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Depletion of endogenous serotonin synthesis with p-CPA attenuates upregulation of constitutive isoform of heme oxygenase-2 expression, edema formation and cell injury following a focal trauma to the rat spinal cord

H. S. Sharma and **J. Westman**

Laboratory of Neuroanatomy, Department of Medical Cell Biology, Biomedical Center, Uppsala University, Uppsala, Sweden

Summary

The possibility that the upregulation of hemeoxygenase (HO) enzyme responsible for carbon monoxide (CO) formation in the spinal cord following trauma is involved in edema formation and cell damage was examined in a rat model. A focal trauma to the rat spinal cord by making an incision into the right dorsal horn of the T10–11 segment resulted in profound upregulation of HO-2 (the constitutive isoform of the enzyme) expression in the T9 and T12 segments 5 h after injury. In these segments a marked increase in edema formation, nerve cell damage, and expression of heat shock protein (HSP 72) were observed. Pretreatment with p-chlorophenylalanine (p-CPA, a serotonin synthesis inhibitor) significantly attenuated the trauma induced edema formation, cell injury, and HSP expression. Upregulation of HO-2 in p-CPA treated traumatised rats was considerably reduced. These observations suggest that (i) spinal cord injury has the capacity to induce an upregulation of HO-2 and HSP expression, (ii) abnormal production of CO as reflected by HO-2 expression is injurious to the cord, and (iii) that endogenous serotonin is involved in HO-2 expression in the cord.

Keywords: Spinal cord injury; heme oxygenase-2; carbon monoxide; edema; cell injury; trauma; serotonin; heat shock protein-72; p-chlorophenylalanine; immunohistochemistry.

Introduction

Trauma to the spinal cord is associated with alterations in fluid microenvironment of the cord and induce abnormal reactions in nerve cells, glial cells, and axons leading to severe long-term disability [4, 10, 19]. The outcome of injury is influenced by several mechanisms: (a) the severity of cell- and microfluid disturbances (edema, haemorrhage) at the site of the primary physical injury, (b) the degree of disruption and block of descending and ascending neuronal pathways including axonal and myelin alterations, (c) additional lesions in the segments cranial and caudal to the primary injury caused by edema, circulatory disturbances, and other factors, so-called perifocal or secondary injuries, and (d) repair mechanisms.

Proposed secondary injury factors include the products of phospholipid hydrolysis, such as polyunsaturated fatty acids, eicosanoids, free radicals, or neuropeptides, monoamines and changes of cations and amino acids [4, 10, 19]. Recently, nitric oxide (NO) and carbon monoxide (CO) which are newly discovered gases involved in neuronal communication, appear to play important roles in the pathophysiology of cell injury following spinal trauma [16, 17]. CO is a by-product of heme degradation and serves as a messenger molecule like NO [1]. There is evidence that CO mimics many actions of NO both in vivo and in vitro [3, 7, 20]. However, details of their biological function in spinal injury are not yet well understood.

CO is synthesised by the enzyme hemeoxygenase (HO) that exists in two different isoforms, i.e., HO-1 (inducible) and HO-2 (constitutive) [1, 17]. HO-1 is induced by many diverse agents, such as heme substances, histamine H_2-receptor antagonists, adrenaline, insulin, glucagon, bacterial toxins, heat shock, oxidative stress, and many other forms of cellular stress [see review by Sharma *et al.*, 17]. On the other hand, the role of HO-2 in cell injury is still not well understood. HO-2 is constitutively expressed and may participate in signal transduction in the central nervous system (CNS) [3, 20].

