Free and Peptide-Bound Amino Acids as Indicators of Ischemic Damage of the Rabbit Spinal Cord

J. Martiniak, Ph.D., K. Saganová, M.Sc., and M. Chavko, Ph.D.

Abstract. The concentrations of free and peptide-bound amino acids aspartate (Asp), glutamate (Glu), glycine (Gly) and gamma-aminobutyric acid (GABA) were studied following ischemia and recirculation in the ventral and dorsal gray matter of the rabbit spinal cord. No changes in the concentrations of amino acids following ten minutes (min) of ischemia and four days of recovery were found. The most significant change after 20 min, and especially 40 min, of ischemia was a decrease in free Asp and GABA levels in both parts of the gray matter. The relatively greater decrease in the concentration of free amino acids in the ventral horns corresponds with the greater morphological damage to this part of spinal cord following ischemia. Following 40 min of ischemia and recirculation decrease in peptide-bound Glu in the ventral horns and Asp and Glu in the dorsal horns was found.

Key Words: Amino acids, bound; Amino acids, free; Ischemia; Spinal cord.

INTRODUCTION

Temporary occlusion of the rabbit abdominal aorta produces severe spinal cord ischemia characterized by selective necrosis of the ventral horns and relative sparing of white matter long tracts (1). The histopathological changes observed correlate closely with the biochemical changes in high energy metabolites (2), lipid metabolism (3), and neurologic function (4).

Spinal rigidity in cats induced by temporary occlusion of the thoracic aorta in the lumbar spinal cord, is primarily attributable to the loss of interneurons (5, 6) accompanied by a marked decrease in tissue levels of aspartate (Asp), glutamate (Glu), glycine (Gly) (5, 6) and gamma-aminobutyric acid (GABA) (6). In addition to their roles in intermediary metabolism, these amino acids serve as neurotransmitters in the spinal cord (7–9) and through their excitatory effects, could exacerbate ischemic damage (10–12) and neurologic dysfunction. On the other hand, the role of peptides, a potential source of these amino acids, in the CNS response to ischemia is unknown.

The aim of this study was to determine whether the levels of these putative neurotransmitter amino acids as well as their peptide-bound concentrations in spinal cord gray matter is affected by ischemia. If so, are they correlated with the degree of ischemic damage sustained by the spinal cord?

MATERIALS AND METHODS

Adult rabbits of either sex, weighing 1–3 kg were used for the experiments. All animals were anesthetized with thiopental, 30 mg·kg⁻¹, intravenously (iv). In the control group (n = 6), the vertebral column corresponding to segments L₅-L₇ was excised. The spinal cord was extruded from the spinal canal with ice cold isotonic saline and immersed into liquid nitrogen.

In the experimental group the aorta was exposed at the level of the left renal artery and occluded for 10, 20 or 40 min (six animals in each ischemic group) (13). After ischemia, the ligature was removed and the animals were allowed to recover from anesthesia and survive

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for four days. Penicillin, 25,000 IU/kg, intramuscularly (im) was administered to all experimental animals postoperatively.

Biochemical Analysis

Spinal cords frozen in liquid nitrogen were allowed to warm to −20°C and one to two mm transverse sections obtained. Gray matter from ventral and dorsal horns was punched out with a needle (1 mm diameter), combined in amounts of 5–10 mg, homogenized at 5°C in 10 volumes of 5% sulphosalicylic acid by ultrasound, at 5°C with a Dynatech Ultrasonic Dismembrator. After storing at 0°C for minimally five hours (h), the homogenate was centrifuged at 12,000 × g for 20 min. The supernatant was evaporated to dryness under vacuum and dry residue dissolved in citrate buffer, pH 2.2, containing internal standard, Norleucine. One aliquot of the sample was applied to an amino acid analyser column (T-339, Mikrotechna) in Li+ cycle and the other aliquot (200 µl) was reevaporated and hydrolyzed in 6 mol hydrochloric acid (HCl) in sealed glass tubes for 22–24 h. The hydrolysates were dried under vacuum, 200 µl of water was added twice and aqueous solution evaporated to insure complete removal of HCl. The concentration of peptide-bound amino acids in each sample was calculated after subtraction of free amino acid from the total concentrations. The results were statistically evaluated by analysis of variance and by the Duncan test.

