Amino Acids (2002) 23: 261–272
DOI 10.1007/s00726-001-0137-z

Amino
Acids
Printed in Austria

A new antioxidant compound H-290/51 modulates glutamate and GABA immunoreactivity in the rat spinal cord following trauma

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Received July 3, 2001
Accepted August 6, 2001
Published online July 31, 2002;

Summary. The involvement of the excitatory amino acid glutamate and the inhibitory amino acid gamma-amino butyric acid (GABA) in the pathophysiology of spinal cord injury is not known in details. This investigation is focused on the role of glutamate and GABA in a rat model of spinal cord trauma using immunohistochemistry. Spinal cord injury produced by a longitudinal incision of the right dorsal horn of the T10–11 segments resulted in profound edema and cell damage in the adjacent T9 segment at 5 h. Pretreatment with H-290/51 (50 mg/kg, p.o.), a potent antioxidant compound, effectively reduced the blood-spinal cord barrier (BSCB) permeability, edema formation and cell injury following trauma. At this time, untreated traumatised rats exhibited a marked increase in glutamate immunoreactivity along with a distinct decrease in GABA immunostaining in the T9 segment. These changes in glutamate and GABA immunoreactivity in traumatised rats were considerably attenuated by pretreatment with H-290/51. These results suggest that (i) oxidative stress contributes to alterations in glutamate and GABA in spinal cord injury, (ii) glutamate and GABA are important factors in the breakdown of the BSCB, edema formation and cell changes, and (iii) the antioxidant compound H-290/51 has a potential therapeutic value in the treatment of spinal cord injuries.

Keywords: Glutamate – GABA – Spinal cord injury – Immunohistochemistry – Antioxidant – H-290/51 – Ultrastructure – Edema – Blood-spinal cord barrier

Introduction

The pathophysiology of spinal cord injury is a complex phenomenon (Schwab and Bartholdi, 1996; Winkler et al., 1998). Clinical or experimental spinal cord injury initiates a series of secondary injury cascade that includes ischemia, disruption of the blood-spinal cord barrier (BSCB) permeability followed by necrosis and inflammation (Sharma and Olsson, 1990; Sharma et al., 1990, 1995a,b, 1998a, Stålberg et al., 1998; Sharma 2000). This secondary injury induced changes are mediated by several neurochemical and biochemical factors (Winkler et al., 1998). Most important biochemical factors are products of membrane breakdown like arachidonic acids, leukotrienes, thromboxane that contribute to lipid peroxidation and formation of free radicals (Cuzzocrea et al., 2001). In addition, several other neurochemicals such as, monoamines, neuropeptides and excitatory amino acids are also involved in these processes. One of the early changes seen within minutes following spinal cord injury is the release of glutamate and aspartate (Liu et al., 1991). It is believed that increased release of glutamate contributes to cell injury via intracellular accumulation of Ca^{2+} and activation of N-methyl-D-aspartate (NMDA) receptors (Schwab and Bartholdi, 1996).

The glutamate acts as a primary afferent neurotransmitter in the spinal cord (Broman, 1994; Danbolt, 2001) in both large myelinated primary afferent fibres (Salt and Hill, 1983), as well as in thinly myelinated and unmyelinated fibres (Schneider and Perl, 1988; Zhong et al., 2000). Using anterograde transport of horseradish peroxidase technique, the presence of glutamate is detected in primary afferent terminals in the laminae I and III–V (Broman et al., 1993) as well as in the deep dorsal horn (Valtschanoff et al., 1994). In these terminals, the levels of glutamate were 2–3 times higher than the levels detected in the inhibitory terminals. The glutamate uptake is diminished in the dorsal horn following dorsal rhizotomy or by blockade of transmission between primary afferent terminals and dorsal horns neurons using excitatory amino acids receptor antagonists (Willis and Coggeshall, 1991; Broman et al., 2000).

In the spinal cord, glutamate is co-localised with many other neurotransmitters (Danbolt, 2001). The glutamate is present together with glycine and GABA in axon terminals of dorsal horn interneurons (Maxwell et al., 1997). High levels of glutamate are also present in the corticospinal terminals in the dorsal horn (Valtschanoff et al., 1993). The glutamate is also present in the spinal cord neurons known to be positive for GABA, monoamines and acetylcholine (Somogyi et al., 1986; Torrealba and Müller, 1999). This indicates that interaction of amino acid neurotransmitters plays important role in physiology or even in the pathology of the spinal cord.

The glutamate is present in nerve cell bodies in brain stem neurons projecting to the spinal cord (Mooney et al., 1990; Nicholas et al., 1992; Liu et al., 1995). In these terminals, apart from monoamines, inhibitory amino acids GABA and glycine are also detected (Ottersen and Storm-Mathisen, 1984, 2000; Holstage and Bongers, 1991). Spinal transection results in a lower level of glutamate (Morrison et al., 1989) and a low density of glutamate-immunoreactive terminals in the spinal cord, caudal to the lesion site (Llewellyn-Smith et al., 1997). However, very little is known regarding changes in GABA levels in the spinal cord following rhizotomy, peripheral nerve lesion or spinal trauma. It seems likely that a balance between excitatory and inhibitory neurotransmission in the CNS plays important role in determining the extent of cell injury or survival under several experimental or clinical conditions.

Recently, oxidative stress is shown to influence glutamatergic neurotransmission in the CNS (Azbill et al., 1997; Kowluru et al., 2001). The oxidative stress is induced following noxious insults to the CNS such as, ischemia, trauma, hypoxia, stroke, and infarction (Cuzzocrea et al., 2001). Upregulation of glutamate receptors and transporters occur in these conditions (Danbolt, 2001). There are evidences that excitatory effects of glutamate and activation of NMDA receptors influence nitric oxide production (Ottersen and Storm-Mathisen, 2000). The nitric oxide is a free radical gas that can induce cell injury either directly or through production of reactive oxygen species and peroxynitrite (Cuzzocrea et al., 2001). Generation of free radicals induces a direct damage to the DNA as well as the cell membranes of neurons, glia and the microvascular endothelium (Danbolt, 2001).

Previous studies from our laboratory demonstrate that upregulation of nitric oxide is injurious to the cord following trauma (Sharma et al., 1996, 1998a,b). These cell injuries and upregulation of nitric oxide following trauma can be prevented by pretreatment with a new antioxidant compound, H-290/51 (Sharma et al., 1998b, 2000a). This indicates that reduction in oxidative stress is neuroprotective in spinal cord injury.

Since, oxidative stress influences glutamatergic neurotransmission (Cuzzocrea et al., 2001; Kowluru et al., 2001), a possibility exists that H-290/51 may induce changes in the glutamate levels in the spinal cord following injury. The glutamate is found co-localised with GABA within the same nerve terminals in the spinal cord (Broman et al., 2000). Thus, it is quite likely that a focal spinal cord trauma will also influence the GABA levels in the cord. Keeping these views in consideration, we examined the glutamate and GABA levels in the spinal cord in the untreated and H-290/51 pretreated traumatised rats using immunohistochemistry at light microscopic level. In addition, microvascular permeability disturbances, edema formation, and cell injury were also investigated in this group of animals.

Materials and methods

Animals

Experiments were carried out on 71 male Sprague Dawley rats housed at controlled room temperature (21 ± 1°C) with 12 h light and dark schedule. The rat food and tap water were supplied *ad libitum* before the experiments.

