Role of the Cerebrovascular and Metabolic Responses in the Delayed Phases of Injury After Transient Cerebral Ischemia in Fetal Sheep

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Background and Purpose—Perinatal hypoxic-ischemic injuries can trigger a cascade of events leading to delayed deterioration and cell death several hours later. The objective of this study was to characterize the cerebral blood flow responses and the changes in extracellular glucose and lactate during the delayed phases of injury and to determine their relationships with the pathophysiological events after hypoxic-ischemic injury.

Methods—Two groups of near-term chronically instrumented fetal sheep were subjected to 30 minutes of cerebral hypoperfusion. In the first group, regional cerebral blood flow was measured over the next 24 hours with radiolabeled microspheres. In the second, cortical extracellular glucose and lactate were measured by microdialysis. Parietal electrocorticographic activity and cortical impedance were recorded continuously in both groups, and the extent of neuronal loss was determined histologically at 72 hours after injury.

Results—Cerebral blood flow was transiently impaired in the cortex during reperfusion, whereas during the delayed phase, there was a marked increase in cerebral blood flow. The severity of cortical neuronal loss was related to the degree of hypoperfusion in the immediate reperfusion period and inversely related to the magnitude of the delayed hyperperfusion. Cortical extracellular lactate was elevated after injury, and both glucose and lactate secondarily increased during the delayed phase of injury.

Conclusions—The delayed phase is accompanied by a period of hyperperfusion that may protect marginally viable tissue. (Stroke. 1999;30:2735-2742.)

Key Words: cerebral ischemia, transient cerebral blood flow, neuronal death, vasodilation

Perinatal hypoxic-ischemic (HI) injuries remain a major cause of neurological morbidity and encephalopathy in newborns. It is now well established that cell injury can occur in 2 phases after an HI insult and that cerebral energy failure and further neuronal loss can occur several hours to days after the primary injury. Apoptosis, accumulation of excitotoxins, and seizures are thought to contribute to this secondary phase. Cerebrovascular responses and mitochondrial dysfunction are also thought to play an important role during the later phases of injury and influence neural outcome.

In a recent study, 2 phases of increased cerebral blood volume (CBV) were measured by near-infrared spectroscopy (NIRS) after 30 minutes of severe ischemia in near-term fetal sheep. The first increase occurred immediately after reperfusion. The second increase started several hours later and spanned the delayed phase of injury, as indicated by the onset of intense seizure activity and subsequent development of cortical cytotoxic edema and decline in the concentration of oxidized cytochrome aa3. The physiological basis of the measured changes by NIRS remains unclear. In particular, it is not clear whether an increase in CBV represents an increase in cerebral blood flow (CBF), vascular pooling, or even an actual increase in CBV. Moreover, the role of these cerebrovascular responses remains uncertain.

We used an established chronically instrumented fetal sheep preparation in which acute and delayed phases of cortical cell dysfunction are observed after 30 minutes of severe cerebral ischemia. A third phase, called the latent phase, has also been identified, spanning the period separating the 2 phases of cell death. Neuronal loss occurs predominantly in the parasagittal cortex and hippocampus. We hypothesized that the changes in CBV previously observed in this preparation reflect changes in blood flow and that these would relate to the neuropathological outcome. A further hypothesis was that there are changes in extracellular glucose and lactate after HI injury that are compatible with mitochon-

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drial dysfunction. Two studies were performed. In the first study, the changes in regional CBF were characterized with the radioactive microsphere technique, whereas the second study determined the time course of the changes in extracellular glucose and lactate by intracerebral microdialysis.

Materials and Methods

Surgical Procedures

These experiments were approved by the Animal Ethics Committee of the University of Auckland. Eighteen Romney/Cross fetal sheep were divided into 2 groups, group 1 (n = 7) and group 2 (n = 11). The fetuses were chronically instrumented at 120 to 127 days gestation age under general halothane anesthesia (2.5%) as previously described. The ewes were given 5 mL Streptopen (Pittman-Moore) IM before surgery.

