Temporal Thresholds for Neocortical Infarction in Rats Subjected to Reversible Focal Cerebral Ischemia

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We investigated the temporal threshold for focal cerebral infarction in the spontaneously hypertensive rat. The right middle cerebral artery and common carotid artery were occluded for 0, 1, 2, 3, 4, or 24 hours, and all the animals were sacrificed 24 hours after the onset of ischemia. Cortical infarct volumes and edema volumes were quantified in serial frozen sections of hematoxylin and eosin-stained tissue using image analysis. Upon occlusion, blood flow in the core of the ischemic zone, measured with laser-Doppler flowmetry, fell to a mean ± standard deviation of 21±7% of the preocclusion baseline value (n=26). During the first hour of ischemia, blood flow in the densely ischemic zone rose to 27±8% of baseline (n=25). Release of the middle cerebral artery and common carotid artery occlusions rapidly restored cortical blood flow to 213±83% of baseline (n=21). Focal ischemia of 1 hour's duration caused little or no infarction, while ischemic intervals of 2 and 3 hours produced successively larger volumes of infarcted cortex. Ischemic intervals of 3-4 hours' duration followed by approximately 20 hours of recirculation yielded infarct volumes that were not significantly different from those after 24 hours of permanent focal ischemia. The results indicate that 3-4 hours of focal cerebral ischemia in this rat model is sufficient to attain maximal infarction and suggest that recirculation or pharmacological interventions after this time will provide little benefit. (Stroke 1991;22:1032-1039)

The period of focal ischemia tolerated by brain without causing infarction and the period beyond which reperfusion fails to reduce infarct size must be considered in the development and testing of new therapeutic agents. Recent interest in thrombolytic therapy for acute stroke,1-2 which may increase the risk of hemorrhage into recently infarcted tissue, makes the knowledge of temporal thresholds for infarction particularly important. In addition, if drugs such as antagonists of voltage-regulated3-5 and ligand-regulated6-7 calcium channels, which reduce cerebral infarct volume in experimental animals, are to be effective in clinical stroke, they must be given before ischemic injury becomes irreversible.

Though controlled studies to address these questions in humans are beyond current technological and practical feasibility, data bearing on the brain's tolerance to focal ischemia8-16 are available for several animal species. In any model of focal ischemia multiple factors influence histological outcome. For example, the degree and distribution of ischemia varies with the species and strain of animal employed,17-20 and changes in physiological factors such as brain temperature,21-23 PacO2,24 and blood glucose concentration25 can further modulate regional cerebral blood flow (rCBF) and the metabolic response to ischemia.

In recent years, several rat models of permanent focal ischemia have been developed in which most physiological variables are well controlled and which result in consistent infarction.20,25,27 Importantly, the improved reproducibility of infarction in these models facilitates experiments with the necessary number of animals to achieve statistically supported conclusions. These models have been modified to allow recirculation to test the brain's temporal tolerance to focal ischemia.
A model of permanent focal ischemia in the spontaneously hypertensive rat (SHR) that produces consistent neocortical infarction in the middle cerebral artery (MCA) distribution has been modified in this study to produce reversible neocortical ischemia. We describe not only the technical modifications of this model but also the temporal characteristics for the inception and maximization of infarction after focal ischemia. Laser-Doppler flowmetry, which has previously been shown to provide accurate continuous measurements of changes in rCBF associated with focal ischemia, was used to assure the severity and reproducibility of the insult in the central zone of ischemia. We have presented a preliminary report of these data.

Materials and Methods

Male SHR (Taconic Farms, Inc., Germantown, N.Y.) weighing 245–310 g were fasted for 18–24 hours. The animals were anesthetized with a mixture of 2.0% halothane and 30% oxygen/70% nitrogen. The tail artery was cannulated for blood pressure monitoring and for serial blood sampling for determinations of pH, PCO2, PO2 (Corning 158 pH/Blood Gas Analyzer, Medfield, Mass.), hematocrit, and glucose concentration (Beckman glucose analyzer, Fullerton, Calif.). The right common carotid artery (CCA) was isolated from adhering tissue and nerves, and a Silastic loop was placed around the vessel for later occlusion and release. Orotracheal intubation was achieved under microscopic visualization with care to avoid injury to the upper airways. The halothane concentration was reduced to 0.5–1% immediately following intubation, and the rats were maintained on a mechanical ventilator (Harvard Rodent Respirator, Millis, Mass.) without curare or atropine because prior experience revealed improved survival after extubation in the absence of these drugs. Though spontaneous respirations sometimes occurred, blood gases were easily maintained within the physiological ranges. Body temperature was kept at 37–37.5°C with a circulating water bath.

