Frequent Episodes of Brief Ischemia Sensitize the Fetal Sheep Brain to Neuronal Loss and Induce Striatal Injury

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ABSTRACT. We examined the neuronal consequences of repeated brief episodes of *in utero* cerebral hypoxia-ischemia. Chronically instrumented fetal sheep were subjected to three 10-min episodes of reversible cerebral ischemia, repeated at either 1-h ($n = 8$) or 5-h ($n = 5$) intervals. Four fetuses were subjected to a single 10-min of ischemia and 17 fetuses to a single 30-min of ischemia. Repeated insults altered the distribution of damage with relatively marked striatal injury compared with isolated episodes of ischemia ($p < 0.01$). Frequent insults were associated with greater neuronal loss ($p < 0.01$) and a failure to restore electrocorticographic activity and resolve cortical cytotoxic edema between insults. Intermittent cortical hyperexcitability developed after repeated insults irrespective of the interval between insults. These findings emphasize that repeated brief episodes of ischemia alter the distribution of damage and sensitize the fetal brain to neuronal injury, particularly if the episodes are frequent. Striatal damage may be a feature of multiple but not single insults. (*Pediatr Res* 33: 61–65, 1993)

Abbreviations

ECoG, electrocorticogram
dB, decibels

Epidemiologic studies have often failed to find a clear association between isolated episodes of fetal asphyxia and neurologic outcome in the newborn (1, 2). This discrepancy may be due, in part, to multiple brief episodes of *in utero* asphyxia being unrecognized but more common than major and identifiable single events. However, few experimental studies have considered the neurologic consequences of multiple insults. Limited evidence from studies in the adult rat and gerbil suggests that brief episodes of ischemia repeated during the period of postischemic hypoperfusion may produce additive degrees of neuronal damage in the cortex, hippocampus, and caudate nucleus and vasogenic edema (3, 4). We have previously shown that the parasagittal cortex and hippocampus are particularly vulnerable to both brief (10-min) and prolonged (30- to 40-min) single episodes of cerebral ischemia or global asphyxia in the fetal sheep (5, 6). However, despite frequent clinical observations of striatal damage with perinatal encephalopathy (7), neither cerebral ischemia nor global asphyxia induced predominantly striatal patterns of injury.

The objective of the study presented here was to determine whether multiple brief episodes of ischemia sensitize the fetal

brain to injury or alter the pattern of damage compared with isolated insults and whether the length of the interval between insults is of importance. We have previously described an experimental preparation of transient cerebral ischemia in the chronically instrumented fetal sheep that induces encephalopathy similar to hypoxic-ischemic brain damage observed in some asphyxiated term infants (8). Therefore, we investigated the electrophysiologic and histologic consequences of isolated and brief episodes of reversible cerebral ischemia repeated at either 1 or 5 h. Fetal peripheral lactate and glucose levels were measured. We demonstrate that repeated insults, particularly if frequent, are associated with an altered distribution of neuronal loss compared to isolated insults, the striatum being particularly sensitive. The frequency of insults appear to have a major effect on the severity of outcome.

MATERIALS AND METHODS

These studies were approved by the Animal Ethical Committee of the University of Auckland. Thirty-four pregnant ewes with fetuses at 119–127 d of gestation underwent operation under 2% halothane/oxygen general anesthesia as described previously (8). Briefly, polyvinyl catheters were placed in each fetal axillary artery and in the amniotic cavity. Through burr holes, two pairs of ECoG electrodes (5 and 15 mm anterior and 10 mm lateral to bregma) and one pair of stimulating electrodes to measure cortical impedance (10 mm anterior and 15 mm lateral of bregma) were placed extradurally over the parasagittal cortex. To restrict cerebral vascular supply to the carotid arteries only, the vertebro-occipital anastomoses between the carotid arteries and vertebral arteries were ligated bilaterally. Two pairs of inflatable cuffs were placed around the carotid arteries.

