

# Adenosine mediates decreased cerebral metabolic rate and increased cerebral blood flow during acute moderate hypoxia in the near-term fetal sheep

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Exposure of the fetal sheep to moderate to severe hypoxic stress results in both increased cortical blood flow and decreased metabolic rate. Using intravenous infusion of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), a selective adenosine A<sub>1</sub> receptor antagonist that is permeable to the blood brain barrier, we examine the role of adenosine A<sub>1</sub> receptors in mediating cortical blood flow and metabolic responses to moderate hypoxia. The effects of DPCPX blockade are compared to controls as well as animals receiving intravenous 8-(*p*-sulphophenyl)-theophylline (8-SPT), a non-selective adenosine receptor antagonist which has been found to be blood brain barrier impermeable. Laser Doppler flow probes, tissue P<sub>O<sub>2</sub></sub>, and thermocouples were implanted in the cerebral cortices of near-term fetal sheep. Catheters were placed in the brachial artery and sagittal sinus vein for collection of samples for blood gas analysis. Three to seven days later responses to a 30-min period of fetal hypoxemia (arterial P<sub>O<sub>2</sub></sub> 10–12 mmHg) were studied with administration of 8-SPT, DPCPX, or vehicle. Cerebral metabolic rate was determined by calculation of both brain heat production and oxygen consumption. In response to hypoxia, control experiments demonstrated a 42 ± 7 % decrease in cortical heat production and a 35 ± 10 % reduction in oxygen consumption. In contrast, DPCPX infusion during hypoxia resulted in no significant change in brain heat production or oxygen consumption, suggesting the adenosine A<sub>1</sub> receptor is involved in lowering metabolic rate during hypoxia. The decrease in cerebral metabolic rate was not altered by 8-SPT infusion, suggesting that the response is not mediated by adenosine receptors located outside the blood brain barrier. In response to hypoxia, control experiments demonstrated a 35 ± 7 % increase in cortical blood flow. DPCPX infusion did not change this increase in cortical blood flow, however 8-SPT infusion attenuated increases in flow, indicating that hypoxic increases in cerebral blood flow are mediated by adenosine but not via the A<sub>1</sub> receptor. In summary, adenosine appears to play a key role in fetal hypoxic defences, acting to increase O<sub>2</sub> delivery via adenosine A<sub>2</sub> receptors and to decrease metabolic rate via A<sub>1</sub> receptors inside the blood brain barrier. These data show for the first time in the mammalian fetus that the adenosine A<sub>1</sub> receptor is an important mediator of brain metabolic rate during moderate hypoxia.

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Compared to the adult, the brain of the mammalian fetus is remarkably tolerant to hypoxic insult (Duffy *et al.* 1975; Mallard *et al.* 1994; Hunter *et al.* 2003*a,c*). During acute global hypoxia, fetal blood flow is centralized to the brain, heart, and adrenal glands at the expense of the peripheral tissues (Jensen *et al.* 1999). This redistribution of flow optimizes oxygen delivery to the vital organs and is a result of increased arterial blood pressure in combination with lowered vascular resistance in these organs (Reuss *et al.* 1982; Jensen *et al.* 1999). The increases in arterial blood pressure during hypoxia are in part a result of elevated sympathetic activity and plasma concentrations of catecholamines (Cohen *et al.* 1982; Iwamoto *et al.* 1983). In contrast to the increased peripheral resistance to blood

flow during hypoxia, cerebral vascular tone is unchanged or decreased, suggesting an important role for one or more mediators of hypoxic cerebral vasodilatation (Jensen *et al.* 1999; Blood *et al.* 2002; Bishai *et al.* 2003; Hunter *et al.* 2003*c*). We recently reported evidence suggesting adenosine is of primary importance in the regulation of fetal cortical vascular tone and increased cortical blood flow during hypoxia (Blood *et al.* 2002).

By increasing cerebral blood flow and oxygen extraction, the fetal brain maintains a normal rate of oxygen consumption during mild to moderate hypoxic stress. However, during severe hypoxic stress (arterial oxyhaemoglobin saturation of ~35 % or less), cerebral metabolic rate is decreased and becomes directly correlated with oxygen delivery (Parer,

1994). In the fetal sheep, the decrease is associated with low energy-consuming EEG states (Chau & Koos, 1999), depression of evoked brainstem potentials (Gunn *et al.* 1991), and increased anaerobic cerebral metabolism (Asano *et al.* 1994). Presently it is not known whether the fall in cerebral oxygen consumption during hypoxia is a result of oxygen starvation or a protective mechanism of adaptive hypometabolism, as has been described in the brain, liver, and whole body of several species (Hochachka *et al.* 1996; Mortola, 1999; Hochachka & Lutz, 2001).

Growing evidence points to adenosine, an endogenously produced purine nucleoside, as a mediator of both increased cerebral blood flow and decreased cerebral metabolic rate during hypoxic stress (Blood *et al.* 2002). Plasma and intracerebral adenosine concentrations increase during hypoxia in the fetal sheep (Kjellmer *et al.* 1989; Koos *et al.* 1997; Suzuki & Power, 1999), newborn lamb (Laudignon *et al.* 1991), and adult rat (Winn *et al.* 1981b; Van Wylen *et al.* 1986), in association with an inhibition of neuronal activity (Fowler *et al.* 1999). Intravenous administration of theophylline, a non-specific adenosine receptor antagonist, abolishes increases in cortical blood flow during hypoxia in the fetal sheep (Blood *et al.* 2002). In adult animals adenosine, acting on the adenosine A<sub>1</sub> receptor, suppresses cerebral metabolic rate during hypoxia/asphyxia in hippocampal slices (Croning *et al.* 1995; Jin & Fredholm, 1997; Fowler *et al.* 1999) and during severe asphyxia *in vivo* (Hunter *et al.* 2003c). In the fetal sheep, breathing movements are inhibited by hypoxia, an adaptation that is abolished by adenosine-receptor blockade at the level of the thalamus (Koos *et al.* 1994a; Chau & Koos, 1999). Thus, there is considerable evidence to warrant the study of the effect of adenosine on fetal cerebral metabolic rate during moderate hypoxia.