Spinal cord injury

Under equithesin anaesthesia (3 ml/kg, i.p.) one segment laminectomy was done at the T10–11 segments. Spinal cord injury (n = 20) was inflicted by making a longitudinal incision into the right dorsal horn of the T10–11 segments (Fig. 1). The wound was covered with cotton soaked in saline at room temperature to avoid a direct exposure of air to the cord (Sharma and Olsson, 1990). The Equithesin anaesthetised normal rats (n = 18) were used as controls. This experimental condition is approved by the Ethical Committee of Uppsala University.

H-290/51 pretreatment

In separate group of rats (n = 33), H-290/51 (Astra-Zeneca, Mölndal, Sweden) was administered 50 mg/kg (p.o.) as described earlier (Mustafa et al., 1995). This dose of the compound is effective in inhibiting lipid peroxidation (Svensson et al., 1993; Mustafa et al., 1995). The spinal cord injury was made in 17 rats 30 min after the treatment. The remaining 16 rats were used as drug-treated controls (Table 1).

Mean arterial blood pressure and physiological variables

The mean arterial blood pressure (MABP) and physiological variables were measured in separate group of animals (Table 1). The MABP was recorded from the arterial catheter implanted into the

right carotid artery. At the time of recording, the arterial catheter was connected to a Strain-Gauge pressure transducer (Statham, P23, USA). The output from the transducer was fed to a chart recorder (Electromed, UK). Immediately before recording of the MABP, a sample of arterial blood wad withdrawn for the measurements of blood gasses and arterial pH using a Radiometer apparatus (Copenhagen) (Sharma and Olsson, 1990).

Blood-spinal cord barrier permeability

The blood-spinal cord barrier (BSCB) permeability was examined using Evans blue (2% in sterile saline solution, pH 7.4) and $^{[131]}$Iodine as protein tracers. The dye was injected (0.3 ml/100 g) together with radioactive iodine (10 μCi/rat) into the right femoral vein through a needle puncture and was allowed to circulate for 10 min. The intravascular tracer was washed-out with 0.9% saline through heart followed by perfusion with the formalin based fixative (Sharma, 1999). The spinal cord tissue was removed, examined and photographed immediately for Evans blue extravasation. The tissue pieces were then weighed and counted in a 3-in well type Gamma counter (Packard, USA) at the energy window 25–50 KeV (Sharma et al., 1990). For high resolution counting, the T9 segment was bisected and divided into several pieces and counted separately. The extravasation of radiotracer was expressed as percentage of blood radioactivity over the tissue radioactivity. Whole blood was withdrawn from the left ventricle immediately before saline perfusion to obtain blood radioactivity (Sharma, 1987). After counting the radioactivity, the Evans blue dye was extracted from the tissue samples by homogenising them in a mixture of n-butanol (pure grade 1.4 ml) and sodium sulphate (0.5% 0.6 ml). The Evans blue dye extracted in the supernatant after centrifugation ($\times$900 g) was measured using a spectrophotometer (620 nm) (Sharma, 1987).

Spinal cord edema formation

Spinal cord edema was measured using water content of the T9 segment. For this purpose rats were killed by decapitation and the T9 segment was removed quickly. The T9 segment was bisected and each half was weighed separately on a pre-weighed filter paper using a precision digital microbalance (Meltzer, sensitivity 0.0001 g) to obtain wet weight of the samples. The filter papers containing spinal cord samples were then placed in an oven maintained at 90°C for at least 72 h to obtained dry weight. The dry weight of the sample was considered final if the three consecutive measurements were constant (Sharma and Olsson, 1990). The water content of the spinal cord was calculated from the difference in the wet weight and the dry weight of the sample (Sharma et al., 1990).

Immunohistochemistry of glutamate and GABA

Immunohistochemistry of glutamate and GABA were examined on free floating vibratome sections (40 μm thick) using commercial antibodies (Sharma et al., 1995b).

Antisera

Polyclonal glutamate (Sigma Chemical Co., USA, 1:2000) and GABA antisera (Calbiochem, USA, 1:2000) were used in this investigation. The binding of antibodies to antigens was visualised using peroxidase-antiperoxidase technique (Sharma et al., 1995b).

Perfusion and fixation

Animals were perfused with cold 2.5% paraformaldehyde containing 0.5% glutaraldehyde and 2.5% picric acid in the phosphate buffer saline (PBS, 0.1 M, pH 7.4) at the rate of 20 ml/min for 10 min. The intravascular blood was washed-out before fixation by 0.1 M PBS (20 ml/min for 5 min). The perfusion pressure was maintained at 100 torr during these procedures (Sharma and Olsson, 1990). After perfusion, the animals were wrapped in aluminium foil and kept overnight at 4°C. On the next day, the spinal cord T9 segment was dissected out and kept in the same fixative at 4°C (Sharma et al., 1995a,b).

Vibratome sections and immunostaining

Tissue pieces from the T9 segment were mounted on a Vibratome (Oxford instruments, UK) and multiple 40 μm thick sections were cut from each block (Sharma et al., 1995b). These multiple sections (3 sections from each block) were processed for Glutamate and GABA immunostaining (Schaffar et al., 1997; Broman et al., 2000). In brief, Vibratome sections were collected in PBS and processed free-floating (Sharma et al., 1995b). The sections were washed in PBS and incubated overnight (18 to 20 h) with glutamate or GABA antiserum (1 : 2000 diluted in PBS containing 0.2% Triton-X) at room temperature under gentle agitation with goat anti-rabbit IgG linked to horseradish peroxidase diluted 1 : 100 in PBS-X (see Fallon and Ciofi, 1990). The sections were washed in PBS-X and then rapidly rinsed in distilled water (30 to 45 sec). The peroxidase activity was revealed by incubation of the sections for 1–3 min in 0.1 M Tris buffer (pH 7.6) containing 0.015% H_2O_2 and 0.03% 3-3′ diaminobenzidine tetra hydrochloride (Sigma Chemical Co., USA). The reaction was stopped by a 1 min wash in distilled water. These sections were then dehydrated, mounted and observed under bright field Leica microscope.

Quality control of immunostaining methods

To understand the specificity of our immunohistochemical methods, in one group of sections, the primary antisera were omitted and rest of the processing was done as described above. These negative controls do not show immunostaining of nerve cells or nerve fibres (Fig. 3) indicating specificity of the antisera used in this investigation.

Microphotography and processing of digital images

Microphotographs were taken on Kodak Supra 100 ASA Colour negative film under a Leica bright field microscope. The digital images (size 52 cm $\times$ 32 cm, 80 pixels/inch) were processed on a CD by Kodak Colour laboratories (Stockholm, Sweden). These digital images were modified using Adobe Photoshop 3.5 programme on a G-4 Macintosh computer. The images obtained from the control, spinal cord injured and/or drug-treated control or injured groups were processed (final image size 8 $\times$ 13 cm, 300 pixels/inch) using identical colour filter and colour balance (Sharma, 1999).

Semiquantitative analysis of immunohistochemical data

The semiquantitative analysis of immunostaining in the control, spinal cord injury, H-290/51 treated injured and control rats were done manually in a blind fashion. The number of immunostained cells per section was counted in each half of the T9 segment obtained from different group of rats for comparison (Fig. 3).

Statistical analysis

The quantitative or semiquantitative data were analysed using ANOVA followed by Dunnett test for multiple group comparison. A P-value less that 0.05 was considered significant.