In group 1 animals, fetal catheters were placed in the left and right axillary arteries, a femoral vein extending into the inferior vena cava, and the amniotic sac. Inflatable cuffs were placed around both carotid arteries. The vertebral-occipital anastomoses were ligated bilaterally to restrict vertebral blood supply to the carotid arteries; the lingual arteries were also ligated to restrict noncerebral blood flow.14 Burr holes for electrode placement were drilled in the skull for placement of electrocorticogram (ECoG) and cortical impedance (CI) electrodes as previously described. The sagittal sinus of 2 fetuses from group 1 was cannulated with an angiocatheter (20 Hz, and sampling at 256 Hz. The CI signal was extracted from the 10 seconds of inflation, followed by an acute rise in the CI signal.

Recordings

Recordings were started at least 72 hours after surgery, 12 hours before the HI injury, and continued for 72 hours after occlusion. Experiments were performed when fetal blood Pao2 and pH (both parameters corrected for the fetal temperature of 39.5°C) were >17.0 mm Hg and >7.35, respectively. Biparietal ECoG data were continuously collected by real-time spectral analysis.4,12 The ECoG signal underwent 10 000 times amplification, low-pass filtering at 30 Hz, and sampling at 256 Hz. The CI signal was extracted from the ECoG signal by a 4-electrode technique as previously described. Briefly, an isolated current source was used to inject a 150-Hz, 1-μA current bilaterally through the parasagittal cortex. The voltage signal generated by this current flowing through the cortical tissue was measured via a second pair of electrodes and extracted from the ECoG signal with a phase-locked loop. The CI technique is an indirect measure of cytotoxic edema because it rises concomitantly as cells depolarize and fluid shifts from the extracellular to the intracellular space. This technique has been validated against changes in MRI in the rat.15 Arterial blood pressure corrected for amniotic pressure, ECoG, and CI were recorded continuously with a custom software package (Labview for Windows V6.12). The fetuses underwent 30 minutes of complete bilateral carotid occlusion induced by inflation of the occluder cuffs. Successful occlusion was confirmed by the onset of an isoelectric ECoG signal within 30 seconds of inflation, followed by an acute rise in the CI signal.

Microsphere Measurements

Regional and global blood flows were measured with 15-μm-diameter microspheres (Dupont NEN) labeled with 1 of 6 different radioisotopes (153Gd, 141Ce, 11Sn, 89Sr, 99Nb, and 46Sc) by the reference sample method previously published. One radioisotope was selected randomly for a measurement at the following time points: −1 hour preocclusion (always made during high-voltage ECoG activity), +10 minutes (+ indicates time after the end of the occlusion), +1.5 hours, +4.5 hours, +12 hours, and +24 hours. These time points were chosen to correspond to the pathophysiological phases after injury, namely, the immediate reperfusion, latent, and delayed phases. The reference sample was withdrawn from the axillary artery at a rate of 4 mL/min for 1.5 minutes via a Harvard infusion-withdrawal pump. In the 2 sheep instrumented with a sagittal sinus catheter, shunting activity in the cortex was simultaneously checked at the above time points by sampling from the sagittal sinus at a rate of 2 mL/min for 1.5 minutes. For each measurement, a suspension of ∼1.5×106 microspheres in saline and 0.01% Tween was sonicated and injected into the inferior vena cava over 20 seconds. A chart recorder (Lineacorder) was used to monitor blood pressure changes during injection. On completion of each microsphere injection (and subsequent blood sampling), the fetus was transfused with 6 mL of maternal or fetal donor arterial blood.

Intracortical Microdialysis

The intracortical microdialysis methodology and its use in the present preparation have been described elsewhere. Microdialysate samples were collected at 30-minute intervals in Eppendorf vials with a refrigerated microfraction collector (Carnegie Medicine CMA/140). Glucose and lactate concentrations were measured with a YSI 2300 Stat glucose/lactate analyzer (Yellow Springs Instrument Co Inc). The relative recoveries used to estimate the extracellular concentrations were determined in vitro for lactate and glucose. The in vitro relative recovery for lactate was 25±15% and for glucose was 22±9%. Changes in extracellular space were estimated from changes in tissue impedance with the Maxwell equation.17

Regional CBF Analysis

The ewe was euthanized 72 hours after fetal injury, the fetus removed, and its brain perfused in situ with 0.9% NaCl followed by 10% phosphate-buffered formalin. One cerebral hemisphere was kept for histological assessment. The other hemisphere was divided into the following cerebral substructures: frontal, parietal, temporal, and occipital cortices, striatum, hippocampus, thalamus, hypothalamus, midbrain, pons, medulla, and cerebellum. These were weighed and counted for radioactivity (cpm) in polyethylene vials (Packard) with a gamma counter (Auto-Gamma 5000 series, Packard). All tissue and reference samples contained ≥500 microspheres.