The present study was designed to test the hypothesis that adenosine A<sub>1</sub> receptor activation mediates both decreased cortical metabolic rate and increased cortical blood flow during moderate hypoxia in the near-term fetal sheep. In order to test this hypothesis late-term fetal sheep were chronically instrumented for measurement of cortical blood flow by laser Doppler flowmetry, cortical heat production, and cortical oxygen consumption. Fetal sheep were subjected to severe hypoxia during intravenous infusion of either DPCPX, a selective adenosine A<sub>1</sub> receptor antagonist that crosses the blood brain barrier (Marston *et al.* 1998), or 8-SPT, a non-selective adenosine receptor antagonist that does not cross the blood brain barrier (Baumgold *et al.* 1992; Coney & Marshall, 1998; Meno *et al.* 2001).

## METHODS

### Surgical preparation

All study procedures were approved by the Loma Linda University Animal Care Research Committee. Western ewes carrying

singleton or twin fetuses were used for the study; in the case of twins only one fetus was studied. Surgical instrumentation was performed between 122 and 128 days gestation (term = 47 days gestation) as previously described (Lan *et al.* 2000). Anaesthesia was induced with 0.5 g thiopental and maintained with 1.5–2.5 % inhaled halothane. A polyvinyl catheter was placed in the femoral vein of the ewe for administration of intravenous fluids during the surgery, as well as administration of a euthanasia solution (Euthasol) at the termination of the experiments. Through a midline maternal incision in the abdomen, the fetal head and neck were exposed. Polyvinyl catheters were placed in a brachial artery for blood gas sampling and arterial blood pressure measurement, in the subclavian vein for drug administration, and in the amniotic sac for measurement of amniotic fluid pressure and administration of antibiotics to the fetus (Blood *et al.* 2002). Then 0.5 mm burr holes were drilled bilaterally in the fetal skull for insertion of a laser Doppler flowmetry probe in the right parietal cortex and a thermocouple (IT-18 thermometer, Physitemp, Clifton, NJ, USA) in the left cortex as described previously (Lan *et al.* 2000; Bishai *et al.* 2003). The laser Doppler flow probe (Oxford Optronix, Oxford, UK) consisted of a four-part composite probe (diameter ~400  $\mu\text{m}$ ) containing emitting and receiving laser Doppler channels, a P<sub>O<sub>2</sub></sub> electrode, and thermocouple. A second thermocouple was inserted into the brachial artery of the fetus together with the polyvinyl catheter, for measurement of arterial temperature. A polyvinyl catheter was also inserted into the sagittal sinus for collection of cerebral venous blood samples. The uterus and abdomen of the ewe were sutured shut in layers and the catheters and probes were exteriorized through the maternal flank and held in a protective pouch between experiments. The ewe was given 900 000 u of intramuscular penicillin for 3 days. The fetus received 500 mg of ampicillin and 40 mg of gentamicin daily in the amniotic fluid, until the termination of the experiment.

Experiments were carried out  $5 \pm 2$  days following the operation, with a minimum of 48 h between the control and experimental protocols conducted on each animal. Experiments were conducted while the ewe was standing in a metabolic cart at room temperature with minimal distractions; alfalfa pellets and water were available to the ewe as desired.

### Experimental protocols

The control protocol consisted of a 60 min baseline period followed by a 30 min period of hypoxia induced by breathing the ewe with 10–12 % O<sub>2</sub> in a balance of nitrogen. This was administered into a bag placed over the head of the ewe with a flow rate of 30 l min<sup>-1</sup>. The hypoxic period was followed by a 60 min recovery period during which the ewe was allowed to breathe room air.

The time course was identical in all experimental protocols except that an adenosine receptor blockade was initiated by intravenous infusion of either 8-(*p*-sulphophenyl)-theophylline (8-SPT) or 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) beginning 30 min prior to the initiation of hypoxia and ending 30 min after completion of hypoxia. 8-SPT is a universal adenosine receptor antagonist that is relatively membrane impermeable (Evoniuk *et al.* 1987; Meno *et al.* 2001). It was diluted in saline to 10 mg ml<sup>-1</sup> and pH was adjusted to 7.4 with sodium hydroxide. The solution was infused intravenously as a 15 mg bolus followed by an infusion of 1.8 mg min<sup>-1</sup> for the remainder of the infusion period. This dose was selected as the greater of two administered in separate studies by Koos *et al.* (1995) and Giussani *et al.* (2001), both of which were reported to attenuate cardiovascular responses to hypoxia in the fetal sheep.

**Table 1. Data for fetal arterial blood pressure and heart rate during the last 10 min of each of the five protocol periods**

		Baseline		Pre-hypoxia infusion		Hypoxia		Recovery with infusion		Post-infusion recovery	
		Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
		Blood pressure (mmHg)	Control	44	1	44	2	53	4*	48	3
	8-SPT	45	2	46	2	54	3*	49	2	48	2
	DPCPX	45	2	51	3†	56	2†	55	3	52	4
Heart rate (bpm)	Control	167	5	166	5	151	8*	176	7	185	4
	8-SPT	170	7	158	8	142	14*	171	8	180	7
	DPCPX	168	5	185	14	172	12	189	11	178	8

\*Significant difference from baseline value ( $P < 0.05$ ); †significant difference from baseline value ( $P < 0.01$ ).

