Time Course of Intracellular Edema and Epileptiform Activity Following Prenatal Cerebral Ischemia in Sheep

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The role of edema in the pathogenesis of hypoxic-ischemic injury in the immature brain is controversial. We studied 15 chronically instrumented fetal sheep following transient cerebral ischemia, to estimate changes in extracellular space using an impedance technique, to quantify the electroencephalogram with real-time spectral analysis, and to assess histologic outcome 3 days after the insult. These measurements were made in the parasagittal cortex. There was a rapid loss of extracellular space from 5±2 minutes after the onset of ischemia. Following 10 minutes of ischemia (n=7) the intracellular edema peaked but then quickly resolved (6±4 minutes), and mild selective neuronal loss was seen. In contrast, the swelling was biphasic after 30–40 minutes of ischemia (n=8). The early edema resolved slowly (28±12 minutes) but incompletely, and secondary swelling began at 7±2 hours and peaked at 28±6 hours. The early swelling was the more severe. Postinsult epileptiform activity began at 8±2 hours and peaked at 10±3 hours; later there was laminar necrosis of the underlying cortex. The secondary decrease of extracellular space indicates that a progressive loss of membrane function started with the onset of postischemic epileptiform activity. The increased metabolic load of the epileptiform activity may have worsened this delayed deterioration. (Stroke 1991;22:516–521)

The role of edema in the pathogenesis of brain damage in asphyxiated term newborns is controversial.1 It has been suggested that early brain edema in neonates is the initial consequence of a hypoxic-ischemic insult and that this edema impairs cerebral blood flow and aggravates brain damage.2 However, other studies have suggested that the immature brain is comparatively resistant to the development of edema,3,4 and it is thought that any edema develops later only as a consequence of necrosis.5,6 If correct, this suggests that early edema is not an important factor in the pathogenesis of perinatal hypoxic-ischemic encephalopathy.7 Therefore, both the time course and role of brain edema in the pathogenesis of perinatal hypoxic-ischemic encephalopathies are unclear.

Several authors have speculated that postischemic epileptiform activity contributes to secondary brain damage.8–9 Postasphyxial seizures are associated with a particularly bad prognosis in very low–birth weight infants, and it has been suggested that the seizures themselves constitute a serious neurologic insult.10 However, some studies tend to contradict this hypothesis since they have not found postischemic epileptiform activity occurring with delayed neuronal loss.11,12 A recent study by us in a fetal sheep preparation has demonstrated that epileptiform activity does not occur with selective neuronal loss but is associated with laminar necrosis of the underlying parasagittal cortex.13 The neurologic consequences of these seizures are not yet known.

Ischemic brain edema is largely the result of increased intracellular water content14,15 and is thought to occur primarily in the glial cells of the gray matter.16 Increases in brain volume are secondary to this intracellular swelling.17 The changes of extracellular space that occur during and after ischemia are closely coupled with the loss and recovery of ion homeostasis.18–21 Intracellular swelling reduces extracellular space and increases tissue impedance, and these changes in impedance can be used to estimate changes of extracellular space.22 Persistent intracellular swelling or increases in impedance following hypoxia-ischemia indicate tissue damage both in vitro23 and in adult animals in vivo.19,24 The purpose of this study was to define the relations among the time course of intracellular edema, the electroencephalogram (EEG), and histologic

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outcome in a defined model of perinatal asphyxia. We recently described an experimental preparation in which a chronically instrumented fetal sheep is subjected to transient cerebral ischemia; this procedure induces an encephalopathy showing characteristics similar to hypoxic-ischemic brain damage of some asphyxiated term infants. Different severities of insult were induced by varying the duration of cerebral ischemia. The parasagittal cortical EEG was continuously quantified with real-time spectral analysis; this technique stores averaged intensity spectra to disc at regular intervals. The changes in size of the extracellular space of the parasagittal cortex were estimated using a four-electrode impedance technique. Cell death was assessed histologically 3 days after the insult.