Table 1. Experimental protocol

Parameters measured	Experimental group (n = 71)			
	Control Group I	H-290/51 Group II	5 h SCI Group III	H-290/51 + SCI Group IV
Immunohistochemistry[a]	6	5	6	5
BSCB[b]	6	5	8	6
Edema	6	6	6	6
Ultrastructure	5[a]	5[a]	5[a]	5[a]
Physiological variables	6[b]	5[b]	8[b]	6[b]
	18	16	20	17

[a] data obtained from same group of animals; [b] data obtained from same group of animals

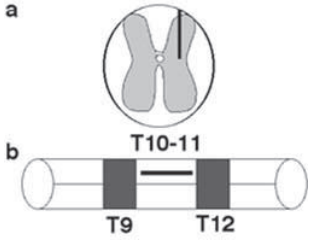

Fig. 1. Spinal cord injury model (**a**). Spinal cord injury was made on the right dorsal horn of the T10–11 segment (horizontal bar = 5 mm, b). The tissue for morphological examination, edema and BSCB permeability was taken from the T9 segment, rostral to the lesion site (3 mm thick) (**b**). Cross section of the spinal cord (**a**) showing depth of lesion (vertical bar = 2 mm). The deepest part of the lesion is usually located between the lamina VII to X (**a**)

Results

Physiological variables

The results are shown in Table 2. A focal trauma to the spinal cord resulted in a significant hypotension (mean 30 mmHg) at 5 h compared to the control group. At this time, the PaO_2 showed a mild increase and the $PaCO_2$ exhibited a slight decline compared to the control group. The arterial pH did not show any significant change at 5 h injury. Pretreatment with H-290/51 did not influence these physiological variables either in normal rats or animals subjected to 5 h cord trauma (Table 2).

Blood-spinal cord barrier permeability

In normal rats, the permeability of Evans blue or $^{[131]}$Iodine in the T9 segment of the cord is very low (Fig. 2). In this group, there was no significant differences in the extravasation of tracers in the right or left side of the cord (Fig. 2). A focal trauma to the spinal cord on the T10–11 segments significantly increased the BSCB permeability to Evans blue and $^{[131]}$Iodine in the T9 segments of the cord (Fig. 2). This increase in BSCB permeability was significantly higher in the ipsilateral (right) cord compared to the contralateral (left) side (Fig. 2). Pretreatment with H-290/51 markedly attenuated the extravasation of these protein tracers in the T9 segment. This effect was most pronounced in the contralateral side of the cord (Fig. 2). However, this drug treatment alone did not alter the BSCB permeability to Evans blue or radioactive iodine in the normal cord (Fig. 2).

Spinal cord edema formation

A focal trauma to the cord on the T10–11 level resulted in a significant increase in the water content in the rostral T9 segment (Fig. 2). This increase in the water content was most pronounced in the ipsilateral side compared to the contralateral half of the cord. Pretreatment with the H-290/51 significantly attenuated trauma induced increase in the water content in the T9 segment (Fig. 2). This effect of the compound was most prominent in the contralateral side compared to the ipsilateral cord (Fig. 2). On the other hand, pretreatment with H-290/51 did not influence the cord water content in normal animals (Fig. 2).

Glutamate immunohistochemistry

Glutamate immunostained neurons were normally present in the gray matter of normal rats (Fig. 3). The dorsal and ventral horns exhibit dense immunostaining of nerve cells containing glutamate (Fig. 3). This pattern of glutamate immunostaining is almost identical in both right and left sides of the cord (Fig. 2).

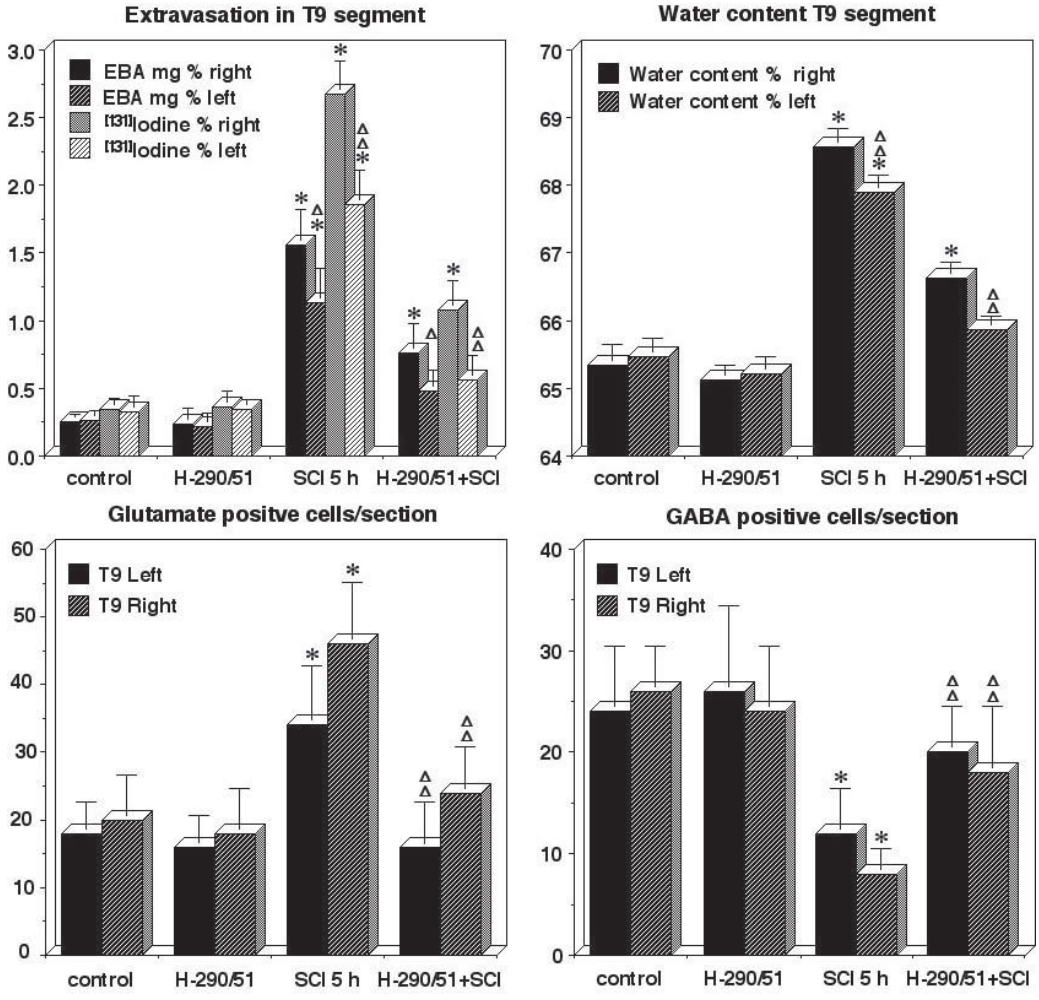

Fig. 2. Extravasation of tracers (upper panel, left) and spinal cord water content (upper panel, right) in the ipsilateral and contralateral sides of the T9 segment of the cord following trauma and their modification with the H-290/51 pretreatment. The lower panel shows semiquantitative analysis of Glutamate (left) and GABA (right) immunostaining in the T9 segment following trauma and its modification with the H-290/51 pretreatment. * = $P < 0.01$ compared from the control group; △ = $P < 0.05$; △△ = $P < 0.01$ compared from the spinal cord injury group, ANOVA followed by Dunnet test for multiple group comparison. *SCI*, spinal cord injury

A focal trauma to the cord markedly increased the glutamate immunostaining in the T9 segment of the cord (Fig. 3). Thus, a marked upregulation of glutamate immunostaining can be seen in the spinal cord ventral horn that was much more intense and compact compared to the control group (Fig. 3). This immunostaining was most pronounced in the ipsilateral right half compared to the contralateral left side (Fig. 2). The glutamate immunostaining was mainly seen in the damaged or distorted nerve cell bodies. However, many nerve fibres in the ventral horn of the cord were also glutamate positive after 5 h injury. The magnitude and intensity of immunostained fibres were much more prominent in the ipsilateral side.

