Acta Neurochir (1997) [Suppl] 70: 130–133

Topical Application of Insulin Like Growth Factor-1 Reduces Edema and Upregulation of Neuronal Nitric Oxide Synthase Following Trauma to the Rat Spinal Cord

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Summary

The neuroprotective effects of insulin like growth factor-1 (IGF-1) on spinal cord injury induced edema formation, cell changes and profound upregulation of constitutive isoform of neuronal nitric oxide synthase (cNOS) was examined in a rat model. A focal spinal cord injury produced by making a lesion (about 2 mm deep and 5 mm long) of the right dorsal horn of the T10–11 segment resulted in a marked edema formation, cell injury and upregulation of cNOS following 5 h after trauma. In separate groups application of IGF-1 (0.1 μg/μl) topically on the exposed spinal cord (T10–11) starting from 30 min before injury (20 μl), immediately before injury followed by 30 min, 60 min and thereafter every 1 h after injury until sacrifice resulted in significant attenuation of edema formation and cell changes. Immunohistochemistry showed a less pronounced expression of cNOS in the T9 and T12 segments of the cord in IGF treated rats compared to untreated traumatised controls. These results for the first time show that IGF treatment is neuroprotective and this effects of the IGF appears to be mediated via inhibition of NOS upregulation.

Keywords: Spinal cord injury; insulin like growth factor; nitric oxide synthase; edema.

Introduction

Insulin-like growth factors (IGF) have numerous actions on neuronal and glia cell functions *in vitro*. However, its role in the CNS *in vivo* situation still remains unknown [4–7]. Brain and spinal cord contains relatively high levels of IGF-1 and there are recent evidences which suggest that the content of IGF is reduced following spinal cord injury [5, 12]. On the other hand human cases of amyotrophic lateral sclerosis (ALS) showed upregulation of IGF-1 binding in the gray matter of spinal cord [1]. There are reports indicating that various growth factors like IGF-1, nerve growth factors (NGF) and ciliray neurotrophic factor (CNTF) can rescue motor neuron atrophy in Alzheimer's diseases, Parkinson diseases, multiple sclerosis and Huntington's diseases [4, 6, 7, 13, 14]. These evidences strongly point out a neuroprotective effect of growth factors in the diseases of nervous system.

Recently the involvement of nitric oxide, a gaseous molecule, in the pathophysiology of cell injury has been suggested [2, 3]. Previous observation from our laboratory showed a marked upregulation of nitric oxide synthase (constitutive and neuronal type, cNOS) following a focal trauma to the rat spinal cord [11]. The number of cNOS positive neurons correlates well with the edematous expansion of the spinal cord and blockade of such activity with cNOS antiserum exhibits neuroprotection indicating an involvement of NO in the pathophysiology of spinal cord edema and cell injury. There are recent evidences indicating that IGF is neuroprotective in ischemic brain injury and experimental autoimmune encephalomyelitis [7, 13]. This investigation was undertaken to examine whether pretreatment with IGF-1 can influence cell changes and edema formation following a focal spinal cord injury in our rat model. Furthermore, in order to clarify the mechanisms of IGF-1 induced neuroprotection we examined the upregulation of cNOS following spinal cord injury using immunohistochemical techniques.

Materials and Methods

Animals

Experiments were carried out on 30 male Wistar rats (body weight 200–300 g) kept under controlled ambient conditions (21 ± 1°C) with 12 h light and 12 h dark schedule. The rat food and tap water were supplied *ad libitum*.

Spinal Cord Injury

Spinal cord injury was inflicted under Equithesin (3 ml/kg, i.p.) anaesthesia by making a longitudinal incision (about 2 mm deep and 5 mm long) into the right dorsal horn of the T10–11 segments [8–10]. The wound was covered with a cotton soaked in 0.9% saline. The rats were allowed to survive 5 h after injury. This experimental condition is approved by the Ethical Committee of Uppsala University.

Treatment with Insulin-like Growth Factor

In a separate group of traumatised rats, IGF-1 (0.1 µg/µl, Kabi Pharmacy, Stockholm) was applied topically on the exposed spinal cord (T10–11) starting from 30 min before injury (20 µl), immediately before injury followed by 30 min, 60 min and thereafter every 1 h after injury until sacrifice.

Spinal Cord Edema

Spinal cord edema was examined using water content of the spinal cord [9, 10]. For this purpose, the spinal cord segments from the T9, T10–11 and T12 were excised, weighed immediately and the placed in an oven maintained at 90°C for 72 h or until the last three measurements of the dry weight were constant. The water content was calculated from the differences between wet and dry weight of the cord.

Nitric Oxide Synthase Immunohistochemistry

For the purpose of NOS immunostaining, the animals were perfused with about 150 ml of 4% paraformaldehyde containing 1.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature after a brief washout of the intravascular blood by 0.1 M phosphate buffer (50 ml) at 90 torr perfusion pressure. After perfusion, the animals were wrapped in an aluminium foil and kept overnight at 4°C in a refrigerator. On the next day, the spinal cord samples were dissected out and placed in the same fixative at 4°C. The c-NOS immunoreactivity was examined on free floating vibratome sections (60 µm thick) using antiserum directed against constitutive isoform of neuronal nitric oxide synthase as described earlier [11].