Studies commenced 3 d after surgery. Fetal ECoG and impedance signals were recorded as previously described (5). Briefly, ECoG spectrum and cortical impedance were collected in real time for 12 h before occlusion and for 72 h afterward. Cortical impedance measurements can be used to estimate the changes in extracellular space that occur concomitantly with cytotoxic edema (9). Reversible cerebral ischemia was achieved by inflating the carotid cuffs bilaterally with sterile saline for a defined period and then reversing by deflation. Fetal blood samples were drawn immediately before and after each occlusion and at postmortem and analyzed for pH, oxygen content, PCO_2 , lactate, and glucose.

Four protocols were followed. In the first protocol, three 10-min carotid artery occlusions were performed at 1-h intervals (3×10 min, 1 h), whereas in the second protocol, three 10-min occlusions were repeated at 5-h intervals (3×10 min, 5 h). Fetuses were randomly assigned to either protocol. In the third protocol, a single 10-min episode of ischemia was induced, and in the fourth protocol, a single 30-min episode of ischemia was induced. Data from the single 10-min and 30-min occlusions have been reported elsewhere (5). In the present study, only histologic outcome from these latter fetuses was considered, as

Received May 26, 1992; accepted August 31, 1992.

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Supported by a program grant from the Health Research Council of New Zealand.

the configuration of electronic processing units previously used does not allow a valid direct comparison. Eight fetuses underwent the first protocol (3×10 min), 1 h, three of which died 60–65 h after the last occlusion due to proven infection. These three fetuses were excluded from final ECoG measurements and histologic analysis. Five other fetuses were subjected to the second protocol (3×10 min, 5 h), four fetuses to a single 10-min episode, and 17 fetuses to a single 30-min episode of ischemia.

Three d after the last occlusion, fetuses were killed by an overdose of sodium pentobarbitone, and the fetal brain was prepared for histologic analysis as previously described (5). Briefly, the brain was perfused *in situ* with formaldehyde, acetic acid, and methanol (1:1:8), paraffin-embedded, and sectioned at 8 μ m. Histologic slides were stained with thionin and acid fuchsin.

Analysis. ECoG intensity was calculated as previously described and normalized with respect to the 12-h reference period (10). Suppression of ECoG intensity postinsult was defined as activity below -5 dB relative to the reference period. Final intensity measurements were based on the last 4 h of the experiment. Hyperexcitability was defined as intensity at least 5 dB above baseline. Cortical impedance was extracted from the ECoG signal, and estimated changes in extracellular space were derived from these measurements as previously described (9). Impedance was normalized and expressed as a percentage of baseline.

Neuronal loss was determined under light microscopy by two independent assessors, one of whom was blind to the experiment. The correlation of neuronal loss scores between the two assessors was $r^2 = 0.92$. Neurons showing ischemic cell change with acidophilic (red) cytoplasm and contracted nuclei were considered dead (5). Damage scores from predetermined regions were as follows: 0 = no dead neurons, 5 = 1–10%, 30 = 11–50%, 70 = 51–90%, 95 = 91–99%, and 100 = 100% dead neurons.

Histologic scores, cortical extracellular space, lactate and glucose measurements, and final 4-h ECoG recordings were subjected to analysis of variance. Region and time were treated as repeated measures as appropriate. When overall differences were found, between-group comparisons were made using the Newman-Keul's multiple comparison test. All data are presented as mean $\pm$ SEM.

RESULTS

3×10 Min ischemia separated by 1 h. During each occlusion, the ECoG became isoelectric, and ECoG activity remained suppressed between occlusions (Fig. 1). ECoG suppression lasted for 5 ± 0.7 h after the initial occlusion. Hyperexcitability developed 9 ± 3 h after the initial occlusion and lasted for 29 ± 8 h. Two animals developed continuous seizure activity, whereas the other six showed discrete episodes of hyperexcitability. By 3 d, ECoG intensity was -4 ± 2 dB, which was not different from baseline.

Cortical impedance increased during each occlusion (Fig. 2). After recirculation, the impedance recovered toward preinsult values but did not fully resolve before the second and third occlusion. Thus, there was an increase in impedance with time, each occlusion resulting in a further increase, which was also reflected in loss of estimated extracellular space ($p < 0.01$, Fig. 3). Cortical impedance returned to baseline after the third occlusion, after which there were no changes except in the two animals with continuous seizure activity that was associated with a secondary rise in impedance.

