Acidic Foci Within the Ischemic Penumbra of the New Zealand White Rabbit

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Background and Purpose: In vivo panoramic imaging of reduced nicotinamide adenine dinucleotide (NADH), intracellular brain pH (pH), and cortical blood flow was used to characterize the ischemic penumbra during focal ischemia. During global ischemia, hypoxia, and status epilepticus, the development of cortical acidic foci has been observed. The hypothesis tested was that during focal ischemia, acidic foci develop, which may lead to recruitment of the ischemic penumbra into infarction.

Methods: Five fasted New Zealand White rabbits underwent middle cerebral artery (MCA) occlusion under 1.5% halothane anesthesia through a retro-orbital approach, and five animals served as controls. Brain pH, and cerebral blood flow were measured with in vivo umbrelliforene fluorescence.

Results: Baseline brain pH, was 6.98±0.05, whereas cortical blood flow and NADH fluorescence measured 52.2±8.7 ml/100 g per minute and 35.5±3.7 gray-scale units, respectively. Fifteen minutes after MCA occlusion, overall brain pH, and cortical blood flow of the ischemic penumbra measured 6.61±0.06 and 31.9±9.2 ml/100 g per minute. Over 3 hours there was normalization of pHi in the majority of the penumbra due to increases in cortical blood flow. Within the ischemic penumbra acidic foci developed with an initial pHi of 6.35±0.09 and cortical blood flow of 18.0±5.7 ml/100 g per minute. These foci remained acidic with increased NADH fluorescence despite being surrounded by cortex that was recovering from ischemia. On light microscopy, these acidic foci had a mixed pattern of neuronal injury.

Conclusions: Within the ischemic penumbra, acidic foci develop that do not follow a vascular distribution and have microscopic evidence of ischemic neuronal injury. This suggests that there is a cortical selective vulnerability regarding pHi, regulation and these acidic foci may lead to recruitment of the ischemic penumbra into infarction. (Stroke. 1993;24:2030-2040.)

Key Words - cerebral blood flow • cerebral ischemia • neuronal damage • rabbits

The original intent of use of the term ischemic penumbra was to describe a zone of electrically silent but structurally intact parenchyma surrounding a core region of severe ischemia or infarction during focal cerebral ischemia.1-5 This concept implied that in the border zone there was reduced but still sufficient cortical blood flow (CBF) to retard irreversible neuronal injury by the preservation of membrane integrity.5 Characterization of the ischemic penumbra has relied primarily on electrophysiological and biochemical measurements made during reduced CBFs by autoradiography,6 microelectrode studies,7 tissue-slice mapping,8 and reduced nicotinamide adenine dinucleotide (NADH) fluorescence.9,10

Topographic studies of the ischemic penumbra have largely depended on CBF or vital dye staining after middle cerebral artery (MCA) occlusion.5,7,11-13 In actuality, clear-cut identification of the ischemic penumbra has been elusive.14 Measurements of CBF in MCA occlusion models often show a sharp distinction between tissue receiving normal blood flow and that receiving low blood flow.15-17 In human embolic stroke, there is usually a clear transition between infarcted tissue and the normal brain.18 In lower animal models, such as that of the Wistar rat or cat, the peri-infarct zone containing scattered necrotic neurons is small, unless the animals are made hyperglycemic before MCA occlusion.19-21 Alternatively, some experiments have demonstrated that there is a gradual transition in CBF across the cortex in a sagittal plane as one traverses from infarcted to normal cortical tissue.15

After MCA occlusion in humans, there is typically a central focus of infarction with adjacent tissue that has varying degrees of neuronal necrosis.18,19 This surrounding zone can be considered the ischemic penumbra. This perifocal area can evolve into an extension of the infarction unless reperfusion is established early. The question then arises as to the mechanisms by which this perifocal zone or ischemic penumbra deteriorates into infarction. It has been proposed that this recruitment occurs through two alternative modes of extension22: (1) the infarction undergoes continuous enlargement until it reaches tissue that has good cerebral perfusion or (2) there is the emergence of "islands" of neuronal necrosis that coalesce and lead to an increase in infarct size. Recent work from this laboratory has demonstrated a variable pattern of intracellular brain pH (pHi) changes during incomplete global ischemia,23 hypoxia,24 and hyperglycemia during status epilepticus.25 In each of...
these experiments, the development of cortical acidic foci did not follow a vascular distribution. These data supported the concepts of brain pH, compartmentalization and differential pH response to injury as originally suggested by Griffith et al. 26