Experimental Protocol

Spinal cord injury (n = 20) was inflicted on the right dorsal horn of the T10–11 segments by making an incision. In one group of animals (n = 10) IGF-1 (0.1 µg/10 µl in phosphate buffer saline) was applied topically 30 min before injury on the exposed spinal cord followed by repeated doses of IGF-1. Normal animals (n = 10) were used as controls. Spinal cord edema was examined in control (n = 5), spinal cord traumatised (n = 5) and IGF treated traumatised (n = 5) rats while NOS immunohistochemistry was done in separate groups of normal (n = 5), spinal cord injured (n = 5) and IGF-1 treated injured (n = 5) rats. Thereafter application of IGF-1 was carried out at every 1 h interval until sacrifice.

Statistical Analysis

The data were analysed statistically using ANOVA followed by Dunnett's test. A p-value less than 0.05 were considered significant.

Results

Spinal Cord Edema

Rats subjected to 5 h spinal cord trauma exhibited profound increase in the spinal cord edema in all the three segments examined. This is apparent with a significant increase in the water content of the T9 (from 66.76 ± 1.23 to $70.23 \pm 1.24\%$, $P < 0.001$), T10–11 (from 66.87 ± 0.34 to $72.17 \pm 0.56\%$, $P < 0.001$) and T12 (from 67.04 ± 0.89 to $71.06 \pm 0.89\%$, $P < 0.01$) segment compared to the control group. Pretreatment with IGF-1 significantly thwarted the edema development in the spinal cord following 5 h after injury. Thus, the water content in the T9 ($68.23 \pm 0.46\%$, $P < 0.05$), T10-11 ($69.38 \pm 0.56\%$, $P < 0.05$) and T12 ($68.81 \pm 0.38\%$, $P < 0.05$) segments was significantly lower compared to the untreated traumatised group ($P < 0.05$).

Spinal Cord NOS Immunostaining

The control rats showed only a few cNOS positive neurons unevenly distributed in the gray matter of the spinal cord of the T9, T10–11 and T12 segments. A focal trauma to the cord significantly upregulated the NOS immunostaining in the traumatised spinal cord after 5 h. This increase in the NOS immunostaining was most marked in the ipsi-lateral side of the cord compared to the contralateral side. The immunostaining can be seen in the cell cytoplasm and occasionally nucleus of the neurons were densely stained.

Pretreatment with IGF-1 markedly attenuated the cNOS immunostaining in the spinal cord. Thus only a few NOS positive neurons can be seen in the IGF treated traumatised rats. Few NOS neurons showed elongated axons within 5 h period, a feature not observed in untreated rats (Fig. 1).

Examination of NOS immunostaining at ultrastructural level showed that the immunostained material is mainly confined within the cell boarders and located within the cytoplasm attached with endoplasmic reticulum as dark black particies (Fig. 2). This increase in immunostaining at ultrastructural level was much less evident in animals received IGF-1 treatment before injury.

Spinal Cord Pathology

Light microscopy of spinal cord injured rats showed profound swelling and edematous expansion of the cord in the T9, T10–11 and the T12 segments of the cord. The neurons were mainly distorted and the distinction between the gray and white matter of the cord in injured animals was not clear. On the other hand, spinal cord general expansion was considerably reduced and the neurons were less distorted in rats re-

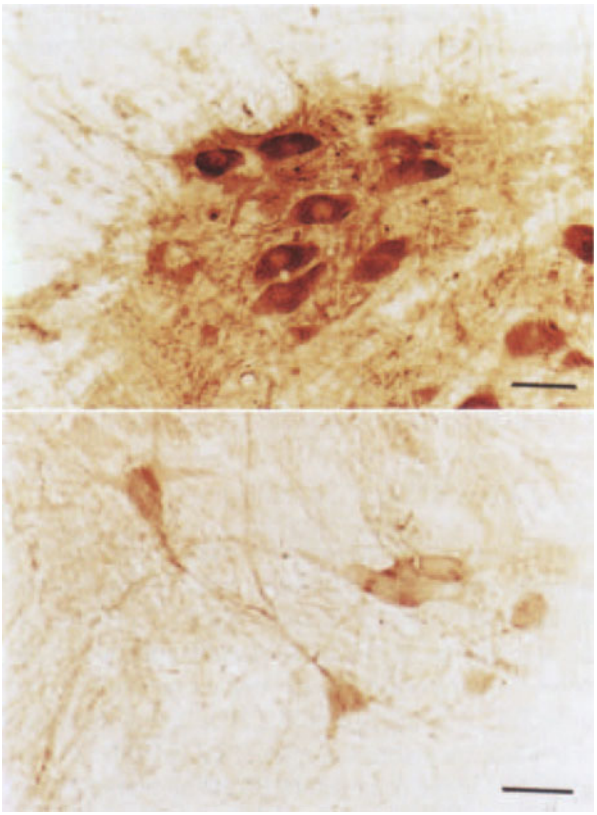

Fig. 1. 5 h spinal cord injury; T9 ventral horn. Light micrograph of cNOS upregulation in the T9 segment of one spinal cord injured rat and its modification with IGF-1 pretreatment (bar = 50 μm). Pretreatment with IGF-1 markedly attenuated the number of NOS positive neurons after spinal cord injury. Upper: untreated; lower: IGF-1 treated