Histology

Tissue slides were prepared from 5-μm coronal sections cut and stained with thionine and acid fuchsin and analyzed as previously described. In group 1, however, the procedure was modified so that the coronal sections corresponded to the same regions as analyzed for regional CBF.

Signal Analysis

Offline time series analyses of the postperfusion ECoG and CI data were performed on the recorded signals median-filtered to minimize the effects of short-term (<20 minutes) fluctuations with Viewdac Data Acquisition software (Keithley Instruments Inc). Epileptiform activity was analyzed with Monitor software (Stellate Systems). For detecting and quantifying epileptiform events, all the default settings of the Monitor software were used except for the maximum coefficient of variation for seizure detection, which was set at 60%. In addition, the detected events were visually examined and confirmed to be seizure events by a pediatric neurologist (S.L.D.). The ECoG intensity and changes in CI were then normalized with respect to the 12-hour
Historical Analysis
In group 1, histological assessment of the brains revealed the highest percentage of neuronal loss in the cortical regions. There was severe neuronal loss and laminar necrosis in the parasagittal region of the cortex, with selective neuronal loss in the more moderately injured cortical and subcortical regions. Hippocampal damage was greatest in the CA3 and to a lesser extent in the CA1, CA2, and CA4 and dentate gyrus of the dorsal horn. Moderate cell loss was seen in the ventral horn, cerebellum, pons, and medulla, and virtually no neuronal death in the midbrain and the hypothalamus. The distribution of neuronal loss in group 2 was the same as reported previously.4

Immediate Reperfusion Phase (+10 Minutes)
In group 1, CI rose to a maximum at 2.4±2.9 minutes after reperfusion (Figure 1). It then fell and had partially resolved by +10 minutes. The ECoG was still suppressed at this time (Figure 1), and global CBF was reduced (96±15 mL·100 g⁻¹·min⁻¹) compared with baseline (138±15 mL·100 g⁻¹·min⁻¹, P<0.05). The flows in the parietal and temporal cortices fell below baseline flows (Figure 2A). The severity of neuronal loss in the cortical regions was inversely related to the levels of blood flow at this time (r=−0.65, P<0.001). In contrast, there was an immediate restoration of blood flow to preischemia levels in the subcortical regions (Figure 2B and 2C). No significant correlation was found between cortical blood flow and CI.

In group 2, CI rapidly increased after occlusion and gradually rose to a peak of 149±9% (P<0.05) after release of the occluders (Figure 3). This corresponds to an estimated decrease in the extracellular space to 75±4% of baseline. Dialysate lactate levels increased 4-fold at the...
end of the HI injury and remained elevated thereafter for 2 hours (Figure 3).

Latent Phase (+1.5 and +4.5 Hours)
In group 1, the acute rise in impedance had largely resolved during this period but remained significantly elevated compared with baseline at +1.5 hours and at +4.5 hours (Figure 1). The ECoG activity had increased slightly at +1.5 hours and at +4.5 hours but remained well suppressed compared with baseline levels (Figure 1). All cerebral regions exhibited normal CBF with the exception of the medulla oblongata, which showed an increase from baseline at +1.5 hours (Figure 2A, 2B, and 2C). No significant changes in regional or global CBF were seen at +4.5 hours.

In group 2, the residual impedance at +2 hours was 109±2% of preischemic levels (P<0.05; Figure 3). The ECoG remained depressed after the insult and progressively increased in intensity from +7±2 hours. Dialysate lactate declined at +4.5 hours, but never to baseline (Figure 3).

Delayed Phase (+12 and +24 Hours)
In group 1, global CBF was increased at +12 hours (186±21 mL · 100 g⁻¹ · min⁻¹) compared with baseline (138±15 mL · 100 g⁻¹ · min⁻¹, P<0.05). The parietal cortex showed an increase at +12 hours (194±27 mL · 100 g⁻¹ · min⁻¹) compared with baseline levels (130±13 mL · 100 g⁻¹ · min⁻¹, P<0.05). At +24 hours, global CBF was exhibited a significant increase in CBF at +24 hours (P<0.05).