Since spinal cord injury (SCI) is associated with marked oxidative stress [13], HSP expression [15], edema formation, and cell damage [11], it seems likely that traumatic insult induces upregulation of HO-2

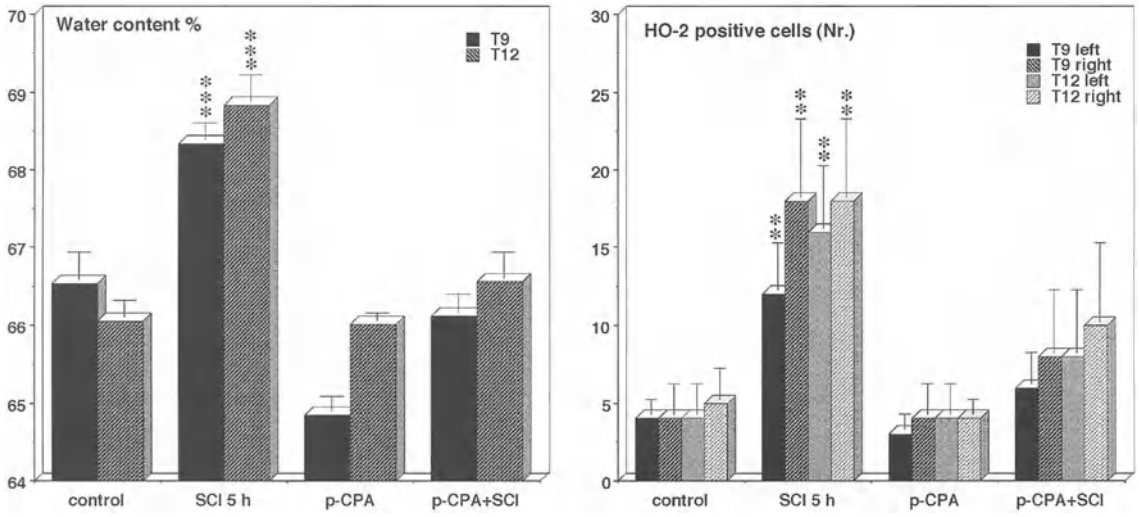

Fig. 1. Spinal cord water content (left panel) and number of HO-2 positive cells (right panel) in the spinal cord at 5 h following injury and its modification by p-CPA. *** $P < 0.001$, Student's unpaired t-test, ** $P < 0.01$, Chi Square test. SCI = spinal cord injury

expression. Previously, serotonin is shown to be a powerful mediator of spinal cord edema formation [14] and HSP response [15]. However, its interaction with the newly discovered neuromodulators NO and CO are not well understood. The present investigation was undertaken to examine, whether inhibition of endogenous serotonin synthesis prior to SCI influences upregulation of HO-2 expression. In addition, cell changes and HSP expression were also examined.

Material and methods

Animals

Experiments were carried out in 48 male Sprague Dawley rats (250 to 350 g) housed at controlled room temperature (21 ± 1 °C) with a 12 h light- and 12 dark schedule. Food and tap water were supplied ad libitum before the experiment.

Spinal cord injury

Under Equithesin anaesthesia (3 ml/kg, i.p.) laminectomy was carried out at the T10–11 segments. SCI was made by an incision into the right dorsal horn (about 2 mm deep and 4 mm long) using a sterile scalpel blade [11]. The wound was covered with cotton soaked in saline to avoid direct exposure of the cord to air. The level of anaesthesia was maintained by repeated administrations of a maintenance dose of Equithesin at regular intervals during the entire period of the experiments. The animals were allowed to survive 5 h after SCI. This experimental protocol was approved by the Ethics Committee of Uppsala University, Uppsala, Sweden. Equithesin anaesthetised normal animals were used as controls.

p-Chlorophenylalanine treatment

p-chlorophenylalanine (p-CPA-methyl ester, Sigma Chemical Co., USA) in powder form was dissolved in distilled water and administered intraperitoneally (100 mg/kg per day) for 3 days [11]. On the 4th day, SCI was made in the animals. One group of p-CPA treated animals were used as drug-treated controls.

Immunohistochemistry

5 h after SCI, the animals were perfused with a formalin based fixative (4% paraformaldehyde in 0.1 M phosphate buffered saline, pH 7.0 at 4 °C) preceded by a washout of blood with phosphate buffered saline [15]. The spinal cord segments from T9 and T12 were dissected out, and 40 µm multiple sections were cut on a Vibratome [15]. Immunostaining for HSP 72 and HO-2 were done on the free floating Vibratome sections according to a standard protocol described earlier [15, 17]. The dark brown reaction product was visualised by light microscopy and counted in a blinded fashion for semiquantitative analysis (see Fig. 1).