**Materials and Methods**

Fifteen fetal Romney/Suffolk sheep were operated on under 2% halothane anesthesia using sterile techniques as previously described. Briefly, catheters were inserted into each axillary artery, and two pairs of shielded stainless steel electrodes were placed bilaterally on the parietal dura via parasagittal burr holes overlying the parietal cortex (skull coordinates relative to the bregma: anterior 5 mm and 15 mm, lateral 10 mm). The mean±SD biparietal width was 5.2±0.3 cm. The vertebro-occipital anastomoses between the carotid arteries and vertebral arteries were ligated bilaterally, thus eliminating vertebral blood supply to the brain. Inflatable occluder cuffs were placed around both carotid arteries. The fetal sheep were returned to the uterus. After the operation the ewe was housed in a metabolic cage at constant temperature (20°C) and humidity (50%) and given free access to hay and water supplemented by sheep nuts and alfalfa. Antibiotics (80 mg gentamicin, 1 g cephalothin) were administered to the ewe daily.

The EEG was recorded on an analog chart at 5 mm/min and analyzed in real time as previously described. Changes in impedance of the parasagittal cortex were measured using a four-electrode technique. One pair of electrodes was used to inject rectangular pulses of ±0.2 μA through the parasagittal cortex from an isolated constant-current source (model S11, Grass Instrument Co., Quincy, Mass.). The voltage-recording electrodes were connected to a high-impedance instrumentation amplifier (10^12 Ω). Driven-shield electrodes minimized capacitive loading on the impedance signal and shielded against cross talk from the stimulation leads.

The subsequent impedance signal was superimposed on the unfiltered EEG signal. This signal was recorded with the current signal on an IBM PC-AT via a DT2801A data acquisition board (Data Translation Inc., Marlboro, Mass.). The stimulator produced 1.2-second trains of 150-Hz biphasic ±0.2-μA pulses. The impedance signal was then extracted by using a phase-sensitive detector implemented in software (Asystant +, Asyst Software Technologies, Inc., Rochester, N.Y.) to remove the asynchronous EEG activity and to reduce background noise.

Experiments were started 72–96 hours after surgery with full approval from the Animal Ethical Committee of the University of Auckland. Fetuses ranged in gestational age from 119 to 127 days (term gestation 147 days). Fetal arterial blood samples were obtained prior to the start of each experiment, and only fetuses exhibiting normal arterial blood gases (pH of >7.30, PaO_2 of >17 mm Hg) were used. Anesthetics were not used during the experiment. After 4 hours of collecting EEG spectral data (reference period), the carotid cuffs were inflated with saline for 10 (mildly damaged group, n=7) or 30–40 (severely damaged group, n=8) minutes. Spectra were then collected continuously for the next 32 hours. The impedance signal was collected at 1-minute intervals for 200 minutes starting 1 hour before the insult. Over the next 60 hours impedance measurements were made at 1,000-second intervals.

Histologic preparation and assessment were done as previously described. Each ewe was killed 3 days postinsult by pentobarbital infusion. The fetus was immediately removed, and the brain was perfused via the common carotid arteries; 500 ml normal saline was followed by 500 ml 10% formalin, then 500 ml 10% formalin/10% sucrose.

The brains were embedded in paraffin, coronally subserially sectioned to 8 μm, and then stained with thionine acid fuchsin. Every fortieth section was mounted and examined by light microscopy. Each selected region was assessed at ×400 magnification before detailed assessment at ×400 magnification. Neurons with ischemic cell change were identified according to the criteria of Brown and Brierley. Cells with acidophilic (red) cytoplasm and contracted nuclei or with just a thin rim of red cytoplasm with pyknotic nuclei were considered dead while all others were considered viable. Each region was scored in multiple preselected areas for the proportion of dead neurons by two independent assessors, one of whom was blinded to the experiment.

The parasagittal cortex was taken to include all of the gyrus bordering the sagittal sulcus and the medial half of the laterally adjacent gyrus corresponding with the positions of the EEG electrodes. The average values from two sections and from both the right and left hemispheres were used. A six-range damage scale was used: 0%, 1–10%, 10–50%, 50–90%, 90–99%, and 100% neurons dead. To enable statistical analysis of this scale, the nominal damage score was taken as the midpoint of each range: 0, 5, 30, 70, 95, and 100, respectively. As these scores are on a ratio scale, the degree of damage could be compared using parametric statistical methods.