DPCPX is a selective adenosine  $A_1$  receptor antagonist that is membrane permeable (Marston *et al.* 1998). It was prepared in 0.1 M NaOH to a concentration of 2.5 mg ml<sup>-1</sup> (final pH ~10) and infused at a rate of 2.5 mg min<sup>-1</sup> for the first 10 min of the infusion period followed by 0.75 mg min<sup>-1</sup> for the remainder of the infusion period. This dose of DPCPX is equivalent to that given to fetal sheep by Koos & Maeda (2001) where it was found to antagonize the effects of the selective adenosine  $A_1$  receptor agonist N<sup>6</sup>-cyclopentyl-adenosine. Both 8-SPT and DPCPX were obtained from Sigma Chemical Co, St Louis, MO, USA.

The total volumes infused were 17.7 ml for the 8-SPT group, 34 ml for the DPCPX group, and 30 ml of saline for the comparable period in the control protocol.

Seven sheep were studied under the control protocol. Because we have found previously that the cortical blood flow responses to hypoxia are consistent when repeated at least 48 h apart (Lan *et al.* 2000), two of these sheep were subsequently (48–72 h later) studied under the 8-SPT protocol and four were studied under the DPCPX protocol. An additional five sheep were studied under the 8-SPT protocol only, and three more were studied under the DPCPX protocol only. The mean fetal gestational age at the time of experiment was 129 ± 1 days (0.89 gestation).

Upon completion of the experiments, the ewe and fetus were killed using Euthasol (17 ml, Western Medical Supply, Arcadia, CA, USA). The location of the probes, thermocouple, and catheters was verified and fetal body weights were recorded.

### Blood sampling

Blood samples (0.5 ml each) were collected into heparinized syringes from the arterial and sagittal sinus catheters during each experiment at 0, 15, 30, 40, 50, and 60 min during the baseline period, 5, 10, 20, and 30 min during the hypoxic period, and 10, 20, 30, 45, and 60 min during the recovery period. The samples were analysed immediately for measurement of blood gases (ABL3, Radiometer, Copenhagen, Denmark), haemoglobin content and oxyhaemoglobin saturation (OSM<sub>2</sub> Hemoximeter, Radiometer), and glucose and lactate concentrations (YSI 2700 analyser, Yellow Springs Instruments, Dayton, OH, USA). A total of 30 blood samples (30 ml) were collected making the total amount of blood drawn during each experiment amount to ~8% of the total estimated fetal blood volume of ~400 ml.

### Electronic data acquisition and handling

Cortical blood flow was measured by use of a laser Doppler flowmeter (Oxford Optronics, Oxford, UK). The perfusion signal

provided an index of blood perfusion in a 1–2 mm<sup>3</sup> hemisphere of tissue around the tip of the laser Doppler probe. Cortical blood flow, mean arterial blood pressure (BP), cortical tissue  $P_{O_2}$  and arterial and cortical temperatures were recorded continuously. Analogue outputs were digitized (sampling rate 100 Hz) and stored using an analogue-to-digital converter (Powerlab 16/SP, ADInstruments, Colorado Springs, CO, USA) and data acquisition software (Chart v.4, ADInstruments). Heart rate was calculated from the pressure wave of the arterial pressure measurements. Cortical vascular resistance was calculated as (mean brachial arterial blood pressure – mean sagittal sinus pressure)/(cortical blood flow). Due to loss of sagittal sinus catheter patency, arterial blood pressure was assumed to be equivalent to cerebral perfusion pressure in three of the sheep studied under each of the three protocols.

Because laser Doppler flowmetry provides a relative, not absolute, measure of flow, flow signals from the baseline period of each experiment were averaged and considered as 100%, with subsequent measurements expressed as a percentage of the baseline values.

Cortical heat production and oxygen consumption were calculated using the Fick principle. Heat production was calculated by multiplying the temperature difference between the artery and the brain by cortical blood flow. For a detailed description of the measurement of fetal brain heat production and metabolism refer to Hunter *et al.* (2003b). Cortical oxygen consumption was calculated by multiplying the arterial–venous difference in oxygen content by cortical blood flow. Both parameters were normalized to a percentage of the baseline period.

### Statistical analyses

Data are presented as means ± S.E.M. For statistical analysis of continuously measured parameters, mean values for the final 10 min of each study period were used. The significance of changes over time was evaluated using a one-way ANOVA with repeated measures and Dunnett's *post hoc* test when a significant difference was detected by ANOVA. The significance of differences between the control and 8-SPT or between the control and DPCPX protocols was evaluated using a two-way ANOVA with a Bonferroni *post hoc* test. The statistical software Graphpad Prism 3.0 (Graphpad Software Inc., San Diego, CA, USA) was used for all statistical analyses.

