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Neurotrophic factors influence upregulation of constitutive isoform of heme oxygenase and cellular stress response in the spinal cord following trauma

An experimental study using immunohistochemistry in the rat

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Summary. The influence of brain derived neurotrophic factor (BDNF) or insulin like growth factor-1 (IGF-1) on spinal cord trauma induced carbon monoxide (CO) production and cellular stress response was examined using immunostaining of the constitutive isoform of the hemeoxygenase (HO-2) enzyme and the heat shock protein (HSP 72 kD) expression in a rat model. Subjection of rats to a 5 h spinal trauma inflicted by an incision into the right dorsal horn at T10–11 segment markedly upregulated the HO-2 and HSP expression in the adjacent spinal cord segments (T9 and T12). Pretreatment with BDNF or IGF-1 significantly attenuated the trauma induced HSP expression. The upregulation of HO-2 was also considerably reduced. These results show that BDNF and IGF-1 attenuate cellular stress response and production of CO following spinal cord injury which seems to be the key factors in neurotrophins induced neuroprotection.

Keywords: Amino acids – Spinal cord injury – Heme oxygenase – Heat shock protein – Carbon monoxide – Growth factors – BDNF, IGF-1 – Immunohistochemistry – Cell injury – Spinal cord edema

Introduction

Neurotrophic factors are well known neuroprotective agents following various kinds of insults to the CNS (Cortman and Berchtold, 1998; Apfel, 1999; Dijkhuizen and Verhaagen, 1999). Long-term treatment with neurotrophins

attenuate ischemia, brain trauma and hypoxia induced neuronal damages (Schwab and Bartholdi, 1996; Lu and Waite, 1999; Oudega and Haag, 1999). Upregulation of neurotrophin receptors occurs following spinal cord injury (Schwab and Bartholdi, 1996), however, the functional significance of such finding is not well understood.

Neurotrophins can influence neurochemical transmission and improve cell to cell communication in order to reduce cellular stress or "shock" phenomena (Skaper and Walsh, 1998; Sharma, 1999). Alterations in secondary injury mechanisms via modulation of signal transduction pathways are also likely by neurotrophins (Oudega and Haag, 1999). Furthermore, neurotrophins can provide trophic support to nerve cells following injury (Lu and Waite, 1999). These properties of neurotrophins are supposed to play important roles in maintaining normal cell metabolism and function which is a key factor to achieve neuroprotection. However, detailed molecular mechanisms of neurotrophins induced neuroprotection is still not well known.

In clinical or experimental situations of trauma, many endogenous neuroprotective or neurodestructive elements are released which altogether play a detrimental role in cell injury (Schwab and Bartholdi, 1996; Stålberg et al., 1998). There are reports that following a focal spinal cord trauma several neurochemicals, ions, enzymes, molecules are released which influence the secondary injury cascade leading to cell injury (Winkler et al., 1998). In addition, oxidative stress, generation of free radicals and lipid peroxidation significantly contribute to neurodegeneration caused by spinal trauma (Tator and Fehlings, 1991; Schwab and Bartholdi, 1996; Sharma et al., 1998a,b). It seems likely that neurotrophins influence secondary injury cascade by attenuating traumatic stress or "shock" phenomena, and/or neutralising the influence of endogenous neurodestructive factors to enhance cell survival.

Recently, carbon monoxide (CO), a newly discovered free radical gaseous molecule is known to influence CNS functions by modulating cell to cell communication (Applegate et al., 1991; Abraham et al., 1996). However, its involvement in the pathophysiology of spinal cord injury is not well known. CO is synthesised from the enzyme hemeoxygenase (HO) which occurs in two isoforms, the constitutive (HO-2) and the inducible (HO-1) type (Ewing and Maines, 1991). Ex-vivo studies using brain slices show that HO inhibitors significantly attenuate edema formation following cortical injury (Fukuda et al., 1996). This indicates that upregulation of HO is harmful in nature.

Upregulation of HO-1 occurs in the glial cells following 6 to 12 h after the fluid percussion injury in the rat brain (Fukuda et al., 1996). An upregulation of HO-2 is seen following 4 h heat stress induced hyperthermia (Sharma et al., 1998c). These observations suggest that upregulation of HO-1 and HO-2 occurs in the CNS following noxious stimuli to the brain. Upregulation of HO is mainly associated with increased CO production (Verma et al., 1993). Since CO is a free radical, it seems likely that increased production of CO following upregulation of HO-1 or HO-2 will somehow contribute to the cell damage.