The method for producing reversible focal ischemia closely follows the procedure described by Brint et al for producing permanent focal ischemia. The temporalis muscle was partially excised, and a 2-mm-diameter burr hole was drilled 2–3 mm rostral to the point of fusion of the zygoma with the temporal bone. Drilling was accompanied by a gentle 0.9% saline drip to prevent warming of the underlying cortex. With the use of a micromanipulator (Narishige Instruments, Tokyo, Japan), an 80-μm-diameter stainless steel hook was placed under the MCA at a point 0–1 mm above the rhinal fissure. This site was proximal to any major MCA bifurcation but distal to the lenticulostriate arteries.

Approximately 60 minutes after the induction of anesthesia and approximately 30–45 minutes after reduction of the halothane concentration to 0.5–1%, focal cerebral ischemia was induced by tightening the Silastic loop around the CCA and by raising the MCA with the micromanipulator approximately 1 mm above the cortical surface. Absence of blood flow in the occluded MCA was visually verified with an operating microscope. Sham-operated rats (n=5) underwent manipulation of the Silastic loop and hook without vessel occlusion.

Focal ischemia in the reversibly occluded animals was maintained for 1 (n=5), 2 (n=6), 3 (n=5), or 4 (n=5) hours, during which time the burr hole and surrounding bone were bathed with a continuous, gentle drip of warmed (37.5°C) 0.9% saline to prevent desiccation of the exposed brain and to permit continuous visualization of the occluded MCA. The hook tension against the MCA was increased as necessary to maintain arterial occlusion. Recirculation was achieved by releasing the Silastic loop around the CCA and lowering the MCA off the hook. In rats exposed to 24 hours of focal ischemia (n=5), permanent middle cerebral artery and common carotid artery occlusion (MCA/CCAO) was achieved by ligating the CCA and severing the MCA by the brief application of an electrocautery needle (Geiger, Philadelphia, Penn.) to the hook. Permanently occluded animals remained anesthetized with 0.5–1% halothane for 4 hours after MCA/CCAO. After awakening and extubation, the rats were closely watched for postoperative respiratory insufficiency, and arterial blood gases were checked 2 hours after extubation. Food but not water was withheld during the 24 hours of recovery.

Changes in rCBF were measured with laser-Doppler flowmetry (TSI, Inc., St. Paul, Minn.) at a single location in the MCA territory known from prior studies to represent the area of densest ischemia in this model. A 1–2-mm burr hole was drilled 4–5 mm dorsal to the site of MCA occlusion in each rat. The dura at the laser-Doppler flowmetry probe site was left intact and, following placement of the hook beneath the MCA but prior to occlusion of the vessel, the 0.8-mm-diameter probe was positioned with a micromanipulator just above the cortical surface at a site relatively free of large pial vessels. The probe site was bathed with warmed (37.5°C) saline to prevent desiccation, brain cooling, and the accumulation of blood beneath the probe. Continuous recordings of rCBF were made beginning prior to the onset of focal ischemia and throughout the periods of cerebral ischemia and reperfusion. If rCBF measurements suggested that blood flow through the MCA was recovering after the initial decline, the hook was raised slightly to ensure complete MCA occlusion.

During the development of this model, increasing attention was directed toward brain temperature as a modulator of ischemic injury. This series was preceded by a pilot study (n=4) to determine the optimal temperature of the saline drip that would maintain brain temperature at 37°C. Rectal temperature was maintained at 37°C as described above. Temperature of the saline drip was measured at the
Physiological variables are presented in Table 1. Glucose concentration was 92±19 mg/dl before occlusion and 101±17 mg/dl 2 hours postoperatively; hematocrits at those times were 44±2% and 52±3%, respectively. The difference in hematocrit was significant (p<0.05 by t test) and probably reflected some degree of dehydration due to mechanical ventilation. No significant differences (by MANOVA) at any time between groups were found comparing rats subjected to 1, 2, 3, 4, or 24 hours of ischemia. Accordingly, the data were pooled for each time of measurement. By MANOVA there were isolated differences between mean arterial blood pressure and pH before and after occlusion, but these differences were small and of no biological significance. Infarct volume enlarged progressively after 1, 2, and 3 hours of ischemia (Figures 1 and 2). There were no significant differences in infarct volume between animals exposed to 3, 4, or 24 hours of focal ischemia. (We conducted two preliminary studies showing a similar temporal pattern for infarction in this model, but these data were deleted because of limitations on manuscript length. Details of these studies are available upon request.)