A progressive metabolic acidosis developed with the occlusions (Table 1). Glucose levels increased after the first occlusion and then stabilized at that level during the second and third occlusions (Table 2). No changes were observed in oxygen content or PCO_2 during the experiment.

The distribution of neuronal loss is shown in Figure 4. Overall neuronal loss was similar to that in fetuses subjected to a single 30-min episode of ischemia ($p = 0.39$), but the distribution of damage differed ($p < 0.001$, Fig. 5). Striatal damage was signifi-

EEG intensity (dB)

Fig. 1. Typical examples of the ECoG intensity changes during and after 3×10 min of ischemia repeated every 1 h (upper panel) and every 5 h (lower panel). The first occlusion is at time zero in both experiments.

cantly increased ($p < 0.01$) and parasagittal cortical damage was significantly decreased ($p < 0.01$) compared with a single 30-min episode of ischemia.

3×10 Min ischemia separated by 5 h. After an isoelectric ECoG during each occlusion, ECoG returned to baseline between insults (Fig. 1). In four of five animals, discrete episodes of hyperexcitability, similar to those in animals subjected to the 3×10 min, 1 h protocol, developed 8 ± 2 h after the first occlusion with a duration of 44 ± 9 h. Final ECoG activity (-0.3 ± 1 dB) was not different from baseline or that in the 3×10 min, 1 h protocol.

There was an increase in cortical impedance during each occlusion, with all three insults resulting in similar increases (Fig. 2). After the third occlusion, cortical impedance returned to baseline, and there was no change in impedance throughout the rest of the experiment. No changes in metabolic parameters were observed (Tables 1, 2).

Overall neuronal loss was slight (Fig. 4) and was similar to that resulting from a single 10-min episode of ischemia ($p = 0.48$, Fig. 5), except for marked striatal damage. Neuronal loss after the 3×10 min, 5 h protocol was less severe than that after the 3×10 min, 1 h protocol ($p < 0.01$) but showed a similar distribution, with striatal damage being predominant in both protocols.

1×10 min ischemia. The electrophysiologic and histologic data are reported in detail elsewhere (5). The distribution of neuronal loss is shown in Figure 5. No postinsult hyperexcitability or prolonged disturbances in cortical impedance or ECoG intensity were observed.

1×30 min ischemia. The electrophysiologic and histologic data are reported elsewhere (5). A period of postischemic continuous seizure activity associated with a secondary rise in cortical impedance was observed. We have previously reported a loss of ECoG intensity of -10 ± 5 dB 3 d after injury (5). The distribution of neuronal loss is shown in Figure 5.

Cortical Impedance (%)

Fig. 2. Changes in cortical impedance relative to baseline (100%) after 3×10 min of transient cerebral ischemia repeated at 1-h (upper panel) and 5-h (lower panel) intervals. Time zero indicates the first occlusion.

Extracellular space (%)

Fig. 3. Maximum decrease of estimated extracellular space during each occlusion relative to baseline (100%). Changes in estimated extracellular space are derived from cortical impedance measurements. ■, 3×10 -min insults, 1 h apart; □, 3×10 -min insults, 5 h apart. **, $p < 0.01$ compared with the first insult.

Table 1. Fetal arterial lactate levels (mmol/L)

	Preinsult 1	Preinsult 2	Preinsult 3
3×10 min, 1 h apart	0.9 ± 0.06	$2.0 \pm 0.2^*$	$2.6 \pm 0.4^*$
3×10 min, 5 h apart	0.8 ± 0.09	0.9 ± 0.2	1.2 ± 0.2

* $p < 0.01$ compared with the first insult.

Table 2. Fetal arterial glucose levels (mmol/L)

	Preinsult 1	Preinsult 2	Preinsult 3
3×10 min, 1 h apart	1.0 ± 0.09	$1.5 \pm 0.1^*$	$1.5 \pm 0.1^*$
3×10 min, 5 h apart	0.9 ± 0.07	1.0 ± 0.1	1.2 ± 0.1

* $p < 0.01$ compared with the first insult.

