DURING CENTRAL NERVOUS SYSTEM ISCHEMIA, there is abrupt loss of neurological function. Since function can be severely compromised while morphological changes are not immediately apparent, some physical or biochemical phenomenon must be responsible for the dysfunction. Because biogenic amines are both neurotransmitters and have potent vasoactive properties, for many years there has been considerable speculation about their influences on the development of injury to the CNS. Many experimental stroke models exist, and many studies have been conducted in which biogenic amine concentrations have been measured during and after CNS ischemia. When focal CNS ischemia has been investigated by such methods, the results are subject to criticism because the distribution of damage is not reliably reproducible in any of the currently known cerebral stroke models. Since the brain is so complex morphologically and neurotransmitter concentrations differ substantially from region to region, comparatively small differences in lesion distribution might produce major differences in the results of biochemical studies. Furthermore, as no gross morphological marker is evident at the early time periods (when the damage is not yet irreversible), sampling from larger areas of brain may include normal or partially ischemic tissue. Consequently, the biochemical measurements may represent an average value from tissues in which very diverse changes are occurring.

To overcome many of these deficits, a rabbit model of spinal cord infarction has been developed. The prime advantages of this model for biochemical purposes are that the lesion is highly reproducible in its distribution and the anatomy of the spinal cord is considerably simpler than that of the brain. Both features facilitate analysis of the results of such studies. Serotonin (5HT) and norepinephrine (NE) are present in substantial concentrations in spinal cord (dopamine and epinephrine are nearly absent). Therefore, it seemed reasonable to investigate the changes that occur in 5HT and NE as a function of the duration of CNS ischemia. In turn, if it could be demonstrated that changes in these biogenic amines are correlated with at least some aspects of CNS infarction, and since many drugs are known to alter the actions and concentrations of these biogenic amines, then it might be possible to develop rational pharmacological strategies to ameliorate the damage produced by CNS ischemia.

Materials and Methods

Forty male New Zealand albino rabbits weighing 2 to 3 kg were fed rabbit chow and water ad lib until the time of surgery. As described in detail previously, a snare ligature was placed around the abdominal aorta of each rabbit just caudal to the more caudal renal artery and the free end of the snare was accessible outside the rabbit. The animals were allowed to recover from surgery to confirm that motor and sensory functions were normal. The snare ligature was then pulled tight to occlude the aorta. Although the animals were fully conscious, none evidenced any discomfort when the aorta was so occluded. Complete paraplegia and anesthesia in the hind quarters ensued in all rabbits within 2 min of the onset of occlusion.

Previous studies indicated that in the rabbit spinal cord ischemia model irreversible neurological damage begins at approximately 9 min of ischemia, causes irreversible damage in half of the animals at 20 min, produces complete irreversible paraplegia at 33 min in half the animals, and causes complete paraplegia in essentially all rabbits by 55 min. Therefore, biochemical studies were performed at 5 min of ischemia to determine if any biochemical changes occurred before permanent damage was present and at 14, 20, 33 and 55 minutes to span the time periods of interest as the clinical damage became progressively more irreversible. At these predetermined times the rabbits received an intravenous bolus of 100 mg of pentobarbital which caused cardiac arrest within 15 sec. The spinal column from the costovertebral angle to the upper sacral vertebrae was removed en bloc and the spinal cord was rapidly pushed out of the spinal canal as described.

SUMMARY A rabbit spinal cord ischemia model was used to study the effects of focal ischemia on the tissue concentrations of serotonin, 5-hydroxyindole acetic acid, and norepinephrine. Ischemia induced by abdominal aorta occlusion caused both serotonin and norepinephrine concentrations to decline in the most ischemic areas of the spinal cord by 55 minutes. In marginally perfused adjacent areas, serotonin concentrations transiently declined at 14 and 20 min. after the onset of ischemia and then returned to normal. The minimum was reached at the same time when previous studies showed damage had become irreversible in more ischemic regions. Concentrations of 5-hydroxyindole acetic acid did not change at any time and norepinephrine declined only in the most ischemic areas after damage was irreversible. Thus, permanent serotonin and norepinephrine decreases occur only in areas destined to be destroyed by infarction, but the serotonin returns to normal in marginal tissue that remains viable. These studies suggest that serotonin may be involved in the early stages of irreversible changes during central nervous system ischemia.

