overwhelming number of endothelial cells (compared with the number of the normal circulating endothelial cells) were injected into the experimental rabbits. We had ascertained that the concentration of circulating endothelial cells in normal rabbits is comparable to that existing in healthy humans. Cerebral embolisms in rabbits was not induced by the injections of endothelial cell-suspensions. Circulating endothelial cells in humans exist both on the arterial and the venous sides.

Increased numbers of circulating endothelial cells in humans are not a factor causing vascular embolism. It is not known why circulating endothelial cells do not cause vascular embolism.

References

Studies of the Influence of Biogenic Amines on Central Nervous System Ischemia

JUSTIN A. ZIVIN, M.D., PH.D., AND UMBERTO DEGIROLAMI, M.D.

SUMMARY Several serotonin inhibitors have been shown to reduce neurologic deficits in experimental CNS ischemia. Using biochemical and histological methods we tested the effects of the serotonin inhibitors cyproheptadine and brom-LSD in a highly reproducible rabbit spinal cord ischemia model. Detailed mapping of regional spinal cord blood flow was used to guide sampling for the biochemical studies. We found that it is possible to study biochemical and morphological aspects of spinal cord ischemia in great detail using a combination of quite precise techniques. However, at this level of resolution there were no substantial changes in biogenic amine concentrations in severely ischemic or marginally perfused tissue after the durations of ischemia that cause the onset of irreversible tissue damage. Treatment with doses of serotonin inhibitors that produce preservation of neurological function did not cause significant alterations of tissue concentrations of biogenic amines or tissue morphology in treated versus untreated animals.

Circ. Vol 17, No 3, 1986

IT HAS BEEN ESTABLISHED that central nervous system (CNS) ischemia is associated with alterations in nervous tissue biogenic amine concentrations.1 Several serotonin (5HT) inhibitors including cyproheptadine and brom-lysergic acid diethylamide (BOL) have been shown to reduce neurologic deficits in a rabbit spinal cord ischemia model.2,3 To further explore how these 5HT inhibitors provided protection, we conducted a series of observations on the biochemical, physiological and morphological effects of these drugs during CNS ischemia.

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Received May 15, 1985; revision #1 accepted November 5, 1985.

Materials and Methods

Surgical Procedure

New Zealand albino male rabbits weighing 2 to 3 kg were fed rabbit chow and water ad lib until the time of surgery. As described in detail previously,4 the animals were anesthetized with ketamine, 15 mg/kg, and open drop ether. Through a midline abdominal incision, the aorta was exposed at the level of the renal arteries. A small diameter plastic line (8 lb test mono-filament fishing line) was threaded through two small plastic buttons surrounding the aorta and then a larger plastic tube to form a snare ligature.5 The incision was closed around the tubing so the free ends were accessible externally. The rabbits were allowed to recover from anesthesia for at least 3 hr. At that time, each animal's motor and sensory functions were apparently normal. The aorta of each animal was occluded by pulling and clamping the fishing line which compressed the aorta between the two buttons. In experiments in which restoration of blood flow was...
desired, the clamp was released and all tubing and fishing line was pulled out of the animal without the need for any further anesthesia, and the small hole in the abdominal wall was closed with surgical clips.

**Blood Flow Measurement Method**

For measurements of spinal cord blood flow (SCBF) we used a previously reported diffusible tracer method. In addition to placement of the snare ligature, a 30 lb test fishing line was wrapped around the aorta just as it emerged on the abdominal side of the diaphragm. The distal ends of this line were led through the incision to the exterior when the incision was closed and the animal was allowed to recover from anesthesia for at least 2 hr. When the blood flow measurement was to be made, an ear artery was exposed and a solution of verapamil, 5 mg/ml dissolved in 10% ethanol, was applied topically to the artery to prevent it from constricting. A small catheter was placed percutaneously in a vein of the opposite ear and 25 μCi of [14C]-iodoantipyrine was injected at a constant rate for 1 min. The exposed artery was cut and arterial blood samples were collected at 5 sec intervals on preweighed filter paper. At exactly 1 min, the 30 lb test fishing line ligature was quickly pulled to sever the aorta and abruptly terminate blood flow to the spinal cord. The caudal 10 cm of spinal cord was rapidly extruded from the spinal canal, quickly frozen with dry ice, cut into 1 mm sections, allowed to thaw slightly and frozen to glass microscope slides. Alternate sections were taken for SCBF determination. These tissue slices along with the blood samples were weighed and solubilized in Protosol and Biofluor (New England Nuclear, Boston, MA). Samples were counted in a scintillation counter using an automatic external standard to correct for quenching. Blood flow was then calculated as previously described.