**Table 2. Data for arterial blood and plasma parameters during each of the five protocol periods**

		Baseline		Pre-hypoxia infusion		Hypoxia		Recovery with infusion		Post-infusion recovery	
		Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
		$P_{O_2}$ (mmHg)	Control	22.4	1.1	22.3	1.2	12.1	0.4†	22.0	1.2
	8-SPT	21.4	1.0	22.1	1.4	11.0	0.6†	22.6	0.9	22.8	1.4
	DPCPX	21.7	1.0	18.3	1.9	10.9	1.0†	19.6	2.6	23.1	1.4
Hb (mg dl <sup>-1</sup> )	Control	8.9	0.5	9.1	0.5	9.3	0.5	8.9	0.5	8.8	0.5
	8-SPT	8.9	0.2	8.5	0.3	9.2	0.2	8.7	0.2	8.5	0.3
	DPCPX	8.8	0.3	9.1	0.3	9.7	0.3	9.6	0.4	8.7	0.4
O <sub>2</sub> content (mM)	Control	3.6	0.3	3.4	0.2	1.7	0.1*	3.3	0.2	3.3	0.2
	8-SPT	3.5	0.2	3.3	0.3	1.4	0.1†	3.2	0.3	3.3	0.3
	DPCPX	3.5	0.2	3.3	0.3	2.0	0.2†	3.4	0.4	3.5	0.3
$P_{CO_2}$ (mmHg)	Control	50.1	1.8	50.1	2.2	44.0	1.9*	48.0	2.6	48.0	2.6
	8-SPT	48.6	1.4	49.2	2.0	44.3	2.0*	48.9	1.5	42.0	1.5
	DPCPX	47.3	1.2	46.7	2.1	46.6	3.2	47.4	2.0	54.0	2.0
pH	Control	7.36	0.01	7.34	0.02	7.34	0.03	7.28	0.03*	7.31	0.03
	8-SPT	7.36	0.01	7.35	0.01	7.37	0.02	7.26	0.02†	7.31	0.02*
	DPCPX	7.37	0.01	7.36	0.02	7.32	0.03*	7.26	0.02†	7.26	0.03†
Glucose (mM)	Control	1.04	0.14	1.13	0.16	1.19	0.11	1.34	0.12†	1.26	0.13†
	8-SPT	0.97	0.22	1.02	0.25	1.20	0.20	1.52	0.21†	1.38	0.25†
	DPCPX	0.98	0.07	1.17	0.09	1.51	0.08†	1.63	0.11†	1.53	0.16†
Lactate (mM)	Control	1.39	0.25	1.79	0.45	3.74	0.93	5.40	1.13†	4.26	1.20*
	8-SPT	1.53	0.22	1.56	0.22	3.54	0.53	6.11	1.06†	4.52	0.97*
	DPCPX	1.06	0.05	2.03	0.32	4.74	0.62	7.10	0.73†	6.61	0.64†

\*Significant difference from baseline value ( $P < 0.05$ ); †significant difference from baseline value ( $P < 0.01$ ).

## RESULTS

### Fetal haemodynamics, blood gases, pH, oxyhaemoglobin saturation, glucose, and lactate values

Fetal blood pressure and heart rate values for the final 10 min of each study period are shown in Table 1. Arterial blood pressure increased significantly in response to hypoxia in all experiments, and then returned to baseline values during recovery. In addition, DPCPX infusion resulted in a significant increase in blood pressure. Heart rate decreased significantly during hypoxia in the control and 8-SPT experiments, but was not significantly altered during the DPCPX experiments. As demonstrated by the mean values for arterial  $P_{O_2}$ , oxyhaemoglobin saturation, and oxygen content (Table 2), each of the three study groups was subjected to a comparable hypoxic insult. This was characterized by a decrease in arterial  $P_{O_2}$  from ~22 to 12 mmHg,  $P_{CO_2}$  from 50–45 mmHg, mild reductions in pH, and subsequent recovery of all variables over the next hour.

### Cortical blood flow and cortical vascular resistance

Cortical blood flow responses are shown in Fig. 1 for all three protocols. In the control experiments hypoxia resulted in a significant increase in cortical blood flow,

with measurements rising steadily during the first 10 min of hypoxia to reach a mean value of  $135 \pm 7\%$  of baseline during the last 10 min of the hypoxic insult. Cortical blood flow then returned to baseline during the recovery period, and remained unchanged during the remainder of the experiment.

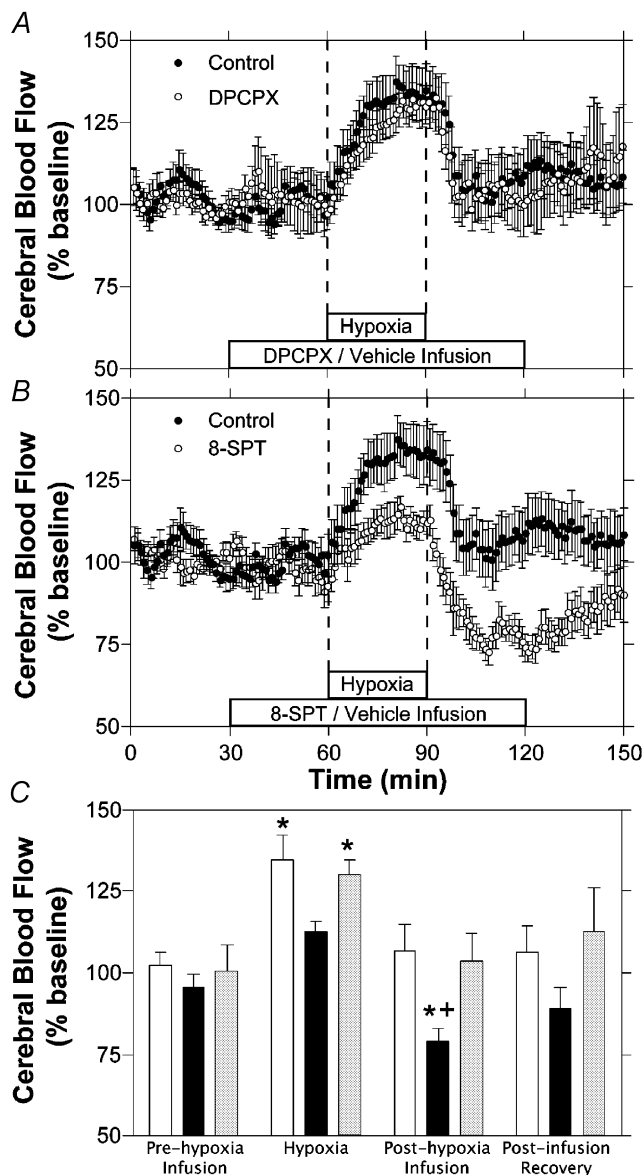
In the DPCPX experiments, there was no significant change in cortical blood flow during the first 30 min of DPCPX infusion. During hypoxia, cortical blood flow rose significantly to a mean of  $130 \pm 5\%$  of baseline levels, a response similar to that observed in control experiments. In the recovery period, cortical blood flow returned to baseline levels within 10 min and remained at baseline levels for the remainder of the DPCPX infusion and recovery period. DPCPX infusion had no significant effect on cortical blood flow compared to control experiments.