Pretreatment with H-290/51 alone did not influence the glutamate immunostaining in normal animals (Fig. 2). However, this drug treatment was able to markedly attenuate the glutamate immunostaining in the cord after 5 h injury (Fig. 3). This effect of the compound was most marked in the contralateral side of the cord (Fig. 2). In these drug-treated traumatised rats, immunostaining of nerve fibres were also considerably reduced compared to the untreated injured group.

GABA immunohistochemistry

GABA immunostained cells were present in the spinal cord gray matter in normal animals (Fig. 3). The

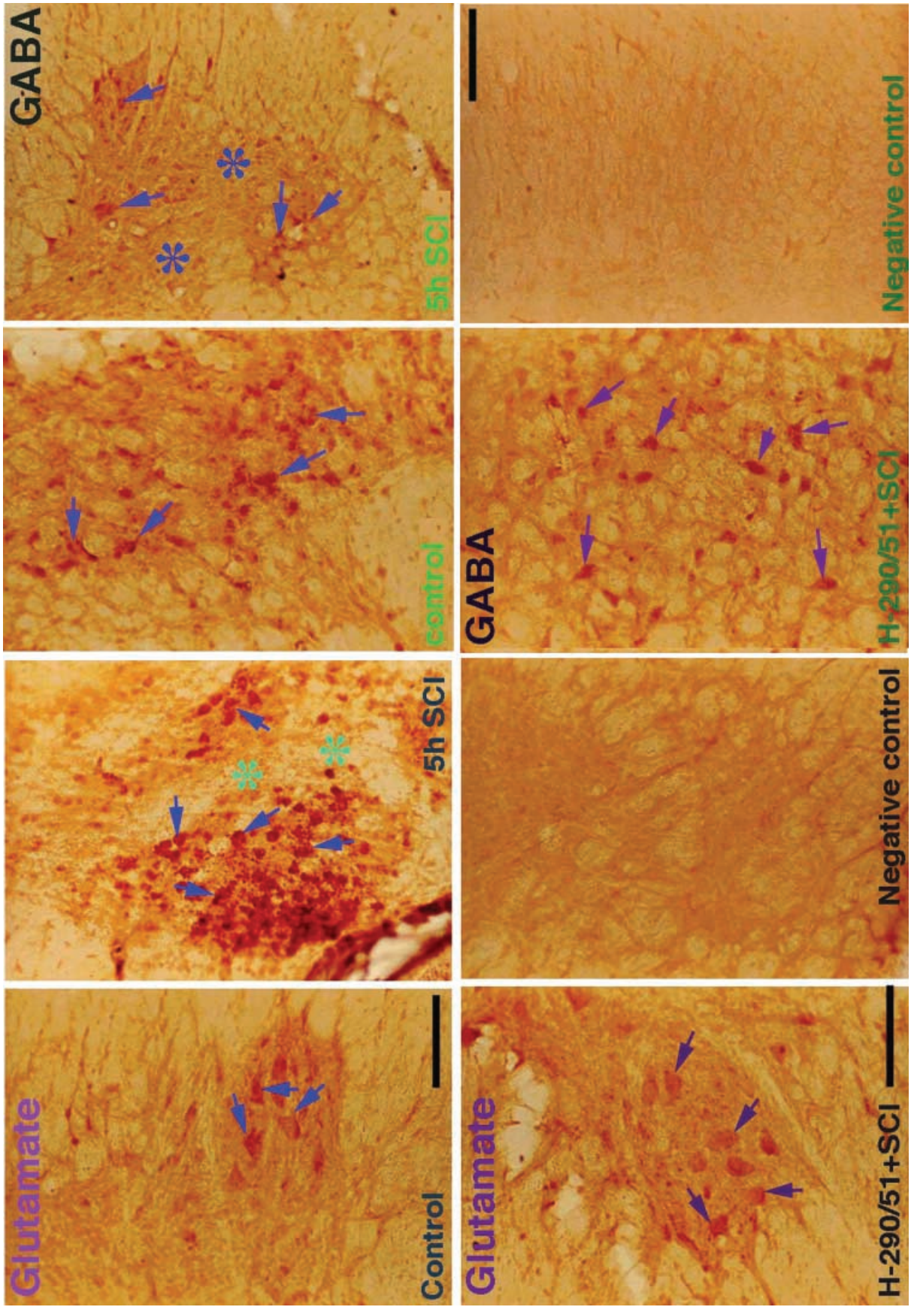

Fig. 3. Shows glutamate and GABA immunostaining (arrows) in the ventral horn in control group and its modification following 5 h spinal cord injury (SCI, upper panel). Influence of H-290/51 treatment on SCI induced changes in Glutamate and GABA immunostaining in the ventral horn are shown in the lower panel. Edematous expansion and sponginess are evident (*) in the spinal cord traumatised rats. Bar = 50 μm

GABA immunostaining is very rich in the dorsal horn. A diffuse staining of GABA positive nerve cells can be seen in the lateral horn. However, many nerve cells in the ventral horn exhibited pronounced GABA immunostaining (Fig. 3).

A focal trauma to the spinal cord resulted in a profound decrease in GABA immunostaining in the spinal cord gray matter (Fig. 3). Thus, in the untreated traumatised rats, the number of GABA positive cells were significantly decreased (Fig. 2). The intensity of decrease in GABA immunostaining was most evident in the ventral gray matter. However, the magnitude of GABA immunostaining was also considerably reduced in the dorsal horn. This decrease in GABA immunoreactivity was most pronounced in the ipsilateral spinal cord compared to the contralateral side.

Pretreatment with H-290/51 in normal animals did not alter the pattern of normal GABA immunostaining (Fig. 2). However, subjection of H-290/51 treated rats to 5 h injury did not result in downregulation of GABA immunostaining in the cord (Fig. 3). Thus, in H-290/51 treated and traumatised rats, the GABA immunostaining was still much intense in the dorsal, lateral and ventral horns of the T9 segment. The intensity of GABA immunostaining was much more evident in the contralateral side compared to the ipsilateral cord in this drug-treated group (Fig. 2).

Gross morphology of the spinal cord

Examination of toludine stained 1 μm thick spinal cord sections from the T9 segment of untreated traumatised rats revealed profound expansion of the cord that was most marked in the ipsilateral side (Fig. 4a). In this group, the distinction between gray and white matter is lost. The spinal cord was spongy in appearance and loss of nerve cells was evident (Fig. 4a).

Pretreatment with H-290/51 in the normal spinal cord did not influence the gross morphology of the cord (results not shown). However, in the H-290/51 treated animals the general expansion of the cord and sponginess were mainly absent 5 h after injury (Fig. 4b). The spinal cord appeared much less swollen compared to the untreated traumatised group. A clear distinction between gray and white matter in the spinal cord in this group can easily be seen. This effect was however, most pronounced in the contralateral side (Fig. 4b).