Histological Analysis

After the predetermined survival time, three animals in each experimental and control group were anesthetized with thiopental, 30 mg/kg, (iv) and transcardially perfused with phosphate buffered 0.9% saline followed by 10% neutral formalin. The spinal cord was removed and placed in the same fixative for 10–14 days. The lumbar and sacral segments (L₇–S₅) were cut in 20 µm transverse sections with a freezing microtome. The sections were impregnated according to the Nauta method (14), using the Laidlaw solution.

Neurological Observations

The neurological function of all animals was evaluated daily. The degree of neurologic impairment was categorized as follows. Grade 0—No neurologic impairment; the animal hopped normally and evaded visual threats. Grade 1—Partial neurologic impairment; the animal dragged its hind limbs with subnormal response to visual threats and painful stimuli (toe or tail pinch). Bowel and bladder function if impaired at all (from barely detectable to severe) were included in this group. Grade 2—Total neurologic impairment; the animals' hind limbs were completely paralyzed and totally unresponsive to painful stimuli. Bladder and bowel function were totally impaired (4).

RESULTS

Neurologic Recovery

After ten min of spinal cord ischemia, the animals improved rapidly and were able to eat one day postischemia. Incomplete neurologic impairment was observed in 30% (2/6) of the rabbits (grade 1). After 20 min ischemia, some of the rabbits (30%) (2/6) were partly paralysed (grade 1) and the rest of animals (4/6) had hind limb paraplegia (grade 2). All animals (6/6) after 40 min ischemia exhibited complete hind limb paraplegia (grade 2).

Neuropathology

Rabbits divided into three groups (three animals per group) after four days survival were used for neurohistological analysis. L₇–S₅ segments of the spinal cord subjected to ten min ischemia and stained by the Nauta method, did not exhibit histopathologic changes (Fig. 1A, B). However, ventral horn motoneurons were more intensely stained than normal. In animals subjected to 20 min of ischemia, the lumbar seg-

Fig. 1. Neuropathological changes in transverse section of midlumbar spinal cord: A. Mild damage of some neurons of gray matter following ten min occlusion. Nauta. ×25. B. Argyrophilia of motoneurons of anterior horn. Nauta. ×75.

ments revealed the formation of asymmetrically localized, multifocal, necrotic cavities in the ventral gray matter (Fig. 2A, B). The incidence and appearance of necrotic foci were characterized by high intra- and intersegmental irregularities. In the foci as well as peripheral zones, the boundaries of which were not strictly defined, many glial fibers had penetrated. The most necrotic foci were localized in the central part of the ventral horns.

Although degenerative changes were not usually found within the superficial layers of the dorsal horn (Fig. 2A, B), in animals subjected to 40 min of ischemia, histopathological changes occurred at segments L₂-S₁. The spinal cord gray matter was densely packed with numerous dark particles (Fig. 3A, B). Microscopic cavities were found in practically all parts of the gray matter in the transverse sections of L₂-S₁ spinal cord. Along the outer circumference of gray matter, there was marked edema and scattered Nauta-impregnated fragments could be identified.

Amino Acid Analysis

Following ten min ischemia and four days survival, no changes in free and peptide-bound amino acids were observed in the ventral horns compared to controls. After 20 and 40 min ischemia and four days survival, there was a marked decrease in the
Asp, Gly and GABA concentrations (Table 1). After 40 min ischemia and recovery Glu level fell as well (Table 1).

Compared to controls, the dorsal horns showed no change in amino acid levels after ten min ischemia and four days of recovery. After 20 min ischemia and four days survival the Asp and GABA concentrations (Table 2) fell. After 40 min ischemia and subsequent survival decrease was observed in Asp, Glu, Gly and GABA levels (Table 2). Relative changes in free amino acid concentrations after ischemia longer than ten min were greater in the ventral compared to the dorsal horns, especially with regard to Asp and Gly (Tables 1, 2).

