Perihematomal Mitochondrial Dysfunction After Intracerebral Hemorrhage

Jeong Sook Kim-Han, PhD; Sarah J. Kopp; Laura L. Dugan, MD; Michael N. Diringer, MD, FAHA

Background and Purpose—Recent measurements in intracerebral hemorrhage (ICH) patients suggest a primary reduction in brain metabolism is responsible for reduced cerebral blood flow and low oxygen extraction surrounding the hematoma. We sought to determine whether reduced mitochondrial respiratory function could account for reduced metabolic demand in ICH patients.

Methods—Brain-tissue samples from 6 patients with acute spontaneous ICH and 6 control patients undergoing brain resection for management of seizure were evaluated. Only tissue removed from the brain adjacent to the hematoma was studied. Specimens were collected in the operating room; mitochondrial studies were begun within 1-hour. Mitochondrial oxygen consumption was measured after the addition of pyruvate, malate, and ADP, followed by oligomycin and carbonylcyanide.

Results—The ICH patients ranged in age from 40 to 54 years; 2 were female and half black. Hemorrhages were located in the temporal lobe (3), cerebellum (2) and parietal lobe (1). The average State 3 (active) O2 consumption for mitochondria from ICH patients was 40% lower than that of control patients (Controls: 129±39 versus ICH: 76±28 nmol O2/min per mg protein). With increasing time from hemorrhage to testing there was a progressive decline in State 3 respiration. Reduced State 3 respiration was evident even at 6 hours, whereas at 72 hours, there was essentially no O2 consumption.

Conclusions—These data support the hypothesis that mitochondrial dysfunction and not ischemia is responsible for reduced oxygen metabolism in ICH. They point to a new direction for investigation and development of therapeutic interventions for ICH patients. (Stroke. 2006;37:2457-2462.)

Key Words: intracerebral hemorrhage ■ mitochondria ■ secondary injury

The pathophysiology of intracerebral hemorrhage (ICH) is incompletely understood. There is general agreement that the acute formation of an intraparenchymal mass lesion (the hematoma itself) produces tissue disruption and displacement.1 A number of secondary pathophysiological processes have been implicated as causes of ongoing injury including ischemia surrounding the hematoma,2–4 the development of cerebral edema,5–7 activation of apoptotic processes8 and toxic effects of components of the hematoma.9,10

The role of ischemia as a cause of secondary injury has recently been investigated with positron-emission tomography. Surrounding the hematoma was a region of reduced cerebral blood flow (CBF), cerebral metabolic rate for oxygen (CMRO2) and oxygen extraction fraction (OEF).11 The OEF finding is of particular importance because it indicates that the state is caused by a primary reduction in brain metabolism rather than a reduction in CBF as would be seen in ischemia.12 When there is a primary reduction in metabolism the fall in CBF is attributable to reduced demand not supply. The OEF is essential in distinguishing these 2 states; in ischemia there is reduction in oxygen delivery and not demand and OEF is high, when demand is reduced, as with mitochondrial dysfunction, then OEF is normal or reduced. Additionally, cerebrovascular autoregulation appears to be intact surrounding the hematoma in acute ICH patients,13 further supporting the argument that ischemia is not a mechanism of secondary injury.

A similar cerebrovascular hemodynamic and metabolic state consisting of low CBF, CMRO2 and OEF is seen in patients with traumatic brain injury (TBI).14,15 Evidence of impaired mitochondrial respiratory function has been reported in brain tissue obtained from acute TBI patients undergoing surgical intervention,16 suggesting a mechanism to explain the cerebrovascular and metabolic state.

In vitro assessment of mitochondrial respiratory function is performed by measuring O2 consumption using a Clark-type oxygen electrode and a closed chamber in which isolated brain mitochondria are introduced. Active (State 3) respiration is initiated by the addition of ADP and metabolic substrates, such as malate and pyruvate, and reflects the
ability of mitochondria to respond to metabolic demand by increasing O₂ consumption to produce ATP. State 4 (resting) respiration occurs when ADP is depleted, thus halting production of ATP and abolishing O₂ consumption by the F₃F₄ATPase; it is typically measured using the F₃F₄ATPase inhibitor, oligomycin. Oxygen consumption during State 4 respiration, which should be negligible in tightly coupled mitochondria, reflects any uncoupling of electron transport chain activity from ATP production, and is associated with inefficient mitochondrial bioenergetics.

We sought to determine whether mitochondrial function was impaired in ICH by assessing mitochondrial respiration in perihematoma tissue removed during evacuation of the acute ICH.

Materials and Methods

Patients

Brain-tissue samples from 6 patients with acute ICH were studied. Samples were obtained under the following circumstances: the decision to perform surgery was made entirely on clinical grounds by an attending neurosurgeon who was not involved in the study; tissue was not removed for the purposes of this investigation but was only available for study if it was resected as part of the surgical procedure; only tissue removed from the brain adjacent to the hematoma was studied.

