An Isolation Method for Assessment of Brain Mitochondria Function in Neonatal Mice with Hypoxic-Ischemic Brain Injury

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Abstract
This work was undertaken to develop a method for the isolation of mitochondria from a single cerebral hemisphere in neonatal mice. Mitochondria from the normal mouse brain hemisphere isolated by the proposed method exhibited a good respiratory control ratio of 6.39 ± 0.53 during glutamate-malate-induced phosphorylating respiration. Electron microscopy showed intact mitochondria. The applicability of this method was tested on mitochondria isolated from naive mice and their littersmates subjected to hypoxic-ischemic insult. Hypoxic-ischemic insult prior to reperfusion resulted in a significant (p < 0.01) inhibition of phosphorylating respiration compared to naive littersmates. This was associated with a profound depletion of the ATP content in the ischemic hemisphere. The expression for Mn superoxide dismutase and cytochrome C (markers for the integrity of the mitochondrial matrix and outer membrane) was determined by Western blot to control for mitochondrial integrity and quantity in the compared samples. Thus, we have developed a method for the isolation of the cerebral mitochondria from a single hemisphere adapted to neonatal mice. This method may serve as a valuable tool to study mitochondrial function in a mouse model of immature brain injury. In addition, the suggested method enables us to examine the mitochondrial functional phenotype in immature mice with a targeted genetic alteration.

Introduction

Immature rodents are commonly used to model brain damage caused by birth asphyxia in human neonates. Several recent studies have demonstrated significant changes in the mitochondrial respiration rates measured in the brain tissue homogenate or ‘crude mitochondrial fractions’ following hypoxia-ischemia (HI) insult in neonatal rats [1, 2]. These reports were first to highlight mitochondrial respiratory dysfunction in a rodent model of HI. However, further dissection of postischemic mitochondrial biochemistry to specify the mechanism of HI-induced respiration deficiency requires a precise control over experimental conditions. This can be achieved only by the use of isolated, relatively pure brain mitochondria. In addition, the availability of genetically modified mice makes the mouse an attractive species to model HI insult. Neonatal mice have been used to model HI encephalopathy [3–5]. However, the small brain size in immature mice is a considerable obstacle to obtaining a sufficient...
amount of mitochondria for ex vivo functional studies. As mitochondria undergo substantial developmental changes in the newborn period [6, 7], studies on isolated postischemic mitochondria may highlight developmental aspects of mitochondrial mechanisms in asphyxia-induced brain damage.

This study was focused on developing a method for the isolation of brain mitochondria from a single hemisphere in neonatal mice and validating this method in a neonatal mouse model of HI insult. This method employs an inexpensive table-top centrifuge and yields brain mitochondria from a single hemisphere in an amount sufficient to perform a spectrum of functional studies.

**Material and Methods**

**Animal Protocol**

All procedures were approved by the Columbia University Animal Care and Use Committee. Three-day-old (p3) mice (C57BL/6) were purchased from Jackson Lab (Bar Harbor, Me., USA) with their dams. On postnatal day 10 the mice were sacrificed by decapitation and the hemispheres were excised and immediately placed into ice-cold isolation buffer (IB) composed of 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 5 mM HEPES-KOH (pH 7.2) and 1 mg/ml of essentially fatty-acid-free bovine serum albumin (BSA). Randomly chosen littermates were subjected to HI insult as described [4] by permanent ligation of the right carotid artery followed (in 60 min) by exposure to 8% O2/92% N2 for 15 min at 37°C. Immediately after HI insult the mice were sacrificed and the hemispheres ipsilateral to the carotid artery ligation side were harvested and immediately placed into ice-cold IB.

**Isolation of Nonsynaptic Brain Mitochondria**

To isolate and purify mitochondria from single hemispheres in neonatal mice, we modified an earlier published protocol combining differential centrifugation and isopycnic centrifugation in Percoll™ gradient [8, 9]. The brain hemispheres were dissected from each other by surgical blade and immediately immersed into 0.7 ml of ice-cold IB in a 5-ml plastic tube. Tissue was homogenized at a shaft rotation rate of 17,000 rpm (regimen 4) for 5 s using the Ultra-Turrax T8 tissue homogenizer (IKA Works Inc., Wilmington, N.C., USA) and transferred into a 1.5-ml microcentrifuge tube. The brain homogenate was centrifuged at 1,100 g for 2 min in a refrigerated (+4°C) table-top centrifuge (Eppendorf 5810R). The supernatant was discarded and the pellet was resuspended in 0.2 ml of the IB and centrifuged again at 1,100 g for 2 min. The pellet was discarded and the supernatant was combined with that from the first centrifugation. The 0.5–0.55 ml of combined supernatants were mixed with 0.07 ml of 80 vol% Percoll solution (about 9.03% Percoll, final) and carefully layered on top of 0.7 ml of 10% Percoll solution and centrifuged at 18,500 g for 10 min. Both the 80 and 10 vol% Percoll solutions were made fresh. To prepare the 80 vol% Percoll solution, 1 M sucrose, 50 mM HEPES and 10 mM EGTA, pH 7.0 with KOH was used. Then 80% Percoll was diluted in IB to prepare the 10% solution. The mitochondria-enriched fraction was collected at the bottom of the tube and resuspended in the 0.7 ml of washing buffer composed of 250 mM sucrose, 5 mM HEPES-KOH (pH 7.2), 0.1 mM EGTA and 1 mg/ml of BSA. The suspension was centrifuged at 10,000 g for 5 min. The final mitochondrial pellet was resuspended in 0.07 ml of washing buffer and stored on ice.

