Bioenergetics of Different Brain Areas After Experimental Subarachnoid Hemorrhage in Rats

Fulvio Marzatico, PhD, Paolo Gaetani, MD, Riccardo Rodriguez y Baena, MD, Vittorio Silvani, MD, Pietro Paoletti, MD, and Gianni Benzi, MD

We studied energy metabolism after experimental subarachnoid hemorrhage in rats. Four different cerebral areas were tested: frontal cortex, occipital cortex, hippocampus, and brainstem. \( V_m \) of the following enzymatic activities was evaluated: in the homogenate: hexokinase, phosphofructokinase, and lactate dehydrogenase for the glycolytic pathway, and glucose-6-phosphate dehydrogenase for the hexose monophosphate shunt; in the purified nonsynaptic mitochondria: NAD\(^+\)-isocitrate dehydrogenase, citrate synthase, and succinate dehydrogenase for the Krebs cycle, and cytochrome oxidase for the electron transfer chain. We also evaluated some parameters related to the respiration of nonsynaptic mitochondria (State 3, State 4, uncoupled state, respiratory control ratio, and ADP:O ratio). Subarachnoid hemorrhage did not significantly affect \( V_m \) of the enzymatic activities related to anaerobic and aerobic metabolism; however, mitochondrial respiration was affected, particularly in the presence of NADH-producing substrates (glutamate + malate). (Stroke 1988; 19:378-384)

In humans, subarachnoid hemorrhage (SAH) from ruptured intracranial aneurysms may produce dramatic events, namely severe central nervous system dysfunction\(^1\) and complications such as cerebral vasospasm, rebleeding, and hydrocephalus responsible for extensive neurologic deterioration and/or irreversible neurologic deficits.\(^2\) Attention has recently been focused on the neuronal damage caused by blood diffusion on the brain surface as an important pattern of events occurring during SAH.\(^3\)

SAH may cause a sequential cellular impairment and even irreversible neuronal damage similar to that occurring in ischemia. Many experimental animal models have been proposed to study the pathogenesis, prevention, and therapy of some complications (such as cerebral vasospasm) without paying marked attention to the neuronal impairment and brain metabolic derangement caused by SAH.

Recently, a suitable rat SAH model was used to define the cerebral blood flow alterations\(^7\) and/or cerebral vasospasm pathogenesis.\(^8\) Using the described SAH rat model, our study was aimed at depicting the regional modification of cerebral energy metabolism.

Materials and Methods

Animals and Surgical Procedure

We used male Sprague-Dawley rats (Charles River strain) weighing 325-350 g. Anesthesia was induced with 3% halothane in a 70:30 N\(_2\)O:O\(_2\) mixture and maintained with 0.75% halothane in the same gas mixture. A scalp incision was made in the dorsal midline. A burr hole was placed at the interparietal/occipital suture in the midline using a refrigerated twist-drill. A small PE-10 polyethylene catheter (0.62 mm o.d.) was inserted into the cisterna magna. Suitable placement of the catheter was assessed by testing the cerebrospinal fluid flowing through the catheter and by observing with magnification the lower distal part of the catheter through the atlanto-occipital membrane. The proximal part of the catheter was fixed to the bone with methyl methacrylate (Lang Dental Mfg. Co., Chicago, Illinois). The external part of the catheter was sealed and sutured subcutaneously to the scalp muscle.

Induction of Subarachnoid Hemorrhage

Three days after the implantation of the cisternal catheter, the rats were anesthetized as described. Arterial blood samples were drawn from the femoral cannula every 15 minutes to measure pH, Paco\(_2\), and Paco\(_2\); arterial blood pressure (Table 1) was monitored with a 446420 indirect blood pressure sensor (Bel-Art Products, Pequannock, New Jersey).

From the femoral artery approximately 0.4 ml autologous arterial blood was collected; 0.35 ml was injected into the cisterna magna via the cisternal catheter within approximately 2 minutes. Before SAH induction, 0.01-0.03 ml cerebrospinal fluid was gently drawn.\(^8\) During SAH induction, the rats were held in a 20° head-down position.

Experimental Groups

The following groups of rats were used: control rats without any manipulation, sham-operated rats subjected to surgical procedure but injected with 0.35 ml saline at 37° C, and rats subjected to SAH by injection of 0.35 ml autologous arterial blood. The biochemical evaluations were performed 1 and 72 hours after SAH.