The present investigation was undertaken to characterise the role of HO in cell injury, and to investigate whether topical application of two potent neuroprotective members of the neurotrophin family, brain derived nerve growth factor (BDNF) and insulin like growth factor-1 (IGF-1) (Apfel, 1999), can influence HO-2 expression in spinal cord injury. In addition, the influence of neurotrophins on trauma induced cellular stress was also examined.

Materials and methods

Animals

Experiments were carried out on 30 male Sprague-Dawley rats (200–230 g) kept at controlled room temperature 21 ± 1°C with 12 h light and 12 h dark schedule. The rat food pellets and tap water were supplied ad libitum.

Spinal cord injury

Under Equithesin anaesthesia (3 ml/kg, i.p.) one segment laminectomy was done over the T10–11 segment. Spinal cord injury was made by making a longitudinal incision of the right dorsal horn at the T10–11 (about 2 mm deep and 5 mm long) (Sharma et al., 1991; 1996). Bleeding from the spinal cord was cleaned with cotton soaked saline and the surface of the exposed cord was covered with the same in order to avoid a direct exposure of the cord to air. Normal rats served as controls. This experimental condition is approved by the Ethical Committee of Banaras Hindu University, Varanasi, India and Uppsala University, Uppsala, Sweden.

Treatment with the BDNF or IGF-1

Separate group of animals were treated with BDNF (n = 5) or IGF-1 topically by applying the growth factor in a concentration of 0.1 μg in 10 μl starting from 30 min before injury, immediately after injury, followed by 30 min, 60 min, 120 min, 180 min and 240 min following trauma as described earlier (Sharma et al., 1997; 1998a,b).

HO-2 immunohistochemistry

Five h after injury, both treated (n = 10) and untreated rats (n = 10) were perfusion fixed and the spinal cord segments comprising T9 to T12 were removed and analysed for HO-2 immunostaining according to the standard protocol (Sharma et al., 1998c; Sharma, 1999). In brief, monoclonal HO-2 antibodies (1:500, StressGene, Canada) were applied on the free floating 40 μm thick vibratome sections obtained from the T9 and the T12 segments with constant agitation at the room temperature. The immunoreaction was developed using peroxidase-antiperoxidase reaction. The HO-2 positive cells, if any, were visualised and analysed by two independent observers and photographed using light microscopy at ×80 to 120 magnification.

Spinal cord edema

Edema of the spinal cord was analysed using water content of the cord from the differences in the wet and dry weight of the sample as described earlier (Sharma et al., 1991).

Blood-spinal cord barrier permeability

The BSCB permeability was examined using Evans blue albumin and $^{[131]}$I-sodium (Sharma et al., 1998a). Both tracers were administered into the right femoral vein through needle puncture. The intravascular tracer was washed-out by perfusion through heart 5 min after its initial administration. Immediately before perfusion, one ml of whole blood was collected from the left ventricle through needle puncture for determination of the blood radioactivity at the time of sacrifice (Sharma, 1999).

Heat shock protein immunoreactivity

In order to determine traumatic stress response and its modification with neurotrophins, heat shock protein (HSP-72) expression in the T9 segment of the spinal cord was examined on free floating Vibratome sections (40 μm thick) using a commercial antiserum (StressGene, Canada; Affiniti, UK) (Sharma and Westman, 1997; Sharma, 1997). The immunostaining was developed using peroxides-antiperoxidase technique and the control, spinal cord injured and neurotrophins treated control or spinal cord injured were processed for HSP immunostaining in parallel.

Statistical analysis

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

Results

Effect of BDNF or IGF-1 on HO-2 immunohistochemistry

Normal rats showed only few HO-2 positive cells in the spinal cord. Subjection of rats to 5 h spinal cord trauma significantly increased the expression of HO-2 immunostaining in the cord compared to the control group (Fig. 1). This increase in HO-2 immunostaining was most pronounced in the ipsilateral cord of the T9, T10–11 and the T12 segments. The HO-2 expression was mainly located in the gray matter. Pretreatment with BDNF or IGF-1 significantly attenuated the number of HO-2 positive nerve cells and dendrites (Fig. 2) compared to untreated traumatised group. However, neurotrophins treated normal rats did not show any change in HO-2 expression (Fig. 1).

Effect of BDNF or IGF-1 on spinal cord edema formation and cell injury

Untreated traumatised rats exhibited profound increase in the spinal cord water content in the T9, T10–11 and the T12 segments. Untreated injured rats show many distorted nerve cells. Edematous expansion and sponginess of the cord is most pronounced in the vicinity of the lesion site.