Fine structure of the spinal cord

Paraffin embedded 3 μm thick sections stained with Nissl or Haematoxyline and Eosin revealed widespread edema, sponginess, nerve cell damage and chromatolysis in the untreated traumatised spinal cord (Fig. 4c). Many nerve cells were swollen and the others were shrunken (Fig. 4c). This effect was most marked in the ipsilateral side compared to the contralateral cord. Microhaemorrhages, edema and sponginess were quite frequent in the gray matter. In the white matter, myelin vesiculation can be seen profoundly in this group.

Pretreatment with H-290/51 markedly attenuated the general expansion, edema and sponginess of the spinal cord. Thus, many nerve cells were quite normal in appearance (Fig. 4d). The dark stained nucleus and the distorted nerve cells were mainly absent in this group of drug-treated traumatised rats (Fig. 4d). This effect was most pronounced in the contralateral side compared to ipsilateral cord. In these animals, microhaemorrhages, signs of myelin vesiculation and widespread damage to the neuropil was much less common (Fig. 4).

Table 2. Physiological variables in control and 5 h spinal cord traumatised rats and their modification with H-290/51 pretreatment (for details see text)

Parameters measured	Control n = 6	H-290/51 n = 5	5 h SCI n = 8	H-290/51 + SCI n = 6
MABP mmHg	110 ± 8	112 ± 6	80 ± 6**	88 ± 8**[a]
Arterial pH	7.38 ± 0.02	7.37 ± 0.04	7.36 ± 0.07	7.36 ± 0.08
PaO_2 mmHg	80.23 ± 0.13	80.08 ± 0.43	81.46 ± 0.64	81.26 ± 0.45
$PaCO_2$ mmHg	34.65 ± 0.21	34.78 ± 0.23	33.23 ± 0.54	33.67 ± 0.43

Values are mean ± SD, ** = $P < 0.01$, [a] significantly different from SCI. $P < 0.05$, Others not significant, ANOVA followed by Dunnet test for multiple group comparison

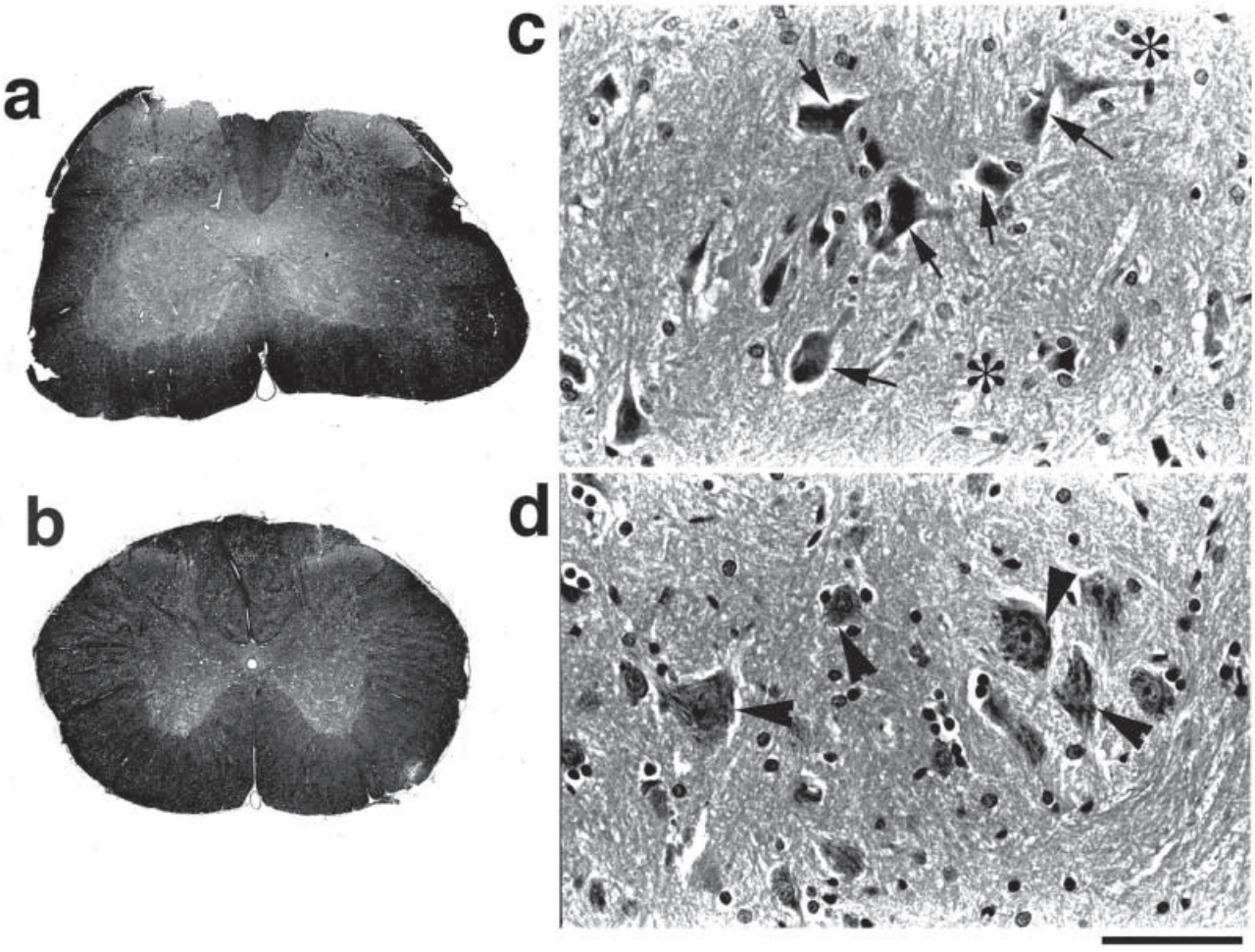

Fig. 4. Gross morphology of the T9 segment of the spinal cord in a 5 h spinal cord injured (**a**) rat and its modification with the H-290/51 pretreatment (**b**). General expansion of the cord in untreated injured spinal cord is clearly apparent (**a**). High power light micrograph shows many distorted and damaged nerve cells (arrows) in the ventral horn of one spinal cord traumatised rat (**c**). In this animal, edema and sponginess is clearly seen (*). Pretreatment with H-290/51 markedly attenuated nerve cell damage (arrow heads). The signs of edema and sponginess are not present (**d**). Bar: **a,b** = 3 mm, **c,d** = 40 μm

Ultrastructural observation of the spinal cord

Ultrastructural changes in the untreated traumatised cord exhibited frequent membrane damage, vacuolation, perivascular edema, swollen nerve cells, glial cells and axons (Fig. 5a). Damage to myelin and distortion of the myelin sheaths were quite frequent in this group of rats. Microhaemorrhages, membrane disruption, myelin vesiculation were most frequent in the dorsal horn (Fig. 5a). However, vacuolation and cell swelling were common throughout the neuropil.

Pretreatment with H-290/51 significantly attenuated myelin vesiculation (Fig. 5b). In this group of animals, vacuolation, perivascular edema, swelling of nerve cells and glial cells were much less frequent. Microhaemorrhages in the neuropil and myelin vesiculation were not much frequent. The neuropil is condensed and membrane disruption was not a common finding. This effect was most marked in the contralateral side of the cord.