C, Time course of changes in regional blood flow in the diencephalon, striatum, and hippocampus in group 1. These regions (except the hypothalamus)
still elevated (255±8 mL · 100 g⁻¹ · min⁻¹, P<0.05), with increased blood flow in all cerebral regions except the hypothalamus and the midbrain (P<0.05; Figure 2A, 2B, and 2C). The ECoG intensity rose during this phase and peaked at +19±8 hours (P<0.05). The magnitude of this rise in ECoG intensity strongly correlated with the rate of seizure-like events (r=0.91, P<0.001; Figure 1). A weak correlation was found between seizure activity and parietal blood flow at either +12 hours or +24 hours. Likewise, the secondary increase in CI started at 18±5 hours and peaked at 44±9 hours (Figure 1). Within the cortex, the severity of neuronal loss was directly related to the magnitude of the secondary rise in CI (r=0.83, P=0.02) and inversely related to the levels of blood flow at +24 hours (r=−0.67, P<0.001).

In group 2, the onset of the secondary increase in CI occurred at +7.5±3.9 hours and gradually reached a peak at +31.6±5.7 hours (Figure 3). This rise in CI corresponded to an estimated reduction in the extracellular space to 78±3%. Lactate rose again from +8 hours with the onset of ECoG epileptiform activity (+7±2 hours) and the secondary rise in CI (+7.5±1.3 hours). Dialysate lactate concentration rose to a peak at +32 hours, occurring after peak epileptiform activity (+12.6±6.9 hours), coincided with peak secondary rise in impedance (+31.6±5.7 hours), and then gradually fell. Similarly, glucose also rose gradually by 2.2-fold to a peak at +10 hours and remained elevated throughout the secondary increase in CI before declining by +72 hours.

**Discussion**

The present studies have characterized the regional CBF and cortical extracellular glucose and lactate responses after 30 minutes of cerebral hypoperfusion in the fetus. There was evidence of impaired cortical reperfusion immediately after relief from the occlusion. This was followed by a marked hyperperfusion and elevation of extracellular glucose and lactate during the secondary phase of injury. The role of these responses is discussed below with respect to the immediate reperfusion, latent, and delayed phases of injury.

**Immediate Reperfusion Phase**

Consistent with previous studies in this model of ischemia,8,13 the primary cortical cytotoxic edema took ~30 minutes to resolve to close to preinsult levels (Figures 1 and 3). In group 1, global CBF at +10 minutes was reduced compared with baseline, in what might reflect impaired reperfusion. The hypoperfusion was restricted primarily to the cortex. Similarly, continuous ultrasonic flowmeter measurements for carotid blood flow in this preparation also show a transient reduction in CBF at this time (R.A.R., unpublished data, 1998). In contrast, a previous study by Marks and colleagues8 indicated an increase in CBV at this time. It is possible that the impaired perfusion is accompanied by pooling of venous blood, resulting in increased CBV as measured by NIRS. These findings suggest that NIRS data may need to be carefully interpreted under reperfusion conditions.

In group 2, the increased cortical extracellular lactate but not glucose levels during reperfusion also suggest that there was ongoing anaerobic metabolism and possibly ischemia. Furthermore, in group 1, there was a strong relationship between the degree of cortical hypoperfusion at this time and the severity of cortical neuronal loss. These data suggest that this hypoperfusion extends the primary injury and exacerbates neuronal damage.

**Latent Phase**

Early in this phase and specifically at +1.5 hours in group 1, ECoG activity remained depressed, yet the acute cortical cytotoxic edema had largely resolved (Figure 1). Global and regional CBF were similar to baseline levels except for increased flow in the medulla oblongata (Figure 2A, 2B, and 2C). However, in a previous study in the same animal preparation, there was a moderate increase in CBV at this time, as detected by NIRS.8 It is likely that the NIRS was detecting the response in this area or even simply reflected a global increase in CBV due to venous pooling without a corresponding increase in flow. At +4.5 hours, although the ECoG was still depressed and although seizures and the secondary rise in impedance had not yet started (Figures 1 and 3), CBF in all regions was similar to baseline (Figure 2A, 2B, and 2C).