Pretreatment with BDNF or IGF-1 significantly attenuated spinal cord water content (Fig. 3) and reduced cell injury. Edematous expansion of the cord and nerve cell distortion were much less pronounced in neurotrophins treated injured rats.

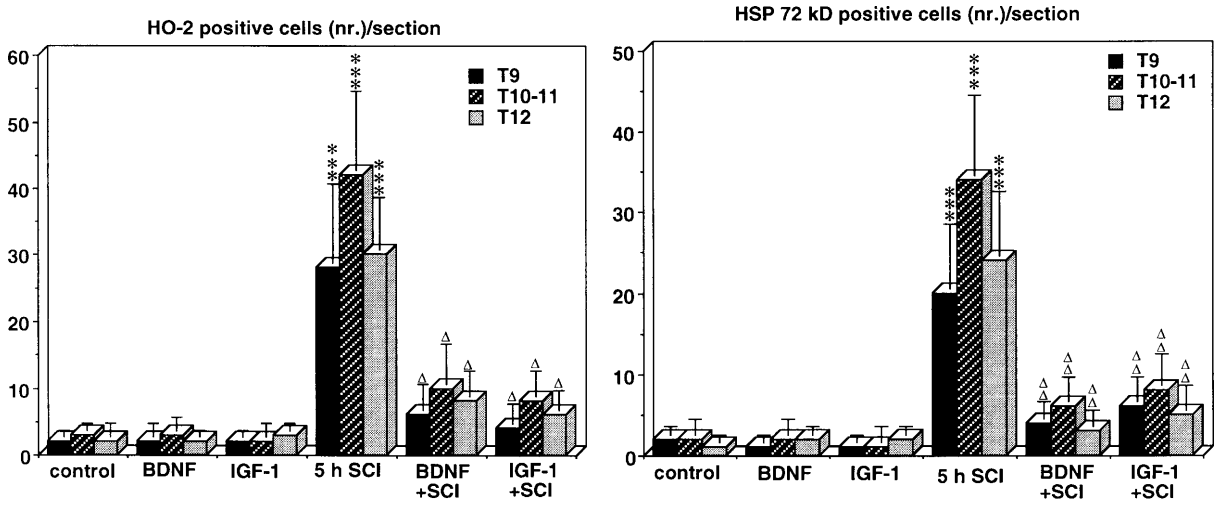

Fig. 1. A semiquantitative analysis of HO-2 immunostaining (left) and HSP immunolabelling (right) in the spinal cord of normal or spinal cord traumatised rats and their modification with BDNF or IGF-1 treatment. ***$P < 0.001$, compared from control group; $\Delta = P < 0.05$, $\Delta\Delta = P < 0.01$, compared from spinal cord injured (*SCI*) group, Student's unpaired t-test

