Key Neuroprotective Role for Endogenous Adenosine A<sub>1</sub>
Receptor Activation During Asphyxia in the Fetal Sheep

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Background and Purpose—The fetus is well known to be able to survive prolonged exposure to asphyxia with minimal injury compared with older animals. We and others have observed a rapid suppression of EEG intensity with the onset of asphyxia, suggesting active inhibition that may be a major neuroprotective adaptation to asphyxia. Adenosine is a key regulator of cerebral metabolism in the fetus.

Methods—We therefore tested the hypothesis that infusion of the specific adenosine A<sub>1</sub> receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), given before 10 minutes of profound asphyxia in near-term fetal sheep, would prevent neural inhibition and lead to increased brain damage.

Results—DPCPX treatment was associated with a transient rise and delayed fall in EEG activity in response to cord occlusion (n=8) in contrast with a rapid and sustained suppression of EEG activity in controls (n=8). DPCPX was also associated with an earlier and greater increase in cortical impedance, reflecting earlier onset of primary cytotoxic edema, and a significantly smaller reduction in calculated cortical heat production after the start of cord occlusion. After reperfusion, DPCPX-treated fetuses but not controls developed delayed onset of seizures, which continued for 24 hours, and sustained greater selective hippocampal, striatal, and parasagittal neuronal loss after 72-hour recovery.

Conclusions—These data support the hypothesis that endogenous activation of the adenosine A<sub>1</sub> receptor during severe asphyxia mediates the initial suppression of neural activity and is an important mechanism that protects the fetal brain.

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Key Words: adenosine ■ antagonists and inhibitors ■ asphyxia ■ fetus ■ ischemia ■ seizures ■ sheep

Since the early 17th century, investigators have observed that fetal and neonatal animals are remarkably tolerant to hypoxia. More important, not only do perinatal animals survive longer than adult animals, but they also tolerate cerebral ischemia or systemic asphyxia without damage for far longer. The precise mechanisms, however, are still unclear. We and others have observed that EEG intensity is rapidly suppressed at the onset of asphyxia in both the fetus and newborn. This suppression could be the result of profound tissue hypoxia, with depletion of energy stores induced by rapid diversion of deoxygenated blood to the brain. However, several lines of evidence suggest that this suppression is an actively mediated protective response and involve inhibitory neuromodulators, particularly adenosine, as likely mediators of this suppression. It is striking that studies by Duffy and coworkers in the asphyxiated newborn dog demonstrated maintenance of cerebral high-energy phosphates and ATP levels for ≈8 minutes after induction of asphyxia even though the EEG became isoelectric within 2 minutes, consistent with active suppression of brain metabolism.

This early suppression is likely mediated by inhibitory neurotransmitters such as adenosine. Adenosine is known to be an important regulator of metabolism and blood flow. The rise in intracerebral concentrations of adenosine during hypoxia is associated with an increase in local cortical blood flow and a decrease in whole-body oxygen consumption in the fetal sheep. Microdialysis studies in the fetal sheep have shown that, in contrast to adult species during ischemia or asphyxia, there is a greater increase in extracellular levels of inhibitory than in excitatory neurotransmitters. Furthermore, adenosine levels in the perinatal brain increase during hypoxia-ischemia well before anoxic depolarization begins, in contrast to amino acid neurotransmitters, which rise only after depolarization. Critically, adenosine receptor blockade prevents hypoxia-induced suppression of electrical activity in rat striatal and hippocampal slices, suggesting that adenosine plays a central role in hypoxic neuronal suppression.

In adult animals, the neuroinhibitory actions of adenosine are mediated primarily through both presynaptic and postsynaptic

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adrenosine A₁ receptors. Administration of adenosine A₁ agonists in focal or global ischemia is generally protective, whereas administration of A₁ antagonists increases neuronal loss. However, there is only limited and contradictory evidence from studies involving the neuroprotective effect of adenosine during hypoxia-ischemia in developing animals.

The rapid suppression of EEG intensity that occurs at the onset of severe asphyxia in the fetal sheep suggests an adaptive suppression of neuronal activity that may be mediated by activation of the adenosine A₁ receptor. Such a mechanism may be a major factor contributing to the profound hypoxic tolerance of the fetal brain to hypoxia-ischemia. Therefore, we tested the specific hypothesis that blockade of the A₁ receptor during asphyxia with the specific antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) would prevent neural inhibition and increase subsequent neuronal injury in a well-characterized model of global asphyxia in the late-term fetal sheep.