Control tissue samples were obtained from patients undergoing elective temporal lobe resection for the treatment of intractable epilepsy. The tissue most distant from any lesion was used for analysis. The study was approved by the Washington University Human Studies Committee.

Isolation of Brain Mitochondria

Mitochondria were isolated using a Percoll gradient as described previously with minor modifications. Human brain specimens were collected in the operating room, and immediately placed in ice-cold isolation buffer (IB). The washed tissue was minced, homogenized in IB in an all-glass Dounce homogenizer (Kontes), transferred to a 15-mL conical tube and centrifuged at 1330 g for 3 minutes. The supernatant was transferred and the pellet resuspended. The homogenate was recentrifuged, the supernatants pooled and centrifuged at 21 200 g for 10 minutes. The pellet was resuspended in IB, mixed with 40% Percoll, and layered onto the discontinuous density gradient. Samples were centrifuged at 30 700 g for 5 minutes. Mitochondria were collected from the interface of the lower 2 Percoll layers, diluted in IB, and centrifuged at 16 700 g for 10 minutes. The supernatant was removed and the pellet washed in 0.1% bovine serum albumin/IB, resuspended in 0.3 mL IB, and stored on ice.

Control Studies on Mouse-Brain Mitochondria After Delayed Isolation

To mimic the delay that occurred between collection of human brain tissue and preparation of isolated mitochondria, a set of control experiments on mouse mitochondria prepared immediately, and to results from control studies performed on human mitochondria isolated with this delay. In Figure 1a shows a typical O₂ consumption trace for mouse mitochondria prepared with this delay. In Figure 1b, O₂ consumption per mg protein was calculated for 5 mice. State 3 (active) respiration (133±36 nmol O₂/min per mg protein) after delayed processing was similar to State 3 respiration (132±15 nmol O₂/min per mg protein) for mitochondria prepared immediately, and to results from control mice in a recent study on TBI, suggesting a 1-hour delay in processing does not alter State 3 respiration. On the other hand, State 4 respiration (32±5 nmol O₂/min per mg protein) was 50% higher in delayed-isolation mitochondria compared with values reported for tightly coupled mouse cortical mitochondria (30 nmol O₂/min per mg protein estimated from figure therein). With delayed processing, the respiratory control ratio (RCR; State 3/State 4 respiration) was also 30% lower than without delay (4.2±0.2, n=5, delayed; 6.5±0.2, n=3, immediate), suggesting the delay increased mitochondrial uncoupling, evidenced by increased State 4 respiration and decreased RCR. We therefore standardized our procedure to process all samples 1 hour after tissue harvest to minimize variability introduced by time-to-process.

Measurement of Oxygen Consumption

Oxygen consumption was measured using a Clark-type electrode in a sealed chamber (Oxygraph; Hansatech). 100 to 200 μL of isolated mitochondria were suspended in 1 mL of reaction mixture (10 mmol/L Tris-HCl, pH 7.4, 100 mmol/L KCl, 75 mmol/L mannitol, 25 mmol/L sucrose, 5 mmol/L phosphate, and 0.05 mmol/L K-EDTA), and after addition of 5 nmol/L pyruvate and 2.5 mmol/L malate, ADP (0.25 mmol/L), State 3 respiration was determined. Oligomycin (2.5 μg/mL) was then added to determine State 4 respiration, and maximal respiration was then obtained after addition of 1 μmol/L CCCP. Protein was determined by BCA assay kit (Pierce).

Statistical Analysis

Mitochondrial respiratory parameters were compared using Student t test, with significance at P<0.05.

Results

Patient Characteristics

The ICH patients ranged in age from 40 to 54 years, 2 were female and half were black and half white. Hemorrhages were located in the temporal lobe (3), cerebellum (2) and parietal lobe (1). The median Glasgow Coma Scale score was 6 (range 4 to 14). In 4 patients samples were obtained within 6 hours of symptom onset, 1 patient each at 48 and 72 hours and the time of onset for 1 was unknown (>96 hours). One of the patients died, 1 was discharged to a nursing home and the remaining 4 were discharge to an inpatient rehabilitation program.

Brain tissue from 6 patients who were undergoing temporal lobe resection for intractable epilepsy was used as controls. Patient ages ranged from 20 to 42 years (average 31), 4 were male, 5 were white, and 1 was black. All tissue samples were from the cortical mantle, and not deep white matter tracts.