Cortical extracellular lactate remained elevated throughout the postreperfusion period despite the nearly complete resolution of cytotoxic edema, suggesting that reduced extracellular space was not a primary determinant of the increases in lactate and glucose. Consistent with other studies in babies, piglets, and rats after HI injury or asphyxia,19-21 this persistently elevated lactate suggests a continued cellular dependence on anaerobic glycolysis, despite restoration of cortical CBF to preinsult levels. A previous study in the present preparation shows that after ischemia, there is a gradual fall in the mitochondrial oxidized cytochrome aa₃ concentration.8 This fall starts shortly after the insult and proceeds throughout this phase. Together, these results suggest that mitochondrial dysfunction is present during this latent phase.

A transient increase in extracellular glucose levels occurred in this phase. Vannucci and colleagues22 have presented evidence of increased expression of glucose transporters across the blood-brain barrier (GLUT1) early in the latent period after HI injury in immature rats. The peak in extracellular glucose is transient, because the increasing ECoG activity may result in increased glucose consumption and hence its restoration toward preinsult levels (Figure 3).

**Delayed Phase**

At +12 hours, epileptiform activity had begun but had not yet peaked in intensity. The impedance had not yet started to rise in group 1 (Figure 1). However, global CBF was increased at this time. In particular, flow was increased in the parietal cortex (Figure 2A). At +24 hours, the epileptiform activity was resolving and the secondary rise in CI in group 1 had started but was still below its maximum (Figure 1). Blood flow at this time was significantly increased in all brain regions (P<0.05) except the hypothalamus and the midbrain.
A previous NIRS study in this preparation indicated a secondary rise in CBV with a time course similar to that in the present study. Taken in conjunction with the present study, these findings suggest the presence of hyperemia in the parietal cortex at +12 hours, increasing in magnitude and spreading to all cerebral areas except the hypothalamus and the midbrain by +24 hours. These results clearly refute the hypothesis that the injury in the delayed phase is due to the development of regional ischemia.

Cerebral hyperperfusion in newborns is associated with brain injury after birth asphyxia and, however, its role remains unclear. The delayed hyperperfusion in the present study might result from a period of increased seizure activity and subsequently increased metabolism because seizures increase metabolic demands. In addition, the earliest and maximal increase in blood flow in the parietal cortex compared with the other cortical regions may correspond to its being a focus of the seizures. However, the rise in CBV previously reported in this preparation preceded the onset of seizure activity and subsequently paralleled the evolution of the secondary edema. Similarly, in another study, a rise in ECoG intensity reflecting the onset of seizure activity was accompanied by an increase in carotid arterial blood flow. However, the peak in ECoG intensity preceded that of carotid blood flow, suggesting that the cerebral hyperperfusion has other determinants. These observations, together with a weak correlation between parietal CBF and seizure-like events, suggest that seizure activity is not the sole factor stimulating the delayed hyperperfusion.

In group 2, the maximal increase in lactate was also similar to the time course of increase in CI, whereas the 2-fold peak in cortical extracellular glucose levels occurred before the peak in seizure activity, remaining elevated for hours thereafter before declining by +72 hours (Figure 3). The time course of changes in systemic blood glucose and lactate concentrations with respect to CI and ECoG changes in previous studies were different from the time course of our microdialysate data. Thus, it is unlikely that the changes observed in the extracellular space were due to changes in the systemic blood concentrations of these metabolites. Given that ischemia and total glucose depletion do not occur and that cerebral oxygen levels are increased at this time, these findings suggest the presence of hyperemia in the parietal cortex at +12 hours, increasing in magnitude and spreading to all cerebral areas except the hypothalamus and the midbrain by +24 hours. These results clearly refute the hypothesis that the injury in the delayed phase is due to the development of regional ischemia.