**Isolation of Total Fraction of Brain Mitochondria**

To isolate both synaptic and nonsynaptic mitochondria, we utilized the same procedure as described above except that IB was supplemented with 0.01% of digitonin at the tissue homogenization step, to release synaptic mitochondria [10].

The *mitochondrial protein content* was measured with a Lowry DC kit (Bio-Rad, Hercules, Calif., USA) according to the manufacturer’s instructions. In randomly chosen samples obtained from ischemic and nonischemic (naïve) hemispheres the level of mitochondria-specific proteins [Mn superoxide dismutase (Mn-SOD) and cytochrome C (Cyt-C)] was assessed by Western blotting. Briefly, isolated mitochondria were lysed and treated with NuPage LDS loading buffer. Samples (5 μg of mitochondrial protein) were loaded in each lane for SDS-PAGE electrophoresis. Then membranes with protein were incubated with anti-mouse antibodies against MnSOD and Cyt-C (Abcam) overnight at 4°C. Following washing and incubation with HRP-conjugated secondary antibodies the immunoreactivity of MnSOD and Cyt-C was identified by chemiluminescence detection assay.

**ATP measurements** were performed in brain homogenates obtained from HI mice and naïve littermates using a Bio luminescence Assay CLS II (Roche) as we described [11].

**Electron microscopy:** neocortical tissue samples obtained at 0 and 12 h of reperfusion and mitochondrial pellets were fixed overnight by incubation in 2.5% glutaraldehyde and 4% parafomaldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C. Then they were postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 1 h, dehydrated and embedded in Epon-Araldite. Ultrathin sections were cut using a Reichert Ultramicrotome, stained with uranyl acetate and lead citrate and examined under a JEOL 1200 electron microscope. Brains obtained from 2 naïve mice, 2 HI mice at 0 h of reperfusion and 2 HI mice at 12 h of reperfusion were used for electron microscopy.

**Mitochondrial respiration** was measured using a Clark-type electrode (Oxytherm, Hansatech, UK). Mitochondria were added at 0.065 mg/ml to 0.7 ml of respiration buffer composed of 200 mM sucrose, 25 mM KCl, 2 mM KHPO₄, 5 mM HEPES-KOH (pH 7.2), 5 mM MgCl₂, 0.2 mg/ml of BSA, 10 mM glutamate and 5 mM malate at t = 32°C. Where indicated, 250 nmol of ADP was added to the mitochondrial suspension to initiate phosphorylating respiration (state 3). The rates of O₂ consumption were expressed in nanomol O₂/milligrams mitochondrial protein/minute. The respiratory control ratio was calculated as the ratio of the state 3 respiration rate to the resting respiration rate (state 4) recorded after the phosphorylation of ADP had been completed and the respiration spontaneously decelerated. The ADP:O ratio was conventionally calculated as the amount of added ADP expressed in nanomol divided by the amount of oxygen atoms (nanomol of O) consumed during ADP-accelerated state 3 respiration. Because it has been reported that the activities of complex I, III and V were impaired in the mitochondrial isolate from the contralateral hemisphere following HI insult in p21 rats [12], mitochondria only from HI hemispheres were studied and compared to that obtained from naïve mice.
Reagents

All reagents were from Sigma-Aldrich (St. Louis, Mo., USA).

Statistical Analysis

Data are expressed as means ± SEM. Student’s t test analysis was used for comparative assessment of respiration parameters in mitochondria obtained from control and HI mice. To compare the cerebral ATP content between naïve and HI mice, 1-way ANOVA with Bonferroni’s post-hoc analysis was used. Values were considered significantly different if p ≤ 0.05.

