model of neuropathic pain and rats were allowed to survive for 8 weeks. Sham operated rats, in which the spinal nerves were exposed but not ligated, served as controls. Ligation of spinal nerves in rats resulted in an upregulation of HO-2 expression which was most pronounced in the ipsilateral gray matter of the spinal cord compared to the contralateral side. In these rats, morphological investigations showed distorted neurons, membrane disruption, synaptic damage and myelin vesiculation. Sham operated rats did not show an upregulation of HO-2 expression and the structural changes in the spinal cord were absent. These observations strongly suggest that spinal nerve lesion is associated with an increased production of CO which is somehow contributing to the neurodegenerative changes in the spinal cord, not reported earlier.

Keywords: Amino acids – Nerve lesion – Neuropathic pain – Heme oxygenase – Carbon monoxide – Cell injury – Immunohistochemistry

Introduction

Chronic neuropathic pain is a serious clinical condition that may arise following nerve lesions due to various causes, including surgery, trauma, infections, spinal or cerebral lesions (Hökfelt et al., 1994; Steel et al., 1994;

Niedbala et al., 1995; Karlsten and Gordh, 1997). Until now, no suitable treatment regimen has been developed. There are previous reports that chronic neuropathic pain following peripheral nerve lesion is associated with trans-synaptic neurodegenerative changes in the spinal cord (Hökfelt et al., 1994; Gordh et al., 1998). Modulation of several neurochemicals in the dorsal horn occurs following lesion of either dorsal nerve roots, ganglia or spinal nerves (Hökfelt et al., 1994). Thus, a possibility exists that neurochemical alterations or their receptor function will somehow lead to the morphological changes in the spinal cord (Dubner and Ruda, 1992). Probably, these neurodegenerative changes can also contribute to the pathobiology of the neuropathic pain (Karlsten and Gordh, 1997). Thus, further studies in chronic neuropathic pain is highly warranted in order to understand the underlying molecular mechanisms.

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; Hökfelt et al., 1994). 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 segment (Steel et al., 1994). It is these peripheral and central neuronal changes which are thought to lead to the hyperalgesia. The specific mechanism(s) underlying this central or peripheral plasticity are unclear but are supposed to involve changes in chemical mediators which influence synaptic transmission in the spinal cord.

Previous studies suggest that models of chronic neuropathic pain can be achieved in experimental animals, e.g., rats by lesion or ligation of peripheral spinal nerves of L5 and L-6 segments (Gordh et al., 1998). These nerve lesioned animals exhibit symptoms of chronic neuropathic pain, such as hyperalgesia, during 4 to 8 weeks after the nerve lesion (Kim and Chung, 1992).

In order to understand the effects of a peripheral nerve lesion on spinal cord neurodegeneration and neuropathic pain, our laboratory is engaged to examine changes in several neurochemicals and their receptor expression using immunohistochemistry, and to relate these changes with morphological alterations in the spinal cord using light and electron microscopy (Gordh et al., 1998).

We observed clear neurodegenerative changes in the spinal cord following nerve lesion (Gordh et al., 1998). Thus, it is interesting to undertake a detailed study underlying the molecular mechanisms of spinal cord neurodegeneration in our rat model. Previously, we have reported involvement of nitric oxide (NO) in the neurodegeneration caused by nerve lesion (Gordh et al., 1998). Our results showed a marked upregulation of nitric oxide synthase (NOS) in the ipsilateral side of the nerve lesion which correlates well with the degenerative changes in the spinal cord. These morphological changes were markedly attenuated by pretreatment with L-NAME indicating that NO is somehow involved in the spinal cord neurodegeneration. By other groups it has been shown that NOS inhibition induce antinociception in neuropathic

pain models (Niedbala et al., 1995) and that pain stimuli is accompanied by an increase in NO production in corresponding spinal cord segments (Steel et al., 1994). These data suggest a possible link between NO production and pain in addition to its involvement in neurodegenerative disease processes.

Since carbon monoxide (CO) has recently been discovered as a similar free radical gas like NO (Chiueh et al., 1994; Dawson and Snyder, 1994; Sharma et al., 1998a; Sharma, 1999), the present study was undertaken to find out whether neurodegenerative changes following spinal nerve lesion are somehow influenced by CO production. There are reports that CO could contribute to neurodegenerative changes in the CNS, if produced either alone or together with NO (Sharma et al., 1997c; 1998a,b). In the present investigation, we examined the constitutive isoform of the heme oxygenase enzyme (HO-2) which reflects an increased CO production using immunohistochemistry following spinal nerve lesion in rats after 8 weeks of survival period. The sham operated group served as controls. In addition, the neurodegenerative changes were also examined in these animals using standard morphological investigation. For comparison, one complete normal group of rats was also included.