Spinal cord edema formation

The water content of the spinal cord of the T9 and T12 segments was determined in separate groups as described earlier [11]. The water content was calculated as the difference in wet and dry weight of the tissue sample as described earlier [13].

Spinal cord cell injury

The spinal cord tissue of the T9 and the T12 segments was processed for standard electron microscopy as described earlier [12]. In a group of rats, lanthanum chloride was added to the fixative to determine blood-spinal cord barrier permeability changes after trauma [11, 13].

Statistical analyses of the data obtained

Student's unpaired t-test was used to evaluate statistical significance of quantitative data obtained. The semiquantitative data were analyzed using non-parametric Chi-square test. A p-value less than 0.05 was considered significant.

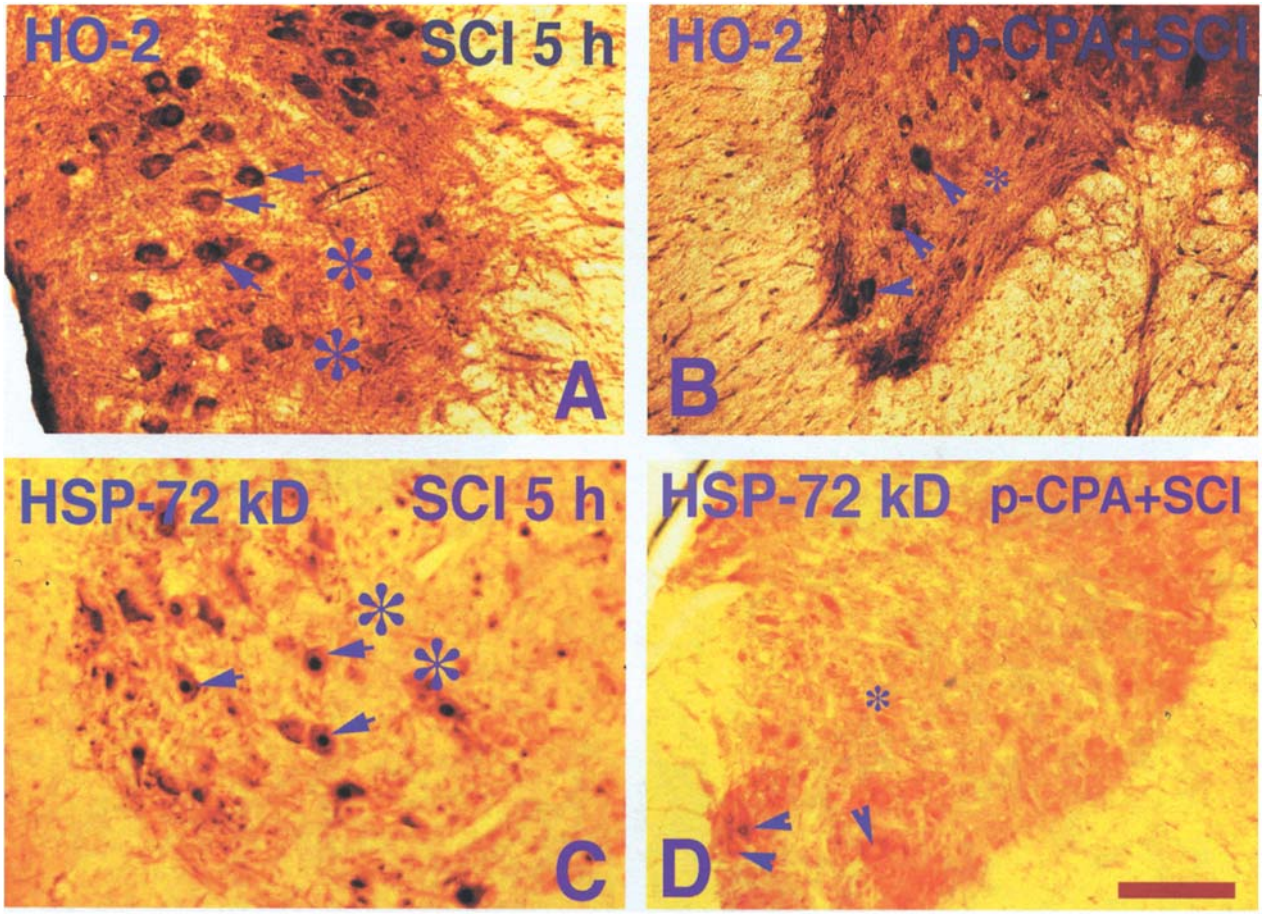

Fig. 2. Immunostaining of HO-2 (A,B) and HSP-72 kD (C,D) following spinal cord injury (*SCI*) and its modification by p-CPA. Many HO-2 (A) and HSP (C) positive cells can be seen following 5 h after SCI (arrows). Edema and sponginess are prominent (*) in these regions. Pretreatment with p-CPA markedly attenuated HO-2 expression (B) and HSP-72 kD upregulation (D) following SCI. In the treated group, only few positive cells (arrowheads) are present and signs of edema and sponginess are much less frequent. Bar: 25 μm

Results

Effect of p-CPA on HO-2 immunostaining