In 8-SPT experiments, baseline cortical blood flow was not changed during the drug infusion administered before hypoxia. During hypoxia, cortical blood flow rose to  $113 \pm 3\%$  of baseline the last 10 min of hypoxia, a value that was not significantly different from baseline and measurably less than in control fetuses. Following hypoxia, cortical blood flow decreased significantly to  $79 \pm 4\%$  of baseline levels in

the last 10 min of the 8-SPT infusion and rose gradually towards baseline levels during the final 30 min of the experiment. Cortical blood flow was significantly reduced during the final 10 min of 8-SPT infusion compared to that of controls from the same time period.

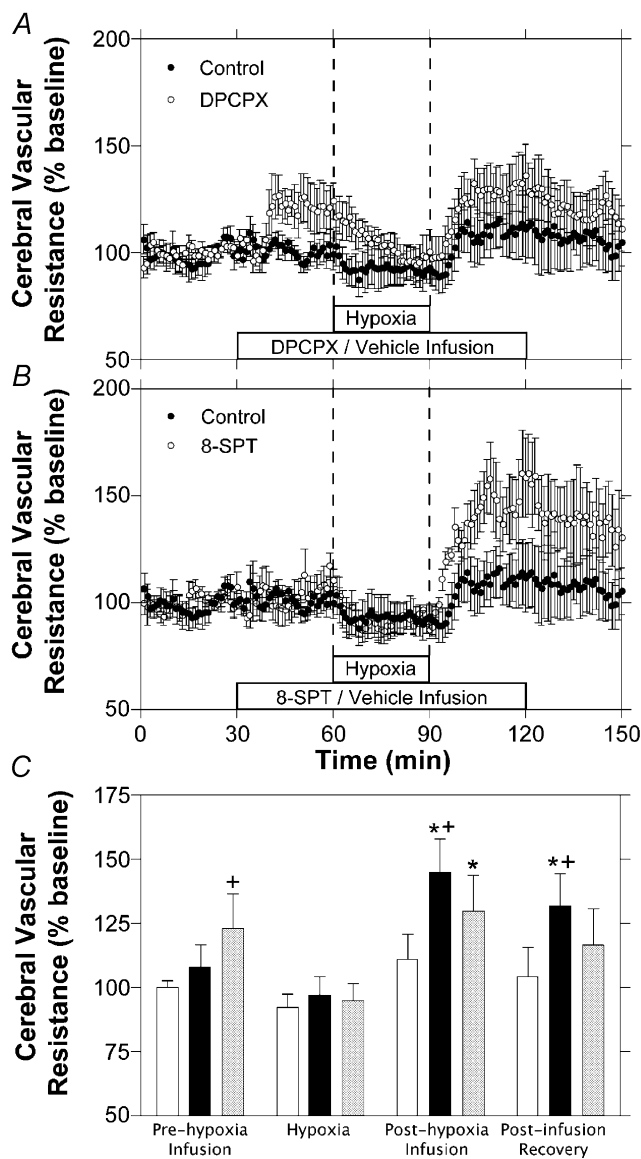
Cerebrovascular resistance to flow did not change significantly relative to baseline values during hypoxia in the control experiments, as shown in Fig. 2. During DPCPX

experiments, mean resistance tended to increase upon initiation of DPCPX infusion and to fall during hypoxia, although neither change was determined to be significantly different from baseline. Following hypoxia, resistance rose significantly to a mean of  $130 \pm 14\%$  during the final 10 min of DPCPX infusion and then returned to baseline values during the final 30 min of the experiment. During 8-SPT experiments, resistance remained unchanged during the hypoxic period, and then rose significantly to a mean of



**Figure 1. Time course of changes in cortical blood flow during hypoxia**

Results are shown for hypoxia with intravenous infusion of vehicle (A and B,  $n = 7$ ), DPCPX (A,  $n = 7$ ) or 8-SPT (B,  $n = 7$ ). C, comparison of cortical blood flow changes from baseline measurements for control (open bars), 8-SPT (black bars) and DPCPX (grey bars) experiments. Bars represent mean values for each of the three experimental groups during the last 10 min of each of the four experimental periods labelled. Error bars indicate S.E.M. \* Significant change from baseline period. + Significantly different from control experiments at that time period.



**Figure 2. Time course of changes in cortical vascular resistance during hypoxia**

Results are shown for hypoxia with intravenous infusion of saline (A and B,  $n = 7$ ), DPCPX (A,  $n = 6$ ) or 8-SPT (B,  $n = 6$ ). C, comparison of changes from baseline in cortical vascular resistance calculations for control (open bars), 8-SPT (black bars) and DPCPX (grey bars) experiments. Bars represent mean values for each of the three experimental groups during the last 10 min of each of the four experimental periods labelled. Error bars indicate S.E.M. \* Significant change from baseline period. + significantly different from control experiments at that time period.