In the 1 hour of ischemia group, microscopic examination revealed scattered irregular patches of injury in most rats, usually adjacent to the subcortical white matter. In all remaining groups, ischemic neurons were identified microscopically in a thin (100–1,200 μm) rim bordering the infarct. The irregular and indistinct margins of these areas precluded their quantification by image analysis.

The development of neocortical edema with increasing durations of ischemia is shown in Figure 2. When measured 24 hours after the onset of reversible or permanent ischemia, no group of reperfused animals had a mean edema volume greater than that of the permanently (24 hours) occluded group. Edema volume did not differ significantly among rats exposed to 3 or 4 hours of reversible ischemia or permanent ischemia (i.e., the groups with maximal infarct volume). The edema volume was linearly proportional to the infarct volume in all groups (r=0.97, Figure 3).

Tandem MCA/CCAO induced a consistent, marked fall in rCBF recorded at a single site in the...
FIGURE 1. Topography of evolving cortical infarction in rats. Tracings of infarcts resulting from 1 (n=5), 2 (n=6), 3 (n=5), 4 (n=5), and 24 (n=5) hours of ischemia are superimposed at four coronal levels from anterior commissure to posterior hippocampus. Cortical infarction in middle cerebral artery territory progresses toward border zone supplied by anterior cerebral (anterior and superior extension) and posterior cerebral (posterior and inferior extension) arteries. Infarction reaches its maximum extent by 3 hours.
ischemic core (Table 2). Upon occlusion rCBF was 21±7% of the preocclusion baseline. No significant differences at any time between groups were found comparing animals subjected to 1, 2, 3, 4, or 24 hours of ischemia. Accordingly, data were pooled for each time of measurement (Table 2); MANOVA applied to the pooled data demonstrated that rCBF rose slightly but significantly ($p<0.05$), from 21±7% to 27±8% of baseline, during the first hour following occlusion. In approximately half the rats, laser-Doppler flowmetry indicated that MCA occlusion was becoming subtotal. In these cases, rCBF promptly returned to levels consistent with complete MCA/CCAO following slight elevation of the hook. Upon release of MCA/CCAO, rCBF rose to a peak value of 213±83% of baseline over 7–10 minutes (Table 2). Single animals in the 2 and 3 hours of ischemia groups failed to reach baseline, but rCBF in these rats rose fivefold and twofold, respectively, from ischemic levels. One animal from the 4 hours of ischemia group was excluded because after the initial hyperemia following release of the MCA occlusion, a marked and persistent decrease in rCBF occurred immediately upon release of the CCA occlusion. In all other rats early-recirculation rCBF exceeded preocclusion rCBF.

FIGURE 2. Relation between neocortical infarction and edema formation resulting from increasing durations of focal cerebral ischemia in rats. Infarct and edema volumes in 1 (n=5), 2 (n=6), 3 (n=5), and 4 (n=5) hours of ischemia groups were all measured at 24 (n=5) hours. Mean infarct and edema volumes increased significantly ($p<0.05$) between 1 and 3 hours of ischemia. Infarct and edema volumes after 3, 4, or 24 hours of ischemia were not significantly different.

FIGURE 3. Neocortical edema volume plotted as function of infarct volume in rats subjected to reversible or permanent focal cerebral ischemia. Each data point represents neocortical infarct and edema volume measured in one animal 24 hours after onset of 1, 2, 3, or 4 hours of reversible or permanent (24 hours) focal ischemia. Edema volume increases in linear fashion with increasing neocortical infarct volume ($r=0.97$).
Discussion

In SHR, tandem MCA/CCAO caused a severe reduction in rCBF in the ischemic core (21±7% of preocclusion baseline), a small attenuation of ischemia during the first hour (27±8% of preocclusion baseline), and upon reversal an acute hyperemia (213±83% of preocclusion baseline). This ischemic insult, if sustained for 1–2 hours, was sufficient to initiate infarction, while 3–4 hours of ischemia produced an infarct volume not significantly different from that observed with permanent MCA/CCAO. The results suggest that the temporal threshold for maximal infarction in this model is 3–4 hours of MCA/CCAO and that recirculation and drug therapy must be instituted earlier to be of any benefit.