The magnitude of cortical hyperperfusion at 24 hours after HI was inversely related to the degree of neuronal loss. Specifically, this suggests that greater hyperperfusion was associated with milder or selective neuronal loss. Previous studies have associated higher blood flow in humans and rats after acute stroke, in fetal sheep after HI injury, and in humans after traumatic brain injury with good tissue outcome. Moreover, attenuation of this hyperperfusion with a nitric oxide synthase inhibitor was associated with exacerbation of neuronal death in fetal sheep. The above evidence combined may suggest a neuroprotective role for the hyperperfusion.

In summary, this study has characterized the CBF responses after an HI insult to the perinatal brain. In addition, the results clarified the relationships between CBV and the pathophysiological responses and histopathological outcome. Our data indicate an early impairment of reperfusion in the cortex and suggest that this may exacerbate the primary injury. Finally, the presence of a marked hyperperfusion during the delayed phases of injury is likely to play a protective role through enhancing nutrient delivery and metabolic waste removal from marginally metabolically viable tissue.

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References

In the animal research study by Abi Raad et al, near-term fetal sheep were subjected to 30 minutes of cerebral ischemia, produced by 4-vessel occlusion. Before and after cerebral ischemia, parietal electrocorticographic activity and cortical impedance were monitored continuously. Regional cerebral blood flow was measured with radioabeled microspheres, and extracellular glucose and lactate were measured via microdialysis. As in their previous investigations, 3 distinct phases of recovery from ischemia are described, including an immediate reperfusion phase (+10 minutes), a latent phase (+1.5 to 4.5 hours), and a delayed phase (+12 to 24+ hours). The most prominent findings include (1) a transiently impaired reperfusion in the immediate phase; (2) a hyperperfusion in the delayed phase; (3) initial suppression of electrocorticographic activity during the early and latent phase, followed by increased activity during the delayed phase; (4) increasing cortical impedance during the delayed phase; and (5) increases in extracellular glucose and lactate during the latent and delayed phases, respectively. Tissue brain damage, assessed histologically at 72 hours of recovery, was focused predominantly on cerebral cortex and hippocampus, with lesser injury in the brain stem and cerebellum.

There are no concerns regarding the experimental design, as the methodologic procedures are reasonable and provide information regarding the cerebrovascular and metabolic responses during recovery from cerebral ischemia in fetal sheep. The division of the recovery interval into 3 phases is reasonable, as the delayed phase appears to correspond with the onset of electrically and clinically apparent epileptiform activity, during which further cerebrovascular and metabolic alterations would be expected to occur. Therefore, it is likely, as the authors suggest, that the hyperperfusion during the delayed phase is the consequence of epileptiform activity. As also has been shown in many animal models, epileptiform activity is associated with increased metabolic demand of brain tissue, with consequent increases in cerebral glucose utilization and tissue lactate formation. Therefore, it is not surprising that during the delayed phase extracellular lactate increases but with a peak value which corresponds more closely to maximal cortical impedance than to maximal electrocorticographic activity. Whether or not the delayed lactate accumulation represents ongoing tissue anaerobic glycolysis is a result of increasing cerebral edema cannot be ascertained from the present study in the absence of
measurements of cerebral glucose utilization or other metabolite concentrations.

The data also show increased extracellular glucose concentrations primarily during the latent phase of recovery from cerebral ischemia. The peak glucose concentrations occur long before the secondary peak in lactate. The authors suggest that this finding is representative of an impairment in oxidative phosphorylation in association with an upregulation of the blood-brain barrier glucose transporter protein. An equally plausible explanation is that during the early and latent phase of recovery, tissue glucose is spared by an inhibition of glycolysis, with tissue lactate—which is also slightly increased—becoming the prominent fuel for oxidative phosphorylation. Under such circumstance, oxidative phosphorylation would proceed normally; any impairment should be associated with alterations in high energy reserves, which were not measured in the present study.

In their summary paragraph, the authors state that the “marked hyperperfusion during the delayed phases of injury is likely to play a protective role through enhancing nutrient delivery and metabolic waste removal from marginally metabolically viable tissue.” A protective role seems unlikely, because the hyperperfusion appears to coincide with maximal epileptiform activity and at least extracellular lactate accumulation. It is difficult to envision how epileptiform activity and its associated cerebrovascular and metabolic alterations would be protective to a penumbral area of ischemic tissue. Indeed, the authors themselves have suggested that epileptiform activity contributes to ischemic brain damage (authors’ reference 5).

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