Similar patterns of changes in peptide amino acids were observed following ischemia and four days survival (Table 3). After 40 min ischemia, bound Asp and Glu (values are calculated by subtraction of free Asp and Glu and free glutamine and asparagine from total concentrations after hydrolysis) decreased in the dorsal part of the gray matter. Bound Glu fell in the ventral part as well; no changes were observed after ten and 20 min of ischemia.

DISCUSSION

Glutamate (Glu), glycine (Gly), aspartate (Asp), glutamine (Glu) and gamma-aminobutyric acid (GABA) occur in highest concentrations in both the brain and
spinal cord (15–17). In the ventral horns, the levels of Asp and Gly are markedly higher than in the dorsal horn, whereas in the dorsal horn, Glu and GABA levels are higher than in the ventral horn.

The distribution of these neurotransmitter amino acids is related to their neurotransmitter function. Distribution analyses and electrophysiological measurement indicate that Glu is the major excitatory neurotransmitter released in the dorsal spinal cord at the primary afferent nerve terminal fibers (18, 19). A few axons from the dorsal root continue into the ventral horns to form monosynaptic contacts with motoneurons (7). GABA is an inhibitory transmitter affecting afferent depolarization of interneurons in the ventral horns (20–22); Gly is a postsynaptic inhibitory transmitter in the ventral horns (23, 24) whereas Asp is an excitatory transmitter released from interneurons (6, 7), or from primary afferent fibers (25).

A loss of interneurons due to injury should be manifested by a decline in the levels of these transmitter amino acids. After ischemia and short durations of recovery (within ten hours) such changes were not detected (26). Therefore, the amino acid composition in spinal cord was studied after four days of recirculation following 10, 20 and 40 min of spinal cord ischemia.

Our results permit the following conclusions: (a) if the decrease in transmitter
### TABLE 1
Free Amino Acid Concentrations in the Ventral Horns of the Rabbit Spinal Cord after Ischemia and Recirculation

<table>
<thead>
<tr>
<th></th>
<th>Asp</th>
<th>Glu</th>
<th>Gly</th>
<th>GABA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>4.896</td>
<td>4.472</td>
<td>4.542</td>
<td>0.825</td>
</tr>
<tr>
<td>±0.246</td>
<td>±0.552</td>
<td>±0.303</td>
<td>±0.088</td>
<td></td>
</tr>
<tr>
<td>Ischemia ten min</td>
<td>5.120</td>
<td>5.268</td>
<td>4.585</td>
<td>0.652</td>
</tr>
<tr>
<td>±0.416</td>
<td>±0.434</td>
<td>±0.207</td>
<td>±0.068</td>
<td></td>
</tr>
<tr>
<td>Recirculation four days</td>
<td>104</td>
<td>118</td>
<td>101</td>
<td>79</td>
</tr>
<tr>
<td>Percent of control</td>
<td>26*</td>
<td>69</td>
<td>57*</td>
<td>54*</td>
</tr>
<tr>
<td>Ischemia twenty min</td>
<td>1.264</td>
<td>3.104</td>
<td>2.593*</td>
<td>0.445*</td>
</tr>
<tr>
<td>±0.173</td>
<td>±0.378</td>
<td>±0.273</td>
<td>±0.035</td>
<td></td>
</tr>
<tr>
<td>Recirculation four days</td>
<td>33*</td>
<td>65</td>
<td>53*</td>
<td>47*</td>
</tr>
<tr>
<td>Percent of control</td>
<td>18†</td>
<td>53*</td>
<td>47*</td>
<td>43*</td>
</tr>
</tbody>
</table>

Values are expressed in mmol·kg⁻¹ of wet tissue and represent means ± SEM. Statistical analysis was performed using analysis of variance: F(3.15) = 70.0752, p < 0.01 for Asp; F(3.15) = 8.606, p < 0.01 for Glu; F(3.15) = 21.945, p < 0.01 for Gly; F(3.15) = 15.37, p < 0.01 for GABA.