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previously. The middle of the lumbar enlargement was identified and the cord was divided into 3 sections: 1. the midlumbar and caudal segment; 2. 1.5 cm of cord above the midlumbar enlargement which included the upper lumbar cord; 3. the next more rostral 1 cm of cord which included the low thoracic structures. The levels were chosen because previous studies had indicated that when infarction was present (documented histologically one week after the occlusion), the low lumbar region was always involved, the upper lumbar region was variably affected, and the low thoracic level never showed signs of morphological damage. In initial studies, pieces of the cervical spinal cord were similarly processed to serve as another control. The spinal cord pieces were then rapidly frozen in blocks of saline ice as described previously. The entire procedure, from the time of pentobarbital injection to embedding the cord segments in ice required less than 5 min. The blocks were then cut transversely in a cryostat to produce 500 μm thick sections which were affixed to glass slides. Morphology was checked grossly to establish that the spinal cord levels had been correctly identified.

Seven consecutive sections were taken from the center of each block. From 6 sections, 500 μm diameter samples (punches) of gray matter were removed as described previously; these came from: 1. anterior horn; 2. lateral gray matter (between the anterior and posterior horn); 3. posterior horn. From the seventh section, the white matter was obtained by dissection with an iris knife as described previously. These particular areas were chosen because our prior histological studies had indicated that the anterior horn in the low lumbar region was more susceptible to infarction than the posterior horn, and that an intermediate lateral gray matter region existed that was variably affected.

In contrast, the white matter was always spared by as much as 1 hr of ischemia. All tissue samples were then placed in small polyethylene tubes containing 100 μl of ice cold 0.1 M perchloric acid and 57 nM 5-hydroxytryptophan. The tubes were closed and immediately spun in a small centrifuge to submerge all tissue in the cold acid. The tubes were then placed in the cup horn of a Branson Sonifier 200 and sonicated at full power for 15 to 30 sec (preliminary studies showed no loss of 5HT or NE after 2 minutes of such treatment). A five μl aliquot was removed for protein determination; the remainder was spun at 5000 × g in a refrigerated centrifuge, the supernate was removed, aliquotted for 5HT and NE assays, and frozen at −80°C. All assays were performed within one week. Serotonin and 5-hydroxyindoleacetic acid (5HIAA) were measured by high pressure liquid chromatography with electrochemical detection; 5-hydroxytryptophan was used as an internal standard. Norepinephrine was measured by a radioenzymatic procedure. These methods are of sufficient sensitivity so that NE, 5HT, and 5HIAA could all be measured on aliquots of each sample. For measurement of 5HT and 5HIAA, tissue sample pools were repeatedly checked throughout each day to ensure that results were reproducible. For 5HT the coefficient of variation for such determinations averaged 2.15%, for 5HIAA the coefficient of variation averaged 3.10%.

The experimental protocol consisted of comparing groups of 4 ischemic animals and 4 control animals in which a snare ligature was placed but not pulled tight. Only one period of ischemia was studied during each experiment so that the related experimental and control animals were exposed to the same uncontrolled variables. Biochemical assays were conducted simultaneously on the corresponding experimental and control animal tissues. Thus, comparison of results of experimental and related control values is more precise than that between different durations of ischemia. As a consequence, the data analysis methods emphasized comparison of the ratio of the experimental results of a given duration of ischemia with the related controls.

Concentrations of the biogenic amines are 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 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.

Results

All animals in which the snare ligature was tightened developed complete paraplegia and anesthesia in the hind quarters within 2 minutes. In preliminary experiments, attempts were made to delineate the extent of the ischemic region by injection of carbon black. Although this procedure clearly showed lack of perfusion in the musculature of the lower abdomen, hind legs, and sacral regions, there was not usually a definite delineation of the carbon black at any level of the spinal cord (DeGirolami and Zivin, unpublished observations). Thus, although the lower lumbar and sacral segments of the spinal cord were unquestionably ischemic, as shown by the abrupt onset of functional deficits and necrotic changes that ultimately appeared, the blood flow is not completely absent in these areas. As a consequence, it was not possible to dissect the spinal cord on the basis of unequivocal knowledge of the precise locus of the blood flow changes, and therefore the division was made on the basis of anatomical landmarks (as indicated in the Materials and Methods Section).