Impedance techniques have limitations for estimating the absolute values of extracellular space. However, by assuming a fixed electrode geometry and a homogeneous media, the changes in extracellular space can be estimated using the Maxwell equation.
The onset, maximum and minimum amplitudes, and timing were measured on the smoothed time series for both the EEG and impedance. A digital Blackman low-pass filter\(^1\) (Asystant, Asyst Software Technologies) with a cutoff of 0.1 cycles/point was applied to the log-transformed EEG intensity data to reduce short-term fluctuations of \(<20\) minutes. Similarly, a cutoff of 0.25 cycles/point was applied to the impedance time series. The onset of epileptiform activity was defined as the time postinsult when smoothed EEG intensity increased above \(-5\) dB.\(^1\) Onset of the acute and delayed increase in impedance was determined by the 10\% point of the rise. Recovery time was defined as the time for the early peak height to fall by 75\%. Median frequency of the EEG activity was calculated over the 5–30-hour period after the insult.

The EEG and impedance parameters were compared using unpaired \(t\) tests with Bonferroni’s correction for multiple comparisons following two-way analysis of variance with time postinsult as a repeated measure. All results are presented as mean±SD. Impedance measurements and extracellular space estimates are expressed as percentages of preinsult levels.

Results

Fetal sheep in the mildly and severely damaged groups had preinsult serum lactate concentrations of 2.8±0.8 and 2.8±0.5 mM, respectively. Detailed descriptions of histologic outcome at 3 days postischemia have been published.\(^1\) Following 10 minutes of ischemia there was no or only mild selective neuronal loss (damage score 5±9) in the parasagittal cortex; neuronal loss was greatest in layer 3. After 30–40 minutes of ischemia there was severe neuronal loss (damage score 87±12) with laminar or full-thickness necrosis of the parasagittal cortex.

We have previously reported the electrophysiological consequences of transient cerebral ischemia.\(^1\) To summarize, after severe cerebral ischemia (30–40 minutes) parasagittal EEG activity was depressed for several hours and then there was a transition to low-frequency epileptiform activity; this hyperactivity gradually resolved and decreased in intensity by 72 hours. This pattern of activity was associated with laminar necrosis of the underlying cortex. In our current study, severe ischemia induced low-frequency epileptiform activity that started at 8±2 hours and then peaked in intensity at 10±4 hours. This activity was associated with a fall in median frequency to 5±1 Hz (5–30 hours postsinsult), whereas the mildly damaged group remained at 10±1 Hz (\(p<0.001\)).

The average preinsult impedance for this electrode geometry was 53±10 \(\Omega\). During ischemia there was a rapid increase of parasagittal impedance from 5±2 minutes after clamping; impedance then appeared to rise more gradually to peak 3±3 minutes after release of the clamps (Figure 1). The initial peak of impedance in the mildly damaged group (125±14\%) was less than that in the severely damaged group (163±22\%) (\(p<0.01\)). The time for this early increase to resolve was 6±4 minutes in the mildly damaged group; resolution took longer (28±12 min) in the severely damaged group (\(p<0.005\)).

The posts ischemic impedance minimum of the mildly damaged group (98±2\%) was less than that of the severely damaged group (107±5\%) (\(p<0.005\)), indicating a residual increase in impedance for the latter. Only in the severely damaged group was there a secondary increase in impedance from 7±2 to 28±6 hours (Figure 1).
Figure 2. Bar graphs comparing time course of change in impedance (lower) and estimated extracellular space (ECS) (upper) following mild (10 minutes) (shaded bars) or severe (30-40 minutes) (filled bars) hypoxic-ischemic insult in fetal sheep. Following severe insult impedance increase was biphasic with both early and secondary increases, whereas mild insult induced only early reversible increase in impedance. Time as hours postischemia. *p<0.05, **p<0.01 different from mild insult by repeated-measures analysis of variance.

FIGURE 3. Comparison between time course of parasagittal impedance (upper) and electroencephalographic activity in same region (middle and lower) of fetal sheep after 30 minutes of ischemia. Onset (middle, total intensity) of low-frequency epileptiform activity (lower, median frequency) coincided with onset of secondary increase in impedance (upper).

hours postinsult (p<0.001); this secondary increase then resolved toward preinsult levels at 60 hours (p<0.05) (Figure 2). The early peak of impedance (163±22%) was greater than the secondary maximum (134±11%) (p<0.05).

Times for the onset of the secondary increase in impedance and the peak epileptiform intensity were not significantly different (Figure 3). However, the peak of the secondary increase in impedance occurred later than the maximum EEG activity (p<0.0001).