Discussion

The salient new findings of the present study show that a 5 h spinal cord trauma has the capacity to induce an upregulation of glutamate in the spinal cord rostral to the lesion site. At this time, there was a marked reduction in the GABA immunostaining in this segment. This segment exhibited profound increase in edema formation, breakdown of the BSCB and cell damage. These observations suggest that an upregulation of glutamate and a downregulation of GABA in the spinal cord are injurious to the cell. Our observations are

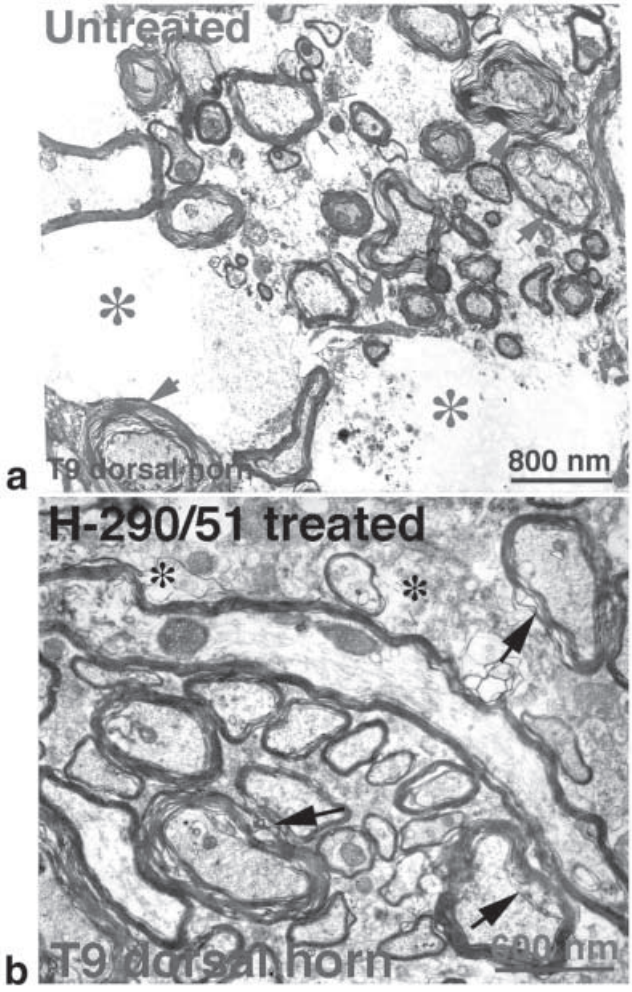

Fig. 5. Low power electron micrograph from the right dorsal horn of the T9 segment of the spinal cord in one untreated 5 h spinal cord traumatised rat (**a**) and its modification with the H-290/51 pretreatment (**b**). Membrane damage, vacuolation (*) and edema is quite frequent in the untreated injured rat. Myelin vesiculation (arrows) is quite common (**a**). Pretreatment with H-290/51 significantly attenuated the membrane disruption, vacuolation (*) and edema. The vesiculation of myelin is less frequent in this drug-treated traumatised rat. Bar: **a** = 800 nm, **b** = 600 nm

the first to show that spinal cord trauma induced increased excitotoxicity and a decrease in the inhibitory activity appears to be important contributing factors in cell injury. Obviously, a balance between excitatory and inhibitory amino acid neurotransmitters is crucial for cell injury or survival.

Another important finding of this study is the observation that pretreatment with the new antioxidant compound H-290/51 was able to restore the balance between glutamate and GABA in the spinal cord after trauma. Thus, in the H-290/51 treated animals, a focal spinal cord injury did not result in glutamate upregulation. Moreover, the downregulation of GABA in the spinal cord in the traumatised animals was also prevented by this drug treatment. These observations suggest that antioxidant compounds are able to influence the glutamate and GABA levels in the cord following injury, not reported earlier.

Spinal cord injury activates calcium-dependent phospolipases leading to phospholipid hydrolysis and release of fatty acids (Janssen and Hansebout, 1987). The rapid accumulation of polyunsaturated fatty acids occurs between 5 and 15 min after trauma (Liu et al., 1991). These elevated levels become normal within 1 h after the insult. However, at 4 h and onwards, tissue fatty acid increased again and reaches its peak level within 24 hours (Katayama et al., 1991). This second increase in fatty acids correlates well with the tissue injury (Faden et al., 1987). Phospholipid hydrolysis results in the release of free radicals followed by ischemia, tissue destruction and disruption of the BSCB.

In the spinal cord injury, oxidative stress plays one of the important roles in inducing free radical formation and production of nitric oxide (Schwab and Bartholdi, 1996; Azbill et al., 1997; Sharma et al., 1998a,b). Free radicals are molecules with unusual reactivity due to the presence of unpaired electrons in the outer orbits. This reactivity further propagates through chain reactions (Del Maestro, 1980). The biological membranes of neurons, glial cells and endothelial cells are highly susceptible to damage by these free radicals.

Thus, any compound that is able to either scavenge the free radicals or to break the chain reaction will induce neuroprotection. In this regard, H-290/51 is the compound of our choice (Svensson et al., 1993; Mustafa et al., 1995; Alm et al., 2000; Sharma et al., 2000a,b). The H-290/51 is able to inhibit lipid peroxidation by breaking the chain reaction (Svensson et al., 1993; Mustafa et al., 1995). This activity of the compound seems to be most important for achieving neuroprotection in spinal cord injury. Previous studies from our laboratory suggest that this compound is also able to induce neuroprotection following hyperthermic brain injury (Sharma 1999). These observations suggest that oxidative stress plays important role in the cell and tissue injuries following trauma or other noxious insults to the CNS.

We observed marked reduction in edema formation and breakdown of the BSCB following trauma in rats pretreated with H-290/51. This indicates that the com-

pound is able to prevent membrane damage of the nerve cells, glial cells and the microvascular endothelium. Apart form a direct membrane damage, several other biochemical and neurochemical factors are involved in edema formation and the microvascular permeability disturbances following cord trauma. A reduction in glutamate level and an increase in GABA activity in the spinal cord following trauma in these drug-treated animals suggest that these amino acids also contribute to the edema formation and disruption of the BSCB.

The glutamate induced neurotoxicity is mediated via formation of reactive oxygen species and lipid peroxidation (Evans, 1993; Hall and Braughler, 1989; Ikeda et al., 1989). In ischemic injury, glutamate release and activation of glutamatergic receptors result in increased accumulation of intracellular Ca^{2+} (Fineman et al., 1993: Globus et al., 1995; Katayama, 1991). Activation of glutamate receptors and increase in the intracellular Ca^{2+} induces the formation of reactive oxygen species and lipid peroxidation (Bondy and LeBel, 1993; Dugan et al., 1995). Increased intracellular Ca^{2+} will act through NMDA receptors and voltage gated-Ca^{2+} channels to impair the mitochondrial electron transport system causing the formation of free radicals. These free radicals will induce a direct damage to the cell membranes, nuclear proteins and phospholipids (Hall, 1993).

Thus, scavengers of free radicals like superoxide dismutase, catalases and glutathione are neuroprotective in several models of traumatic and ischemic brain injuries. Furthermore, transgenic mice overexpressing superoxide dismutase gene exhibit potential resistance to ischemia injury and glutamate neurotoxicity in vitro (Chan, 1992). These observations suggest that reactive oxygen species and superoxide play important roles in cell death probably via glutamate induced neurotoxicity (Beal, 1995; Azbill et al., 1997).

Our present findings in spinal cord injury are in line with the above hypothesis. Thus, similar to ischemic injuries, release and accumulation of glutamate also occur in the spinal cord following trauma. This observation indicates that glutamate excitotoxicity plays major role in spinal cord injury induced free radical formation and cell damage. An increased glutamate may act through the NMDA receptors and induce intracellular Ca^{2+} accumulation (Balentine, 1988) resulting in formation of free radicals.