### Materials and Methods

#### Surgical Procedures

Sixteen fetal sheep (gestation, 118 to 126 days) were operated on using sterile techniques under halothane anesthesia (2%). Through a midline abdominal incision, the uterus was opened. ECG electrodes were placed to record heart rate. Polyvinyl catheters were inserted into each axillary artery, an auxiliary vein, and the amniotic space for recording of pressure, blood sampling, and drug infusions. A reversible inflatable occluder (In Vivo Metric) was placed around the umbilical cord. Two pairs of EEG electrodes (AS633-5SSF, Cooner Wire Co) were placed on the dura bilaterally over the parasagittal parietal cortex (5 and 10 mm anterior to the bregma and 5 mm lateral). To record cortical impedance, a third pair of electrodes (AS633-3SSF, Cooner Wire Co) was placed on the dura 5 mm lateral to the EEG electrodes. A catheter was placed in the sagittal sinus to measure oxygen content.

In a composite probe, consisting of a laser Doppler channels, a Po2 electrode, and a thermocouple was placed in the right parietal cortex approximately 5 mm lateral to the midline and 5 mm posterior to the coronal suture, to a depth of 5 mm below the dura. A 3-mm ultrasonic flow probe (Transonic Systems Inc) was placed on the right carotid artery near the angle of the jaw. A thermocouple (IT-18 thermometer, Physitemp) was inserted into the lingual artery to measure arterial blood temperature.

After surgery, ewes were allowed to recover for 3 days before fetal recordings were begun. The study was approved by the Animal Ethics Committee of the University of Auckland.

#### Recordings

Fetal arterial blood pressure corrected for amniotic fluid pressure, carotid arterial flow (T208 Ultrasonic Flowmeter, Transonic Systems Inc), local cortical blood flow (laser Doppler flowmetry, Oxyflow, Oxford Optronics Inc), cortical oxygen tension (Oxylite, Oxford Optronics Inc), fetal cortical temperature (Oxylite, Oxford Optronics Inc), lingual arterial temperature, and fetal parietal EEG and impedance were recorded continuously (256 Hz). The EEG signal was low-pass filtered at 30 Hz, and then the intensity spectrum and impedance signal were extracted as reported previously. The total EEG intensity (power) was normalized by log transformation (dB; 20 log [intensity]).

Cortical impedance was measured with a 2-electrode technique. The impedance of a tissue rises concomitantly as cells depolarize and fluid shifts from the extracellular to the intracellular space; thus, impedance is a measure of cytotoxic edema. Carotid blood flow was measured as an index of changes in global cerebral blood flow, whereas laser Doppler signals were taken as an index of local cortical flow.

### Brain Heat Production With the Fick Principle

Heat production of the cortex was calculated as an index of local cerebral metabolism with the Fick principle. The difference between the temperature of arterial blood (Tbrain) supplying the brain and the brain tissue itself (Tbrain) gives the temperature increase resulting from brain metabolism. Multiplying this difference by blood flow provides the heat production in the local region of the brain in which the laser Doppler probe and temperature sensor are placed. The following is the equation for heat production (cal in min⁻¹): heat flow = (Tbrain - Tblood) × SHblood. Where SHblood is specific heat of blood (0.89 cal · mL⁻¹ · °C⁻¹). Because laser Doppler measures relative changes in cerebral blood flow, heat production is expressed as percent change from baseline.

#### Experimental Protocol and Drug Treatment

DPCPX was dissolved in 0.1N NaOH (2.5 mg/mL). Fetuses were randomized to receive either vehicle (n=8) or DPCPX (n=8) infused into the right brachiocephalic vein at a rate of 3.6 mg · min⁻¹ for 10 minutes and 0.75 mg · min⁻¹ for 60 minutes, a dosage regimen known to block the bradycardia induced by cyclopentyl adenosine, a selective adenosine A₁ agonist. After infusion for 60 minutes, asphyxia was induced by complete occlusion of the umbilical cord for 10 minutes. Infusions were discontinued at the end of the occlusion period.

#### Histological Analysis

Three days after the asphyxial insult, the sheep were killed with an intravenous overdose of pentobarbital (3.5 g), and histological analysis was performed. The fetal brain was perfusion-fixed in situ with 0.9% saline solution followed by 500 mL of 10% phosphate-buffered formalin. Neuronal loss was scored by light microscopy on 8-μm-thick coronal sections stained with thionin and acid fuchsins by an assessor blinded to treatment group. The proportion of neurons showing ischemic cell change in preassigned areas was scored on a 0-10 scale: 0 = no dead neurons; 5 = 50% to 90%; 7 = 70% to 100%; 9 = 90% to <100%; and 10 = 100% dead neurons. Average scores were calculated for each region.