**Biochemical Methods**

After local SCBF rates were determined, the remaining sections were used for biochemical measurements. Areas of interest were determined on the basis of the blood flow studies and the location of the ischemic tissue was identified. It was also possible to identify marginally ischemic tissue at the edge of the ischemic zone and normally perfused tissue in more rostral areas. Tissue slices from the areas of interest were dissected with an iris knife as previously described and gray matter was separated from white matter. These tissues were then placed in small polyethylene tubes containing 100 μl of an ice cold solution which was composed of 0.1 M perchloric acid and 0.1 mM ascorbic acid. This solution contained 0.1 mM N-methyl serotonin and 0.1 mM 3,4 dihydroxybenzyllamine hydrobromide to serve as internal standards for indoleamine and catecholamine assays. The tubes were covered and immediately spun in a small centrifuge to submerge all tissue in the cold acid solution. The tubes were then placed in the cup horn of a Branson Sonifier 200 (Branson Sonic Power Co., Danbury, CT) and sonicated at full power for 60 to 90 sec (preliminary studies showed no loss of biogenic amines after more than 120 sec of such treatment). A 5 μl aliquot was removed for protein determination. The remainder was spun at 5000 X g in a refrigerated centrifuge, the supernate was removed, aliquotted, and frozen at –80° C. Serotonin and 5-hydroxyindole-acetic acid (SHIAA) were measured by high pressure liquid chromatography with electrochemical detection and norepinephrine (NE) was measured in a similar separate assay. These methods were sufficiently sensitive so that all assays could be performed on each sample. Standards were checked repeatedly throughout each day to ensure that results were reproducible.

**Data Analysis Techniques**

Concentrations of the biogenic amines were normalized in terms of protein concentration in the homogenate. Results were analyzed by a three-factor analysis of variance (case I). The differences among cell means were determined by the Newman-Keuls procedures and results demonstrable at the 5% confidence level (p < 0.05) were considered significant. With a complex factorial design, an occasional result may be lost (in this study, fewer than 1%). Such data were handled by standard methods and the degrees of freedom were appropriately reduced.

**Neuropathological Studies**

A separate set of animals was designated for the neuropathological studies. Four rabbits were treated with BOL, 1.5 mg/kg 10 min prior to aortic occlusion and underwent occlusion for 30 min. An additional animal was treated with BOL and made ischemic for 60 min. Another group of animals was pretreated with cyproheptadine, 1.0 mg/kg and underwent occlusion for 30 min. After the animals were made ischemic, they were observed for evidence of neurologic deficits at several time intervals over the ensuing week. Their clinical status was recorded as normal (N), paretic (P) or paraplegic (T) as described in detail previously. The animals selected for the neuropathological study were allowed to survive for seven days so as to allow us to study the distribution and histology of irreversible lesions and the effect of treatment on these lesions. Animals were killed by cervical disarticulation. The aorta was known to be patent in all animals because, if it becomes permanently occluded for any reason, the animal will not survive for more than 2 days. The spinal column was removed en block from the mid-thoracic vertebrae to the sacral termination and immersed in 10% phosphate-buffered formalin for one week. The spinal cord was removed by dissection and immersed in a fresh solution of the same fixative for an additional week. The tissue was then processed for light microscopy as follows: 1) each cord was embedded whole in celloidin; 2) the celloidin block was then sectioned through the dorsal root ganglia at about 10 segmental levels. Sections through each block were then stained with cresyl violet.
Protocol
All animals were made ischemic for predetermined periods. At the end of that time the animal was either killed for biochemical studies or the occlusion was removed and the neurological conditions of the animals were assessed at 2 hr, 18 hr and 5 d after the occlusion for the pathological study. The drugs used were cyproheptadine (a gift from Merck, Sharp and Dohme Research Laboratories, West Point, PA) or BOL (supplied by NIDA). The cyproheptadine was dissolved in distilled water, the BOL was soluble in isotonic saline. Concentrations were adjusted such that the animals received 1 ml/kg. The drugs were injected intravenously 10 to 15 min prior to the onset of ischemia in doses that were previously demonstrated to reduce neurological deficits.2,3 Control rabbits received 1 ml/kg isotonic saline.