In normal animals, only few spinal cord nerve cells were positive for HO-2 immunostaining (Fig. 1). The focal trauma of the spinal cord induced a pronounced upregulation of HO-2 immunostaining in the T9 and the T12 segments (Fig. 1). The intensity of HO-2 immunostaining was most marked in the injured side of the cord as compared to the contralateral side (Fig. 1). In most cases, the nerve cytoplasm exhibited marked HO-2 immunostaining as compared to the cell nucleus. Pretreatment with p-CPA significantly attenuated the trauma induced HO-2 expression in the spinal cord (Fig. 1 and 2B). The effect of p-CPA on inhibition of HO-2 immunostaining was most marked in the contralateral side as compared to the ipsilateral cord (Fig. 1). In p-CPA treated traumatised animals, HO-2 expression was confined to only few sporadic nerve cells while in these regions, edema and expansion of the cord was markedly attenuated (Fig. 2B).

Effect of p-CPA on HSP immunostaining

The focal trauma was markedly increasing the number of HSP positive nerve cells in the spinal cord (Fig. 2C). HSP immunoreactivity was seen in both the nerve cell nucleus and the nerve cell cytoplasm. Cells exhibiting HSP immunoreactivity were often located in the edematous region of the cord. Pretreatment with p-CPA markedly reduced HSP expression in the traumatised cord (Fig. 2D). In this group of rats, the edematous expansion of the cord was considerably reduced. Thus, only few cells exhibited a HSP positive reaction with the cell nucleus remaining unstained (Fig. 2D).