The purpose of this experiment was to first identify the ischemic penumbra through the use of in vivo imaging of brain pH, and cortical blood flow via umbelliferone fluorescence. After this confirmation of the ischemic penumbra, the hypothesis tested was that acidic foci exist within the ischemic penumbra and that these foci might determine in part whether tissue in the ischemic penumbra progresses to infarction.

**Materials and Methods**

**Animal Preparation**

After the protocol was reviewed and approved by the Institutional Animal Care and Use Committee, 10 overnight-fasted New Zealand White rabbits weighing between 3.5 and 4.5 kg were induced, operated, and studied under 4.0%, 2.5%, and 1.5% halothane anesthesia, respectively. A tracheostomy was performed and the animals were placed on a Harvard respirator. The animals were given 0.15 mg/kg pancuronium bromide to abolish respiratory efforts. Catheters were inserted into the right femoral artery and vein for monitoring blood pressure, sampling arterial blood gases, and administering drugs. A catheter was inserted into the right lingual artery for retrograde delivery of the indicator umbelliferone into the internal carotid artery.

The skin, subcutaneous tissue, and muscle were excised over the right supraorbital ridge and parietal area. With the aid of an operating microscope, a craniectomy exposing the majority of the right hemisphere was performed with a high-speed air drill. The dura was removed and the cerebrum covered with Saran Wrap, which kept the brain moist and prevented surface oxygenation. The trunk of the MCA was exposed via a retro-orbital approach. The contralateral common and cervical internal carotid arteries were ligated. Five animals served as controls and five underwent MCA trunk occlusion with a microclip.

After surgical preparation, the animal was moved from the operating table and placed on an intravital-type microscope stand. The microscope was focused on an area centered around the suprasylvian gyrus. Arterial blood pressure was recorded on a polygraph. The animals were kept normothermic by the use of a heating blanket, and body temperature was monitored with a rectal digital thermometer. Arterial PCO₂, PO₂, and pH (pHᵢ) were measured with a London radiometer blood gas analyzer. Serum glucose, serum lactate, and hematocrit levels were also determined before each measurement.

**In Vivo Video Fluorescent Instrumentation**

Instrumentation was designed to perform serial panoramic video imaging of cortical brain pHᵢ and focal cortical blood flow with umbelliferone fluorescence. 24

The optical characteristics were such that the majority of the entire hemisphere could be studied simultaneously through a large craniectomy. The use of umbelliferone as a noninvasive in vivo technique for measuring brain pHᵢ and cortical blood flow has been previously described. 24-28

Umbelliferone is nontoxic, fat soluble, and freely diffusible across the blood-brain barrier, and it rapidly equilibrates across cell membranes and is distributed through the cytoplasm as an uncharged molecule. 24

Umbelliferone was prepared for injection by dissolving 0.2 g of indicator in 200 mL of a 5% glucose-saline solution at 90°C for 30 minutes. The solution was then filtered through a 0.22-μm mesh filter before injection. The volume of injectate was 1.5 mL in this study.

The pH-sensitive indicator umbelliferone has two fluorophors, anionic and isobestic. The anionic and isobestic forms are excited at 370 nm and 340 nm, respectively, and have a common emission at 450 nm. The fluorescence of the anion varies directly with pH, whereas the fluorescence of the isobestic form varies directly only with the indicator concentration. Therefore, it is possible to create a nomogram from the ratio of 340-nm to 370-nm excitations to determine brain pHᵢ. Acquired images were corrected for background NADH fluorescence before processing. NADH fluorescence images were stored for later analysis of mitochondrial function. The images from the 340-nm excitation were processed to compute CBF using the 1-minute initial slope index. The CBF image was then displayed and stored on tape for final analysis. For processing of the pHᵢ image, ratios of the paired images from the 340-nm and 370-nm excitations were made, and the resultant pHᵢ image was then displayed and stored on tape for final analysis.
Because of anatomic variation in the microvasculature from animal to animal, single points along an x,y coordinate cannot be averaged frame by frame from different animals at the same time. Therefore, measurements of regional pH_i, CBF, and NADH fluorescence were made in areas devoid of major vessels. Measurements were made over these relatively avascular areas by averaging...
2755 pixels (55 780 \( \mu \)m\(^2\)), and the mean and standard deviation were tabulated. Statistical significance was determined by Student's \( t \) test for paired data.