Materials and methods

Animals

Experiments were carried out on 14 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; Gordh et al., 1998). 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

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.

Parameters measured

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

HO-2 immunohistochemistry

The HO-2 immunohistochemistry was done on L-5 segment of the spinal cord using free floating Vibratome sections (60μm thick) using a monoclonal antibody raised against constitutive isoform of HO enzyme (StressGene Canada) (n = 5) (for details see Sharma et al., 1997c; 1998a).

Morphological study

Some tissue pieces from the L-5 segment of the cord (n = 4) were embedded in epon for routine light and electron microscopy for structural investigation as described earlier (Sharma et al., 1998a,b). In brief, for high resolution light microscopy, about 1μm thick sections were cut and stained with toluidine blue and examined under a 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., 1998a).

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

Effect of spinal nerve lesion on HO-2 immunohistochemistry

Ligation of spinal nerves in rats resulted in an upregulation of HO-2 expression which was most pronounced in the ipsilateral gray matter of the spinal cord compared to the contralateral side. Sham operated rats did not show HO-2 upregulation (Fig. 1). The immunostaining was confined within the neuronal cytoplasm, however occasionally, the nerve cell nucleus was also stained (Fig. 2).

Effect of spinal nerve lesion on spinal cord morphology

Morphological investigations showed marked neurodegenerative changes in the spinal cord following spinal nerve lesion. These changes were most pronounced in the ipsilateral gray and white matter (Fig. 3). Thus, vacuolation of neuronal cytoplasm, degeneration of myelin and distorted neurons were quite frequent in the lesioned animals compared to sham operated group.

Discussion

Clinically it is well known that peripheral or central lesions in sensory neural structures may induce so called neuropathic pain (Dubner and Ruda, 1992). It is characterised by sensory disturbances in the affected part of the body, and also spontaneous burning pain, and pain evoked by normally non-painful

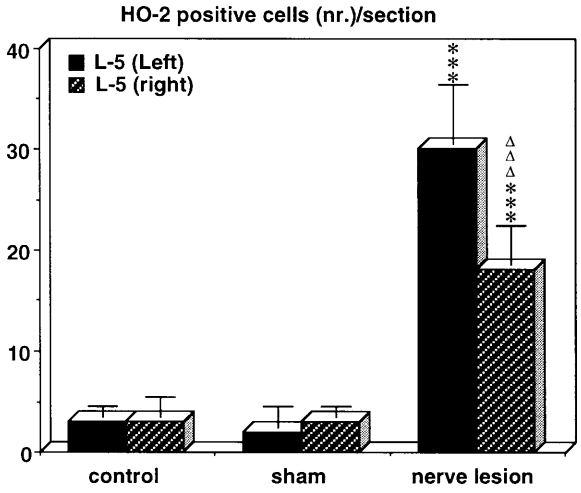

Fig. 1. Semiquantitative analysis of HO-2 immunostaining in the spinal cord following spinal nerve lesion. *** = $P < 0.001$, compared from control group; ΔΔΔ = $P < 0.001$ compared from ipsilateral lesioned side

stimuli, such as touch or temperature changes (Karlsten and Gordh, 1997). Neuropathic pain does not respond well to conventional analgesics, and is often resistant also to strong opioids such as morphine. Neuropathic pain sometimes can be treated by antidepressants associated with increasing CNS serotonin and norepinephrine levels, or by antiepileptics which are well known sodium channel blockers (Karlsten and Gordh, 1997). But in many clinical cases it is not possible to relieve the pain. Thus, new treatment strategies must be sought.

Keeping these views in consideration, we have used a rat model for neuropathic pain to analyse changes in spinal cord morphology induced by peripheral nerve lesion in relation with expression or contribution of several neurochemicals or neuromodulators. The salient new findings of the present study revealed that increased production of CO is somehow associated with the neurodegenerative changes in the experimental model of chronic neuropathic pain. To our knowledge, this is the first report which showed an involvement of CO in the pathophysiology of chronic spinal nerve lesion. We used immunohistochemical expression of HO-2 enzyme to evaluate formation of CO in the spinal cord (Ewing and Maines, 1991; Fukuda et al., 1996). Previous reports clearly show that upregulation of HO is associated with CO production (Verma et al., 1993; Sharma et al., 1998a; Sharma, 1999). Thus it seems likely that increased formation of CO in spinal cord following nerve lesion is associated with neurodegenerative changes in the cord.