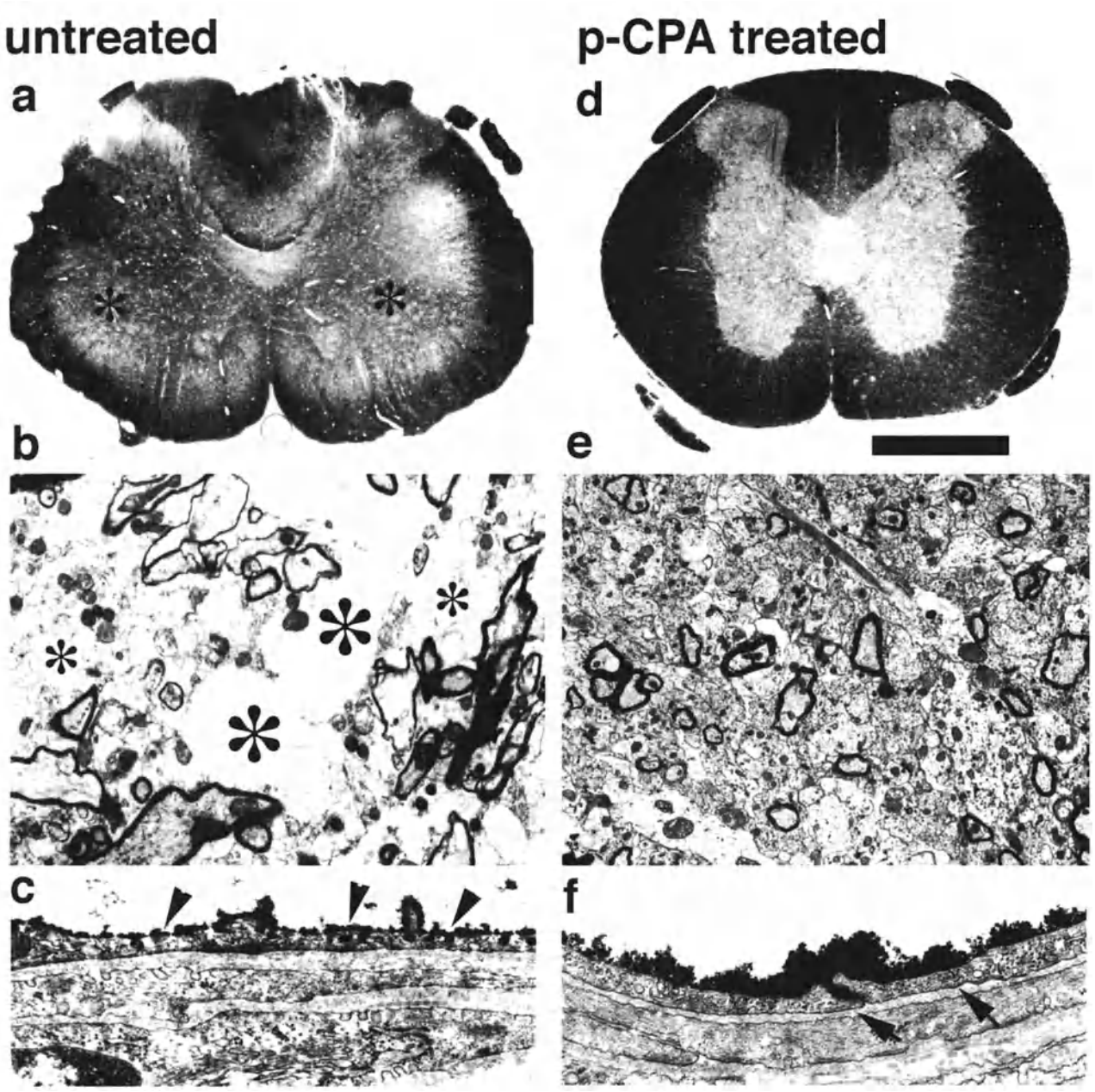

Fig. 3. Morphological alterations in the spinal cord and blood-spinal cord barrier following SCI and its modification by p-CPA. The gross pathology of the spinal cord following SCI shows profound expansion, of the cord, sponginess and edema (*) in the untreated injured animal (a). Pretreatment with p-CPA markedly attenuated the gross pathology of the spinal cord after trauma (d). The low power electron micrograph of the ventral horn of the spinal cord of one untreated rat exhibits membrane damage, vacuolization and edema (*) in the neuropil (b). These changes were much less apparent in the p-CPA treated traumatised rat (e). The high power electron micrograph of an endothelial cell of the dorsal horn of the spinal cord in an untreated injured rat shows several microvascular profiles loaded with lanthanum within the endothelial cytoplasm (arrowheads) (c). In a p-CPA pretreated injured animal, lanthanum is mainly confined to the vessel lumen, and its passage is stopped by the tight junction (arrow) (f). Bar: a,d = 5 mm; b,e = 1 μm, c,f = 600 nm (Modified after Sharma and Olsson 1990)

Effect of p-CPA on edema formation

Measurement of the water content of the spinal cord tissue revealed a profound increase of the T9 and T12 segments (Fig. 1). The edematous expansion of the cord is evident in the untreated traumatised animals (Fig. 3a), whereas pretreatment with p-CPA markedly attenuated the trauma induced edematous

expansion indicative of swelling of the spinal cord (Fig. 3b).

Effect of p-CPA on cell injury

In the untreated traumatised rats, the breakdown of the blood-spinal cord barrier (BSCB) was quite widespread, as visualized by the lanthanum tracer in several regions of the T9 and T12 segments of the cord (Fig. 3c). Thus, exudation of lanthanum across the endothelial cell was quite common in the untreated traumatised rats, exhibiting edema, membrane disruption, vacuolization, and damage of the neuropil (Fig. 3b, c).