Region

Fig. 4. Distribution of histologic damage in fetuses subjected to 3×10 -min insults at 1-h (upper panel) or 5-h (lower panel) intervals. Animals were killed 72 h after the last insult. PsCx, Parasagittal cortex; LtCx, lateral cortex; Str, striatum; DG, dentate gyrus; CA4, CA4 region of the hippocampus; CA3, CA3 region of the hippocampus; CA1/2, CA1 and CA2 regions of the hippocampus; Thal, thalamus; and Amg, amygdala.

DISCUSSION

Choreoathetosis secondary to striatal injury is one of the classic syndromes of cerebral palsy and is presumed to be a result of striatal injury in the perinatal period (7). However, despite its clinical importance, little is known about the etiology of such perinatal injury (11). Attempts to correlate pregnancy risk factors with the various patterns of cerebral palsy have not been successful (12). Similarly, attempts to relate immediate perinatal events to the different clinical outcomes have not been elucidatory.

After repeated short episodes of ischemia, irrespective of the

Fig. 5. Histologic outcome in selective regions 3 d after transient cerebral ischemia. *PsCx*, Parasagittal cortex; *Str*, striatum; and *CA3*, CA3 region of the hippocampus. ■, 1 × 30 min; □, 3 × 10 min, 1 h apart; ▨, 1 × 10 min; ▩, 3 × 10 min, 5 h apart. **, $p < 0.01$ compared with a single 30-min episode of ischemia.

interval between insults, striatal damage was a dominant histologic finding. In contrast with these results, we have previously shown that a single 10-min episode of ischemia in the fetal sheep causes trivial cerebral damage, whereas an isolated, more prolonged episode of ischemia (30 min) results in severe neuronal loss in the hippocampus and infarction of the parasagittal cortex but little damage in the striatum (5, 13). Although these previous studies used the same protocol, it is possible that conditions were not identical, which may have influenced the outcome. In other experimental approaches to isolated episodes of *in utero* asphyxia, such as umbilical cord occlusion, we have demonstrated particular vulnerability of the hippocampus (6). Therefore, it appears that the distribution of damage may be dependent on the temporal sequencing as well as the specific nature of the insult. Marked striatal damage may be a feature of multiple, but not isolated, *in utero* insults.

A number of mechanisms have been implicated in hypoxic-ischemia cell loss, including intracellular sodium and water accumulation leading to lysis, intracellular calcium accumulation, and toxic effects of excitatory amino acids (14). Injury to hippocampal neurons after ischemic insults has been associated with excessive release of glutamate (15). However, whether similar mechanisms apply to striatal damage after ischemia is not clear. In developing rats, the striatum and hippocampus contain a high density of postsynaptic glutamate receptors (16). Immediately after severe global asphyxia in exteriorized fetal sheep, the concentration of extracellular excitatory amino acids increases markedly, particularly in the striatum (17). Interactions between corticostriatal glutamatergic pathways and nigrostriatal dopaminergic afferents have also been suggested to play a role in postischemic striatal damage (18). Creating lesions in the substantial nigra

prevented the marked postasphyxial increase in striatal dopamine and attenuated striatal neuronal loss (19). Striatal neurons, particularly small- to medium-sized ones, have been shown to attain ischemic cell change more rapidly than cortical and hippocampal neurons (20). This early degeneration of small- to medium-sized striatal neurons has been related to changes in striatal dopamine neurohistochemistry (21). It may be that repeated insults enhance these early ischemic cell changes, sensitizing striatal neurons in particular.

The magnitude of neuronal loss after multiple episodes of ischemia in the present study was related to the interval between occlusions, cerebral damage being greater after more frequent insults. Three 10-min insults repeated at 1-h intervals showed an amount of overall damage similar to that in a single 30-min ischemic insult, whereas three 10-min insults 5 h apart resembled a single 10-min ischemic insult. These results are similar to those in studies of adult rats demonstrating more damage in the hippocampus after three 3-min four-vessel occlusions delivered hourly than after a single 9-min occlusion (4). Similarly, repeated cerebral ischemia in the adult gerbil caused greater neuronal damage after three 5-min episodes of ischemia at 1-h intervals than 15 min of ischemia produced as a single insult (3). The increased damage in those experiments was suggested to result from repeated insults coinciding with the time of postischemic cerebral hypoperfusion (3), although other mechanisms must be considered. For example, the cytotoxic milieu, as a result of free radical production, excitatory amino acid and Ca^{2+} release, and changes in extracellular volume may place the neuron in a more vulnerable state when episodes of ischemia are repeated more frequently. Conversely, homeostatic mechanisms, including the release of inhibitory neuromodulators such as adenosine and the gradual resolution of cytotoxic accumulation, may mean that greater protection is offered by less frequent insults. Indeed, infrequent episodes of ischemia in the adult gerbil have been shown to be neuroprotective (22).