Frequency histograms were made from entire video frames at each time interval of pH\(_i\), CBF, and NADH fluorescence. Surface vasculature and the margin of the
Fig 2. A, Time-course plot of intracellular brain pH (pHi) before and during focal cerebral ischemia. Region 1 (\(\cdots\cdots\cdot\cdots\circ\)) demonstrated an initial reduction in brain pHi to 6.61±0.06 after middle cerebral artery (MCA) occlusion. Although brain pHi in region 1 remained acidic for approximately 45 minutes, by 3 hours it had normalized. This is opposite to that observed in region 2 (\(\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\)), in which brain pHi was initially 6.35±0.09. At 3 hours, brain pHi in region 2 remained acidic, measuring 6.55±0.10. Nonischemic control rabbits demonstrate the stability of the preparation (\(\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cd\end{array}\)
Histology

After the final measurements of pH and CBF were made, the animals underwent a thoracotomy followed by intracardiac perfusion of 10% formaldehyde for in situ fixation. With a fine needle, the corners of the photographic overlays of pH and CBF were marked on the brain surface with trypan blue before brain removal. The brains were then sectioned coronally in 10-µm increments from a rostral to caudal direction. The locations of cortical regions of interest were referenced by measuring the distance from the trypan blue markings. In this way, microscopic sections within the proximity of these regions of interest were then processed with hematoxylin and eosin staining.

Results

Systemic Parameters and Video Acquisition

There were no significant differences between control and ischemic animals in PaCO₂ (39.7±1.9 mm Hg); pHx (7.32±0.02); PaO₂ (225.4±10.3 mm Hg); mean arterial blood pressure (83.5±3.7 mm Hg); and levels of glucose (164.5±12.1 mg/dL), lactate (1.72±0.3 mmol/L), or hematocrit (34.5±1.4%). Depicted in Fig 1 is a composite video picture of a typical experiment. After MCA occlusion two zones were immediately apparent: a zone of no blood flow, which was subsequently shown to be an area of evolving infarction via histology, and a second surrounding zone of reduced CBF that was associated with intracellular acidosis. This second zone was defined in this experiment as the ischemic penumbra, and it extended from the suprasylvian gyrus posteriorly to involve the majority of the parietal lobe. Within this ischemic penumbra there were two regions: region 1 was the majority of the ischemic penumbra, whereas region 2 consisted of acidic foci immediately apparent at 15 minutes after occlusion within region 1. These two distinct regions of the penumbra were analyzed individually. The control nonischemic animals had stable pHx and CBF measurements throughout the experiment, thereby confirming the stability of the preparation. These data are included on the graphs for comparison but are not discussed below.

Cortical pH (Figs 2A and 3A)

Preocclusion. Baseline brain pHx was relatively homogeneous over the exposed cortex, measuring 6.98±0.05. Before MCA occlusion the average pHx values of the cortex destined to become the two penumbral regions were 6.98±0.05 (region 1) and 6.98±0.05 (region 2).

Ischemia. After 15 minutes of MCA occlusion, the pHx of the majority of the ischemic penumbra, or region 1, was 6.61±0.06 ($P<.005$). Within this penumbra was an area of severe acidosis or acidic foci termed region 2, with a pHx of 6.35±0.09 ($P<.005$). The brain pHx of region 1 remained acidic for 45 minutes but returned to normal after 3 hours despite MCA occlusion. The pattern of brain pHx recovery within the overall penumbra followed an avascular distribution, in that the tissue closest to collateral vessels became normalized first.