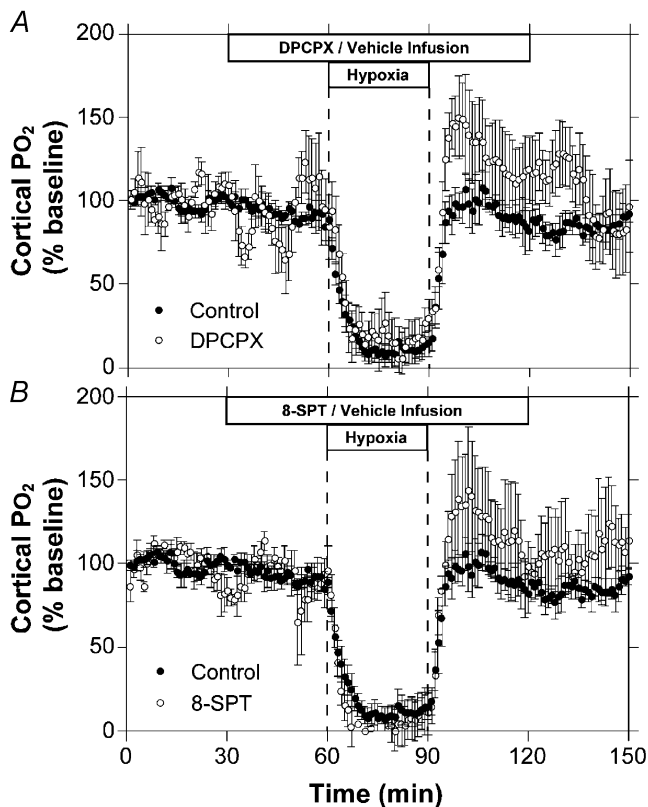
145 ± 13% of baseline during the final 10 min of 8-SPT infusion and remained elevated at 132 ± 12% during the final 10 min of the experiment. Compared to control experiments, 8-SPT infusion resulted in significantly increased resistance during the post-hypoxic period of the study, while DPCPX infusion resulted in significantly increased cerebrovascular resistance only during the initial 30 min of the infusion period.

### Cerebral cortical $P_{O_2}$

Cortical tissue  $P_{O_2}$  ( $tP_{O_2}$ ) is presented in Fig. 3 as a percentage of baseline. During the baseline period mean  $tP_{O_2}$  for the control ( $n = 6$ ), DPCPX ( $n = 6$ ), and 8-SPT ( $n = 4$ ) groups was  $9 \pm 1$ ,  $7 \pm 2$ , and  $6 \pm 2$  mmHg, respectively. During hypoxia,  $tP_{O_2}$  fell rapidly to a nadir of 1–2 mmHg for each of the three study groups and there were no significant differences between responses of the 8-SPT, DPCPX, or control experiments. Finally, during the recovery period,  $tP_{O_2}$  rapidly returned to baseline values in all three groups for the remainder of the experiment.

### Cortical heat production and oxygen and glucose consumption

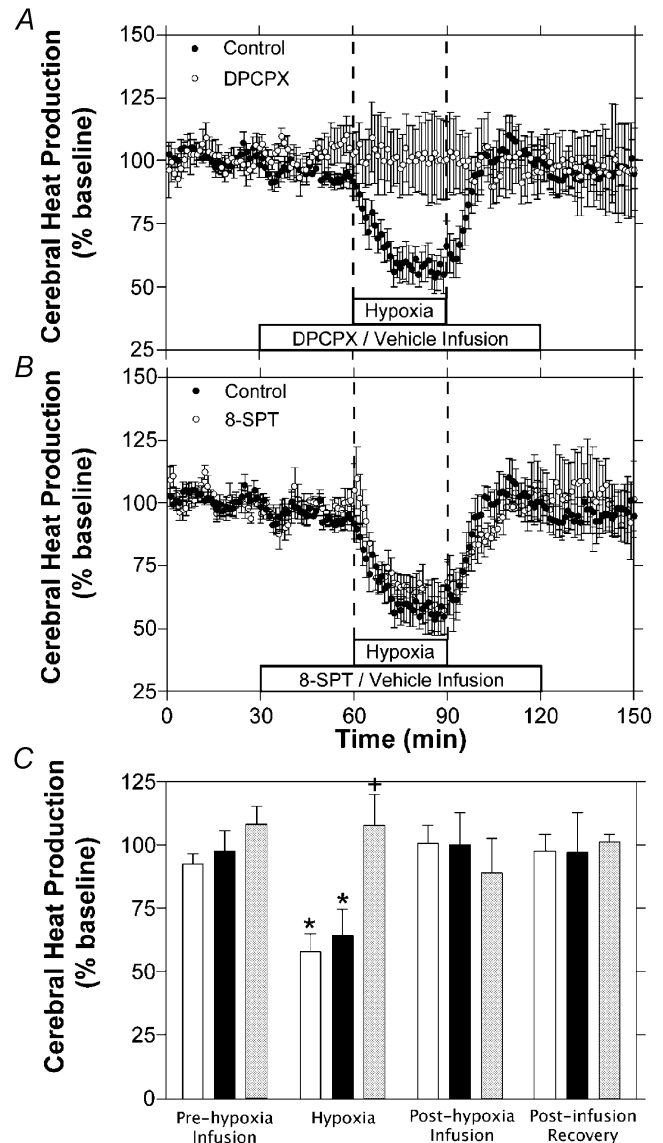
Cortical heat production data are presented in Fig. 4. Vehicle, DPCPX, and 8-SPT infusions had no effect on heat production during the initial 30 min of infusion.



