Energy Metabolism During Brain Ischemia

STABILITY DURING REVERSIBLE AND IRREVERSIBLE DAMAGE

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Abstract:

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The permissible duration of brain ischemia without sustaining damage is short. Less clear are the mechanisms accounting for the vulnerability of brain to ischemic insults. Neurochemical factors implicated include impairment of energy synthesis by mitochondria and of energy-dependent processes such as synaptic transmission, ATPase activity, membrane conductance and altered protein and lipid synthesis. To clarify the vulnerability of energy metabolism, we investigated energy availability and synthesis in our model of global cerebral ischemia. Our studies evaluated in vitro mitochondrial ATP synthesis and the in vivo quantitation of the cortical adenylate pool. Results of our investigations support a growing body of evidence showing the energy state to be relatively stable to ischemia. We conclude that an energy-dependent process of brain is primarily vulnerable to ischemia.

Additional Key Words

oxidative phosphorylation

ATPase activity

energy charge

global ischemia

anoxia-ischemia

ATP

ADP

Neurochemical factors accounting for ischemic vulnerability of the brain are incompletely understood. Despite the high, obligate energy demands of the brain and the previously demonstrated susceptibility of brain mitochondria in vitro to ischemia, recent studies with in vivo brain ischemia disclose a relatively stable energy metabolism. Our investigations confirm and extend these observations by correlating simultaneous in vitro and in vivo parameters of energy metabolism with functional recovery. In our model of global ischemia, ischemia was created by combining systemic hypotension with hypoxia of varying periods followed by circulatory restoration. Energy metabolism was evaluated with in vitro mitochondrial ATP synthesis and the in vivo cortical adenylate pool.

Methods

BRAIN ISCHEMIA AND RECOVERY

Random-bred New Zealand white rabbits weighing between 4 and 5 kg were used. The rabbits were given a rapid-acting anesthesia, Ketamine (cyclohexylamine), intubated, and paralyzed with succinylcholine. Ventilation was performed mechanically with a Harvard pump. Two dural electrodes were implanted to record the electroencephalogram. The femoral artery was catheterized for constant blood pressure recording and the collection of blood for gases and pH measurements. A detailed description of procedures and the dosage of various pharmacological agents used have been reported. Hypotension was induced with Arfonad (trimethaphan). When the mean arterial blood pressure stabilized at 20 to 25 mm Hg, 4% oxygen with 96% nitrogen was introduced through the Harvard pump. The onset of an isoelectric EEG after the induction of hypotension was an average 4.5 ± 0.7 minutes. The severity of ischemia was equated to the duration of the isoelectric EEG before blood pressure was restored and 100% oxygen was introduced.

PREPARATION OF TISSUE EXTRACTS

Serial, three and occasionally four randomly selected cortical brain biopsies weighing 50 to 100 mg were obtained through a craniectomy with a rongeur, and quickly immersed into liquid nitrogen (-195°C). The rongeur was not kept in liquid nitrogen prior to biopsy since otherwise excessive brain tissue became adherent. The time interval from biopsy to immersion into liquid nitrogen was approximately two to three seconds. This delay in tissue freezing may account for our slightly reduced levels of ATP. Metabolites were extracted from the brain using a modified procedure of Lowry et al. The samples were powdered in a mortar and chilled in liquid nitrogen. The brain powder in liquid nitrogen was placed on top of 0.3 ml of 3 M HClO4 in a 12-ml centrifuge tube, weighed before and after the addition of the powder and kept in a Dry Ice-acetone bath.