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TABLE 1. Physiological Parameters in Rats Subjected to Subarachnoid Hemorrhage Caused by Cisternal Injection of 0.35 ml Autologous Arterial Blood

<table>
<thead>
<tr>
<th>Time</th>
<th>MABP (mm Hg)</th>
<th>pH</th>
<th>$P_aO_2$ (mm Hg)</th>
<th>$P_aCO_2$ (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>130 ± 4</td>
<td>7.40 ± 0.02</td>
<td>114 ± 7</td>
<td>36.6 ± 2</td>
</tr>
<tr>
<td>SAH</td>
<td>138 ± 5</td>
<td>7.41 ± 0.01</td>
<td>110 ± 8</td>
<td>37.7 ± 1</td>
</tr>
<tr>
<td>30 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>125 ± 4</td>
<td>7.39 ± 0.02</td>
<td>120 ± 10</td>
<td>36.4 ± 1</td>
</tr>
<tr>
<td>SAH</td>
<td>120 ± 6</td>
<td>7.38 ± 0.02</td>
<td>108 ± 7</td>
<td>37.8 ± 1</td>
</tr>
<tr>
<td>60 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>130 ± 6</td>
<td>7.39 ± 0.01</td>
<td>113 ± 6</td>
<td>37.6 ± 2</td>
</tr>
<tr>
<td>SAH</td>
<td>122 ± 8</td>
<td>7.38 ± 0.02</td>
<td>110 ± 5</td>
<td>36.8 ± 1</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. MABP, mean arterial blood pressure; SAH, subarachnoid hemorrhage.

Mitochondrial Isolation and Biochemical Evaluations

One or 72 hours after SAH, the rats were decapitated. The brain was quickly removed (in approximately 15 seconds) from the skull and immediately immersed in a cold isolation medium of 0.32 M sucrose plus 1 mM EDTA at pH 7.4. The brain areas (frontal cortex, occipital cortex, hippocampus, and brainstem) were dissected in a precooled (−5°C) box in approximately 1–1.30 minutes. All subsequent manipulations were carried out between 0°C and 4°C. An aliquot of the homogenate was used to measure $V_{\text{max}}$ of the following enzyme activities: for the glycolytic pathway, hexokinase (EC 2.7.1.1), phosphofructokinase (EC 2.7.1.11), and lactate dehydrogenase (EC 1.1.1.27); for the hexose monophosphate shunt, glucose-6-phosphate dehydrogenase (EC 1.1.1.49). The remaining homogenate was submitted to subcellular fractionation to obtain the nonsynaptic mitochondrial fraction using a Ficoll density gradient. $V_{\text{max}}$ of the following enzymatic activities was evaluated in the nonsynaptic mitochondrial fraction: for the Krebs cycle, NAD⁺-isocitrate dehydrogenase, citrate synthase, and succinate dehydrogenase, for the electron transfer chain, cytochrome oxidase. It should be stressed that in vitro, with excess substrate and under conditions abolishing metabolic autoregulation, $V_{\text{max}}$ does not define exactly the actual enzymatic activity in situ. $V_{\text{max}}$ really depicts enzymatic or metabolic pathway potential activity rather than an absolute measure of any cerebral metabolic flux. Nevertheless, the potential rate of a metabolic flux can be measured by $V_{\text{max}}$ of the regulatory enzymes that catalyze irreversible reactions or reactions far from equilibrium. Detailed discussions on the theoretical and experimental bases of this approach have been published. The reaction rate at its maximum depends on the enzyme concentration in the sample. Anoxic-ischemic episodes markedly influence the synthesis of cerebral proteins and could affect differently the proteins with different turnover rates and can produce selective gene expression, with synthesis of new (stress) proteins related to various reductions of cerebral blood flow.

Mitochondrial respiratory parameters (State 3 [oxygen consumption in presence of exogenous ADP], State 4 [oxygen consumption in absence of exogenous ADP], State 3U [uncoupled state], respiratory control ratio, and ADP:O ratio [moles ADP phosphorylated per atom oxygen consumed]) were evaluated with glutamate + malate or succinate + rotenone as oxidizable substrates using an isotonic medium in the presence of 5 mM K⁺ at 23°C. It is well known that State 3 reflects the oxygen uptake capacity of the mitochondria and is well correlated with the functional state of the electron transfer chain, depending on oxidizable substrates. State 4 reflects the dissipation rate of the mitochondrial electrochemical gradient and indirectly gives information about selective properties of the mitochondrial membrane.

Protein content was evaluated in the homogenate in toto and in the nonsynaptic mitochondrial fraction. Statistical significance was assessed with analysis of variance.

Figure 1. Variation of State 3 (•) and State 4 (□) respiration in brain areas of rats after 1 and 72 hours of subarachnoid hemorrhage compared with sham-operated rats.
hippocampus, or brainstem of control rats compared with sham-operated rats. Nonsynaptic mitochondria obtained from the frontal cortex of sham-operated rats showed a significant decrease in the respiratory control ratio (Table 3).