The brain pHx of region 2 remained acidic for the duration of the experiment, measuring 6.55±0.10 at 3 hours ($P<.005$). As depicted in Fig 1, this cortical area of severe acidosis coalesced over the period of observation. This pattern of reduction did not clearly follow an avascular distribution.

The microfoci of acidosis were most apparent visually on analysis of the cortical brain pH images. In addition to this visual observation, illustrated in Fig 3A is a three-dimensional histogram of cortical pHx before and during the ischemic insult. This histogram demonstrates that...
before occlusion, brain pH, was relatively homogeneous. On occlusion of the MCA, there was tremendous heterogeneity in brain pH across the cortical surface. There was one peak at approximately 6.65, which persisted throughout the duration of the experiment. There was also a peak at approximately 6.35, indicative of the acidic foci. Since this histogram reflects not only brain pH, but also the volume of cortical tissue expressing that brain pH, the acidic foci are more easily detected on the video images as opposed to the pH histogram. With progression of the ischemic insult, a large area of cortical tissue recovers, with eventual normalization in brain pH.

Regional CBF by Umbelliferone (Figs 2B and 3B)

Baseline CBF measured 52.2±8.7 mL/100 g per minute. After 15 minutes of MCA occlusion, there was a spectrum of CBF reductions in the penumbral region. Region 1 had an overall CBF of 31.9±9.2 mL/100 g per minute (P<.025), and region 2 measured 18.0±5.7 mL/100 g per minute (P<.005). By 3 hours, region 1 CBF had normalized, while it remained improved but still significantly low in region 2, measuring 31.9±2.4 mL/100 g per minute (P<.005). Therefore, there were areas within the penumbra with compromised blood flow surrounded by the remainder of the penumbra with normal blood flow. This pattern did not fit the cortical vascular anatomy.

Illustrated in Fig 3B is a cortical blood flow histogram. Contrary to the homogeneity in brain pH, before occlusion, there was a greater heterogeneity in blood flow. After MCA occlusion the reduction in blood flow was also quite heterogeneous.

NADH Fluorescence Levels (Figs 2C and 3C)

Preeclusion. Baseline NADH fluorescence over the exposed cortex measured 35.5±3.7 gray-scale levels. The baseline NADH fluorescence in the two regions as defined previously were 35.5±3.7 (region 1) and 36.4±4.5 (region 2).

Ischemia. After 15 minutes of focal cerebral ischemia, NADH fluorescence increased significantly in the two regions, to 74.1±28.4 (region 1) (P<.05) and 92.6±27.2 (region 2) (P<.01). NADH fluorescence declined in both regions as ischemia progressed but remained significantly elevated compared with control values. The difference in NADH fluorescence between regions 1 and 2 was significant throughout the experiment (P<.05). The NADH three-dimensional histogram (Fig 3C) demonstrates that before occlusion NADH fluorescence was quite homogeneous across the cortical surface. Immediately after MCA occlusion there was significant heterogeneity in NADH fluorescence, similar to that observed for brain pH. However, there was a more uniform recovery of NADH across the cortical surface, which began to occur 45 minutes into the ischemic insult.

Histology

After the final measurements of pH, and CBF were made, the animals underwent a thoracotomy followed by intracardiac perfusion of 10% formaldehyde for in situ fixation; these procedures have previously been outlined in “Materials and Methods.”

Examination of the cortical area corresponding to the zone of no blood flow after MCA occlusion was consistent with infarction (Fig 4A). This confirms that the lack of umbelliferone fluorescence in this zone during MCA occlusion was due to a lack of blood flow as opposed to a technical artifact.

Region 1 contained normal-appearing neurons (Fig 4B), whereas the cortex corresponding to region 2, or the acidic foci, demonstrated islands of pyknotic cells, consistent with ischemic neuronal injury (Fig 4C).

Discussion

The present study using serial in vivo umbelliferone fluorescent imaging identified a zone of altered acid-base homeostasis surrounding an infarct produced by MCA occlusion. This zone of altered acid-base homeostasis surrounding the core ischemic zone is referred to in this study as the ischemic penumbra. This nomenclature, reflective of the terminology of Astrup et al,1 was adopted to avoid introducing an additional term or neologism to denote a territory surrounding a core ischemic zone. This generalization, as recently discussed by Strong et al,9 seems appropriate with the introduction of a real-time in vivo umbelliferone fluorescent imaging technique that facilitates rapid, serial measurements of brain pH, CBF, and NADH fluorescence to investigate this region.