The tubes were agitated in an alcohol-H2O (1:3)-Dry Ice bath (~15°) until the acid was liquefied and had penetrated the powder (about five minutes). To each sample was added 1.2 ml of 1 mM EDTA (K+). After repeated mixing at 4° for ten minutes, the tubes were centrifuged at 27,000 X g for ten minutes. The supernatant fluid was removed and placed in a 12-ml centrifuge tube. The precipitate was resuspended in 0.1 ml of 3 M HClO4, and mixed at 4° for five minutes. This was followed by the addition of 0.4 ml of 1 mM EDTA (K+) mixed for five minutes, and centrifuged at 27,000 X g for ten minutes. The supernatant fluid was removed and combined with the previous extraction and the solution was neutralized with 0.9 ml of cold 2 M KHC03. After CO2 bubbling had stopped (about ten minutes), the solution was centrifuged at 27,000 X g for ten minutes. The supernatant...
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The tissue was homogenized in a glass homogenizer (0.2 mm needles) and was determined by the method of Lowry et al. 10 and was usually 8 to 10% more than the control. The enzymes were obtained from the Boehringer Mannheim Corporation and approximately one unit of each enzyme was used for each assay.

PREPARATION OF BRAIN MITOCHONDRIA

After varying periods of ischemia, 4 to 6 g of brain tissue were taken through a cranectomy. The tissue was quickly immersed in and washed with cold SMH solution (0.07 M NaCl, 0.03 M HEPES buffer, pH 7.4, 2 mM L-glutamate, 0.002 M ADP, 0.002 M 32Pi, 4 X 10^6 cpm per milliliter, and the mitochondrial preparation equivalent to 1 mg protein per milliliter. The reaction was carried out in a 22 X 100 mm glass tube usually containing 3 to 5 ml of basic reaction medium. After initial incubation for five minutes at 30°C in a shaker bath for temperature equilibration, mitochondria were added to the medium to start the reaction. At every time interval, 0.5 ml aliquot was drawn from the reaction mixture and quickly delivered into a test tube containing 0.5 ml 20% trichloroacetic acid. One milliliter of acetone was then added and the tube was stirred on a vortex mixer for 30 seconds. After ten minutes, 1 ml of isobutanol-benzene (1:1)-saturated water and 7 ml of water-saturated isobutanol-benzene were added. The tube was vigorously shaken to extract acetone from the lower aqueous phase into the upper organic phase. Ammonium molybdate (5% in 2 M H2SO4), 0.25 ml, was slowly added along the test tube wall into the lower aqueous phase. The tube was gently swirled to mix ammonium molybdate with the aqueous phase. After ten minutes the tube was vigorously shaken to extract the ammonium phosphomolybdate complex into the organic phase which was subsequently removed by suction into a trapping flask. One drop of 80 mM K2HPO4 was added as carrier to remove excess molybdate reagent. The tube was shaken by hand, followed by the addition of 7 ml of water-saturated isobutanol-benzene. After five minutes the tube was again vigorously shaken, and the organic phase was removed as before. Two more extractions with organic solvents were sufficient to remove essentially all unreacted phosphate. Aliquot of 0.5 ml from the remaining aqueous phase was added to 5 ml of scintillation fluid (Aquasol, New England Nuclear) and was counted in a liquid scintillation counter. The amount of ATP formed was expressed as micro moles per milligram of protein of mitochondria. The authenticity of ATP synthesized in this assay system was substantiated by the enzymatic determination described in the section on Analytical Methods.

Results

CORTICAL GLUCOSE CONCENTRATIONS DURING ISCHEMIA AND RECOVERY

Following systemic hypotension and hypoxia and five minutes of an isoelectric EEG, cortical glucose levels decreased from a control value of 2.40 μmoles per milligram wet brain to a plateau of approximately 0.9 μmole per milligram wet weight. During the recovery phase, cortical glucose contents rose to control levels in five minutes and reached almost twice the control concentrations in 14 minutes. Serial brain biopsies over a 12-minute period in control rabbits without hypotension or hypoxia showed no change in the cortical level of glucose. These results indicate that the biopsy procedure itself does not alter glucose levels.

IN VITRO BRAIN MITOCHONDRIAL ATP SYNTHESIZING ACTIVITY FOLLOWING ISCHEMIA

Results of ATP synthesizing ability from control and five-minute ischemic rabbits are shown in figure 1. Brain mitochondria following five-minute ischemia synthesized 43% and 100% more ATP than control mitochondria following 20 and 40 minutes' incubation, respectively.