Statistical difference between ischemia group and controls was calculated by Duncan test.
* = p < 0.05; † = p < 0.01.

Asp: Aspartate.
GABA: Gamma-amino-butyric acid.
Glu: Glutamate.
Gly: Glycine.

### TABLE 2
Free Amino Acid Concentrations in the Dorsal Horns of the Rabbit Spinal Cord after Ischemia and Recirculation

<table>
<thead>
<tr>
<th></th>
<th>Asp</th>
<th>Glu</th>
<th>Gly</th>
<th>GABA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>3.291</td>
<td>5.682</td>
<td>3.232</td>
<td>1.468</td>
</tr>
<tr>
<td>±0.182</td>
<td>±0.517</td>
<td>±0.208</td>
<td>±0.104</td>
<td></td>
</tr>
<tr>
<td>Ischemia ten min</td>
<td>3.284</td>
<td>5.558</td>
<td>3.685</td>
<td>1.203</td>
</tr>
<tr>
<td>±0.251</td>
<td>±0.370</td>
<td>±0.121</td>
<td>±0.033</td>
<td></td>
</tr>
<tr>
<td>Recirculation four days</td>
<td>100</td>
<td>98</td>
<td>114</td>
<td>82</td>
</tr>
<tr>
<td>Percent of control</td>
<td>100</td>
<td>98</td>
<td>114</td>
<td>82</td>
</tr>
<tr>
<td>Ischemia twenty min</td>
<td>1.827</td>
<td>3.994</td>
<td>2.627</td>
<td>0.858*</td>
</tr>
<tr>
<td>±0.389</td>
<td>±0.313</td>
<td>±0.251</td>
<td>±0.104</td>
<td></td>
</tr>
<tr>
<td>Recirculation four days</td>
<td>56*</td>
<td>70</td>
<td>81</td>
<td>58*</td>
</tr>
<tr>
<td>Percent of control</td>
<td>56*</td>
<td>70</td>
<td>81</td>
<td>58*</td>
</tr>
<tr>
<td>Ischemia forty min</td>
<td>1.566</td>
<td>3.627*</td>
<td>2.362*</td>
<td>0.637†</td>
</tr>
<tr>
<td>±0.354</td>
<td>±0.423</td>
<td>±0.254</td>
<td>±0.096</td>
<td></td>
</tr>
<tr>
<td>Recirculation four days</td>
<td>45*</td>
<td>64*</td>
<td>73*</td>
<td>43*</td>
</tr>
<tr>
<td>Percent of control</td>
<td>45*</td>
<td>64*</td>
<td>73*</td>
<td>43*</td>
</tr>
</tbody>
</table>

Abbreviations: Same as Table 1.

Values are expressed in mmol·kg⁻¹ of wet tissue and represent means ± SEM. Statistical analysis was performed using analysis of variance: F(3.15) = 8.571, p < 0.01 for Asp; F(3.15) = 6.381, p < 0.01 for Glu; F(3.15) = 11.707, p < 0.01 for Gly; F(3.15) = 16.08, p < 0.01 for GABA.

Statistical difference between ischemia group and controls was calculated by Duncan test.
* = p < 0.05; † = p < 0.01.

TABLE 3
Bound Amino Acid Concentrations in the Ventral and Dorsal Horns and the Rabbit Spinal Cord after Ischemia and Recirculation

<table>
<thead>
<tr>
<th></th>
<th>Ventral horns</th>
<th>Dorsal horns</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Asp</td>
<td>Glu</td>
</tr>
<tr>
<td>Controls</td>
<td>7.520</td>
<td>7.074</td>
</tr>
<tr>
<td></td>
<td>±1.035</td>
<td>±1.028</td>
</tr>
<tr>
<td>Ischemia twenty min</td>
<td>4.122</td>
<td>5.717</td>
</tr>
<tr>
<td>Recirculation four days</td>
<td>±0.633</td>
<td>±0.806</td>
</tr>
<tr>
<td>Ischemia forty min</td>
<td>3.432</td>
<td>4.748*</td>
</tr>
<tr>
<td>Recirculation four days</td>
<td>±0.616</td>
<td>±0.225</td>
</tr>
</tbody>
</table>

Abbreviations: Same as Table 1.

