Role of monoamines in experimental spinal cord injury in rats

Relationship between Na\textsuperscript{+}-K\textsuperscript{+}-ATPase and lipid peroxidation

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A spinal cord injury was produced in Wistar rats by extradural compression of the cord with a Sugita aneurysm clip for 5 seconds. During a 2-week observation period following the injury, the tissue norepinephrine (NE), dopamine (DA), and serotonin (5-HT) concentrations decreased uniformly at and below the injured site. The chemical denervation of NE or 5-HT neurons produced by the intraspinal injection of 6-hydroxydopamine (6-OHDA) or 5,7-dihydroxytryptamine (5,7-DHT) 2 weeks before the injury did not cause a marked difference in the extent of hemorrhagic necrosis of the spinal cord after trauma as compared to control animals without pretreatment. In the rats pretreated with 6-OHDA, NE was decreased to less than 30% of control (non-pretreated) values, and, beginning at 5 days after injury, motor performance (assessed quantitatively with the inclined-plane method) was significantly improved compared to results in the non-pretreated control rats. The rats pretreated with 5,7-DHT showed no change from control animals. Spinal cord samples from non-pretreated control animals obtained at the injury site 30 minutes after the compression injury showed a marked decrease in the activity of synaptosomal Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (adenosine triphosphatase) of about 50%, and an increase in both thiobarbituric acid reaction substance (about 170%) and cyclic guanine monophosphate (about 150%). The NE-denervated rats showed no significant changes in these three parameters.

The results indicated that NE released after crush injury may impair the neuronal cell membrane around the lesion site by induction of lipid peroxidation. The possible mechanisms by which released NE may alter membrane function are discussed.

KEY WORDS • experimental spinal cord injury • monoamine • Na\textsuperscript{+}-K\textsuperscript{+}-ATPase • adenosine triphosphatase • lipid peroxidation • chemical denervation • rat

The hypothesis of norepinephrine (NE) metabolism alterations in experimental spinal cord injury, first proposed in 1971 by Osterholm and Mathews,\textsuperscript{23-25} remains controversial. On reanalyzing the tissue levels of NE, Alderman, et al.,\textsuperscript{1} recently reversed their previous claim that central NE increased fourfold at the lesion site 1 hour after cord injury. However, their other finding that inhibitors of NE synthesis, such as alpha-methyl tyrosine, could increase motor recovery by decreasing the accumulation of NE around the lesion site may be valid, and this critical issue should be resolved by further investigation.

Demopoulos, et al.,\textsuperscript{6} and others,\textsuperscript{9,14,15} suggested that cell damage following ischemia or cord injury was induced by free-radical reaction and lipid peroxidation. It is also well known that some membrane-bound enzymes require phospholipids for maintenance of their activities. In particular, Na\textsuperscript{+}-K\textsuperscript{+}-activated ATPase (adenosine triphosphatase) is very susceptible to free-radical reaction and lipid peroxidation, because of two lipid moieties in its structure.\textsuperscript{36}

The present study was performed to investigate the hypothesis of NE metabolism changes following spinal cord injury proposed by Osterholm and Mathews,\textsuperscript{23-25} and to evaluate the contribution of monoamines to the pathophysiological mechanism of cell damage after injury by simultaneous determination of synaptosomal Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity and liperoxides.

Materials and Methods

**Animal Preparation and Injury Procedure**

Male Wistar rats, weighing 280 to 320 gm each, were used for this study. All surgical procedures were per-
formed under anesthesia induced by intraperitoneal injection of pentobarbital sodium (40 mg/kg).

The chemical denervation of spinal cord NE or serotonin (5-HT) neurons was produced by 6-hydroxydopamine (6-OHDA) or 5,7-dihydroxytryptamine (5,7-DHT) pretreatment. The denervation agents were dissolved in 0.9% NaCl containing 0.1% ascorbic acid. Two weeks before trauma, the solution mixture (2 μg/μl) of 6-OHDA or 5,7-DHT was injected stereotaxically into the central gray matter bilaterally at the C-4 vertebral level (coordinates: 1.0 mm lateral to the midline and 1.0 mm deep from the dorsal surface of the spinal cord) at a rate of 1 μl over 3 minutes.

Spinal cord injury was produced by extradural compression of the exposed spinal cord at the T-2 level for 5 seconds with a Sugita aneurysm clip, which produces a closing force of 120 gm on the cord. Clinical motor function was evaluated by the inclined-plane method described by Rivlin and Tator at 1, 3, 5, 7, 10, and 14 days after injury. Some uninjured control animals underwent a laminectomy only, and were treated with pentobarbital anesthesia alone or with pentobarbital and 6-OHDA to determine if these treatments exerted an influence on the biochemical factors being measured.

