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

Christian J. Hunter, PhD; Laura Bennet, PhD; Gordon G. Power, PhD; Vincent Roelfsema, BS; Arlin B. Blood, PhD; Josine S. Quaedackers, BS; Sherly George, PhD; Jian Guan, PhD; Alistair J. Gunn, MBChB, PhD

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.

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 implicates 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
adenosine 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. How-mia. Therefore, we tested the specific hypothesis that block-
found hypoxic tolerance of the fetal brain to hypoxia-ische-
mechanism may be a major factor contributing to the pro-

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-ische-

Therefore, we tested the specific hypothesis that block-

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 axillary vein, and the amniotic fluid space for recording of pressure, blood sampling, and drug infusions. A reversible inflatable occluder (In Vivo Metric) was placed around the base of the umbilical cord. Two pairs of EEG electrodes (AS633-SSSF, 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 over the dura 5 mm lateral to the EEG electrodes. A catheter was placed in the sagittal sinus to measure oxygen content.

In 12 fetuses, a composite probe (diameter,~400 μm) containing 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. 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 axillary vein, and the amniotic fluid space for recording of pressure, blood sampling, and drug infusions. A reversible inflatable occluder (In Vivo Metric) was placed around the base of the umbilical cord. Two pairs of EEG electrodes (AS633-SSSF, 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 over the dura 5 mm lateral to the EEG electrodes. A catheter was placed in the sagittal sinus to measure oxygen content.

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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.

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 on 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).

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![Figure 1](image-url)  
**Figure 1.** Time sequence of changes in cortical tissue PO₂ in control and DPCPX-treated fetuses. Note profound tissue hypoxia during umbilical cord occlusion in both groups with rapid rebound after release of occlusion.
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 $A_1$ receptor. Administration of the adenosine $A_1$ 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,28
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
hypoxia5,29; however, it has been unclear whether this sup-
pression is a direct effect of profound tissue hypoxia or an
active suppression mediated by inhibitory neuromodulators
such as GABA and adenosine.4,12,30,31 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 is striking parallels in the adults of
vertebrate species such as the freshwater aquatic turtle that
can tolerate severe hypoxia32 that is medi-
ated by adenosine A1 receptor activation and other inhibitory
amino acids.33

The accelerated rise in impedance during asphyxia in the
DPCPX group in the present study supports this interpreta-
tion. Because a rise in cortical impedance reflects cytotoxic
edema, this denotes a more rapid development of hypoxic
cellular depolarization, probably because of accelerated de-
pletion 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.5

Impaired acute adaptation to asphyxia was followed by
histological and electrophysiological evidence of increased
injury in the DPCPX group. All the DPCPX animals demon-
strated delayed postasphyxial seizures after cord occlusion.
Seizures are well recognized clinically as a marker of
asphyxial damage.2 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.34 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 al18 reported that direct administration of an
adenosine A1 agonist in 7-day-old rats during hypoxia-ische-
mia 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 compro-
mise cerebral perfusion. Bona et al19 found that pretreat-
ment 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 al20 found that increasing the strength of adenosine
binding to the A1 receptor significantly reduced hypoxic-is-

Figure 5. Left, Photomicrographs of striatum and of CA1 region of the hippocampus. Animals treated with DPCPX show selective cell
death in the striatum and diffuse cell death in the CA1 region of the hippocampus, changes that are absent in control animals. Arrows
indicate examples of dead cells. Right, Quantification of neuronal loss in DPCPX-treated and control fetuses. Values are mean±SEM.
*P<0.01 DPCPX vs control fetuses.
pheric damage in the newborn rat. On balance, the weight of earlier evidence and the current study strongly suggest that the adenosine A₁ receptor is of major neuroprotective importance.

Conclusions
The finding that administration of an adenosine A₁ 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 markedly 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 A₁ receptor during severe asphyxia in the near-term fetus provides important protection against neuronal injury.

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