Results
The blood flow method we used was sufficiently sensitive to allow detailed analysis of the lesion. Figure 1 demonstrates a typical profile of regional SCBF in an ischemic spinal cord. Measurement of blood flow at the caudal end of the cord during occlusion of the aorta resulted in flow rates that averaged 0.542 ± 0.086 ml/100 g/min (n = 8, mean ± S.E.). This was approximately 2% of the normal blood flow rate in this region. The ischemic segment started at the caudal tip of the spinal cord and extended rostrally for approximately 4 cm. The next 2 to 3 cm was marginally perfused and frequently (but not invariably) included a slightly hyperemic region at its rostral end. Rostral to the ischemic tissue, thoracic spinal cord blood flow was within the normal range that we previously reported.6 When the aortic occlusion was released, the previously ischemic region became hyperperfused as shown in figure 2.

Measurement of biogenic amines revealed that in normal spinal cord there was an approximately 50% increase in 5HT and 5HIAA concentrations in the gray matter of the lumbar enlargement compared with other gray matter regions in the cord as shown in figure 3. Since the length of the ischemic region varied somewhat from animal to animal, and the rostral edge was frequently located in the lumbar enlargement, it was impossible to compare the concentrations of 5HT at the edge of the lesion with normal controls to determine if there were alterations of 5HT or 5HIAA in the gray matter. Concentrations of 5HT and 5HIAA did not vary from control in the most ischemic tissue or in white matter at any location and NE was not significantly different from normal at any level in the spinal cord. Concentrations of these biogenic amines in the thoracic spinal cords of ischemic animals were not significantly different from normal.

We then compared animals that were made ischemic without treatment with animals that were made ischemic and treated with cyproheptadine, 1 mg/kg i.v., 10 min prior to the onset of ischemia. As shown in tables 1 and 2, after 25 or 60 min of ischemia, treatment did not alter spinal cord blood flow significantly at any spinal cord level when compared with the rabbits that were made ischemic for identical periods but not given any cyproheptadine. Similarly, we could not detect any alterations in the biogenic amines that were measured at any location in treated ischemic animals versus untreated ischemic rabbits.
There were two exceptions. Cyproheptadine treated animals. Results are expressed as mean ± SE, n = 4.

<table>
<thead>
<tr>
<th></th>
<th>SCBF</th>
<th>SHT</th>
<th>SHIAA</th>
<th>NE</th>
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<tbody>
<tr>
<td></td>
<td>LL</td>
<td>RI</td>
<td>MP</td>
<td>LT</td>
</tr>
<tr>
<td></td>
<td>25 Min Occlusion</td>
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</table>

At 25 min of occlusion, SCBF (ml/100 g/min) was measured throughout the length of the spinal cord and 4 regions of interest were selected based on the regional blood flow. The low lumbar (LL) area was very ischemic, the rostral edge of the most ischemic (RI) area had low flow, the marginally perfused area (MP) had intermediate flow, and the low thoracic cord (LT) was normally perfused. Similar measurements were made in both untreated (U) and cyproheptadine treated (T) animals. No significant change in perfused. Similar measurements were made in both untreated (U) and cyproheptadine treated (T) animals. No significant change in biogenic amine concentrations could be observed in untreated versus treated animals. Results are expressed as mean ± se, n = 4.

We conducted similar pilot studies in untreated animals after ischemia lasting 25 or 60 min followed by 30 or 60 min of reperfusion. In those instances, spinal cord blood flow was increased in the previously ischemic regions, as shown in figure 2, but no substantial changes in biogenic amine concentrations could be demonstrated.

Table 3 summarizes the results obtained from neuropathological observations. All animals except one showed total infarctions of the gray matter of the cord (grade 2 lesion) at one or more segmental levels. One animal treated with BOL showed multifocal small infarcts (grade 1 lesion). Paralyzed animals showed grade 2 lesions and paretic animals grade 1 lesions. There were two exceptions. Cynoheptadine treated animals 6 and 7 had grade 2 lesions and were neurologically normal throughout the period of observation. In all animals the lesions were confined to the gray matter (fig. 4). They always involved the mid-lumbar cord and variably extended to the adjacent upper and lower lumbar segments. As regards the histological appearance of the 7 day old lesion three general grades were defined: 0: normal; 1: multifocal gray matter infarction without cavitation; 2: total gray matter infarction. There was no difference in the range of histologic abnormalities between these animals treated with BOL and cyproheptadine and previously reported non-treated animals. In addition, no difference could be demonstrated between the animals treated with the two drugs. When we compared the current results with previous investigations of animals not treated with BOL or cyproheptadine, we found that in treated animals the correlation between the severity of the clinical deficits versus the topographical extent and histological completeness of tissue destruction at 7 d after aortic occlusion was not as close as in untreated animals.