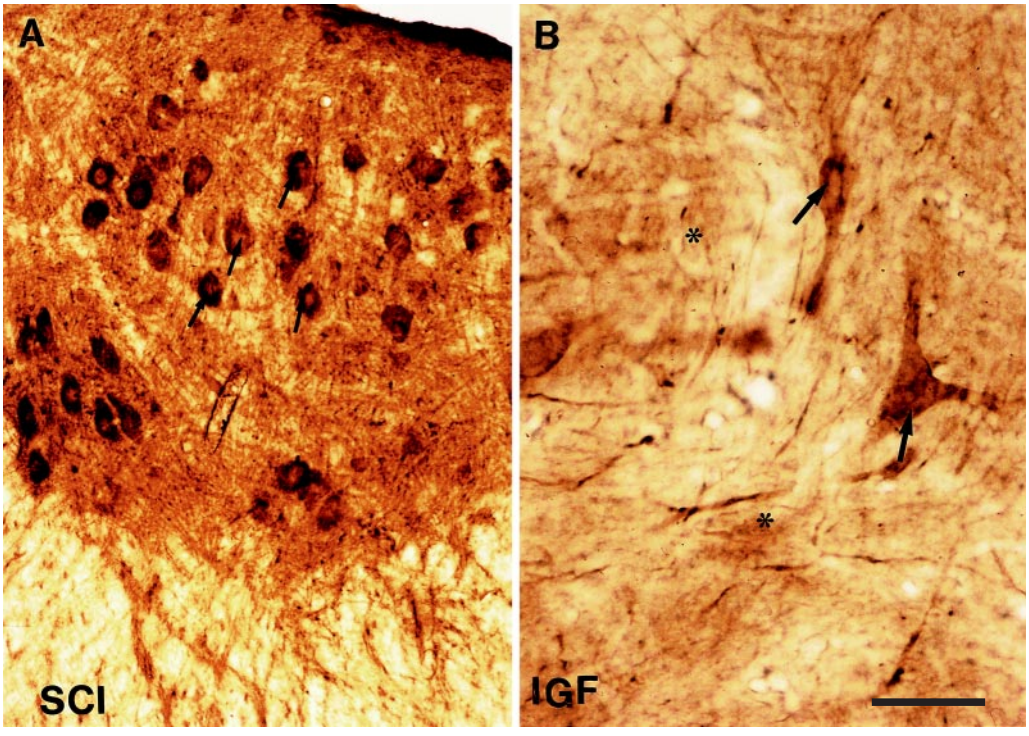

Fig. 2. A representative example of HO-2 immunostaining (arrows) in the spinal cord of a 5h untreated traumatised (*SCI*) rat (**A**) and its modification with IGF-1 (*IGF*) pretreatment (**B**). HO-2 labelling is mainly absent (*) in the IGF treated traumatised rat (bar = **A** = 40μm; **B** = 25μm)

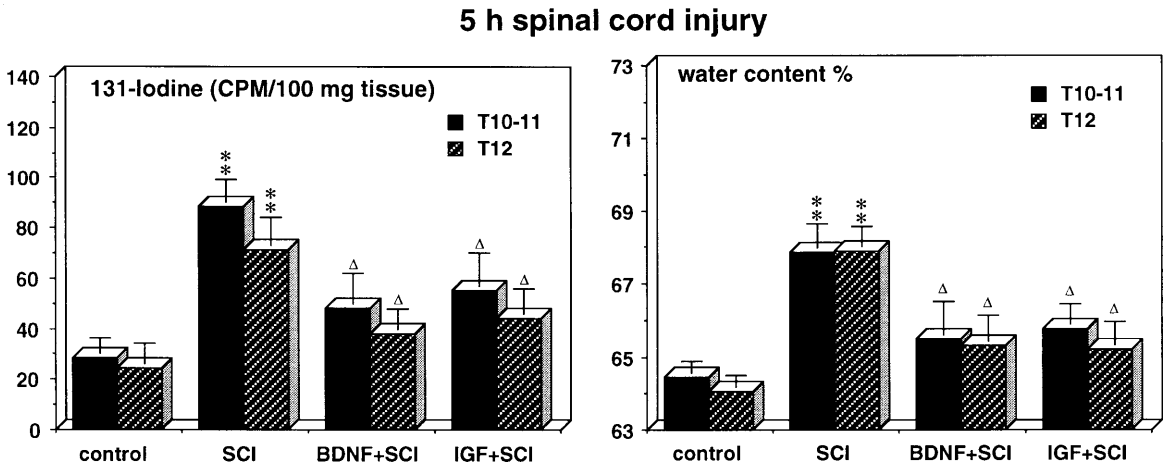

Fig. 3. Effect of BDNF or IGF-1 pretreatment on the blood-spinal cord barrier (BSCB) permeability (left) and spinal cord water content (right) following spinal cord injury. **P < 0.01, compared from control; $\Delta = P < 0.05$, compared from spinal cord injury (*SCI*); Student's unpaired t-test

Effect of BDNF or IGF-1 on blood-spinal cord barrier permeability

Untreated traumatised rats exhibited a profound increase in the permeability of radioactive iodine in the T9, T10–11 and the T12 segments of the spinal cord. This increase in the radiotracer extravasation is significantly reduced by pretreatment with neurotrophins (Fig. 3).

Effect of BDNF or IGF-1 on HSP immunohistochemistry

Spinal cord injury markedly upregulated HSP 72 expression in the spinal cord. This expression of HSP was most pronounced in the nerve cells and occasionally nerve cell nucleus was also stained. Topical application of BDNF or IGF-1 significantly attenuated the HSP expression in the spinal cord following trauma (Fig. 2). Thus only a few spinal cord axons and dendrites could be seen labelled with HSP immunostaining, whereas nerve cell reaction of HSP expression is markedly absent in neurotrophins treated and traumatised rats (Fig. 4, see p. 358).

Discussion

The present results show that BDNF or IGF-1 has the capacity to induce neuroprotection following a focal spinal cord injury. Our observations suggest that neurotrophins apart from ischemia, brain injury and hypoxia (Schwab and Bartholdi, 1996), can enhance cell survival following trauma to the spinal cord. Taken together, these observations indicate that the mechanisms of cell injury occurring either following ischemia, hypoxia or trauma are quite similar in nature.