However, in vivo situation, no single chemical compound is alone responsible for the cell injury or survival. Several endogenous neurodestructive and neuroprotective agents are released simultaneously in the CNS injuries. A balance between these neurodestructive and neuroprotective elements will finally determine the extent of cell injury or cell survival in such conditions.

Spinal trauma induces a release of GABA in the cord to neutralise the excitotoxicity of glutamate. However, a decline in the GABA activity occurs 5 h after trauma. Thus, the glutamate induced excitotoxicity is no longer neutralised or counteracted by the low level of GABA activity present in the spinal cord at this time. An increased excitotoxicity and a decrease in inhibitory activity will thus contribute to cell injury. This idea gets further support by the results obtained with the H-290/51 treatment in this study. Pretreatment with this compound significantly restored the balance between the excitatory and inhibitory amino acids in the cord after trauma. Thus, in this drug treated animals no *increase* in glutamate or no *decrease* in GABA was observed. Obviously, without glutamate excitotoxicity, the sequence of events leading to the formation of free radicals and cell damage may not take place. Since this drug-treatment attenuated the glutamate release, it appears that production of free radicals or lipid peroxidation is also reduced in spinal trauma. A decrease in edema formation and reduction in the BSCB permeability are in line with this idea. Obviously, in the absence of edema formation and breakdown of the BSCB permeability the cell changes are considerably reduced.

In conclusion, our results clearly suggest that in the spinal cord injury, the balance between glutamate and GABA is essential to minimise cell injury. A focal trauma upregulates glutamate immunostaining and simultaneously downregulates the GABA activity in the cord. The antioxidant compound H-290/51 is able to restore the balance between excitotoxicity and inhibitory activity in the spinal cord following trauma by inhibiting the glutamate activity and by facilitating the GABA neurotransmission in the traumatised cord. This effect of the compound appears to be another important factor in inducing neuroprotection in the traumatised spinal cord. Taken together, our results strongly indicate that the compound H-290/51 has a potential therapeutic value in the treatment of spinal cord injuries in the future.

Acknowledgements

This investigation is supported by Grants from the Swedish Medical Research Council (2710); Astra-Zeneca, Hässle, Mölndal, Sweden; The Humboldt Foundation, Bonn, Germany and The University Grants Commission, New Delhi, India. The technical assistance of Kärstin Flink, Kerstin Rystedt, Katjya Deparade, Angela Jan is highly appreciated.

References

Alm P, Sharma HS, Sjöquist O, Westman J (2000) A new antioxidant compound H-290/51 attenuates nitric oxide synthase and heme oxygenase expression following hyperthermic brain injury. An experimental study using immunohistochemistry in the rat. Amino Acids: 19: 383–394

Azbill RD, Mu X, Bruce-Keller AJ, Mattson MP, Springer JE (1997) Impaired mitochondrial function, oxidative stress and altered antioxidant enzyme activities following traumatic spinal cord injury. Brain Res 765: 283–290

Balaentine JD (1988) Spinal cord trauma: in search of the meaning of granular axoplasm and vesicular myelin. J Neuropathol Exp Neurrol 47: 77–92

Beal M (1995) Aging, energy, and oxidative stress in neurodegenerative disease. Ann Neurol 38: 357–366

Bondy SC, LeBel CP (1993) The relationship between excitotoxicity and oxidative stress in the central nervous system. Free Radic Biol Med 14: 633–642

Broman J (1994) Neurotransmitters in subcortical somatosensory pathways. Anat Embryol (Berl) 189: 181–214

Broman J, Anderson S, Ottersen OP (1993) Enrichment of glutamate-like immunoreactivity in primary afferent terminals throughout the spinal cord dorsal horn. Eur J Neurosci 5: 1050–1061

Broman J, Hassel B, Rinvik E, Ottersen OP (2000) Biochemistry and anatomy of transmitter glutamate. In: Ottersen OP, Storm-Mathisen J (eds) Glutamate. Handbook Chem Neuroanat 18: 1–44

Chan PH (1992) Antioxidant-dependent amelioration of brain injury: role of CuZn-superoxidedismutase. J Neurochem 9 [Suppl 2]: S417–S423

Cuzzocrea S, Riley DP, Caputi AP, Salvemini D (2001) Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. Pharmacol Rev 53: 135–159

Danbolt NC (2001) Glutamate uptake. Prog Neurobiol 65: 1–205

Del Maestro R (1980) An approach to free radicals in medicine and biology. Acta Physiol Scand 492: 153–168

Dugan LL, Sensi SL, Canzoneiro LM, Handran SD, Rothman SM, Lin TS, Goldberg MP, Choi CW (1995) Mitochondrial production of reactive oxygen species in cortical neurons following exposure to N-methyl-D-aspartate. J Neurosci 15: 6377–6388

Evans PH (1993) Free radicals in brain metabolism and pathology. Br Med Bull 49: 577–587

Faden AI, Chan PH, Longar S (1987) Alterations in lipid metabolism, (Na^+, K^+)-ATPase activity, and tissue water content of spinal cord following experimental traumatic injury. J Neurochem 48: 1809–1816

Fallon JH, Ciofi P (1990) Dynorphin-containing neurons. Handbook Chem Neuroanat 9: 1–286

Fineman I, Hovda DA, Smith M, Yoshino A, Becker DP (1993) Concussive brain injury is associated with a prolonged accumulation of calcium: a ^{45}Ca autoradiographic study. Brain Res 624: 94–102

Globus MY, Alonso O, Dietrich WD, Busto R, Ginsberg MD (1995) Glutamate release and free radical production following brain injury: effects of posttrumatic hypothermia. J Neurochem 65: 1704–1711

Hall ED (1993) Neuroprotective actions of glucocorticoid and nonglucocorticoid steroids in acute neuronal injury. Cell Mol Neurobiol 13: 415–432

Hall ED, Braughler JM (1989) Central nervous system trauma and stroke II. Physiological and pharmacological evidences for involvement of oxygen radicals and lipid peroxidation. Free Radic Biol Med 6: 303–313

Holstage JC, Bongers CMH (1991) A glycine projection from the ventromedial lower brainstem to motoneurons. An ultrastructural double labeling study in the rat. Brain Res 566: 308–315

Ikeda Y, Brelsford KL, Ikeda K, Long DM (1989) Oxygen-free radicals in traumatic brain oedema. Neurol Res 11: 216–231

Janssen L, Hansebout RR (1987) Pathogenesis of spinal cord injury and newer treatments. Spine 14: 23–32

Katayama Y, Kawamata T, Tamura T, Hovda D, Becker D, Tsubokawa T (1991) Calcium-dependent glutamate release concomitant with massive potassium flux during cerebral ischemia in vivo. Brain Res 558: 136–140

Kowluru RA, Engerman RL, Case GL, Kern TS (2001) Retinal glutamate in diabetes and effect of antioxidants. Neurochem Int 38: 385–390

Liu D, Thangnipon W, McAdoo DJ (1991) Excitatory amino acids rise to toxic levels upon impact injury to the rat spinal cord. Brain Res 547: 344–348

Liu RH, Fung SJ, Reddy VK, Barnes CD (1995) Localization of glutamatergic neurons in the dorsolateral pontine tegmentum projecting to the spinal cord of the cat with a proposed role of glutamate on lumbar motoneuron activity. Neuroscience 64: 193–208