Results and Discussion

The yield of mitochondrial protein from a single p10 mouse hemisphere weighing 107 ± 8 mg was 0.17 ± 0.07 mg per hemisphere. Electron microscopy of the isolated mitochondrial pellet revealed a well-preserved morphological integrity of the outer and inner membranes (fig. 1a–d). The total fraction of mitochondria isolated with 0.01% digitonin was essentially devoid of synaptos-
somes (fig. 1a, b), whereas nonsynaptic mitochondria contained some synaptosomes (fig. 1c, d). The digitonin treatment may be preferable when an increased yield of mitochondria preparations is required. However, the use of digitonin can result in potential experimental artifact. It is well known that digitonin breaks vesicular membranes by binding with the membrane cholesterol [12]. Whereas it helps to release mitochondria from synaptosomes, one should keep in mind that the outer mitochondrial membrane also contains a significant amount of cholesterol and, therefore, it can also be damaged by digitonin [14, 15]. It has been reported that digitonin-isolated guinea pig brain mitochondria exhibited nonstandard patterns of response to the respiratory chain inhibitors [16] and rat brain mitochondria isolated with digitonin exhibited altered Cyt-C release and Ca$^{2+}$ handling properties [15]. Therefore, we have selected nonsynaptic mitochondria to validate our isolation method in a neonatal mouse model of HI brain injury, although functional assessment of the nonsynaptic mitochondria only
has potential limitation, as this does not reflect the heterogeneity of the cerebral mitochondrial population in vivo.

The oxygen consumption measurements revealed that the phosphorylating (state 3, fig. 1e) respiration rate was significantly slower in nonsynaptic mitochondria isolated immediately after HI insult as compared to mitochondria obtained from naïve mice. The state 4 respiration rate (fig. 1f) was not affected by HI insult. As a result of a decreased state 3 respiration rate, mitochondria isolated from the ischemic hemisphere exhibited a significant (p < 0.0001) decrease in respiratory control ratio (fig. 1g) compared to mitochondria isolated from the nonischemic (naïve mice) hemisphere. Because the HI insult did not change the ADP/O ratio (fig. 1h), we can conclude that HI insult, at 0 h of reperfusion, significantly inhibits phosphorylating respiration rather than resulting in uncoupling of oxidative phosphorylation in brain mitochondria. Theoretically, similar results could be obtained if the amount of metabolically active mitochondria differed between the compared (HI vs. naïve) samples. However, Western blot analysis revealed that the amount of MnSOD (marker for the integrity of mitochondrial matrix) and Cyt-C (marker for the integrity of the outer membrane) was similar in mitochondrial isolates from ischemic and nonischemic brains (fig. 1i, k). This indicates that the difference in respiration between mitochondria obtained from HI mice and naïve littermates cannot be accounted for by variability in mitochondrial content in our isolates. Figure 2a demonstrates characteristic patterns of respiration measured in cerebral mitochondria from ischemic and nonischemic mice. Thus, the presented dataset indicates that following HI insult, prior to reperfusion, the mitochondrial respiratory chain is significantly dysfunctional. This can be supported by a profound depletion of cerebral ATP content in the ischemic hemisphere immediately after HI insult compared to that in naïve mice (fig. 2b). Earlier, Gilland et al. [1] and Puka-Sundval et al. [2], using brain tissue homogenate and 'crude mitochondrial fraction', reported that HI insult resulted in a significant decrease in mitochondrial respiration rate only at state 3, which led to reduction of respiratory control ratio immediately and at 1 h after the index event in p10 rats. Our results are in strong agreement with these data, thereby providing an additional validation to using isolated mitochondria from p10 mice. In the presented experiments we used only glutamate-malate as a substrate for mitochondrial respiration because the use of other substrates (pyruvate-malate) in testing cerebral mitochondria from neonatal HI rats [2] demonstrated a pattern of mitochondrial dysfunction similar to that observed in our experiments on HI mice.

Although, mitochondrial dysfunction was detectable immediately after HI insult, no morphological damage to mitochondria could be detected in brain tissue at this time point (fig. 2d, g compared to 2c, f). At 12 h of reperfusion, however, electron microscopy revealed compelling morphological evidence of mitochondrial damage such as mitochondrial swelling and partial or complete loss of cristae (fig. 2e). There was also a nuclear damage clearly manifested at this time point as condensation of the chromatin and nuclear shrinkage (fig. 2h vs. fig. 2f, g) in neurons. As the main purpose of this study was to develop a method for mitochondrial isolation suitable for a HI model in neonatal mice, we did not further examine reperfusion-induced changes in mitochondrial respiration.

This is the first report to demonstrate morphological and functional outcome on brain mitochondria isolated from a single hemisphere in neonatal p10 mice. A functional analysis of isolated mitochondria is a powerful method in the assessment of damage after insults and in revealing molecular details of the damage. One should, however, be aware that cerebral mitochondrial fractions consist of a mixed entity of different cellular origins that might respond differently to insults. Neurons are known to contain more mitochondria compared to glia. Given that in p10 mice the glia is underdeveloped, it can be assumed that in the developing brain there is no less than 50–60% of mitochondria of neuronal origin in the isolated fraction, as it has been shown that the percentage of neurons in the adult mouse brain is about 50–60% [17].

In conclusion, in this work we have reported a new, reproducible and simple method for the isolation of cerebral mitochondria from a single hemisphere in neonatal mice. We strongly believe that the presented approach to mitochondrial isolation will become very helpful in studies of the molecular mechanisms of tissue damage in the postischemic developing brain.

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References