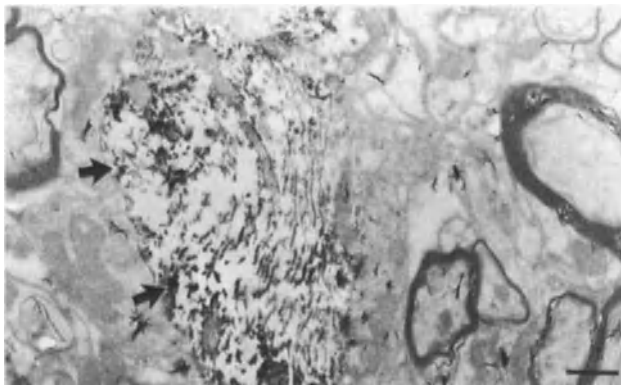

Fig. 2. cNOS immunostaining; T9 ventral horn. Low power electron micrograph shows NOS immunostaining in the cytoplasm of one nerve cell (arrows) attached to the endoplasmic reticulum (dark black particies) following 5 h spinal cord injury in the dorsal horn of the T9 segment (bar = 1 μm)

ceived IGF-1 compared to the untreated injured group. The gray and white matter were distinctly separated and the neuropil appears quite preserved.

Discussion

The most salient new finding of the present study is that repeated application of IGF-1 on the traumatised cord significantly attenuated the edematous swelling and reduced the cell changes of the cord, a feature not reported earlier. Our results are the first to show a significant neuroprotective effects of IGF-1 in *in vivo* situation following acute spinal cord injury.

The spinal cord is very rich in IGF levels and spinal cord injury reduces the IGF content [5, 7]. Our results showed a marked cell injury in the spinal cord of untreated animals. This suggests that a possible decrease in the IGF-1 level following spinal cord injury deprives the needed growth factors to neurons resulting in atrophy or cell death [6, 7, 13, 14]. In the present study, topical application of IGF-1 apparently protects the nerve cell changes and further suggests that if the growth factors are applied exogenously following trauma, they can induce neuroprotection. The probable mechanisms of IGF induced neuroprotection is unclear. However, it may be that loss of the endogenous growth factor can be restored with exogenous application of the IGF-1. To further confirm this measurement of the growth factor in untreated and IGF-1 treated rats are needed, which may be the subject for additional investigation. Another possibility of the neuroprotective mechanisms of IGF-1 could be due to its effect on attenuation of the trauma-induced stress reaction in the neurons by making an enriched microenvironment and thereby reducing the signs of cell damage [7, 14].

The second most important finding from this study is that IGF-1 has the capacity to thwart cNOS upregulation following spinal cord injury. This observation suggest that growth factors may mediate their neuroprotective effects via inhibition of NOS regulation. This study is the first to provide immunohistochemical evidences that NOS upregulation following spinal cord injury are reduced by repeated topical applications of the IGF-1. The possible mechanisms underlying IGF-1 induced neuroprotection is not known in all its details [7, 13, 14]. However, a possibility exists that the beneficial effects of IGF-1 in spinal cord injury is mediated via inhibition of NOS upregulation [2, 3].

The mechanisms by which IGF induced downregulation of cNOS following spinal cord injury is unclear from this study. It may be that IGF-1 influences the intracellular cascade following trauma by modifying signal transduction and intracellular accumulation of

Ca^{2+} and thus influencing NOS upregulation [2, 3], a feature which require additional investigation. Another possibility is that IGF somehow attenuates the intensity of cellular stress caused by trauma [7], a feature which require additional investigation using specific markers of stress reaction such as heat shock protein and heme oxygenease in IGF-1 treated traumatised rats.

Acknowledgements

This study is supported by grants from Swedish Medical Research Council nrs. 2710, 9459, 9710, 11205, Göran Gustafsson Foundation, Torsten and Ragner Söderberg Foundation, Sweden; Alexander von Humboldt Foundation, Bonn, Germany; The University Grants Commission, New Delhi, India. The technical assistance of Kärstin Flink, Ingamarie Olsson, Inga Hjörte, Ulla Johansson; photographic assistance of Frank Bittkowski and the secretarial assistance of Aruna Misra and Gun Britt Lind are highly appreciated.

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