**Figure 3.** Time course of changes in cortical tissue  $P_{O_2}$  during hypoxia

Results are shown for hypoxia with intravenous infusion of saline (A and B,  $n = 6$ ), DPCPX (A,  $n = 5$ ) or 8-SPT (B,  $n = 5$ ). Error bars indicate S.E.M.

During control experiments, mean cortical heat production decreased significantly to  $58 \pm 7\%$  of baseline values during the hypoxic insult, rapidly returned to baseline values following termination of hypoxia, and remained unchanged for the remainder of the experiment. During the DPCPX experiments, mean cortical heat production did not vary significantly from baseline values at any point throughout the experiments. During 8-SPT experiments, mean cortical heat production decreased



**Figure 4.** Time course of changes in cortical heat production during hypoxia

Results are shown for hypoxia with intravenous infusion of saline (A and B,  $n = 7$ ), DPCPX (A,  $n = 7$ ) or 8-SPT (B,  $n = 7$ ). C, comparison of cortical heat production changes from baseline measurements for control (open bars), 8-SPT (black bars) and DPCPX (grey bars) experiments. Bars represent mean values for each of the three experimental groups during the last 10 min of each of the four experimental periods labelled. Error bars indicate S.E.M. \* Significant change from baseline period. + Significantly different from control experiments at that time period.

**Table 3. Data for cerebral oxygen and glucose uptake ( $\dot{V}$ ) during each of the five protocol periods**

		Baseline		Pre-hypoxia infusion		Hypoxia		Post-hypoxia infusion		Post-infusion recovery	
		Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
		$\dot{V}_{O_2}$ (% baseline)	Control	100	–	110	14	64	10†	137	24
	8-SPT	100	–	125	18	69	7†	131	27		9
	DPCPX	100	–	106	10	87	19	137	37	107	30
$\dot{V}$ glucose (% baseline)*	Control	100	–	109	4	93	8	107	7	127	19
	8-SPT	100	–	120	5	108	13	115	6	172	10†
	DPCPX	100	–	117	3	111	6	79	8‡	122	11

\*Cortical uptake of glucose was calculated as the product of cortical flow and the arteriovenous difference. †Significant difference from baseline value ( $P < 0.05$ ); ‡significant difference from control for the same time period ( $P < 0.05$ ).

significantly to  $65 \pm 10\%$  of baseline values during the hypoxic insult, rapidly returned to baseline values following hypoxia, and remained unchanged for the remainder of the experiment. 8-SPT infusion had no significant effect on cortical heat production compared to the control experiments. In contrast, DPCPX infusion prevented the fall in cortical heat production during the hypoxic insult compared to the control experiments.

Relative changes in cortical oxygen uptake and glucose consumption, calculated by the Fick principle, are shown in Table 3. Hypoxic insult resulted in a significant decrease in cortical oxygen consumption in the control and 8-SPT experiments. In contrast, there was no significant change in cortical oxygen consumption during hypoxia in the DPCPX experiments.

Cortical glucose consumption was unchanged by infusion of either 8-SPT or DPCPX during the baseline period, and did not change significantly during the hypoxic insult for any of the three experimental groups. Glucose consumption remained unchanged from baseline values throughout the recovery for the control and DPCPX groups, although consumption was significantly lower in the DPCPX group compared to controls during the recovery period while DPCPX infusion was continued. Glucose consumption was significantly increased during the recovery period in the 8-SPT group.

## DISCUSSION

The principal findings of this study are that adenosine is an important mediator of decreased cerebral metabolic rate during moderate hypoxia and that this effect on metabolism is mediated by the adenosine  $A_1$  receptors located within the brain. The fact that hypoxic decreases in metabolism are abolished by infusion of DPCPX, a selective adenosine  $A_1$  antagonist, is consistent with the idea of adaptive hypometabolism of the fetal brain in response to hypoxic stress. The  $A_1$  receptors mediating this adaptive hypometabolism appear to be located inside the

blood brain barrier, since intravenous infusion of 8-SPT, a non-selective adenosine receptor antagonist with poor blood brain barrier permeability, resulted in no significant change in the fall in fetal cortical oxygen consumption or heat production during hypoxia. In addition, adenosine appears to mediate increased cerebral blood flow by its actions on  $A_2$  but not  $A_1$  receptors during hypoxia because cerebral blood flow increases were markedly attenuated by infusion of 8-SPT but not DPCPX.

Adenosine is a purine nucleoside produced principally from dephosphorylation of AMP and to a lesser extent from hydrolysis of *S*-adenosyl-homocysteine. In the fetal sheep, both hypoxia and asphyxia result in increased plasma concentrations of adenosine (Koos & Doany, 1991; Kubonoya & Power, 1997). Microdialysis measurements also show increased adenosine concentrations in the interstitial fluid of the fetal sheep brain with hypoxia (Koos *et al.* 1997) and cord occlusion (Watson *et al.* 2002).

Adenosine mediates various physiological processes acting via at least four different adenosine receptor types named  $A_1$ ,  $A_{2A}$ ,  $A_{2B}$ , and  $A_3$ . The receptors are all coupled to G-proteins and have been found in a wide range of species and types of tissues (for review, see Fredholm *et al.* 2001).  $A_1$  receptor expression is notably high in the brain, adrenal gland, atria, skeletal muscle, and liver. In the brain, the activation of the  $A_1$  receptor results in neuronal membrane stabilization and decreased neuronal firing (Croning *et al.* 1995; Fowler *et al.* 1999). The  $A_{2A}$  and  $A_{2B}$  receptors can be found in cerebral vascular smooth muscle where both mediate vasodilatation in response to hypoxia (Coney & Marshall, 1998) and neuronal stimulation (Meno *et al.* 2001).