Sample Preparation

Animals were sacrificed by decapitation at appropriate times after injury. The spinal cord was removed and 1-cm samples were taken from the injury site (at the T-2 level), and from above and below the lesion. The sample from the injury site was divided into three blocks, two of which were immediately frozen and stored, sealed in a -80°C freezer for assays of monoamines, cyclic guanine monophosphate (GMP), and lipoperoxides. The third block was immediately frozen and 1.0 mm deep from the dorsal surface of the spinal cord and was stored in a -80°C freezer for assay of ouabain-sensitive Na+-K+-ATPase.

Histological analysis of the extent of hemorrhagic necrosis of the central gray matter was also made using an image analysis system.

Determination of Monoamines

The concentration of NE and dopamine (DA) in spinal cord segments was measured simultaneously by the method of Kawano, et al., using a gas chromatograph equipped with an electron capture detector. The frozen samples pooled from four rats were homogenized in 0.4 N perchloric acid containing 0.05% EDTA (ethylene-diaminetetra-acetic acid) and alpha-methyl dopamine (100 ng/100 μl 0.01 N HCl) as the internal standard before being centrifuged at 18,000 G for 15 minutes at 2°C. The NE and DA in the supernatant were extracted according to the method of Wang, et al.

The concentration of 5-HT in the deproteinized supernatant was measured using a high-performance liq-

uid chromatograph with an electrochemical detector according to the modified method of Reinhard, et al.

Synaptosomal Na+-K+-ATPase Activity

The frozen synaptosomal pellet was defrosted and resuspended in 1 ml cold homogenizing buffer. Then, 150-μl aliquots (40 to 80 μg protein) of the homogenate were incubated at 37°C in 350 μl reaction mixture (50 mM Tris-HCl, 3 mM MgCl2, 100 mM NaCl, 10 mM KCl, 1 mM EDTA, and 3.3 mM Tris-ATP). After 15 to 30 minutes of incubation, the reaction was terminated by the addition of 0.5 ml of 12% trichloroacetic acid. Inorganic phosphate released from ATP was determined by the method of Allen as modified by Nakamura and Mori. The value of Na+-K+-ATPase activity was calculated from the difference between that activity in the absence (total-ATPase) and presence (Mg-ATPase) of 1 mM ouabain. Protein levels were determined by the Folin phenol reagent method described by Lowry, et al. Activity of Na+-K+-ATPase was expressed as μM Pi/mg protein/hr.

Determination of Lipoperoxides

Lipid peroxidation in injured spinal cord was estimated by the thiobarbituric acid reaction method for malondialdehyde (TBA-RS) described by Ohkawa, et al. The reaction mixture was extracted with the mixture of n-butanol and pyridine. The fluorescence of the organic layer was measured in a spectrophotofluorometer, with excitation 515 nm and emission 553 nm.

Assay of Cyclic GMP

Acetylated cyclic GMP was determined by radioimmunoassay according to the method of Steiner, et al. Protein levels in the trichloroacetic acid pellet were determined by the method of Lowry, et al. Cyclic GMP content was expressed as pmoles/mg protein.

Results

Alteration of Monoamine Content

Two weeks after 6-OHDA pretreatment, the concentration of NE below the C-4 vertebral level was reduced to 28% of the non-pretreated control values (412 ± 47 ng/gm wet weight). The 5-HT content was slightly decreased, but the reduction was not statistically significant and the DA level was not altered (control: 18.6 ± 2.4 ng/gm wet weight). In the 5,7-DHT-pretreated cord, the concentration of 5-HT was decreased to approximately 35% of the non-pretreated control values (466 ± 58 ng/gm wet weight) and the levels of the other monoamines, NE and DA, were not altered (Fig. 1).

The alteration of monoamine content in control rat spinal cord following injury is shown in Fig. 2. Depletion of NE levels began 2 hours after injury at and below the injury site and was reduced to approximately 50% at 1 week after injury. The DA and 5-HT concentrations also decreased beginning 2 hours after injury and remained depressed during the 2-week observation...
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FIG. 1. Alteration of monoamine levels in the spinal cord below C-4 2 weeks after 6-OHDA or 5,7-DHT pretreatment showing the effect of chemical denervation on spinal cord monoamines. Control values for norepinephrine (NE): 412 ± 47 ng/gm wet weight; for dopamine (DA): 18.6 ± 2.4 ng/gm wet weight; and for serotonin (5-HT): 466 ± 58 ng/gm wet weight. Columns and vertical bars represent mean values ± standard error of the mean for 10 samples. Student's t-test was used to test the significance of differences from control values (* = p < 0.01).

FIG. 2. Alterations of monoamine content at the injury site at stated times after injury. The columns and vertical bars represent the mean percentage of control values ± standard error of the mean for 10 samples. The concentration of monoamines at the injury site was reduced starting 2 hours after injury and remained depressed during 2 weeks of observation. The Student t-test was used to test the significance of differences from control values (* = p < 0.05; ** = p < 0.01; and *** = p < 0.001).