Preliminary experiments also indicated that the blood pressure in the thoracic aorta did not change from normal while the abdominal aorta was occluded, but the pressure in the femoral arteries dropped to undetectable levels (Roizen and Zivin, unpublished observations).

Serotonin

The average 5HT concentrations in control rabbits at low thoracic and lumbar levels were: posterior gray
matter, 5.65 ± 0.25 ng/mg protein (mean ± s.e., n = 60); lateral gray matter, 12.67 ± 0.57; anterior gray matter, 13.46 ± 0.59; and white matter, 5.28 ± 0.36. Table 1 summarizes the results seen in ischemia; the table gives the ratios of experimental to control values at the three spinal cord levels and in the four areas as a function of duration of ischemia. Asterisks indicate values that are significantly different from unity. It seemed possible that further significant effects might be hidden in the noise level of the assay. Therefore, to increase precision, statistical pooling was considered. Analysis of variance indicated that, since the appropriate interaction terms were not significant, it was legitimate to pool the gray matter serotonin concentrations at each spinal cord level (results were pooled, not the tissues). Figure 1 is a graphical representation of the results of this analysis; asterisks again indicate significant changes from control.

Table 1 demonstrates that significant 5HT concentration changes occurred in a number of individual areas. At the low lumbar level, 5HT declined only in the anterior horn at 33 and 55 min of ischemia. However, when the gray matter areas were pooled at that level no significant changes were found, as shown in figure 1. In the upper lumbar region several individual gray matter areas were significantly reduced as shown on table 1, but analysis of the pooled gray matter indicates the pattern more clearly; figure 1 shows that in the upper lumbar region pooled gray matter 5HT declined significantly at 14 and 20 min of ischemia and then returned to normal. At the low thoracic level, 5HT in two gray matter areas was significantly reduced at 14 min and the pooled gray matter at that time was also decreased. In white matter, table 1 shows a significant increase in 5HT in the low lumbar level at 14 min and a significant decrease at 20 min in the low thoracic level.

At 14, 33 and 55 min of ischemia, 5HT measurements were made on anterior horn, lateral gray, posterior horn and white matter specimens from cervical spinal cord. No significant changes from control values were found at any of these times.

5-Hydroxyindoleacetic Acid

Measurements of 5-HIAA were made simultaneously with serotonin in each sample. Posterior gray 5-HIAA concentration from control animals at the low thoracic and lumbar levels averaged 2.35 ± 0.20 ng/mg protein (n = 60), lateral gray was 3.00 ± 0.11, anterior gray was 2.92 ± 0.15, and white matter was 2.21 ± 0.17. No significant changes from control values were found at any time of ischemia.

Norepinephrine

Norepinephrine was also measured in all of the same tissue samples that were tested for 5HT. The average NE concentrations in control rabbits were: posterior gray matter, 2.26 ± 0.11 ng/mg protein (n = 60); lateral gray matter, 2.08 ± 0.10; anterior gray matter, 2.92 ± 0.10; and white matter 1.56 ± 0.13. Table 2 summarizes the results seen in ischemia; the table gives the ratios of the experimental to control values as

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Ratios of experimental to control groups. Time is minutes of ischemia. *indicates significant change from control.

FIGURE 1. Serotonin concentrations in various levels of spinal cord gray matter at progressively longer durations of ischemia. Triangles indicate low lumbar (LL) points, squares indicate upper lumbar (UL) points, and circles indicate low thoracic (LT) points. Each point is the average of posterior, lateral, and anterior gray matter at that level from 4 animals. The dashed line indicates control values. Asterisks indicate points that are significantly different from control.
Changes from controls.
The dashed line indicates control values. The asterisk indicates the only point that is significantly less than control.