Values are expressed in mmol·kg⁻¹ of wet tissue and represent means ± SEM. Statistical analysis was performed using analysis of variance: F(3,15) = 16.2439, p < 0.01 for Asp in ventral horns; F(3,15) = 47.196, p < 0.01 for Asp in dorsal horns.

Statistical difference between ischemia group and controls was calculated by Duncan test. * = p < 0.05.

Amino acid concentration in spinal cord is related to the loss of interneurons then the maximal ischemic period without functional injury to the spinal cord lies somewhere between ten and 20 min. (b) The percentage decrease in amino acid levels in the ventral horns is higher than in the dorsal horns. The heterogeneous decrease in amino acids in both dorsal and ventral parts of the gray matter might correspond to differences in the severity of ischemic injury as manifested by energy metabolism (2) and structure (1). (c) In the two parts of the gray matter, the concentrations of the amino acids are reduced. Because of the hypothesis of the neurotoxicity of the excitatory amino acids, especially Glu, in selective brain injury following ischemia (11, 27) and with the distribution of Glu in the spinal cord, a higher degree of damage to neurons might have been expected in the dorsal horn. However, greater damage occurred in the ventral horn suggesting that factors other than the effects of excitatory amino acids may be involved in ischemic injury of the spinal cord.

The present experiments do not enable us to differentiate between the decrease of amino acid in various pools in the spinal cord. We do not postulate a generalized fall in all amino acids in the spinal cord after four days of recovery because the concentration of some nontransmitter amino acids, that we have measured (threonine, ileucine and leucine) did not exhibit any significant alteration (unpublished data). Therefore, the decrease of Glu, Asp, Gly and GABA in the spinal cord following more than ten min of ischemia probably reflects, at least partly, changes in the neurotransmitter pool of these amino acids.

Postischemic changes in peptide-bound amino acids were similar in both the dorsal and ventral regions of the spinal cord. Following ten and 20 min of ischemia and recovery, there were no detectable changes in peptide-bound amino acids. After 40 min of ischemia, however, a marked reduction in peptide-bound Asp and Glu in dorsal horns occurred. Bound Glu also fell after 40 min of ischemia and recovery in the ventral gray matter. This may be the basis for the postulated relationship between the fall in peptide-bound amino acids and irreversible spinal cord injury as manifested morphologically and biochemically (2).

Since the decrease in peptide-bound amino acids observed at the end of the survival
period was limited only to two amino acids these changes are not expected to reflect alterations of total proteolytic activity but only alterations in some specific enzymes. In addition to the other peptides there are also low molecular weight peptides present in the brain, which may possess transmitter function (28). A large amount of Asp (30–40%) and Glu (20–30%) is released from these peptides upon hydrolysis and these may, therefore, supply free amino acids for neurotransmission (29).

As for the free amino acids, the fall in peptide-bound Asp and Glu may be related to the degenerative changes in the spinal cord and loss of neurons, where the peptides serve as neurotransmitters. Degenerative changes induced by mechanical injury of the spinal cord are accompanied by reductions in transmitter substances such as serotonin (30), Glu (31), Asp and Glu (32), substance P (33) and also a decrease in a poorly characterized peptide containing Glu and Asp which is present in the spinal cord (34). The amount of Glu and Asp released by hydrolysis corresponds to a ratio of 1:2 amino acids in the peptide. This was an exact ratio of the decrease in the amount of the peptide-bound Asp and Glu in ventral and dorsal horns (4.3:2.3 and 5.0:2.5 respectively) following 40 min ischemia and four days survival in the present experiments. If these peptides were identical in both cases, their decline in the spinal cord could serve as a marker of degenerative changes.

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