Clinical Performance

The motor function after injury, evaluated by the inclined-plane method, is shown in Fig. 3. Although the non-pretreated and the 5,7-DHT-pretreated rats revealed similar paraparesis after injury that remained for 14 days, the 6-OHDA-pretreated rats demonstrated a moderate improvement beginning 5 days after injury. At 14 days after injury, they revealed a remarkable recovery as compared to non-pretreated and 5,7-DHT-pretreated rats (the rats adjusted to an inclined plane of up to 66.4° ± 3.0°, 52.6° ± 3.1°, and 54.5° ± 2.6°, respectively); this recovery was statistically significant (p < 0.01).

Central Gray Hemorrhagic Necrosis

No significant difference was observed in the extent of central gray hemorrhagic necrosis at the injury site between pretreated and non-pretreated spinal cords at 30, 60, and 120 minutes after injury. As shown in Table 1, the extent of necrosis in non-pretreated control cords measured by the image analysis system at 7 days after injury occupied 56.32% ± 2.46% of the entire area of the cord section, and the necrotic area in 6-OHDA- and 5,7-DHT-pretreated cords measured 53.44% ± 1.59% and 54.39% ± 2.39%, respectively.

Synaptosomal Na⁺-K⁺-ATPase Activity Posttrauma

Figure 4 left shows that neither pentobarbital anesthesia nor 6-OHDA pretreatment influenced synaptosomal Na⁺-K⁺-ATPase activity at the T-2 spinal cord level before injury. As shown in Fig. 4 right, at 30
FIG. 3. Clinical performance after injury as evaluated by the inclined-plane method. Symbols represent an average of the maximum angle ± standard error of the mean for 20 rats. At 1 day after injury, non-denervated rats adjusted to an inclined plane up to 38.2° ± 4.1°; norepinephrine (NE)-denervated rats to 40.2° ± 3.3°; and serotonin (5-HT)-denervated rats to 42.3° ± 3.8°. At 14 days after injury, non-denervated rats adjusted to an inclined plane up to 52.6° ± 3.1°; NE-denervated rats to 66.4° ± 3.0°; and 5-HT-denervated rats to 54.5° ± 2.6°. Student's t-test was used to test the significance of differences from the values of non-denervated or 5-HT-denervated rats (* = p < 0.05; and ** = p < 0.01).

TABLE 1

<table>
<thead>
<tr>
<th>Study Group</th>
<th>No. of Rats</th>
<th>Extent of Necrosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>no denervation</td>
<td>8</td>
<td>56.32 ± 2.46</td>
</tr>
<tr>
<td>NE denervation</td>
<td>8</td>
<td>53.44 ± 1.59</td>
</tr>
<tr>
<td>5-HT denervation</td>
<td>8</td>
<td>54.39 ± 2.37</td>
</tr>
</tbody>
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* The extent of central hemorrhagic necrosis at 7 days after injury measured by the image analysis of histological findings as a percentage of the entire section of cord. None of these data showed a statistically significant difference as calculated by Student's t-test (p < 0.05).

NE = norepinephrine; 5-HT = serotonin.

minutes after injury the activity of this enzyme at the injured site of non-pretreated rats was reduced to 51.72% of corresponding control values (laminectomy only: 3.27 ± 0.39 μM Pi/mg protein/hr) — a statistically significant change (p < 0.01). On the other hand, in the 6-OHDA-pretreated rats, Na⁺-K⁺-ATPase was protected. It was not decreased significantly as compared to corresponding control values (6-OHDA-pretreatment plus laminectomy: 3.03 ± 0.27 μM Pi/mg protein/hr).

Alteration of TBA-RS Content

The TBA-RS content at the injury site decreased to 83.15% of untreated control values (67.20 ± 6.17 nM/gm protein) on administration of pentobarbital anesthesia before injury. At 30 minutes after injury, the TBA-RS level increased to 169.51% of control values, which was a significant difference (p < 0.001). However, 6-OHDA pretreatment neither altered the content of TBA-RS before injury, nor increased it at 30 minutes after injury. Thus, 6-OHDA pretreatment also protected the increase of TBA-RS following injury (Fig. 5).

Alteration of Cyclic GMP Content

Cyclic GMP content at the injury site of non-pretreated rats increased to 151.53% of control values (laminectomy only: 0.42 ± 0.05 pM/mg protein) at 30 minutes after injury. However, no significant alteration was detected in 6-OHDA-pretreated rats (Fig. 6).
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![Graph](image)

**Fig. 5.** Alteration of lipoperoxides (TBA-RS) at the injury site 30 minutes after injury. *Columns and vertical bars* express the mean percentage of untreated control values ± standard error of the mean for five samples (67.20 ± 6.17 nM/gm protein). Although pentobarbital slightly decreased the TBA-RS content before injury (*p < 0.05), after injury the content of TBA-RS at the injury site of non-pretreated rats (without NE-denervation) was significantly increased (**p < 0.001), and was markedly higher than that of pre-treated rats with norepinephrine (NE)-denervation (p < 0.001). Statistical significance was measured by Student’s t-test.