Brain temperature was not measured in this study, as earlier infrared microscopy studies had shown only small decreases (<1.5°C) in brain temperature during ischemia in a squirrel monkey model of focal ischemia.29 It is known that brain tissue is a poor conductor of thermal activity. Therefore, in a model of focal ischemia where small volumes of tissues are affected, there would be much smaller variations in brain temperature compared with that of global ischemia.

Acidosis and Cell Death

Lactic acid accumulation in cerebral ischemia has been implicated in the evolution of brain infarction.30–32 In vivo, brief exposures of neurons and astrocytes to high concentrations of extracellular hydrogen ion (pH=5.3) have been shown to produce cell death.33 Recently, Nedergaard et al30 addressed this question of prolonged acid exposure over a range of pH values and concluded that cell death was an inverse function of the duration of hydrogen ion exposure.

In this experiment a portion of the penumbra (region 1), with initially altered acid-base homeostasis was able to normalize pH. The rate of change in pH was maximal between 120 and 140 minutes, with a slope of 0.192 pH unit/h compared with 0.072 pH unit/h between 15 and 120 minutes. This rapid reduction in extracellular hydrogen ions occurred when pH ranged between 6.7 and 6.8, suggesting that the homeostatic mechanisms for pH regulation are impaired when pH falls to approximately 6.7. Nedergaard et al30 also recently reached a similar conclusion regarding the significance of pH in the regulation of acid-base homeostasis.

The rapid normalization of pH in region 1 when pH reached 6.8 suggests that the level of initial intracellular acidosis (pH of 6.66±0.06) in the penumbra after MCA trunk occlusion was not detrimental. In our model, simultaneous measurements of intracellular and extracellular pH were not performed. However, other investigators have demonstrated that after acidification of the extracellular space, a similar level of intracellular acidification may also occur.30 These observations suggest that limited exposure to an intracellular acidosis of
intracytoplasmic vesicles. An explanation for the potential neuronal protective effect of intracellular acidosis is that increased hydrogen decreases glutamate neurotoxicity, possibly through a reduction in the N-methyl-D-aspartate-associated channel conductance.

Islands of severe acidosis, or acidotic foci, were identified within the penumbra with an initial pH of 6.35 ± 0.09. Histological analyses (by light microscopy) of these regions were consistent with a mixed pattern of ischemic neuronal injury. Therefore, these acidotic foci represent cortical regions within the penumbra that have sustained ischemic damage, a concept that has also been proposed by other authors. These acidotic foci might correspond to the type 2 ischemic penumbra, in which blood flow reductions are associated with repetitive transient increases in K⁺ and variable neuronal loss. Evidence to support this hypothesis comes from both morphological studies and in vivo experiments. In a histopathologic study of the penumbra in a cat model of focal ischemia, Strong et al. noted the presence of microfoci of ischemic cell injury. In our study the ischemic cell change observed in the acidotic foci region, although not pathognomonic of cell death, indicated that these areas suffered a significant insult. Another morphological parallel has been described in minor chronic cerebral infarction, in which islands of neuronal injury were observed adjacent to the infarct and, after an initial improvement in pH, began to deteriorate. Furthermore, in both complete and incomplete global cerebral ischemia, hypoxia, and hyper-

approximately 6.6 does not cause irreversible injury. Interestingly, as an initial response to ischemia in other tissues such as the hepatocyte, mild cytoplasmic acidification occurs through the release of hydrogen ions from the cell. This extracellular pH (pHᵢ) of 6.6 does not cause irreversible injury. The reason why this peak is not more apparent is that the acidotic foci were actually small in terms of cortical area and therefore are poorly depicted on the histogram as compared with the video images of Fig. 1.