Pretreatment with p-CPA markedly attenuated extravasation of the lanthanum tracer (Fig. 3f), and damage of the neuropil (Fig. 3e). Signs of vacuolization, membrane damage, edema and distorted myelin were much less intense compared to the untreated traumatised rats (Fig. 3e).

Discussion

The new finding of this study is the pronounced increase of HO-2 expression in the perifocal segments of the cord at 5 h following SCI. This indicates that SCI induces upregulation of the constitutive isoform of the HO enzyme. At this time, breakdown of the BSCB, edema formation, and cell injury were prominent in the cord, indicating that secondary injury mechanisms, as leakage and spread of edema fluid in the cord contribute to HO-2 expression. The HO-2 immunoreactivity in the damaged and distorted nerve cells which were located in the edematous region of the cord supports this idea.

It appears that an increased expression of the constitutive isoform of HO is injurious to the cell, while it has been shown earlier that expression of the inducible isoform is neuroprotective [5]. Expression of HO-1 coincides with glial scar formation occurring during regeneration in the CNS [10]. An increased HO-2 expression at 5 h after SCI suggests that secondary injury factors, as the release of several neurotransmitters and cell injury play an important role. The spinal cord trauma induced microhaemorrhages can also be considered to enhance HO-2 expression.

Expression of HO-2 is associated with the production of CO that causes induction of LTP, a feature commonly known to mediate neuronal injury in many pathophysiological conditions [1, 7, 8]. Another possibility of HO/CO induced neuronal damage may be due to the release of free iron which acts as a catalyst for the production of free radicals thereby amplifying neuronal damage [8, 20]. Thus, generation of CO from over-expression or HO-2 may be associated with cell injury.

Oxidative stress, haemorrhage and/or ischemia are important factors in the generation of the HO response following fluid percussion brain injury in rats [1, 5, 9, 18]. Since SCI is markedly associated with ischemia, microhaemorrhages, and oxidative stress [13], these factors may somehow influence HO-2 induction in the cord following SCI.

In an *in vitro* model of anoxia and in fluid percussion injury, HO inhibitors metalloporphyrins (Sn-PP or Zn-PP) are neuroprotective. These inhibitors exert some anti-inflammatory effects which are apparent from the finding that Zn-PP attenuates infarct size and edema following cerebral ischemia [6]. In the present study, p-CPA treatment was markedly reducing SCI induced expression of HO-2 and cell damage. This supports that upregulation of HO-2 is associated with neurodestruction by generation of CO [for review see 17].

Trauma of the spinal cord induces a profound increase in plasma and cord tissue serotonin [14] indicating that an elevated level of tissue and blood serotonin influences HO-2 expression in the cord. In p-CPA treated traumatised rats, an elevation of the endogenous serotonin level was mainly absent [11]. In the absence of an endogenous serotonin elevation, the cellular and molecular mechanisms may not be operating causing overexpression of HO-2 in the cord. A direct inhibitory effect of serotonin on HO-2 expression in the spinal cord is unlikely, because depletion of serotonin with p-CPA in normal rats does not influence HO-2 expression.

The abnormal expression of HSP in the spinal cord following trauma suggests that cellular stress plays an important role in tissue damage. The absence of HSP expression in p-CPA treated and traumatised rats indicates that a serotonin dependent cellular stress response was somehow responsible for the HO-2 expression and cell injury. The reduction in HSP response following p-CPA treatment is indicative of a reduction of the trauma induced stress response, rather than of a direct inhibitory influence of p-CPA on HSP expression [12, 15, 21]. Thus, it seems likely that the SCI induced cellular stress plays a significant role in upregulation of HO-2 expression, a feature that requires additional investigation.

Conclusion

Our observations suggest that (i) an increased production of CO, as evident by upregulation of the HO-2 expression, is contributing to the spinal cord pathology and (ii) that endogenous serotonin is involved in the trauma induced overexpression of HO-2 in the cord, which has not been reported earlier.

Acknowledgments

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Correspondence: Hari Shanker Sharma, Dr. Med. Sci., Frödingsgatan 12, App: 28, SE-75421 Uppsala, Sweden. e-mail: Sharma@medcellbiol.uu.se