Mitochondrial respiration was, however, affected by SAH (Table 4, Figure 1). Mainly, in the presence of glutamate + malate, the respiratory control ratio decreased compared with sham-operated rats in all brain areas tested. This decrease in the frontal and occipital cortices 1 hour after SAH may be related to a decrease of State 3 respiration while State 4 respiration was unaffected; 72 hours after SAH, State 3 reached the value of sham-operated rats, while State 4 respiration increased. In mitochondria from the brainstem, State 4 increased 1 hour after SAH without any change in State 3 respiration. The increase in State 4 respiration remained significantly higher 72 hours after SAH (Table 5, Figure 1).

In the hippocampus, the respiratory control ratio with glutamate + malate as substrates decreased 1 hour after SAH, reaching significance 72 hours after SAH.

### TABLE 3. Mitochondrial Respiratory Activity in Nonsynaptic Mitochondria Isolated From Brain Areas of Rats Subjected to Subarachnoid Hemorrhage Caused by Cisternal Injection of 0.35 ml Autologous Arterial Blood

<table>
<thead>
<tr>
<th>Brain area</th>
<th>Glutamate + malate</th>
<th>Succinate + rotenone</th>
<th>Glutamate + malate</th>
<th>Succinate + rotenone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>State 3</td>
<td>State 4</td>
<td>RCR</td>
<td>State 3</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>Control</td>
<td>92.4±11.7</td>
<td>19.1±2.7</td>
<td>4.9±0.2*</td>
</tr>
<tr>
<td></td>
<td>Sham-operated</td>
<td>90.6±12.3</td>
<td>20.8±2.5</td>
<td>4.33±0.21</td>
</tr>
<tr>
<td>Occipital cortex</td>
<td>Control</td>
<td>74.1±9.8</td>
<td>20.5±3.0</td>
<td>3.7±0.2</td>
</tr>
<tr>
<td></td>
<td>Sham-operated</td>
<td>80.1±7.1</td>
<td>21.5±2.5</td>
<td>3.71±0.15</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>Control</td>
<td>72.5±13.8</td>
<td>21.1±5.1</td>
<td>3.8±0.3</td>
</tr>
<tr>
<td></td>
<td>Sham-operated</td>
<td>82.0±6.9</td>
<td>26.6±2.3</td>
<td>3.25±0.47</td>
</tr>
<tr>
<td>Brainstem</td>
<td>Control</td>
<td>84.4±19.6</td>
<td>26.5±5.8</td>
<td>3.7±0.4</td>
</tr>
<tr>
<td></td>
<td>Sham-operated</td>
<td>82.0±3.5</td>
<td>24.0±2.9</td>
<td>3.42±0.36</td>
</tr>
</tbody>
</table>

Values are mean ± SEM nat.O2/min/mg protein in groups of 5–7 rats for State 3 and State 4. RCR, respiratory control ratio (rates of oxygen consumption in States 3 and 4).

*Significantly different from sham-operated rats.
The respiratory control ratio with succinate + rotenone as substrates in all brain areas was unchanged after SAH (Table 4).

SAH also affected the ADP:O ratio in the frontal and occipital cortices in the presence of NADH- and FADH$_2$-producing substrates (Table 4).

**Discussion**

In humans, SAH from ruptured intracranial aneurysms is characterized by different pathogenetic aspects, leading to derangement of central nervous system integrity. SAH affects the intracranial pressure responsible for diffuse hypoperfusion. In 20-30% of patients, local or diffuse prolonged spasm leads to clinical symptoms ranging from mild functional and metabolic disturbances of diencephalic structures to massive cerebral ischemic damage. Morever, the direct neuronal effect of blood has been proposed as the trigger of cellular membrane dysfunction. This event probably supports the neuronal metabolic alteration and the long-term cognitive impairment of patients.

As far as SAH-induced alteration is concerned, several experiments on rats were designed to define cerebral blood flow modifications, arterial diameter variations, morphologic and structural changes of the arterial wall, and the role of the adrenergic, dopaminergic, and serotonergic pathways in the pathogenesis of vasospasm. However, few data have been available concerning the modification of cerebral energy metabolism by SAH in any case without paying attention to the alterations of enzyme activities related to energy metabolism and/or to modification of mitochondrial respiration.

In our research, the enzymatic activities of different rat brain areas are unaffected by SAH. On the contrary, other experimental conditions (e.g., complete ischemia) markedly change the same enzymatic activities related to energy metabolism.