In table 1. Again, analysis of variance indicated that all gray matter areas at a given spinal cord level could be statistically pooled and results are shown in figure 2. Pooled gray matter NE was significantly reduced from control only in the low lumbar area at 55 min. No other significant changes were detected in pooled gray matter at a given level. There were some individual gray matter areas (as shown in table 2) that did change from control at various other times but no pattern was apparent. In white matter, the NE concentration was significantly elevated in the lumbar level at 14 min of ischemia and significantly decreased at 55 min. At 14, 33 and 55 min of ischemia, NE measurements in cervical spinal cord sections from animals subjected to ischemia at more caudal levels showed no significant changes from controls.

Discussion

The findings of this study support the contention that spinal cord biogenic amine concentrations change during the early stages of the infarction process. The 5HT changes in gray matter indicate that in the most severely damaged region, there is a modest decline in 5HT after the damage becomes permanent. In less ischemic regions adjacent to the infarcted areas, changes in serotonin are more complex. During the times when irreversible changes are occurring in more ischemic areas, there is approximately a 35% decrease in 5HT. However, when the damage finally becomes permanent in the more ischemic regions, 5HT in the "marginal areas" returns to normal. There is a significant decrease in the concentration of 5HT in gray matter atrostal as the low thoracic area at 14 min of ischemia. Since there was no similar change in the cervical sections at that time, the 5HT changes were not generalized. Therefore, it is probable that the "marginal region" extended a bit more rostrally briefly or that the low thoracic sections were cut a bit too caudally in this particular set of animals. No consistent pattern of changes in 5HT occurred at more distant sites or at other times. The importance of white matter 5HT changes is not clear. The NE in the white matter near the ischemic gray matter increases to 160% of control during the early stages of ischemic but latter declines to less than 50% of control. Consistent gray matter decreases become clear only at 55 min of ischemia, which is after the neurological damage has become permanent in essentially all animals. We did not find any generalized changes in 5HT, 5HIAA, or NE which indicates that our methods are valid for use as a screening procedure.

The method of dissection was chosen to correlate with morphological changes we observed previously. Since more damage had been seen in the anterior horns than in other areas after brief periods of ischemia, we thought there might be major biochemical differences among these regions. The lack of significant interaction terms in the analysis of variance indicated that all the gray matter areas at a given level were changing in essentially the same way and pooling such data might

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Ratios of experimental to control groups.
Time is minutes of ischemia.
*indicates significant change from control.
give more reliable results because the sample size was larger. With the possible exception of the anterior horn 5HT changes detected at the low lumbar level, there does not appear to be any recognizable or consistent pattern to the changes in isolated gray matter areas where pooled data did not indicate significant differences. Thus, the pooled results (shown in figures 1 and 2) appear to be more meaningful indicators of the pattern of gray matter changes (i.e., significant changes seen in individual areas does not guarantee that they are scientifically important).

There have been a number of other studies of the effects of strokes on biogenic amine concentrations. Welch et al. measured 5HT concentrations in cortex of gerbils 3 and 4 hours after unilateral carotid ligation. They reported decreased concentrations on the infarcted side, but the time periods studied are long after irreversible damage is done. Furthermore, gerbils are highly subject to seizures during infarction, which would have unpredictable effects on neurotransmitter concentrations. Members of this group reported that unilateral common carotid artery occlusion in gerbils resulted in decreased NE by one hour after occlusion in the ischemic hemisphere. They also reported that after occlusion of one carotid, there was a decrease in 5HT within 5 min in both hemispheres which persisted essentially unchanged for up to one hour. Subsequently some of the members of this same group reported that, one hour after occlusion of one hemisphere with multiple large emboli, NE increased in both hemispheres, 5HT remained unchanged and, 5HIAA increased.

Brown et al. reported decreased 5HT and NE concentrations during global ischemia produced by raised intracranial pressure. These decreases were observed after 7.5 min of ischemia and the decline continued after 30 min of subsequent restoration of flow. The "bloodless" global ischemia produced by raising intracranial pressure is unlike the focal ischemia which is the most common medical problem. Calderini et al. exposed rats to bilateral carotid occlusion plus hypovolemia for 15 min and found no changes in NE, 5HT and 5HIAA, but NE and 5HT declined in some groups during 30 min of subsequent recirculation.