Llewellyn-Smith IJ, Cassam AK, Krenz NR, Krassioukov AV, Weaver LC (1997) Glutamate- and GABA-immunoreactive synapses on sympathetic preganglionic neurons caudal to a spinal cord transection in rats. Neuroscience 80: 1225–1235

Maxwell DJ, Kerr R, Jankowska E, Riddell JS (1997) Synpatic connection of dorsal horn group II spinal interneurons: synapses formed with the interneurons and by their acon collaterals. J Comp Neurol 380: 51–69

Mooney RD, Bennett-Clarke CA, King TD, Rhoades RW (1990) Tectospinal neurons in hamster contain glutamate-like immunoreactivity. Brain Res 537: 375–380

Morrison SF, Callaway J, Milner TA, reis DJ (1989) Glutamate in the spinal sympathetic intermediolateral nucleus: licalization by light and electron microscopy. Brain Res 503: 5–15

Mustafa A, Sharma HS, Olsson Y, Gordh T, Thoren P, Sjöquist P-O, Adem A, Nyberg F (1995) Vascular permeability to growth hormone in the rat central nervous system after focal spinal cord injury. Influence of a new anti-oxidant H-290/51 and age. Neurosci Res 23: 185–194

Nicholas AP, Pieibone VA, Arvidsson U, Hökfelt T (1992) Serotonin-, substance P- and glutamate/aspartate-like immunoreactivities in medullo-spinal pathways of rat and primate. Neuroscience 48: 545–559

Ottersen OP, Storm-Mathisen J (1984) Handbook of chemical neuroanatomy, vol 3. Classical transmitters and transmitter receptors in the CNS, Part II. Elsevier, Amsterdam, pp 141–246

Ottersen OP, Storm-Mathisen J (2000) Glutamate. Handbook Chem Neuroanat 18: 1–287

Salt TE, Hill RG (1983) Neurotransmitter candidtaes of somatosensory primary afferent fibers. Neuroscience 10: 1083–1103

Schaffar N, Rao H, Kessler J-P, Jean A (1997) Immunohistochemical detection of glutamate in rat vagal sensory neurons. Brain Res 778: 302–308

Schneider SP, Perl ER (1988) Comparison of primary afferent and glutamate excitation of neurons in the mammalian spinal dorsal horn. J Neurosci 8: 2062–2073

Schwab ME, Bartholdi D (1996) Degenration and regeneration of axons in the lesioned spinal cord. Physiol Rev 76: 319–370

Sharma HS (1987) Effect of captopril (a converting enzyme inhibitor) on blood-brain barrier permeability and cerebral blood flow in normotensive rats. Neuropharmacology 26: 85–92

Sharma HS (1999) Pathophysiology of blood-brain barrier, brain edema and cell injury following hyperthermia: New role of heat shock protein, nitric oxide and carbon monoxide, an experimental study in the rat using light and electron microscopy. Acta Universitatis Upsaliensis 830: 1–94

Sharma HS (2000) A Bradykinin BK_2 receptor antagonist HOE-140 attenuates blood-spinal cord barrier permeability following trauma to the rat spinal cord. An experimental study using Evans blue, $^{[131]}$I-sodium and lanthanum tracers. Acta Neurochir (Wien) [Suppl 76] 159–163

Sharma HS, Olsson Y (1990) Edema formation and cellular alterations following spinal cord injury in rat and their modification with p-chlorophenylalanine. Acta Neuropathologica (Berlin) 79: 604–610

Sharma HS, Olsson Y, Dey PK (1990) Early accumulation of serotonin in rat spinal cord subjected to traumatic injury. Relation to edema and blood flow changes. Neuroscience 36: 725–730

Sharma HS, Olsson Y, Pearsson S, Nyberg F (1995a) Trauma induced opening of the blood-spinal cord barrier is reduced by indomethacin, an inhibitor of prostaglandin synthesis. Experimental observations in the rat using ^{131}I-sodium, Evans blue and lanthanum as tracers. Restorative Neurology and Neuroscience 7: 207–215

Sharma HS, Olsson Y, Westman J (1995b) A serotonin synthesis inhibitor, p-chlorophenylalanine reduces the heat shock protein response following trauma to the spinal cord. An immunohistochemical and ultrastructural study in the rat. Neuroscience Research 21: 241–249

Sharma HS, Westman J, Olsson Y, Alm P (1996) Incvolvement of nitric oxide in acute spinal cord injury: an immunohistochemical study using light and electron microscopy in the rat. Neuroscience Research 24: 373–384

Sharma HS, Nyberg F, Gordh T, Alm P, Westman J (1998a) Neurotrophic factors attenuate neuronal nitric oxide synthase upregulation, microvascular permeability disturbances, edema formation and cell injury in the spinal cord following trauma. In: Stålberg E, Sharma HS, Olsson Y (eds) Spinal cord monitoring. Springer, Wien New York , pp 118–148

Sharma HS, Westman J, Nyberg F (1998b) Pathophysiology of brain edema and cell changes following hyperthermic brain jnjury. In: Sharma HS, Westman J (eds) Brain functions in hot environment. Progress in Brain Research 115: 351–412

Sharma HS, Alm P, Sjöquist P-O, Westman J (2000a) A new antioxidant compound H-290/51 attenuates upregulation of constitutive isoform of heme oxygenase (HO-2) following trauma to the rat spinal cord. Acta Neurochir (Wien) [Suppl] 76: 153–157

Sharma HS, Nyberg F, Gordh T, Alm P, Westman J (2000b) 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. Amino Acids 19: 351–361

Somogyi P, Halasy K, Somogyi J, Storm-Mathisen J, Ottersen OP (1986) Quantification of immunogold labelling reveals enrichment of glutamate in mossy and parallel fibre terminals in cat cerebellum. Neurosciene 19: 1045–1050

Stålberg E, Sharma HS, Olsson Y (1998) Spinal cord monitoring. Basic principles, regeneration, pathophysiology and clinical aspects. Springer, Wien New York, pp 1–527

Svensson L, Borjeson I, Kull B, Sjöquist PO (1993) Automated procedure for measuring TBARS for in vitro comparison of the effect of antioxidant on tissues. Scand J Lan Invest 53: 83–85

Torrealba F, Müller C (1996) Glutamate immunoreactivity of insular cortex afferents to the nucleus tractus solitarius in the rat: a quantitative electron microscopic study. Neuroscience 71: 77–87

Valtschanoff JG, Weinberg RJ, Rustioni A (1993) Amino acid immunoreactivity in corticospinal terminals. Exp Brain Res 93: 95–103

Valtschanoff JG, Phend KD, Bernardi PS, Weinberg RJ, Rustioni A (1994) Amino acid immunocytochemistry of primary afferent terminals in the rat dorsal horn. J Comp Neurol 346: 237–252

Willis WD, Coggeshall RE (1991) Sensory mechanisms of the spinal cord, 2nd edn. Plenum Press, New York

Winkler T, Sharma HS, Stålberg E, Westman (1998) Spinal cord bioelectrical activity, edema and cell injury following a focal trauma to the spinal cord. An experimental study using pharmacological and morphological approach. In: Stålberg E, Sharma HS, Olsson Y (eds) Spinal cord monitoring. Springer Wien New York, pp 281–348

Zhong J, Gerber G, Koji'c L, Randi'c M (2000) Dual modulation of excitatory synaptic transmission by agonists at group I metabotropic glutamate receptors in the rat spinal dorsal horn. Brain Res 887: 359–377

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