#### Data Analysis

The effect of DPCPX on histology and continuous variables such as cortical blood flow were evaluated by 2-way analysis of variance. Cerebral region and changes over time were treated as repeated measures. When an effect of time or treatment was found, data were compared with baseline through the use of Dunnett’s post test. Data are shown as mean±SEM.

#### Results

The mean gestational age of the animals at time of experiment was 123±1 days for controls and 125±1 days for the DPCPX group. Fetal weight at autopsy was 3.35±0.21 kg in the control group and 3.19±0.22 kg in the treatment group (P=NS).

#### Effect of Infusion of DPCPX or Saline on Baseline Cardiovascular Values

Infusion of vehicle or DPCPX was not associated with any significant change in blood pressure, heart rate, local cortical blood flow, or carotid blood flow compared with the baseline period. Infusion of DPCPX but not vehicle was associated with a small rise in plasma lactate from 0.68±0.05 to 1.17±0.1 mmol/L (P<0.01) but no change in arterial pH, Po2, PCO2 (the Table), or glucose (data not shown).

#### Blood Gases and Tissue Po2

Initiation of cord occlusion was associated with severe fetal acidosis, hypercapnia, and hypoxemia that were not different
between groups (the Table) and a reduction in cortical Po2 from baseline values in both groups to essentially 0 mm Hg (P<0.01; Figure 1). Immediately after termination of occlusion, cortical Po2 in both groups increased transiently above baseline (Figure 1).

**Hemodynamics**

There were no significant differences between control or DPCPX-treated fetuses in the pattern of changes in mean arterial blood pressure, heart rate, local cortical blood flow, and carotid blood flow before, during, or after occlusion (Figure 2). In both groups, occlusion of the umbilical cord was followed by sustained bradycardia, with initial hypertension followed by a progressive fall in blood pressure. After release of the occluder, there was a rapid, transient overshoot increase in blood pressure in both groups (Figure 2). Cortical blood flow fell similarly in both groups (from 100±4% to 61±6% in control and from 103±5 to 65±5% in fetuses treated with DPCPX; P<0.01 versus baseline) (Figure 2). Similarly, there was no difference in the pattern of changes in carotid blood flow (Figure 2).

**EEG, Impedance, and Cortical Heat Production**

In control fetuses, occlusion was associated with rapid suppression of EEG activity by 2 minutes. In contrast, after DPCPX treatment, occlusion was associated with a sharp elevation of EEG intensity in the first minute (P<0.01), followed by a slower reduction over 5 minutes (Figure 3 and 3b). The cortical impedance of the DPCPX-treated animals began to rise more rapidly than in controls and reached a greater peak amplitude (P<0.01; Figure 3a). There was a rapid decline in cortical heat production after initiation of cord occlusion in controls that was significantly attenuated by DPCPX treatment (28.1±5.9% versus 42.9±2.6% of baseline; P<0.01; Figure 4).

**Electrophysiological Recovery**

In control fetuses, EEG intensity recovered progressively to baseline values over 6 to 8 hours after asphyxia. In contrast, all DPCPX-treated fetuses but no controls developed delayed-onset seizures, characterized by stereotypic high-voltage/low-frequency activity with increases in nuchal muscle activity, heart rate, blood pressure, local cortical blood flow, and decreased local cortical tissue Po2 lasting for up to 10 minutes. These acute cortical seizures developed 6 to 12 hours after release of occlusion and in all cases resolved by 24 hours. Final EEG intensity at 72 hours was reduced in the DPCPX group compared with controls (−3.5±0.7 versus −0.4±0.2 dB; P<0.01).
Histology

There was a significant overall increase in neuronal loss in the DPCPX group (P<0.01; Figure 5). In the DPCPX group, significant increases in neuronal loss were seen in the Cornu Ammonis hippocampal regions, parasagittal cortex, and striatum (Figure 5).

Discussion

The present study demonstrates for the first time that early suppression of EEG activity is a central aspect of hypoxic tolerance in the perinatal brain and that this suppression is mediated by activation of the adenosine A1 receptor. Administration of the adenosine A1 receptor antagonist DPCPX was associated with an acute increase in and then delayed suppression of the EEG in contrast with rapid suppression in controls during cord occlusion (Figure 3). This delayed EEG suppression was associated with evidence of earlier and greater hypoxic cortical depolarization, as shown by cortical impedance, and with attenuated suppression of cortical heat production (Figures 3 and 4). These acute changes were followed by evidence of greater cerebral damage with severe electrographic and clinical seizures, reduced recovery of EEG intensity, and greater neuronal loss after 72 hours of recovery. The effect of the adenosine receptor blockade was not due to...
hypotension, impaired cerebral perfusion, or reduced oxygen delivery (Figure 2).