Mrsulja and associates measured neurotransmitters in cerebral tissue of gerbils during infarction produced by unilateral carotid occlusion. Serotonin and NE concentrations were shown to decrease relative to those of the opposite hemisphere. These results are difficult to interpret because the concentrations of monoamines in the perfused hemisphere may change after infarction of the opposite side. In another paper this group found that, after 15 min of cortical ischemia produced by bilateral carotid occlusion in gerbils, 5HT declined to 78% of control in frontal cortex but was unchanged in basal ganglia. During that same period NE declined to 78% in cortex and 50% in basal ganglia.

Zervas and associates measured NE after 24 hours of unilateral common carotid ligation in gerbils. Norepinephrine was shown to be decreased on the infarcted side. These measurements were made long after irreversible changes occurred. Robinson et al. showed ipsilateral decrease in NE in rat cortex or brainstem after arterial occlusion many hours after irreversible changes occurred.

Kogure et al. reported NE declined for at least 4 hours in both hemispheres of rats after carotid infarction produced by injection of microemboli. Again, the biochemical measurements were made long after damage was irreversible.

Harrison and associates studied 5HT, 5HIAA and NE in gerbils 3.5 hours after carotid ligation and found decreased concentrations of all these substances in both the infarcted and control hemispheres. These studies were conducted long after damage was irreversible.

To summarize, although a number of groups have studied various aspects of the effects of biogenic amines on CNS ischemia, none has used a model of focal ischemia that is as reproducible as the rabbit spinal cord infarction model. Many of the previously reported results suggest, as does the present paper, that substantial rapid declines in 5HT and NE occur. But, most of these previous studies were not designed to evaluate the early time periods when the changes were becoming irreversible and therapeutic intervention might be expected to be most effective.

These prior studies were hampered by the complexity of the cerebral models that were used, and it was particularly difficult to examine charge occurring in any marginally viable tissue that may border irreversibly damaged regions. Screening studies have been successful in other circumstances when large and uncomplicated changes occur. However, the biogenic amine changes during ischemia appear to be more subtle. Therefore, we hoped that use of a simpler system would help to reduce the complexity of the problem. We recognized that such screening tests are rarely definitive, especially when we had no a priori hypotheses concerning which changes might occur or their locations. We were primarily searching for suggestions of the types and locations of changes that might indicate more rigorous follow up studies.

The rapid alterations of biogenic amines reported in this paper may have substantial implications for understanding the pathophysiology of ischemia and suggesting possible therapeutic manipulations. The absolute size of the changes may be larger than was apparent in this study because the precise location of the structures involved has not yet been completely established. For example, if large changes occur in very localized areas, they may have been missed in this study which was a survey. Also, it is possible that substantial changes in turnover rates of biogenic amines are occurring. Since a steady state is not present during the early stages of infarction, such turnover studies are currently impossible. However, it is clear that 5HT is not being rapidly degraded to 5HIAA since the 5HIAA concentrations do not change.

The lack of rapid changes in the most ischemic areas is not surprising because, at a low blood flow rate,
rapid alterations in tissue concentrations of biogenic amines cannot occur unless they are degraded. We have shown that such metabolism of 5HT did not occur and apparently the same is true of NE although catecholamine metabolites were not measured in this study. Whether there are alterations in the location of these substances (for example, release from neurons or platelets to other locations) cannot be established by the methods used in this study.

The rapid 5HT changes in the border zone of the lesion that occur at precisely the time that damage is becoming irreversible are particularly interesting. Reversible changes occur at the lesion edge, and this suggests that pharmacological manipulations could potentially alter these responses. The viability of the tissue at the lesion edge is presumably precarious during ischemia and tissue in this region which might otherwise be irreversibly damaged could be potentially salvaged if 5HT is causally related to the destruction process. The relatively larger changes that occur in this transitional region compared with the more modest changes in completely infarcted tissue and at more distant sites suggest that 5HT is actively participating in the development of the lesion; whether these effects are harmful or helpful is still not clear. Furthermore, since the damage becomes irreversible so quickly, only a pharmacological manipulation would have any reasonable chance of success in clinical situations (for example, surgical intervention in most cases would be impractical). There are a large number of drugs that can alter 5HT actions, thus, further investigation is justified by these findings.