Our observation is limited to the survival period of 8 weeks. Thus it is not clear from this study whether these neurodegenerative changes or CO production 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

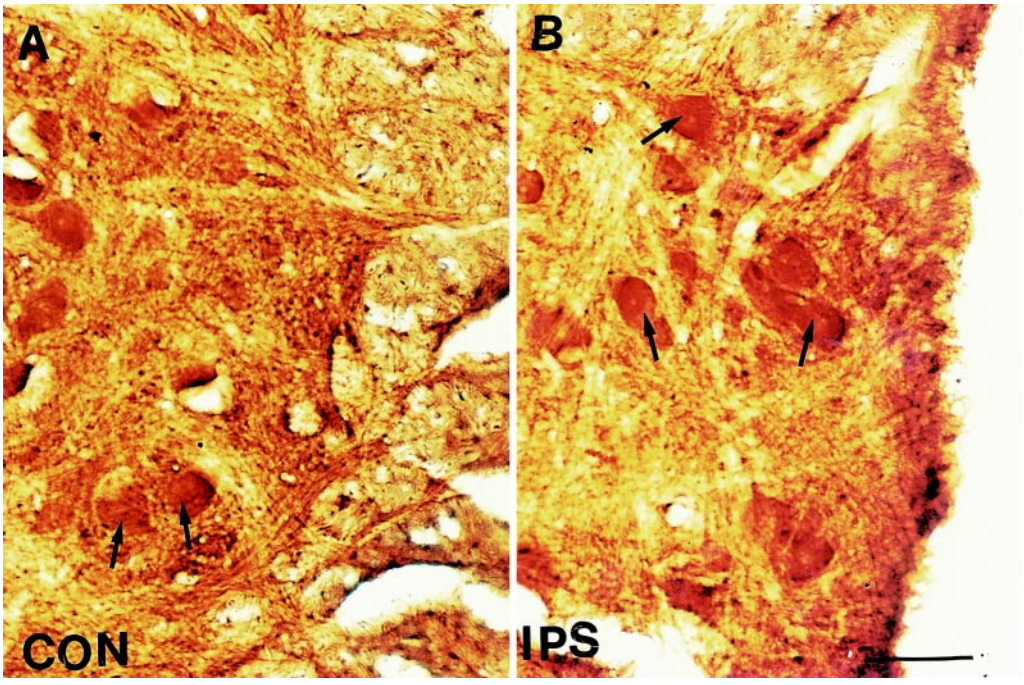

Fig. 2. A representative example of HO-2 immunostaining (arrows) in the contralateral (*CON*) side (**A**) and in the ipsilateral (*IPS*) side (**B**) of the L-5 segment of the spinal cord following spinal nerve lesion (bar = 30 μm)