In nonsynaptic mitochondria, experimental SAH induces extensive impairment of respiration, which plays a crucial role in energy metabolism regulation during anoxic-ischemic conditions. In nonsynaptic mitochondria isolated after complete or incomplete ischemia, State 3 respiration is reduced with less impairment of State 4 respiration. We observed different alterations caused by SAH in nonsynaptic mitochondria isolated from different brain areas. Thus, in the frontal and occipital cortices and in the hippocampus 1 hour after SAH, State 3 respiration decreases probably because of the approximately 40% decrease in cerebral blood flow, suggesting general incomplete ischemia. In the frontal and occipital cortices, in the hippocampus 72 hours after SAH, and in the brainstem 1 and 72 hours after SAH, an increase in State 4 respiration occurs, suggesting marked impairment of the proton electrochemical gradient, probably related to a dielectric breakdown of mitochondrial membranes.

### TABLE 5. Mitochondrial Respiratory Activity in Nonsynaptic Mitochondria Isolated From Brain Areas of Rats Subjected to Subarachnoid Hemorrhage Caused by Cisternal Injection of 0.35 ml Autologous Arterial Blood

<table>
<thead>
<tr>
<th>Brain area</th>
<th>Glutamate + malate</th>
<th>Succinate + rotenone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>State 3</td>
<td>State 4</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>92.4 ± 11.7</td>
<td>19.1 ± 2.7</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>90.6 ± 12.3</td>
<td>20.8 ± 2.5</td>
</tr>
<tr>
<td>1-hour SAH</td>
<td>67.4 ± 5.7</td>
<td>22.2 ± 2.1</td>
</tr>
<tr>
<td>72-hours SAH</td>
<td>82.2 ± 7.9</td>
<td>27.3 ± 2.9</td>
</tr>
<tr>
<td>Occipital cortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>74.1 ± 9.8</td>
<td>20.5 ± 3.0</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>80.1 ± 7.1</td>
<td>21.5 ± 2.5</td>
</tr>
<tr>
<td>1-hour SAH</td>
<td>70.5 ± 8.1</td>
<td>22.7 ± 3.3</td>
</tr>
<tr>
<td>72-hours SAH</td>
<td>85.9 ± 12.7</td>
<td>35.8 ± 7.6</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>72.5 ± 13.8</td>
<td>21.1 ± 5.1</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>82.0 ± 6.9</td>
<td>26.6 ± 2.3</td>
</tr>
<tr>
<td>1-hour SAH</td>
<td>65.4 ± 5.2</td>
<td>26.6 ± 3.2</td>
</tr>
<tr>
<td>72-hours SAH</td>
<td>77.4 ± 10.2</td>
<td>34.1 ± 4.6</td>
</tr>
<tr>
<td>Brainstem</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>84.4 ± 19.6</td>
<td>26.5 ± 5.8</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>82.0 ± 3.5</td>
<td>24.0 ± 2.9</td>
</tr>
<tr>
<td>1-hour SAH</td>
<td>86.9 ± 9.6</td>
<td>37.4 ± 5.7*</td>
</tr>
<tr>
<td>72-hours SAH</td>
<td>108.3 ± 10.9</td>
<td>38.3 ± 3.6*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM nat.O$_2$/min/mg protein in groups of 5-7 rats for State 3 and State 4. SAH, subarachnoid hemorrhage.

*Significantly different from sham-operated rats.
### Results

Table 2 shows how SAH affected $V_{\text{max}}$ of enzymatic activities related to anaerobic and aerobic metabolism in different brain areas. Phosphofructokinase activity was significantly reduced in the frontal cortex 1 and 72 hours after SAH and in the occipital cortex 1 hour after SAH. There were no differences in State 3 respiration, State 4 respiration, or respiratory control ratio of the nonsynaptic mitochondria from the occipital cortex,
Our results suggest that SAH impairs mitochondrial respiration, probably by two different mechanisms. During the acute stage of SAH there is inhibition of the NADH-oxidizing branch of the electron transfer chain since State 3 respiration decreases with glutamate + malate as substrates but does not change with succinate + rotenone as substrates. This modification is probably localized in the NADH-ubiquinone reductase complex. In the late phase of SAH, the increase in State 4 respiration with glutamate + malate as substrates suggests an impairment of mitochondrial membrane function, perhaps the result of cascade reactions started by Ca2+ influx caused by the hemorrhagic insult.52

In this respect, the theory of SAH neuronal disruption by calcium52 involves movement of Ca2+ from the extracellular to the cytoplasmic compartment, resulting in a sequence leading to cell membrane dysfunction caused by different biological compounds (e.g., free oxygen radicals, arachidonic acid, prostaglandins, and leukotrienes).53,54 This fact suggests the use of drugs such as calcium channel blockers capable of ameliorating the long-term evolution of SAH patients without significantly reducing the incidence of vasospasm.

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KEY WORDS • enzymes • mitochondria • rats • subarachnoid hemorrhage
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