**Discussion**

Since Osterholm and Mathews23–25 postulated that hemorrhagic necrosis in the central gray matter following spinal cord injury was produced by vasoconstrictive action of released NE, many studies have been performed on the role of monoamines in the pathogenesis of spinal cord injury.3,10,11,15,19 Osterholm and Mathews based their hypothesis mainly on two findings: the increase of NE content around the lesion site after impact injury in cats, and the dramatic effect of administration of alpha-methyl tyrosine (tyrosine hydroxylase inhibitor) on the pathological changes and motor recovery. Their hypothesis still remains controversial.

The present study has failed to confirm either the findings or the conclusions proposed by Osterholm and Mathews. The conflicting results may be partially caused by differences in the method of producing the injury, in analyzing the content of monoamines, and in species used in experimental models.3,7,10,11,13,19 Alderman, et al.,1 reevaluated and reanalyzed the results of Osterholm and Mathews.23,24 They reported a decrease of NE at 60 and 120 minutes after injury in the same model as reported by Osterholm and Mathews, and discussed artifacts in the biochemical analysis of catecholamine in the original publication. Our results show a decrease in the concentrations of NE, DA, and 5-HT at and below the injury site; these data are in agreement with those reported by Alderman, et al.,1 Hinwood, et al.,11 and Bingham, et al.1 However, our finding that 6-OHDA-pretreated rats in which NE was decreased to less than 30% of the control value showed improved function as compared to the non-pretreated controls and to the 5,7-DHT-pretreated rats, leads us to consider that released NE may still play a role in the pathogenesis of the spinal cord lesion caused by trauma. Our other finding that the extent of hemorrhagic necrosis in the central gray matter after injury in 6-OHDA-pretreated cords does not show a significant difference from that of non-pretreated control and 5,7-DHT-pretreated rats suggests that released NE may have an important direct influence on cellular function rather than playing a role in production of the central gray hemorrhagic necrosis.

Demopoulos, et al.,6 reported that cell membrane damage in the central nervous system following cerebral ischemia and spinal cord injury may be induced by free-radical reaction and lipid peroxidation. Synaptosomal Na+-K+-ATPase which maintains the resting membrane potential physiologically has been demonstrated to be a phospholipid-dependent membrane-bound enzyme26 and is very susceptible to free-radical reaction and lipid peroxidation.34 Clendenon, et al.,5 reported that the activity of this enzyme decreased as early as 5 minutes after spinal cord impact injury in dogs. Recently, Braughler and Hall10 also demonstrated the relationship between the inhibition of activity of this enzyme and lipid peroxidation following spinal cord injury. In our investigation, both the decrease in Na+-K+-ATPase activity and the increase in the generation of lipoperoxides after trauma could be prevented in rats with 6-OHDA pretreatment, which may suggest that released NE impairs the neuronal cell membrane by induction of lipid peroxidation. The increase in cyclic GMP, which was used as an index of injury-
induced free-radical reaction by Hall and Braughler,² may also support this suggestion.

The following possible mechanisms of NE in the generation of free radicals and lipoperoxides should be considered. First, the enzymatic lipid peroxidation mechanism may be initiated during the synthesis process of prostaglandin-like substances secondary to enhanced phospholipase A₂, and increase in intracellular cyclic adenosine monophosphate (AMP) content¹⁷ or Ca++.²⁶ Gullis and Rowe¹⁰ have shown the stimulation of phospholipase A₂-acylation system of synaptic membrane by cyclic nucleotides. Levine and Moskowitz¹¹ have stated that stimulation of arachidonic acid metabolism occurs via NE mediated by alpha- or beta-receptors. The protective effect of alpha-adrenergic receptor blocker and alpha-methyl tyrosine on the motor recovery observed by Hedeman, et al.,¹⁰ or Osterholm and Mathews²³-²⁵ may be explained by this mechanism.

Second, the non-enzymatic lipid peroxidation mechanism may be propagated when the released NE is metabolized by degradation enzymes, monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT). Quinone, one of the degradation products, is considered to produce free radicals. Furthermore, as Seregi, et al.,³⁰,³¹ have pointed out, MAO also produces hydrogen peroxide which stimulates endoperoxide synthesis.

We would like to emphasize that, in the pathogenesis of spinal cord injury, both ischemia induced by microcirculatory disturbance from released NE and NE itself may be noxious to neuronal cell membrane by the generation of free radicals and lipoperoxides following injury.

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