The threshold concept implies an all-or-none response and, while useful descriptively, is somewhat artificial as applied to any single variable in ischemic brain injury since many of these variables are interactive. Efforts to define the so-called temporal thresholds for infarction in focal ischemia are complicated by the fact that the duration and the degree of ischemia in the rat have been developed that yield relatively consistent infarction of the caudate26 or neocortex20,27 after MCA occlusion. Our results and those of others13–15 suggest an earlier appearance of initial and complete infarction in the rat than in the large animals discussed above. He et al13 reversibly occluded the MCA and both CCAs in Long-Evans rats and found infarcts after 30 minutes of ischemia and apparent maximization of infarction resulting from 90 minutes of ischemia. Preliminary results of Xue et al14 indicate a time course in the unanesthetized SHR subjected to permanent CCA occlusion and reversible MCA occlusion that closely equal in severity to those seen with permanent occlusion after 6–8 hours of ischemia. Sundt et al10 reported that 3–6 hours of MCA occlusion were required before infarction began to develop in the squirrel monkey, but the time to achieve maximal infarction was not addressed. Jones et al11 noted small infarcts after 15–30 minutes of ischemia and “moderate to large” (not quantified) infarcts after 2–3 hours in the macaque monkey. These authors attempted to correlate the temporal threshold for infarction with rCBF and found that tissue with rCBF below 10–12 ml/100 g/min infarcted after 2–3 hours and that tissue with rCBF below 17–18 ml/100 g/min went on to infarction if occlusion was permanent.

In awake cats subjected to MCA occlusion, Weinstein et al12 found no infarcts with ischemia lasting <4 hours; ischemia lasting 5–8 hours produced infarcts significantly smaller than those seen after permanent occlusion. Animals reperfused after 24 hours of ischemia had infarcts similar in size to infarcts in permanently occluded animals. Sundt et al10 reported that infarcts were significantly smaller in cats subjected to 6 hours of MCA occlusion with reperfusion than in permanently occluded animals.

The rat is an attractive species for the study of focal ischemia because handling is easier and expense is less than with primates and other larger animals and because models of permanent focal ischemia in the rat have been developed that yield relatively consistent infarction of the caudate26 or neocortex20,27 after MCA occlusion. Our results and those of others13–15 suggest an earlier appearance of both initial and complete infarction in the rat than in the large animals discussed above. He et al13 reversibly occluded the MCA and both CCAs in Long-Evans rats and found infarcts after 30 minutes of ischemia and apparent maximization of infarction resulting from 90 minutes of ischemia. Preliminary results of Xue et al14 indicate a time course in the unanesthetized SHR subjected to permanent CCA occlusion and reversible MCA occlusion that closely

### Table 2. Relative Regional Cerebral Blood Flow in Rats Exposed to Focal Ischemia and Reperfusion

<table>
<thead>
<tr>
<th>Group</th>
<th>Upon occlusion</th>
<th>1 hr</th>
<th>2 hr</th>
<th>3 hr</th>
<th>4 hr</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hr</td>
<td>5</td>
<td>24±8</td>
<td></td>
<td></td>
<td></td>
<td>276±93</td>
</tr>
<tr>
<td>2 hr</td>
<td>6</td>
<td>18±5</td>
<td>24±9</td>
<td>23±7</td>
<td></td>
<td>154±51</td>
</tr>
<tr>
<td>3 hr</td>
<td>5</td>
<td>25±7</td>
<td>28±5</td>
<td>29±9</td>
<td>33±7</td>
<td>210±89</td>
</tr>
<tr>
<td>4 hr</td>
<td>5</td>
<td>21±6</td>
<td>24±4</td>
<td>27±10</td>
<td>25±7</td>
<td>27±4</td>
</tr>
<tr>
<td>24 hr</td>
<td>5*</td>
<td>19±8</td>
<td>25±10</td>
<td>31±11</td>
<td>34±6</td>
<td>35±7</td>
</tr>
<tr>
<td>Pooled</td>
<td></td>
<td>21±7</td>
<td>27±8†</td>
<td>30±8†</td>
<td></td>
<td>213±83†</td>
</tr>
<tr>
<td>n</td>
<td>26</td>
<td>25</td>
<td>20</td>
<td>14</td>
<td>9</td>
<td>21</td>
</tr>
</tbody>
</table>