Discussion

Previous studies have led to conflicting conclusions regarding the role of the edema that occurs in association with perinatal hypoxic-ischemic encephalopathies. Studies by Brann and Myers in asphyxiated term primates suggest that early edema impedes reperfusion and thus worsens outcome. In contrast, other researchers have suggested that early brain edema does not occur and therefore cannot play an important role in the pathogenesis of hypoxic-ischemic encephalopathies in the developing brain. The preparation we used shows similarities to the encephalopathies occurring in some asphyxiated term infants. Although impedance measurements tend to underestimate changes of extracellular space (particularly due to the limitations of assuming a homogeneous medium), our study clearly demonstrates that intracellular edema is biphasic following a severe hypoxic-ischemic insult.

This confusion appears to have arisen because of the differing definitions of brain edema and the biphasic time course of the swelling. Traditionally, brain edema has been defined as an increase in brain volume and is thus assessed with specific gravity techniques. However, ischemic brain edema has been shown to be predominantly intracellular initially, and brain volume increases only secondarily to these intracellular changes. We directly assessed changes of intracellular edema whereas Rice et al looked at the secondary changes in brain water content, Tweed et al investigated increases in extracellular fluid or supposed vasogenic edema, and Lupton et al measured the increases in intracranial pressure that occur secondary to gross increases in brain volume.

The onset of the early intracellular swelling appeared to have two distinct stages (Figure 1). First, there was rapid swelling beginning 5 minutes after
the start of ischemia; this is a consequence of energy failure, leading to depolarization and loss of ion homeostasis. The onset latency in fetal sheep was longer than that observed in adult cats and may reflect the increased resistance of the immature brain to hypoxia-ischemia. This phase of rapid swelling shows properties similar to spreading depolarization in the cortical gray matter, and the rapidly increasing intracellular edema is thought to result from water influx following sodium and chloride influxes. After this rapid increase, there was continued gradual swelling that peaked soon after the end of the insult (Figure 1).

The profile of the early intracellular swelling is similar to that seen in mature brains, and its existence confirms observations of early brain edema seen in asphyxiated term monkeys. This early edema has been shown to be a consequence of intracellular swelling, demonstrating a loss of extracellular space and altered ion homeostasis, and it occurs predominantly in the gray matter. The reduction of extracellular space in the cerebral cortex immediately after prolonged partial asphyxia in newborn monkeys (-55%) was similar to that seen at the end of severe ischemia (-35±9%).

Failure to recover extracellular space indicates tissue damage and cell death in adults. Thus, the residual increase in impedance seen after the longer insult implies that some cell death occurred during ischemia and early reperfusion. The existence of intracellular edema during early reperfusion suggests that cells were likely to have been vulnerable to osmolytic damage and oxygen free radical damage. Furthermore, it has also been suggested that extensive glial swelling can impede oxygen transport to neurons and impair their capability to take up extracellular glutamate. Work by Thiringer et al shows that short-term recovery of asphyxiated newborn lambs was improved by rapid infusion of a mixture of antiedema agents, calcium channel blockers, and free radical scavengers during early reperfusion. Thus, the rate of resolution of this early intracellular edema may be an important factor determining the degree of final damage.

The secondary intracellular swelling (Figure 2) that occurred with laminar necrosis indicates a delayed deterioration of membrane function and suggests secondary tissue damage in the parasagittal cortex. Similarly, secondary cell swelling is coupled with cell death following transient ischemia in vitro. A secondary deterioration of cortical cells with loss of ion homeostasis, edema, and neuronal death is seen following transient ischemia in some adult animal preparations. Secondary tissue edema is also associated with necrosis, at least in adult animals.

We have shown in a previous study that distinct electrophysiological phases of depression, epileptiform activity, and subsequent loss of intensity are associated with laminar necrosis of the underlying cortex. Intense epileptiform activity per se can cause intracellular edema in neonatal rat cortex. Intense epileptiform activity can lead to persistent depolarization, intracellular edemas in vitro, and eventually cell death in the adult brain. Focal cortical seizures can cause neuronal swelling and cell death locally and at remote thalamic sites in adult rats. Indeed, others have suggested that brain edema plays a pathogenic role in epileptic brain damage. The increased metabolic load of epileptiform activity is likely to have accentuated the secondary intracellular swelling.

We demonstrate that intracellular edema was most severe during early reperfusion; this may interfere with recovery. The delayed epileptiform activity is likely to have worsened the secondary intracellular edema. Further studies in this preparation in which the edema or epileptiform activity is prevented therapeutically may define causal relations.

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