Discussion

Spinal cord blood flow can be measured with excellent resolution by the methods that were employed, and we were able to confirm previous findings that SCBF was markedly reduced in the ischemic regions although flow was not entirely eliminated. It appears that spinal cord and brain react similarly when subjected to ischemic damage. The region of relative hyperemia adjacent to the ischemia in the spinal cord lesion appears to be analogous to "luxury perfusion" which is frequently seen adjacent to cerebral lesions. Also, increased SCBF is present during the first hour after restoration of blood flow to previously ischemic regions in spinal cord as has been frequently demonstrated after similar cerebral insults. We did not conduct studies at even later times during reperfusion to determine whether a subsequent hypoperfusion state would develop in the spinal cord although this phenomenon is observed after cerebral ischemia. The blood flow method we used is most appropriate for detailed studies of regional differences because it yields excellent spatial resolution of SCBF which was necessary for biochemical correlations. This diffusible tracer technique is not optimal for repeated measurements over time and, therefore, extensive studies were not con-

### Table 2 60 Min Occlusion

<table>
<thead>
<tr>
<th></th>
<th>SCBF</th>
<th>SHT</th>
<th>SHIAA</th>
<th>NE</th>
</tr>
</thead>
<tbody>
<tr>
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<td>LL</td>
<td>RI</td>
<td>MP</td>
<td>LT</td>
</tr>
</tbody>
</table>

At 60 min of occlusion, SCBF and biogenic amine concentrations were measured as in table 1. An additional set of measurements was made at the rostral end of the marginally perfused region (RM). As in table 1, no significant changes were detected in untreated versus treated animals.
ducted. We also found that treatment with cyproheptadine at doses that were previously shown to have CNS protective effects did not significantly alter SCBF in the ischemic region. This result is similar to our previous finding that BOL did not alter SCBF during ischemia. Thus, we have further evidence to suggest that 5HT inhibition causes decreased neurological deficits by some mechanism that does not alter blood flow to the ischemic regions of the CNS.

The results of the biogenic amine studies did not help to elucidate the mechanisms of how they influence ischemic damage. Many previous reports have indicated that alterations in these substances do occur, although most measurements were made long after infarction had occurred (see review). Thus, they did not explore the mechanisms of irreversible damage production. Our previous studies showed that at 25 min of ischemia approximately half of the animals developed irreversible paraplegia and by 1 hr all animals sustained infarction. Also, the 5HT concentration transiently decreased at the rostral edge of the lesion at 14 and 20 min after the onset of ischemia in gray matter and the concentration of NE did not change until 1 hr after the onset of ischemia when it declined only in infarcted gray matter. In that study relatively large amounts of tissue were included in each measurement because determination of the location of ischemic tissue was guided by anatomical landmarks and not by actual knowledge of regional blood flow. The results of the present study are not in conflict with these earlier findings, but differences may have been obscured by the relatively large variance introduced by the increased resolution of our newer methods. The localized increase of 5HT concentration in the lumbar enlargement of the spinal cord caused particular problems in this regard. In our previous study of comparatively large tissue samples, the biogenic amine concentration changes that were found did not exceed 50% of control. The naturally occurring 50% increase in 5HT concentration in the lumbar enlargement where the rostral edge of the lesion frequently but not invariably appeared may have obscured alterations produced by ischemia. The rostral edge of the lesion was located in the lumbar segments where 5HT concentration is normally elevated in some animals and caudal to this high concentration area in other animals. Any elevations of 5HT that may have been caused by ischemia at the rostral edge of the lesion were therefore difficult to detect. Nevertheless, if any ischemia induced alterations of 5HT were present, they cannot have been large. Furthermore, in preliminary studies we produced spinal cord ischemia in rabbits for 25 min or 1 hr and then allowed restoration of flow for various periods of up to 1 hr, but we did not observe large alterations in biogenic amine concentrations. Therefore, although biogenic amines may play a role in lesion development and at later stages of CNS infarction, measurement of the tissue concentrations does not give a clear picture of the pathogenesis of the process during its early phases.