Three-dimensional histograms of cortical blood flow (rCBF) as a function of intracellular pH (pHi) and duration of ischemia. Contrary to that demonstrated with brain pH, in NADH fluorescence is a greater heterogeneity in cortical blood flow across the brain surface before MCA occlusion. This heterogeneity persisted throughout the experiment after onset of ischemia. Overall there was general improvement in CBF due to collateral circulation.
glycemia during status epilepticus, a variable pattern of brain pH changes has been demonstrated supporting the concepts of brain pH compartmentalization and an apparent cortical selective vulnerability to a pathological insult.23-26 The development of these cortical acidic foci did not follow an avascular distribution similar to that observed with the aforementioned pathological conditions. These data support the concepts of brain pH compartmentalization and differential pH response to injury as originally suggested by Griffith and colleagues.26 It is intriguing to consider that these acidic foci may play an important role in the recruitment of the ischemic infarction into the penumbra.

Glucose metabolism in the penumbral region has been found to be increased.6,38 In the study by Peek et al.,6 the penumbra with an acidosis of 6.87 had an increased local cerebral metabolic rate for glucose, which was primarily utilized in anaerobic glycolysis. It is possible that the acidic foci were a result of glucose delivery under anaerobic conditions, leading to increased lactic acid production.31,32 Support for this concept comes from the observation that acidic foci will develop with hyperglycemia during status epilepticus.25

**NADH Fluorescence**

These measurements are considered to reflect changes in mitochondrial NADH levels, with the NADH signal originating from the superficial 400 to 500 μm of the cortex.24 The pattern of response in NADH fluorescence in the penumbral zone, which has also been described by other authors,9 was a transient increase in fluorescence followed by a return to a slightly elevated level at 3 hours. After 15 minutes of occlusion, signal intensity was elevated in all penumbral regions. Overall, the highest levels of NADH fluorescence came from the evolving infarction and the region of acidic foci. The slow decline in NADH fluorescence despite ongoing ischemia may have been due to acid-promoted destruction of NADH.39

Welsh and colleagues10 initially demonstrated a columnar pattern of NADH fluorescence during cerebral ischemia, possibly due to local vascular anatomy. In those experiments the brain was sliced coronally for fluorescence. It is possible that our cortical topographic pattern of pH and NADH changes in the penumbra were imaging these columns from a perpendicular perspective.

**Regional CBF**

Within the penumbra, regional CBF, like acid-base homeostasis, was heterogeneous, with a reduction in CBF in all regions during ischemia and reperfusion. Other authors have also observed a reduction in CBF in the peri-infarct zone. In the cat model of focal ischemia, the penumbral region was considered to be situated in the marginal gyrus, with reduced blood flow in this region.2 Serial panoramic imaging demonstrates that overall blood flow and metabolic recovery are dependent on the cortical vascular anatomy. However, despite the overall improvement in CBF within the penumbra, the islands of acidosis had decreased blood flow. This pattern of blood flow reduction did not follow a vascular anatomic distribution. It is possible that the severe acidosis in these islands resulted in glial edema, with

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**Fig 4.** A, Histological sample from the animal imaged in Fig 1 showing a hematoxylin and eosin–stained section through the core region of ischemia, which had no umbelliferone penetration. Although this preparation underwent only 3 hours of ischemia, there is obvious infarction with pannecrosis (original magnification ×147). B, Hematoxylin and eosin–stained section through penumbral region 1, which had normalization of intracellular brain pH and cerebral blood flow over 3 hours. Neuronal morphology is normal on light microscopy (original magnification ×235). C, Hematoxylin and eosin–stained section through penumbral region 2, the acidic foci. There is evidence of neuronal injury with a mixed pattern of pyknotic and normal-appearing neurons (original magnification ×235).
extravascular capillary bed compression and subsequent CBF reductions.\textsuperscript{31,40}

**Conclusions**

This experiment confirms that an ischemic penumbra does develop during focal cerebral ischemia, which can be visualized with panoramic imaging of umbelliferone fluorescence. Although the majority of the ischemic penumbra in this model normalizes in terms of brain pH, homeostasis, the development of acidic foci does not occur in a vascular distribution. Despite normalization of the majority of the ischemic penumbra these acidic foci persist, and on light microscopy have evidence of ischemic neuronal injury. These data support the hypothesis that there is a cortical selective vulnerability regarding pH, regulation and that these acidic foci may lead to recruitment of the ischemic penumbra into infarction.

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