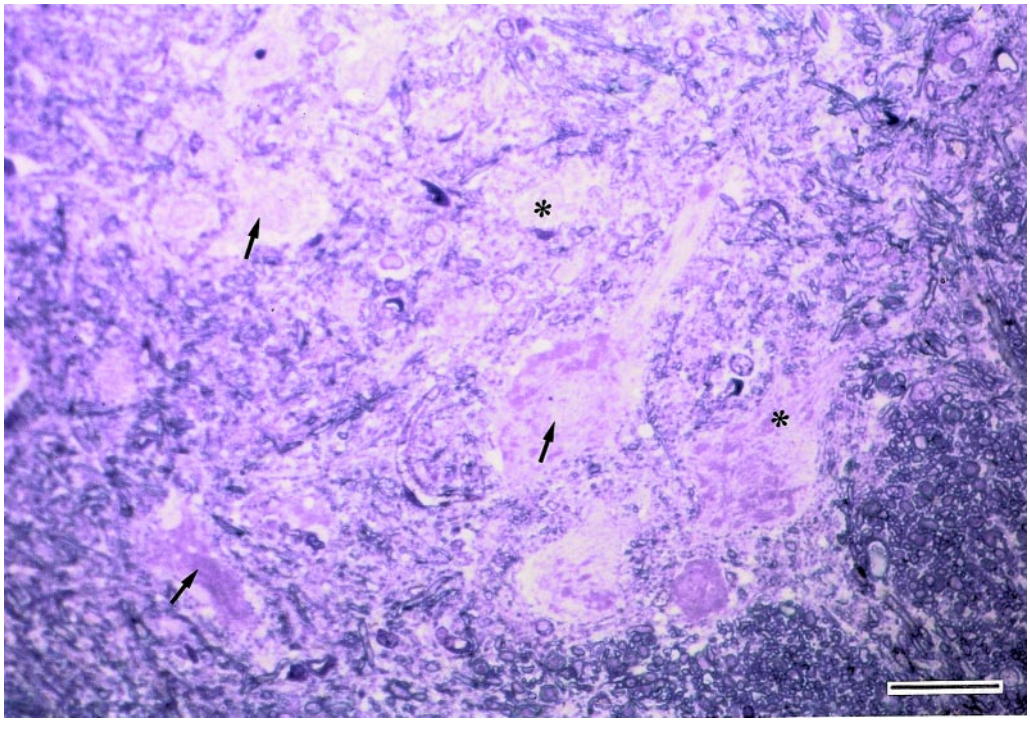

Fig. 3. High power light micrograph from ipsilateral ventral horn in one spinal nerve lesioned rat. Many neurodegenerative changes (arrows) and signs of membrane damage and vacuolation (*) are evident (bar = 100 μm)

further added the information that the spinal cord itself shows marked cell changes corresponding to the nerve lesioned 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 in the spinal cord would represent a general phenomenon or if it is limited to a focal change in the cord, a feature which require further investigation.

Clearly, we do not yet know the full story of the neurochemical pathology in the CNS, which must be the underlying cause of the neuropathic pain. A peripheral nerve lesion gives rise to pronounced changes in the expression of neuropeptides and other transmitters substances of the dorsal root ganglion, as well as in the dorsal horn cells (Hökfelt et al., 1994). The lesion actually gives rise to a new phenotype in the lesioned nerve cell, and also transsynaptically. The "neurochemical key" to neuropathic pain probably is to be found in changes of the expression of transmitters and receptors, and a deeper insight should be the basis for development of new treatment modalities. It may be that the changes seen are unrelated to the pain, and could be due to the responses to nerve lesion as such. Thus future studies must combine morphological, neurochemical and behavioural data to understand this complex problem.

The possible mechanisms by which CO is involved in neurodegeneration is not clear from this study. However, available evidences suggest that CO is a free radical gas which can induce a direct membrane damage or induce a series of events leading to lipid peroxidation which may cause neurodegenerative changes in the cord (Ewing and Maines, 1991; Verma et al., 1993). To further testify this use of antioxidants drugs are needed in this model to understand the contribution of oxidative stress, free radicals and lipid peroxidation in cell injury following spinal nerve lesion. In earlier studies, we have found that NOS is significantly upregulated in the dorsal horn following such an experimental nerve lesion, paralleling the hypersensitivity, and that drugs inhibiting NOS gives "pain relief" in this model.

Like CO, NO is a free radical gaseous molecule which is synthesised by the enzyme NOS (Chiueh et al., 1994; Dawson and Snyder, 1994; Dawson and Dawson, 1996). An upregulation of NOS is considered to be mainly responsible for NO production (Verma et al., 1993). There are reports that activated NOS thus produce NO which is mainly contributing to the cell injury (Dawson and Dawson, 1996; Sharma et al., 1998a). The idea that NO is contributing to cell damage was further examined in this model by using chronic L-NAME treatment. L-NAME which an inhibitor of NOS enzyme when given repeatedly, upregulation of NOS and cell injury were significantly reduced (Gordh et al., 1998). These observations clearly suggest that production of NO is related with neurodegeneration in the spinal cord.

Since, in in vivo situations, no single chemical compound or factor is solely responsible for cell injury, it appears that many factors are working in synergy to produce cell damage in a clinical or experimental situation involving nerve lesion (Sharma, 1998; 1999). Thus, the present results show that upregulation of HO-2 expression which reflects an increased production of CO is contrib-

uting to cell damage in the spinal cord. Taken together, our results suggest that in the peripheral nerve lesion the induced upregulation of NOS and HO are probably working in synergy to induce neurodegeneration, a feature which however, require further investigation by using selective HO inhibitors. Since pronounced upregulation of HO takes place after nerve lesion, CO may have an important role in the pathophysiology of neuropathic pain. However, before this is proven as a causal factor, we must first establish that drugs which increase or reduce CO production really affects pain behaviour in the neuropathic pain models. Known HO inhibitors comprising metalloporphyrins have many undesirable and serious side effects (Fukuda et al., 1996; Sharma et al., 1998a). Thus, their use in vivo situation is quite limited at the moment.

In conclusion, our observations strongly indicate a putative role of CO as evident with HO-2 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 suitable HO inhibitors may be of some use in the treatment of chronic nerve lesion or pain mechanisms as well as in neurodegeneration.

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