The fetus and newborn are able to withstand short periods of hypoxia-ischemia that would be severely injurious to the brain of adults of the species. Whereas the fetus can fully maintain oxidative metabolism during moderate hypoxia by increasing blood flow and oxygen extraction by the brain, during profound asphyxia, oxygen metabolism cannot be maintained because of the concomitant profound fall in tissue oxygen availability, whereas cortical blood flow increases only modestly and transiently (Figures 1 and 2). Therefore, during profound hypoxia, additional mechanisms to reduce metabolic demands would seem to be required to prevent injury.

We and others have consistently reported that the EEG activity of perinatal animals is rapidly suppressed with severe hypoxia; however, it has been unclear whether this suppression is a direct effect of profound tissue hypoxia or an active suppression mediated by inhibitory neuromodulators such as GABA and adenosine. The present work extends this finding by showing that this depression is initially mediated by adenosine A1 receptors and can be blocked for a period despite rapid-onset, profound tissue hypoxia. This finding has striking parallels in the adults of vertebrate species such as the freshwater aquatic turtle that can tolerate severe hypoxia that is mediated by adenosine A1 receptor activation and other inhibitory amino acids.

The accelerated rise in impedance during asphyxia in the DPCPX group in the present study supports this interpretation. Because a rise in cortical impedance reflects cytotoxic edema, this denotes a more rapid development of hypoxic cellular depolarization, probably because of accelerated depletion of energy reserves by anaerobic metabolism (Figure 3). Similarly, cortical heat production was rapidly suppressed in control animals, strongly suggesting a regulated reduction in brain metabolism (Figure 4). This fall was markedly blunted in DPCPX-treated animals, consistent with loss of adenosine-mediated suppression of cerebral metabolism. These data are consistent with previous data in newborn dogs showing that cerebral ATP levels can be maintained during asphyxia for several minutes after the EEG becomes isoelectric.

Impaired acute adaptation to asphyxia was followed by histological and electrophysiological evidence of increased injury in the DPCPX group. All the DPCPX animals demonstrated delayed postasphyxial seizures after cord occlusion. Seizures are well recognized clinically as a marker of asphyxial damage. We have previously shown that complete suppression of postischemic seizures with the potent N-methyl-D-aspartate antagonist MK-801 has only a limited effect on cell loss in the near-term fetal sheep. Thus, although it is possible that the delayed seizures may have potentiated the primary injury caused by asphyxia, these seizures overall are likely a reflection of injury, not the primary cause.

There have been only limited and conflicting previous studies of the role of A1 receptor activation in the perinatal brain. Aden et al reported that direct administration of an adenosine A1 agonist in 7-day-old rats during hypoxia-ischemia was not neuroprotective. These investigators found impaired coupling between the adenosine A1 receptor and its G protein second messenger in the neonatal rat, suggesting an important developmental species difference. Furthermore, adenosine led to profound bradycardia, which could compromise cerebral perfusion. Bona et al found that pretreatment with DPCPX did not affect hypoxic-ischemic brain damage in 7-day-old rats; however, there was a very high mortality associated with the DPCPX vehicle (Tween 80). In contrast, Halle et al found that increasing the strength of adenosine binding to the A1 receptor significantly reduced hypoxic-is-
chemic damage in the newborn rat. On balance, the weight of earlier evidence and the current study strongly suggest that the adenosine A<sub>1</sub> receptor is of major neuroprotective importance.

Conclusions

The finding that administration of an adenosine A<sub>1</sub> antagonist resulted in greater electrocortical activity for the first 5 minutes of asphyxia, coupled with attenuated suppression of heat production and a marked increase in cerebral damage compared with controls, supports the hypothesis that the very rapid initial suppression of neuronal activity and metabolic rate during severe asphyxia in the fetal lamb is not due to profound tissue anoxia but is actively mediated by adenosine release. This period of neural suppression appears to mark-edly extend the period during which residual anaerobic reserves can prevent hypoxic depolarization as shown by the onset of cortical depolarization. These data demonstrate for the first time that endogenous activation of the adenosine A<sub>1</sub> receptor during severe asphyxia in the near-term fetus provides important protection against neuronal injury.

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