Postischemic hypoperfusion has been related to cerebral edema in the adult rat (23), and vasogenic edema was found 24 h after ischemic insults repeated at 1-h intervals in gerbils (3). However, these studies did not measure the acute cytotoxic edema formation during insults. In the fetal sheep, we have previously reported transient loss of extracellular space due to cytotoxic edema during ischemic insults (9). The intracellular swelling is understood to result from increased Na^+ and Cl^- influx and uptake of osmotic water (24). *In vitro* studies of hippocampal cells have suggested that cellular swelling plays a role in neuronal death (25). Our data show that frequently repeated insults lead to progressively worse cytotoxic edema during the insult period. The exact neuronal consequences of this phenomenon are unclear but are likely to reflect longer-lasting defects in cellular integrity. This incomplete restoration of membrane function could leave the neuron more sensitive to the ion and water fluxes associated with the frequently repeated insults.

We found that there was a prolonged depression of ECoG activity after occlusions at 1-h intervals, whereas ECoG activity recovered between insults when they were separated by 5 h. After isolated transient ischemic insults in the fetal sheep, prolonged ECoG depression is associated with cortical injury (5). Although the underlying mechanisms are not well understood, depression of ECoG activity reflects loss of electrical activity. Electrical activity and trophic factors such as nerve growth factor and IL-1 have been suggested to regulate neuronal survival *in vitro* (26, 27). We have recently shown that other neurotrophic factors such as IGF-I are induced after hypoxic-ischemic injury in the rat brain and that postasphyxial central administration of IGF-I is neuroprotective (28). Possibly, neuronal loss in the present study was diminished after less frequent insults because the ECoG activity recovered between insults, allowing for induction of neurotrophic action.

We have previously shown that continuous seizure activity after 30 min of ischemia in the fetal sheep is associated with

infarction of the underlying parasagittal cortex (8) and that intermittent hyperexcitability develops after uterine artery occlusion only when mild to moderate neuronal loss is found in the parasagittal cortex (29). However, suppression of these seizures with an N-methyl-D-aspartate receptor antagonist, MK-801, improves neuronal outcome in the lateral cortex and the hippocampus but not in the parasagittal cortex (13). Similarly, in this study, discrete episodes of hyperexcitability were associated with mild to moderate degrees of cortical neuronal loss after repeated insults.

Metabolic factors have long been considered to have some effect on outcome of cerebral hypoxic-ischemic insults. Systemic hyperglycemia before ischemia is known to worsen outcome in adult rats (30) but may be neuroprotective in immature rats (31). Similarly, elevated systemic lactate has long been thought to play a role in the development of ischemic brain damage in monkeys (32). We observed increased plasma lactate and glucose levels after the initial occlusion, and these changes were more pronounced in the protocol with 1-h intervals. However, the peripheral acidosis and hyperglycemia observed and presumed to reflect cerebral concentrations were only mild, and it is doubtful that such small changes could have a central role in the differences in outcome found in this study.

The present study shows that brief episodes of ischemia, repeated before full recovery of ECoG and intracellular edema, sensitize neurons to damage. Repeated insults, independent of the interval between the insults, alter the pattern of brain injury. This implies that the pattern rather than the nature of the insult may be important in certain neuropathologies. In particular, striatal damage appears to be a feature of multiple but not single insults. These observations offer at least a partial explanation of epidemiologic difficulties linking perinatal events with subsequent outcome.

Acknowledgment. The authors thank Dr. B. M. Johnston for her helpful advice.

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