ANALYTICAL METHODS

Metabolites in the rabbit brain extracts were analyzed according to the procedure of Lowry et al. 11 with slight modification. The Eppendorf Photometer 1100 M with Fluorescence Attachment 1030 was used to measure the appearance of NADPH or the disappearance of NADH upon addition of appropriate enzymes. The assay mixture in the 1-ml cuvette was read twice: initially after the sample had been added, and a second time after adding the required enzyme or enzymes and incubating for the predetermined time interval for the completion of the reaction. The standards were analyzed simultaneously and performed in duplicates. A solution without added brain powder was used as a blank. The analytical conditions for glucose, ATP, ADP and AMP determinations were basically those described by Lowry et al. 12 Glucose and ATP were assayed with the coupling of hexokinase and G6P dehydrogenase, ADP by the pyruvate kinase-lactate dehydrogenase system and adding adenylate kinase to the above for AMP assays. Only the amount and source of enzymes used differed from those of Lowry et al. 12 The enzymes were obtained from the Boehringer Mannheim Corporation and approximately one unit of each enzyme was used for each assay.

MEASUREMENT OF MITOCHONDRIAL ATP SYNTHESIS

The assay was based on the incorporation of 32Pi into ADP and was determined according to a modification of the methods of Ernster et al. 13 and Nielsen and Lehninger. 14 The basic reaction medium contained the following: 0.15 M NaCl, 0.03 M HEPES buffer, pH 7.4, 2 mM L-glutamate, 0.002 M ADP, 0.002 M 32Pi, 4 X 10^6 cpm per milliliter, and the mitochondrial preparation equivalent to 1 mg protein per milliliter. The reaction was carried out in a 22 X 100 mm glass tube usually containing 3 to 5 ml of basic reaction medium. After initial incubation for five minutes at 30°C in a shaker bath for temperature equilibration, mitochondria were added to the medium to start the reaction. At every time interval, 0.5 ml aliquot was drawn from the reaction mixture and quickly delivered into a test tube containing 0.5 ml 20% trichloroacetic acid. One milliliter of acetone was then added and the tube was stirred on a vortex mixer for 30 seconds. After ten minutes, 1 ml of isobutanol-benzene (1:1)-saturated water and 7 ml of water-saturated isobutanol-benzene were added. The tube was vigorously shaken to extract acetone from the lower aqueous phase into the upper organic phase. Ammonium molybdate (5% in 2 M H2SO4), 0.25 ml, was slowly added along the test tube wall into the lower aqueous phase. The tube was gently swirled to mix ammonium molybdate with the aqueous phase. After ten minutes the tube was vigorously shaken to extract the ammonium phosphomolybdate complex into the organic phase which was subsequently removed by suction into a trapping flask. One drop of 80 mM K2HPO4 was added as carrier to remove excess molybdate reagent. The tube was shaken by hand, followed by the addition of 7 ml of water-saturated isobutanol-benzene. After five minutes the tube was again vigorously shaken, and the organic phase was removed as before. Two more extractions with organic solvents were sufficient to remove essentially all unreacted phosphate. Aliquot of 0.5 ml from the remaining aqueous phase was added to 5 ml of scintillation fluid (Aquasol, New England Nuclear) and was counted in a liquid scintillation counter. The amount of ATP formed was expressed as micro moles per milligram of protein of mitochondria. The authenticity of ATP synthesized in this assay system was substantiated by the enzymatic determination described in the section on Analytical Methods.

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Mitochondrial synthesis of ATP from control and five-minute ischemic brains. These results are representative of six other similar experiments.