Control Studies on Mouse-Brain Mitochondria After Delayed Isolation

Because of the time (1 hour) needed to obtain tissue samples, bring them out of the operating room, and transport them to the laboratory, we performed a set of control experiments on mice to determine the effect of this delay on mitochondrial respiration. Figure 1a shows a typical O₂ consumption trace for mouse mitochondria prepared with this delay. In Figure 1b, O₂ consumption per mg protein was calculated for 5 mice. State 3 (active) respiration (133±36 nmol O₂/min per mg protein) after delayed processing was similar to State 3 respiration (132±15 nmol O₂/min per mg protein) for mitochondria prepared immediately, and to results from control mice in a recent study on TBI, suggesting a 1-hour delay in processing does not alter State 3 respiration. On the other hand, State 4 respiration (32±5 nmol O₂/min per mg protein) was 50% higher in delayed-isolation mitochondria compared with values reported for tightly coupled mouse cortical mitochondria (30 nmol O₂/min per mg protein estimated from figure therein). With delayed processing, the respiratory control ratio (RCR; State 3/State 4 respiration) was also 30% lower than without delay (4.2±0.2, n=5, delayed; 6.5±0.2, n=3, immediate), suggesting the delay increased mitochondrial uncoupling, evidenced by increased State 4 respiration and decreased RCR. We therefore standardized our procedure to process all samples 1 hour after tissue harvest to minimize variability introduced by time-to-process.
substantially lower O₂ consumption after ICH. ICH patients had a 40% decrease in State 3 respiration compared with controls (Figure 2b). The average State 3 O₂ consumption for mitochondria from control patients was nearly identical to that of control mice, and was somewhat higher than State 3 respiration reported for the 2 control patients in another study (110±8 nmol O₂/min per mg protein), suggesting good preservation of State 3 respiration throughout the isolation procedure. State 4 respiration in control patients was 35±4 nmol O₂/min per mg protein, similar to our values for State 4 in control mice (Figure 1b), indicating that mitochondria obtained from patients should reflect their in vivo

Figure 1. Control studies on mouse brain mitochondria isolated after a 1-hour delay. O₂ consumption was measured in isolated mouse brain mitochondria prepared as described in Methods. The mitochondrial suspension (1 mL) was introduced into the oxymetry chamber, and substrates (5 mmol/L pyruvate, 2.5 mmol/L malate) and ADP (0.25 mmol/L) were added to initiate State 3 respiration. After 2 minutes, oligomycin (2.5 µg/mL) was added to measure State 4 respiration. After an additional 2 minutes, CCCP (1 µmol/L) was added to determine maximal O₂ consumption by the respiratory chain. Rates were normalized to mitochondrial protein. a, Typical oxygen electrode trace; b, average State 3, State 4 and maximal (CCCP-induced) respiration for isolated mouse brain mitochondria (n=5). The amount of mitochondrial protein loaded was similar between samples, but for statistical analysis, values were normalized to mitochondrial protein.

Figure 2. Respiration in isolated human brain mitochondria. Samples were prepared and assayed as described in the legend for Figure 1. a, Oxygen electrode traces for representative control and ICH patients; b, State 3 and State 4 respiration for control versus ICH patients; c, RCR for control and ICH patients (n=6 per group). Values are mean±SEM; P=0.04 by t test.
function. State 4 respiration in cerebrocortical ICH patients, however, was 117±81 O$_2$/min per mg protein, a 3-fold increase over controls. O$_2$ consumption during State 4 respiration reflect O$_2$ consumption that is not being used by the F$_{1}$F$_{0}$ATPase to generate ATP, and therefore reflects inefficient mitochondrial energy production. Mitochondria isolated from ICH patients also had a lower RCR (Figure 2c), again indicating significant uncoupling after ICH. The only other study we identified which measured respiration in isolated mitochondria from human brain examined patients after TBI and found a >50% loss of State 3 respiration. Interestingly, their data suggested that dysfunction was reversible and might be attributable to calcium overload.

**Time Course of Mitochondrial Metabolic Compromise**

Mitochondria isolated from patients within 2 hours after hemorrhage retained reasonable State 3 and State 4 respiration, but mitochondria isolated after longer periods were extremely impaired. Oxymetry traces for 5 of 6 ICH patients are presented in Figure 3. The ICH patient who had tissue removed >96 hours after the hemorrhage had no mitochondrial respiratory response to substrate/ADP, and was not included for clarity. The tracings indicate that there is a progressive decline in State 3 respiration with time from hemorrhage to surgery both in cortical and cerebellar ICH. In cortex, mitochondria from tissue removed 6 hours after hemorrhage still demonstrated State 3 respiration, albeit with some evidence of uncoupling (ie, O$_2$ consumption before addition of pyruvate/malate/ADP), and little response to ADP. By 72 hours, there was minimal O$_2$ consumption during either State 3 or State 4, suggesting that O$_2$-dependent production of ATP by the F$_{1}$F$_{0}$ATPase was extremely impaired, despite the presence of excess metabolic substrates and O$_2$.