The reasons for the early increase in 5HT in white matter is not clear. However, the white matter is not infarcted within the time periods studied in this model. Thus, further studies will be necessary to confirm and clarify this finding.

The NE changes are of a different character. In gray matter there is a gradual decline in the infarcted region that only becomes statistically significant at 55 min, i.e., after damage is irreversible. In white matter, there is an initial increase in NE concentration that only becomes statistically significant at 55 min, i.e., after damage is irreversible. In white matter, there is a gradual decline in the infarcted region compared with the more modest changes that occur in the CNS during ischemia, for example, energy metabolism is markedly altered. The time and course of these changes does not match the occurrence of irreversible deficits, and therefore it is not clear that energy metabolism changes are involved in initiating the irreversible damage. Similarly, in this paper it was shown that norepinephrine changes occur somewhat after the onset of clinical injury. However, these energy metabolism and NE changes probably eventually play a role in tissue destruction. Further investigations clearly indicated are studies of the precise correlation of spinal cord blood flow with biochemical and morphological changes, and pharmacological manipulations which alter biogenic amine concentrations. It may then be possible to more completely establish a cause-effect relationship between the biochemical changes observed and the ultimate neurological and histological consequences of CNS infarction.

Acknowledgment

We would like to thank John Venditto for excellent technical assistance. This project was supported by PHS grant NS 00456 and NS 15827.

References


Ischemic Brain Edema With and Without Reperfusion: An Experimental Study in Gerbils

FAUSTO IANNOTTI, M.D. AND JULIAN HOFF, M.D.

SUMMARY Tissue water and rCBF from the same area of brain was measured in gerbils with cerebral ischemia. In one experiment we related the severity of ischemia that developed after one hour of carotid occlusion to the amount of edema which formed. In a second experiment brain made ischemic for one hour was reperfused for one hour to assess the effect of reperfusion of ischemic tissue upon edema formation. We identified a critical threshold (10–14 ml·100g·min⁻¹) for the reversibility of the ischemic process, above which edema can resolve upon reperfusion. When postocclusion rCBF was less than 10 ml·100g·min⁻¹, edema was maximal at the end of occlusion and did not resolve with reperfusion. Autoregulation was preserved in ischemic tissue in which the edema process resolved with reperfusion.

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RESTORATION OF BLOOD FLOW to ischemic brain is now clinically feasible.¹ Logic dictates that reperfusion of ischemic brain tissue should improve brain function and might improve outcome from an ischemic event, but clinical and laboratory experience indicate that edema formation associated with ischemia may actually be aggravated by reperfusion.¹ ² This conflict between logic and experience poses a practical question for clinicians: Should ischemic tissue be reperfused and, if so, under what circumstances?

Both the duration and the severity of ischemia influence the effects of reperfusion.² The duration of ischemia from which recovery of function can be expected varies from species to species, and is affected by temperature, drugs, anatomical variants, age and other factors. The severity of ischemia is influenced by the degree of perfusion of a core of tissue with its own exclusive capillary bed and by the perfusion of that tissue through collateral vessels able to react to the ischemic event nearby. Thus, acute occlusion of a major cerebral artery may cause severe or even complete ischemia in a small area, moderate ischemia in a larger adjacent area, and no ischemia in peripheral areas perfused normally by abundant collateral vessels.

We developed an animal model that provides a spectrum of low flow states for one hour after acute carotid occlusion.³ ⁴ Measurement of rCBF and tissue water from the same area of brain in this preparation has allowed us to relate the severity of ischemia to the severity of edema which forms during the first hour after occlusion and to identify the effects of reperfusion upon rCBF and edema in the same tissue.⁵ In earlier studies, brain water content was closely related to the severity of ischemia. The rCBF threshold for edema formation, identified in our gerbil model, coincided with that found in the primate model by Symon, et al.⁶ The ischemia threshold for edema formation we demonstrated was slightly higher than that...
The effect of ischemia on biogenic amine concentrations in the central nervous system.
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