EFFECT OF BRAIN ISCHEMIA AND RECOVERY ON IN VIVO ADENYLATE CONCENTRATIONS

Results of adenylate measurements are summarized in figures 2 and 3. Each time period of ischemia (fig. 2) or recovery (fig. 3) represents the sum of three rabbits and a maximum of four serial cortical biopsies from each animal. Each line represents a best-fit curve for the set of observations and the range of values indicated. Zero time commences with the isoelectric onset of the EEG and the length of ischemia is equated to its duration. Moderate or three-minute ischemia (fig. 2) is associated with a decline in the ATP concentration. In severe or five-minute ischemia (fig. 2) ATP decreases further and levels off at about 0.8 μmole per milligram wet weight. A mild rise in ADP and AMP occurred under both conditions as indicated by the bottom pair of lines. As with cortical glucose measurements, serial brain biopsies over a 12-minute period in control rabbits showed no change in the cortical levels of ATP, ADP and AMP, thus indicating that the biopsy procedure itself did not affect the cortical adenylate concentrations.

Figure 3 shows the cortical adenylate concentrations during circulatory restoration following ischemia. After sustaining either three or five minutes of an isoelectric EEG, the blood pressure was restored and 100% oxygen was introduced. With three minutes of ischemia, the ATP levels returned to control levels in about ten minutes, whereas after five minutes of ischemia the rise was slower, reaching approximately 80% of the control levels after 14 minutes.

In our model, reduction of cerebral glucose may reflect decreased glucose delivery from impaired blood flow. The possible role of accelerated glycolysis, however, cannot be excluded. For example, Drewes et al.\(^3\) have shown a 50% reduction in cortical glucose in the perfused dog-head preparation following two minutes of anoxia (97% N\(_2\) and 3% CO\(_2\)). Despite enhanced glucose transport, reduced cerebral glucose levels resulted from a fivefold increased rate of glycolysis due to facilitation of hexokinase, phosphofructokinase, fructokinase, and glycogen phosphorylase activities. In our model of global ischemia, however, the degree of hypoxemia (PaO\(_2\) of 19 to 20 mm Hg) was similar to Salford et al.\(^13\). In their investigations, if the PaO\(_2\) were maintained by 21 mm Hg, cerebral glucose was not reduced.

Discussion

The permissible duration of brain ischemia without sustaining irreversible damage is disputed, but is recognized to be short by Plum,\(^14\) Brierley et al.,\(^15\) and
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Hossmann and Kleihues. Less clear are the mechanisms accounting for brain vulnerability. Primary factors implicated are: (1) microcirculatory alterations, (2) edema, of both brain and vessels, and (3) neurochemical impairment. The last includes ATP synthesis by mitochondria and energy-dependent processes, such as synaptic transmission, ATPase activity, membrane conductance, and altered lipid and protein synthesis.

More recent studies have explored alterations in energy metabolism during brain ischemia prompted by the high, obligate energy requirements of the brain. In vitro mitochondrial studies of Ozawa et al. showed brain mitochondria to be more vulnerable to anoxic insults than mitochondria from other organs. Since other brain functions were not studied in parallel, Ozawa's findings do not clarify the molecular basis of in vivo vulnerability. More recent studies have explored energy metabolism utilizing various models of in vivo cerebral ischemia, and they indicate that energy synthesis remains relatively unimpaired despite severe brain ischemia.

Our present investigations were designed to extend these observations by specifically correlating changes in energy metabolism with functional impairment following cerebral ischemia. Our model of global cerebral ischemia provides such an opportunity.

Energy metabolism was investigated by both mitochondrial ATP synthesis and the cortical adenylate pool. ATP synthesis by isolated mitochondria following varying periods of in vivo brain ischemia gave a direct measure of oxidative phosphorylation, while analysis of the cortical adenylate pool gave an index of the in vivo energy state. As indicated in figure 1, the rate and quantity of ATP synthesis by mitochondria taken from five-minute ischemic rabbits were higher than control. These findings show that mitochondria were intact and may be more tightly coupled following moderately severe ischemic conditions. These results suggest that mitochondria are not primarily vulnerable to ischemia. Our findings and conclusions are in agreement with the studies of Schutz and his colleagues. They studied mitochondria obtained from cats subjected to cerebral exsanguination produced by raising the cerebrospinal fluid pressure and found that after ten minutes of severe ischemia, mitochondria displayed tight coupling, demonstrated by a respiratory control ratio of 12.9. Although functional brain recoveries were not correlated systematically by these authors, functional impairment is anticipated from similar experiments on rats.