Comparison of ischemic animals that received 5HT inhibitors with ischemic animals that were not treated was a somewhat more readily analyzed experimental

### Table 3: Neuropathology Results

<table>
<thead>
<tr>
<th>Animal #</th>
<th>Occlusion Time</th>
<th>Treatment*</th>
<th>Clinical†</th>
<th>Neuropathology‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2h 18h 5d</td>
<td></td>
<td>LL§ ML UL</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>60 min</td>
<td>BOL</td>
<td>T T T</td>
<td>2 2 2</td>
</tr>
<tr>
<td>2</td>
<td>30 min</td>
<td>BOL</td>
<td>N N T</td>
<td>2 2 2</td>
</tr>
<tr>
<td>3</td>
<td>30 min</td>
<td>BOL</td>
<td>P P T</td>
<td>2 2 2</td>
</tr>
<tr>
<td>4</td>
<td>30 min</td>
<td>BOL</td>
<td>N P P</td>
<td>1 1 1</td>
</tr>
<tr>
<td>5</td>
<td>30 min</td>
<td>Cyp</td>
<td>T T T</td>
<td>2 2 0</td>
</tr>
<tr>
<td>6</td>
<td>30 min</td>
<td>Cyp</td>
<td>N N N</td>
<td>2 2 0</td>
</tr>
<tr>
<td>7</td>
<td>30 min</td>
<td>Cyp</td>
<td>N N N</td>
<td>2 2 0</td>
</tr>
<tr>
<td>8</td>
<td>30 min</td>
<td>Cyp</td>
<td>P P T</td>
<td>0 1 2</td>
</tr>
<tr>
<td>9</td>
<td>30 min</td>
<td>Cyp</td>
<td>P P T</td>
<td>2 2 1</td>
</tr>
</tbody>
</table>

*BOL = Brom-LSD; Cyp = cyproheptadine.
†T = paralysis; P = paresis; N = normal.
‡Grade of lesion: 0 = no lesion; 1 = multifocal infarction; 2 = total infarction.
§Cord levels: LL = low lumbar; ML = mid-lumbar; UL = upper lumbar.
design. In this set of experiments it was possible to directly contrast the various aspects of lesion development at comparable anatomical sites relative to the ischemic areas. Our prior pharmacological study indicated that the average duration of ischemia required to produce irreversible paraplegia was increased from approximately 25 to 40 min, but by 1 hr of ischemia even the treated animals would nearly all have sustained irreversible damage. Again the lack of any clear substantial alterations of tissue biogenic amine concentrations demonstrates that, although the pharmacological studies we previously performed appear to implicate the serotoninergic system in lesion production, even quite detailed tissue measurements of these substances does not explain the protective effects of these drugs.

The results we obtained from the biogenic amine studies does not imply that biogenic amines are not causally related to production of CNS infarction, and alterations may be occurring in a fashion that cannot be detected by the methods that were employed in this study. For example, if ischemia causes rapid release of biogenic amines and they move from the presynaptic storage locations to the postsynaptic neurons causing toxic effects, measurement of tissue concentrations which include both presynaptic and postsynaptic regions might not demonstrate any changes until well after the onset of irreversible changes. It is possible that tissue concentrations of the biogenic amines were constant but turnover rates were markedly altered. However, the lack of increases in 5HIAA makes it unlikely that such changes are occurring in 5HT regulation. Any released 5HT or NE would probably have to be attached to another tissue near the site of release since, if they could be easily washed away, ischemia followed by restored flow should have resulted in decreased tissue concentrations of these substances.

The findings of the pathological studies suggests that although 5HT inhibition results in preservation of neurological function, in some animals, the cause of this beneficial effect is not apparent on the light microscopic examination of the tissue. Such lack of clear correlation between neuropathological and clinical findings have also been observed in other CNS ischemia models. The reason for this interesting finding is not yet clear to us.

In conclusion, we found that it is possible to study biochemical and morphological aspects of spinal cord ischemia in great detail by using a combination of very precise SCBF measurements, in conjunction with very sensitive biochemical measurement methods and appropriate histological techniques in a highly reproducible and well characterized CNS ischemia model. We confirmed that during and after ischemia, SCBF responds in a fashion similar to brain exposed to ischemic conditions. At this level of resolution, there are no substantial changes in biogenic amine concentrations in ischemic or marginally perfused tissue after lengths of ischemia that would be expected to cause infarction during the early stages of irreversible tissue damage. Similarly, treatment with 5HT inhibitors that have been shown to exert beneficial protective effects in this model system did not significantly alter biogenic amine tissue concentrations. Neuropathological studies did not demonstrate differences in lesion morphology that would account for the improved neurological function. These results suggest that studies of tissue biogenic amine concentrations during the early stages of ischemia and infarction are unlikely to shed any additional light on the mechanisms of how biogenic amines alter tissue injury during CNS ischemia.

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Studies of the influence of biogenic amines on central nervous system ischemia.
J A Živin and U DeGirolami

Stroke. 1986;17:509-514
doi: 10.1161/01.STR.17.3.509

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