**Discussion**

CBF is reduced in the perihematomal region in experimental models and patients with ICH. This has led to the belief that ischemia is an important contributor to secondary injury after ICH. Recent work, however, has questioned that assumption. Positron-emission tomography studies demonstrating low oxygen extraction have suggested that low CBF is attributable to a primary reduction in cerebral metabolism rather than ischemia. A similar pattern of low CBF, CMRO$_2$ and oxygen extraction has been reported in head injury, which has been linked to mitochondrial dysfunction. Thus, if the ability of mitochondria to metabolize oxygen use is reduced, measured CMRO$_2$ will be low and there will be a secondary fall in CBF with normal or low oxygen extraction.

We sought to determine whether mitochondrial dysfunction could explain the cerebrovascular hemodynamic and metabolic picture seen in ICH patients. Not only did we find that mitochondrial function was impaired after ICH but also that the dysfunction appears to worsen over time.

Mitochondria, the main source of ATP in eukaryotic cells, are regulated by many factors including diet, temperature, and energy demands. The driving force for ATP production is the proton electrochemical gradient, established by the electron transport chain across the inner mitochondrial membrane. Dissipation of the proton electrochemical gradient without concomitant ATP synthesis is termed “uncoupling”.

Our data show reduced State 3 respiration in ICH patients. This indicates the mitochondria are using substantially less O$_2$ despite the presence of high concentrations of substrates. This mirrors the lower OEF observed in ICH patients in the perihematomal region. Increased O$_2$ consumption during State 4 respiration, therefore, reflects uncoupling of electron transport from ATP production, and could be associated with increased production of reactive oxygen species (ROS) by mitochondria, although mild uncoupling can also lead to decreased mitochondrial membrane potential, which can reduce mitochondrial ROS production. This degree of uncoupling might decrease basal mitochondrial ATP production, but would almost certainly impair mitochondrial ATP generation under stress, despite adequate levels of O$_2$ and metabolic substrates.

Uncoupling of mitochondria can be mediated by a number of mechanisms, including activation of mitochondrial uncoupling proteins. Protein-mediated uncoupling activity has been recognized in brown adipose tissues newborns and hibernating mammals, which help maintain the body temperature, and appears to be attributable to a specific mitochondrial protein, uncoupling protein 1. This novel protein generates heat instead of ATP. UCPs are normally expressed in brain, and in central nervous system injury they are postulated to cause mild uncoupling and thereby reduce ROS production by mitochondria.

Furthermore, although mitochondrial respiration is abnormal as early as 2 hours after ICH, there is a progressive decline in function with longer periods after the ICH, with
negligible respiration by mitochondria isolated from tissue removed >72 hours after the hemorrhage. The cause(s) of such a decline could be manifold. One possibility, which has previously been reported in animal models of ICH27 and TBI,28 and human ICH patients,29 is enhanced ROS production by impaired mitochondria which then interacts with metals released from blood to produce oxidative damage. Other potential mediators include inflammatory80,31 or metalloproteinase31 mediated damage.

There is the possibility that an initial ischemic event led to subsequent mitochondrial dysfunction, because our patients were not examined immediately after their hemorrhage. We do not think that the mitochondrial functional defect is attributable to prior ischemia, because in patients with acute cerebral ischemia, areas of increased OEF are found in the majority of patients for many hours (100% within 9 hours, 83% within 12 hours, and 57% within 24 hours) after the ischemic event, yet in ICH patients, we never saw increased OEF, even at these relatively early time points.11 In addition, in most patients with acute ischemic stroke who do not have increased OEF, CBF is elevated, and this was also not observed in ICH patients.11 The progressive deterioration of mitochondrial function over days without progressive infarction is also not consistent with an early transient ischemic event.

One other important question that is raised is whether our controls are likely to have normal mitochondrial function. We only included patients who underwent lobectomy, as opposed to focal resection of a seizure focus, and chose tissue samples that were from as far away from any lesion (>1.5 cm) as possible. A previous study on mitochondrial electron transport chain activity in mitochondria from temporal lobe epilepsy patients32 suggested that there was decreased Complex I activity at the seizure focus, but not in tissue outside the seizure locus. Thus, we feel that mitochondria isolated from tissue remote from the epilepsy focus are likely to be as near to control mitochondria as possible.

These preliminary findings have important potential implications. They add to the growing body of literature indicating that cerebral ischemia is a contributor to secondary injury in ICH patients. This, along with the observation that cerebral autoregulation is intact globally and in the perihematomal region, alleviates one of the important concerns about treating hypertension in ICH patients. In addition, they suggest that neuroprotective strategies focusing on cerebral ischemia are unlikely to be effective in ICH patients. Finally, they point out a new direction for investigation and development of therapeutic interventions for ICH patients.

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**Disclosures**

None.

**References**