Brierley et al. found vacuolation in brain mitochondria as the earliest morphological change following ischemia or anoxia. Our findings of preserved mitochondrial ATP synthesis in the presence of ischemic brain damage suggest two interpretations of Brierley's observations: first, mitochondrial alterations are reversible, or second, and more likely, functional impairment occurs prior to identifiable morphological alterations.

Our studies on cortical adenylate pool were designed to complement in vitro mitochondrial assays. As indicated in figures 2 and 3, ATP and total adenylate concentrations decrease with the duration of ischemia. The relative maintenance of ATP levels is noteworthy despite the onset of an isoelectric EEG. This finding suggests the operation of mechanisms which operate to reduce or modulate neuronal activity during ischemia in order to preserve the energy state. Similar observations were witnessed with anoxia and hypoglycemia. The decline of ATP and its recovery are similar to those reported by Drewes and Gilboe in their anoxic, isolated dog-head perfusion model. In their experiments, the effect on brain metabolism of six minutes, anoxia (97% N2 and 3% CO2 with PaO2 10 mm Hg) was studied. The energy charge fell from 0.98 to 0.56 and returned to normal in about five minutes. Our results show a decline in total adenylates in our model of ischemia, perhaps reflecting a greater additional insult from hypotension.

Atkinson has suggested that the energy charges of the adenylate pool rather than their absolute concentrations function as a metabolic regulator. It was of interest, therefore, to determine the energy charge changes during and after cerebral ischemia. The energy charge of the adenylate system (ATP + 0.5 ADP)/(ATP + ADP + AMP) was calculated from the data in figures 2 and 3, and the results are plotted in figure 4. With three-minute ischemia, the energy charge decreased from 0.90 to 0.79 and declined further to 0.68 in five-minute ischemia. The similarity between the recovery slopes of both three and five minutes of ischemia is noteworthy. Marked global ischemia of five minutes' duration, compatible with functional cerebral damage, shows only a modest decrease in energy charge.

Energy charge of the adenylate pool during and following three and five minutes of global cerebral ischemia and circulatory restoration plus 100% oxygen.

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of energy charge in the face of declining total adenylates is accomplished by a relative depletion of AMP. This may occur by one of two mechanisms: activation of adenylate deaminase or AMP-nucleotidase. In addition to these mechanisms, preservation of adenylates may be accomplished by suppression of ATP utilization. Siejö and Ljunggren investigated the energy charge after cerebral ischemia was produced by raising intracranial pressure of 1 to 15 minutes' duration and found the energy charge declined from a control of 0.95 to values less than 0.4. With restitution of circulation after 7.5 minutes' ischemia, for example, the energy charge rose to more than 0.94 within five minutes. Despite this apparent recovery, the authors are cautious in its interpretation, believing it unwarranted to conclude that energy metabolism is reconstituted after ischemia of more than five minutes.

We believe our combined in vitro and in vivo studies on brain energy metabolism during moderate and severe ischemic insults offer a reasonable basis to conclude that energy metabolism is not primarily vulnerable to ischemic insults. Preserved in vitro mitochondrial function following five minutes of ischemia shows that the machinery for energy synthesis remains intact. The relative maintenance and restitution of cortical energy-charge during five minutes of ischemia and following circulatory restoration further support the stability in vivo of energy metabolism. In addition, a mechanism appears to operate in maintaining the energy state during substrate deprivation. A critical depression of the energy state, however, may be reached that is sufficient to initiate irreversible damage.

The permissible duration of ischemia compatible with recovery depends upon the severity of ischemia and the specific brain function assessed. In our experimental model, severe cerebral ischemia was produced by combining hypotension with hypoxia in awake animals and was correlated with functional recovery. Our results support the contention that the permissible duration of brain ischemia is relatively short. We interpret our studies on energy metabolism to support its relative stability to ischemic insults. On the basis of these conclusions, we suggest that energy-dependent neuronal processes account for the exquisite vulnerability of the brain to ischemia.

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