Values are mean±SD percentage of preocclusion baseline (100%) as measured with laser-Doppler flowmetry. Reperfusion is peak value recorded over 7–10 minutes following release of occlusion. *n=4 for ischemic intervals 1, 2, 3, and 4 hr. †p<0.05 different from occlusion by repeated-measures analysis of variance.
corresponds to the time course for infarction in SHR reported in our study.

Two descriptions have appeared recently of rat models that occlude the MCA intraluminally using nylon suture or a silicone rubber cylinder introduced via the carotid system extracranially.15,16 Zea Longa et al13 found that infarct volumes following 2 hours of occlusion were not significantly different from those in permanently occluded animals. While the ability to avoid intracranial surgery is appealing, both models resulted in exceptionally high mortality in permanently occluded animals, making studies of infarct maturation difficult. Moreover, failure to monitor the adequacy of cerebral reperfusion in these studies undermines control of a singularly important variable in reversible ischemia models.

The differences in temporal thresholds observed in different species are probably due in part to inherent differences in resistance to ischemia and, most importantly, to marked differences in the volumes of densely ischemic tissue in the models employed. Interspecies differences that may influence brain tissue tolerance to ischemia are the metabolic rate and the body/brain temperature, both of which are higher in smaller animals.31 A higher cerebral metabolic rate and a higher body/brain temperature may increase vulnerability to ischemia. Current rat models of focal ischemia, including the model described by Tamura et al29 in which the MCA and lenticulostriate arteries are occluded, are all based on occlusions of more than one vessel to reduce variability in postocclusion rCBF. Focal ischemia produced in rats has been shown to be more consistent15,16,20,27 than that seen with MCA occlusion in cats35 and monkeys,33 and within the rat species focal ischemia is most severe and reproducible in the SHR strain.

There was a high positive correlation between the volumes of neocortical infarcts and edema in rats subjected to reversible or permanent focal ischemia (Figures 2 and 3). Reperfusion has been reported to aggravate edema formation,10,34 perhaps due to disruption of the blood–brain barrier35,36 and an unopposed pressure head in tissue that has lost autoregulation. Although we observed no accentuation of the edema volume in reperfused versus permanently ischemic brains, we cannot exclude the possibility that cerebral edema formed more rapidly and perhaps excessively during the early recirculation period. However, by 24 hours the edema volume did not reflect the period of recirculation but depended entirely on the size of the infarct. In Sprague-Dawley rats, 2 hours of MCA occlusion followed by 2 hours of recirculation caused a smaller decrease in brain tissue specific gravity (i.e., less edema formation) than 4 hours of permanent occlusion.35 Although histology was not studied in that experiment, the benefits of recirculation early after ischemia onset appear to outweigh the potential for aggravating edema formation. Data from this and other studies8–14 of temporary focal ischemia suggest that reperfusion of previously ischemic brain does not contribute to injury as does reperfusion of ischemic heart muscle.

In summary, we used a model of reversible focal ischemia in SHR to determine the durations of ischemia required for the initiation and production of maximum infarction. These time points define a "therapeutic window" for this model: establishing reperfusion within 1 hour after the onset of ischemia will result in little injury, while treatment or reperfusion instituted later than 3–4 hours after ischemia will be of little or no benefit. This information should prove useful in designing experiments to test potential treatments for focal cerebral ischemia.

Cautious extrapolation of these and other data derived from animals to clinical stroke is warranted. We do not imply that treatment of focal cerebral ischemia in humans is fruitless beyond 3–4 hours after stroke onset, especially in view of the interspecies differences in the temporal profile of infarct evolution. Nevertheless, therapies aimed at limiting the extent of brain injury from focal ischemia should be initiated without delay since cerebral infarction may maximize within a few hours after stroke.

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