We have applied growth factors repeatedly over the exposed surface of the spinal cord. Topical application of BDNF or IGF-1 will result in a direct access into the spinal cord tissue and thus enhance the delivery of these neurotrophins to the cells exposed to the trauma induced adverse cellular or fluid microenvironment. Since topical application of growth factors in the present study exhibited profound neuroprotection, it appears that delivery of neurotrophic factors within the traumatised zone is necessary for the beneficial effects (Sharma et al., 1998a; Oudega and Haag, 1999). Previously, continuous delivery of growth factors through minipumps implanted into the skin, or infused directly into the blood stream over long periods, are needed to attain neuroprotection (Dijkhuizen and Verhaagen, 1999; Lu and Waite, 1999). Our study suggest that topical application of growth factors in a much less quantity is equally effective. Thus, topical application or local administration of growth factors seems to be a promising approach for neuroprotection in CNS injuries, a feature which require additional investigation.

Our study used only two growth factors BDNF and IGF-1 in relatively high doses in the vicinity of the traumatised zone and we got good neuroprotection in our model of spinal cord injury. Thus, high concentration of growth factors at the injury site is important in inducing neuroprotection with growth factors. Continuous slow infusion of BDNF and IGF may have some neuroprotective effects in the CNS following injury, however, other growth factors administered in same way such as NGF, NT-3 or NT-4, GDNF or CTNF may have some other effects as well (Schwab and Bartholdi, 1996; Stålberg et al., 1998).

Normally growth factors have some access across the BBB however (Dijkhuizen and Verhaagen, 1999). It is not certain whether the permeability of growth factors can be rendered less effective in case of edematous swelling or microhaemorrhages within the CNS tissues (Sharma et al., 1996). Alternatively, systemic injection of growth factors will also be vulnerable to metabolic degradation rapidly (Dijkhuizen and Verhaagen, 1999). Our study demonstrates that maintenance of a high concentration of growth factors is crucial at injury site in exhibiting neuroprotection. Our study, however, does not shed any light on the additional factors which may influence the binding of these growth factors to the traumatised or untraumatised cells containing receptors and thereby influencing secondary signal transduction mechanisms.

It seems quite likely that neurotrophic factors influence secondary injury mechanisms following trauma. Previous studies from our laboratory suggest that neurotrophic factors attenuate NOS upregulation in the spinal cord (Sharma et al., 1998a,b). Upregulation of NOS which is known to generate NO, a well known free radical, will induce a direct damage to the cell (Verma et al., 1993; Hökfelt et al., 1994). Neurotrophins pretreatment attenuated the NOS upregulation following spinal cord injury (Sharma et al., 1997; 1998a,b). Thus it seems feasible that neurotrophins induced attenuation of NO production is related with beneficial effects of neurotrophins. The present results further suggest that similar neurotrophins treatment have also attenuated CO production following trauma. These observations clearly suggest that like NO, CO is also involved in the cell injury mechanisms following trauma to the spinal cord

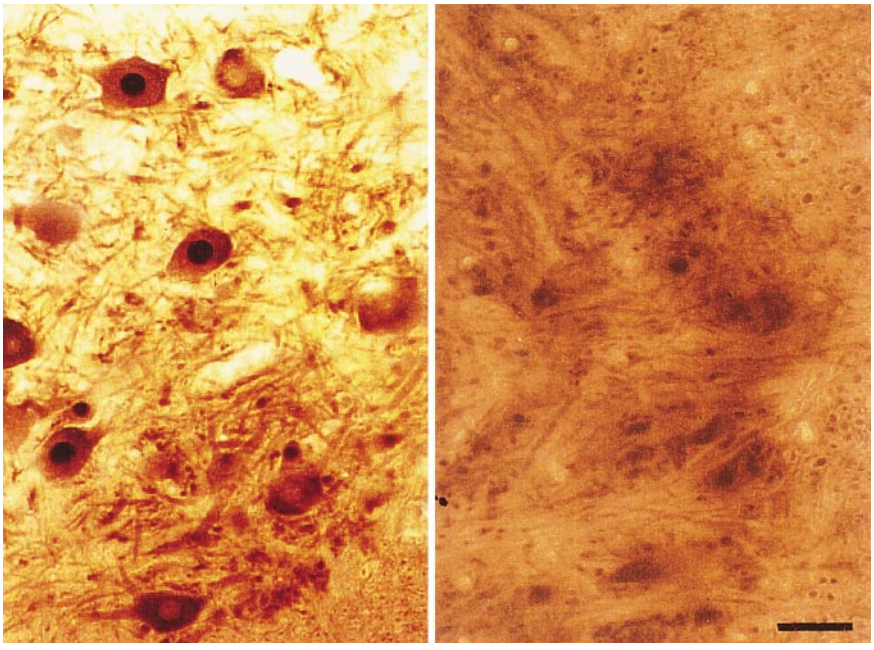

Fig. 4. HSP 72 kD immunostaining in 5 h spinal cord injury and its modification with BDNF pretreatment (bar = 30 μm)

(Sharma et al., 1998c). Since CO is also a free radical gas and neurotrophins induced downregulation of HO-2 expression seems to be related with the neurotrophins induced neuroprotective activity.

The mechanisms of spinal cord injury induced upregulation of HO-2 expression is not clear from this study. However, it appears that release of several neurochemicals, oxidative stress, generation of free radicals and lipid peroxidation following spinal cord trauma play important roles (Abraham et al., 1996; Sharma, 1999). Increased expression of HO-1 occurs following 6 to 12 h after brain injury (Fukuda et al., 1996). This increase in HO-1 expression seems to be related with cellular stress caused by trauma (Sharma and Westman, 1997). This assumption gets further support with the fact that one member of the stress protein family, the heat shock protein 27 (HSP 27) is very similar to HO-1 regarding its molecular characteristics, structure and function (for details see Sharma, 1998). Thus, trauma induced cellular stress could be one of the important triggering factors for HO induction in the cord.

Since neurotrophins are able to improve cell to cell communications and modulate neurochemical transmission within the CNS, a possibility exists that application of neurotrophins will alter fluid microenvironment of the cord as well as cellular stress reaction following trauma. That neurotrophins influence

spinal microfluid environment following trauma is clearly evident from the results obtained with BSCB permeability investigations in the present study. A significant reduction in the BSCB permeability with neurotrophins treatment following trauma suggest that neurotrophins somehow influence spinal cord fluid microenvironment following injury.

A breakdown of the BSCB following trauma may result either from release of several neurochemicals via neurochemical receptors, or due to a direct damage of endothelial cell membrane following release of free radicals, and or due to activation of specific intracellular signal transduction mechanisms activated by cellular stress (Schwab and Bartholdi, 1996; Sharma et al., 1998a). Cellular stress is one of the potent factor in disruption of the BBB permeability following several emotional stressors, ischemia and brain injury (Sharma et al., 1998c). Thus, it seems likely that cellular stress is playing equally important role in trauma induced breakdown of the BSCB permeability (Tator and Fehlings, 1991). Obviously, a reduction in stress response by neurotrophins will result in a significant reduction in the BSCB permeability.

That pretreatment with neurotrophins reduces cellular stress response following trauma is further supported by the observations of HSP 72 kD expression following spinal cord injury. The HSP immunoreaction is a well known measure of cellular stress and upregulation of HSP 72 kD occurs following brain or spinal cord trauma (Sharma and Westman, 1997). A significant attenuation of HSP expression following trauma to the cord in neurotrophins treated animals suggest that neurotrophins are able to attenuate trauma induced cellular stress considerably. Obviously, a reduction in cellular stress will reduce the consequences of trauma induced events leading to cell injury.

The influence of neurotrophins on oxidative stress or NO or CO production is not examined in this study. Previous reports from our investigations suggest that neurotrophins somehow attenuate production of NO by inhibiting NOS upregulation following spinal cord trauma (Sharma et al., 1998a,b). The present results further suggest that neurotrophins are also able to attenuate HO-2 upregulation. This indicates that neurotrophins may somehow also inhibit CO production, not reported earlier. These observations suggest that probably NO and CO are involved synergistically in cell injury caused by spinal trauma. Taken together, our results suggest that CO is injurious to the cell in the spinal cord following trauma.

In conclusion, our results for the first time show that neurotrophins are associated with inhibition of HO-2 expression in spinal cord following trauma. Our observations suggest that upregulation of HO-2 which is responsible for CO production is injurious to the spinal cord and neurotrophins offer significant neuroprotection in spinal cord following injury. Further studies in our laboratory are in progress to find out the suitable time frame when application of neurotrophins after injury is still neuroprotective. This will allow us to examine the therapeutic potential of neurotrophins for the treatment of spinal cord injuries in future.

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