The exact mechanisms by which adenosine concentrations increase during hypoxia are not fully understood. In the guinea-pig heart (Decking *et al.* 1997) as well as the rat brain (Lynch *et al.* 1998), it appears that hypoxia-stimulated increases in adenosine concentrations result from decreased phosphorylation of adenosine by

adenosine kinase (Decking *et al.* 1997), the enzyme that facilitates the major pathway of adenosine metabolism in the rat brain (Lloyd & Fredholm, 1995). In contrast, the breakdown of ATP does not appear to be a major contributing factor to increases in adenosine concentrations during hypoxic stress (Doolette, 1997). In the brain of the fetal sheep, however, evidence exists that the primary hypoxic increases in extracellular adenosine concentration are a result of increased degradation of extracellular 5'-AMP (Koos *et al.* 1997).

Thus, there is evidence that adenosine concentrations increase in response to hypoxia and may be involved in regulating both increased cerebral blood flow and decreased neuronal activity to optimize both oxygen delivery and usage.

### Effect of adenosine on cerebral metabolic rate during hypoxia

It has been nearly 30 years since adenosine was first reported to inhibit neuronal firing in isolated brain slices (Kuroda *et al.* 1976; Phillis & Edstrom, 1976). Since then, there have been numerous reports supporting a neuro-inhibitory role for adenosine (for review see Dunwiddie & Masino, 2001). In the fetal sheep brain, hypoxic/anoxic stress results in a marked suppression of electrocortical activity (Bocking & Harding, 1986; Gunn *et al.* 1991), an effect that is attenuated in the presence of DPCPX (Hunter *et al.* 2003b). Likewise, adenosine has been implicated in the inhibition of fetal breathing movements during hypoxic stress by its action on receptors in the thalamus (Koos *et al.* 1994b; Chau & Koos, 1999). Significantly, recent work in the near-term fetal sheep has shown that adenosine A<sub>1</sub> receptor activation during anoxia is a major mediator of the pronounced hypoxic tolerance in the fetal sheep. This work by Hunter *et al.* demonstrated that inhibition of the A<sub>1</sub> receptor during a 10 min period of complete cord occlusion resulted in significantly increased neuronal cell death compared to controls, delayed the suppression of EEG activity and resulted in increased cerebral metabolism as evidenced by continued heat production (Hunter *et al.* 2003a).

In the present study, cortical oxygen consumption and heat production decreased as fetal arterial P<sub>O<sub>2</sub></sub> fell below 11–12 mmHg during both control and 8-SPT experiments. In contrast, cerebral metabolic rate was not reduced during hypoxia with DPCPX infusion, providing strong evidence that the adenosine A<sub>1</sub> receptor mediates the reduction in cerebral metabolic rate in response to hypoxic stress. These data, taken together with previous work, support the presence of a mechanism of adaptive hypometabolism in the brain of the fetal sheep such that oxygen consumption is actively reduced during hypoxic stress, rather than being reduced by oxygen starvation.

Since 8-SPT is a relatively non-selective receptor antagonist that inhibits the same adenosine A<sub>1</sub> receptors as DPCPX, it is of interest that cerebral metabolic rate was not altered by 8-SPT administration as with DPCPX. One explanation for this finding may be due to the relatively plasma membrane-impermeable properties of 8-SPT resulting in its inability to cross the blood brain barrier. 8-SPT has been shown to not cross the blood brain barrier of the adult mouse (Baumgold *et al.* 1992) and rat (Coney & Marshall, 1998; Meno *et al.* 2001), as evidenced by a lack of significant cerebrospinal fluid (CSF) concentrations with significant plasma concentrations following intraperitoneal injection (Baumgold *et al.* 1992; Meno *et al.* 2001). Although cerebrospinal concentrations of 8-SPT were not determined in the present study, the blood brain barrier of the fetal sheep is intact as early as 0.6 gestation (Stonestreet *et al.* 2000; Harris *et al.* 2001). Exclusion of 8-SPT by the blood brain barrier in the present experiments would indicate that the effects of DPCPX on metabolic rate are due to blockade of adenosine receptors located inside the blood brain barrier. Alternatively, it is possible that the hypometabolism observed during 8-SPT infusion is due to the lack of increased cerebral blood flow compared to controls, such that the observed decrease in metabolic rate may be due to oxygen starvation rather than hypometabolism.

The finding of hypoxic adaptive hypometabolism in the brain is not new, as it has also been reported as a protective mechanism in the brains of other animals. For example, exposure of the freshwater turtle to anoxia results in a five-fold decrease in cerebral energy turnover with no significant decrease in cerebral ATP stores (Hochachka & Lutz, 2001). Likewise, in the rat, hypoxia results in a strong inhibition of neuronal firing (Croning *et al.* 1995; Fowler *et al.* 1999). In both instances, the investigators provided strong evidence suggesting these responses are mediated by activation of the adenosine A<sub>1</sub> receptor. In the adult mouse, knockout of the adenosine A<sub>1</sub> receptor results in significantly decreased neuronal tolerance to hypoxia (Johansson *et al.* 2001).

### Regulation of cerebral blood flow during hypoxia

Intravenous infusion of theophylline, a non-selective adenosine receptor antagonist, results in a lack of increased cortical blood flow during hypoxia in the fetal sheep (Blood *et al.* 2002). Theophylline crosses cell membranes freely, resulting in blockade of adenosine receptors on either side of the blood brain barrier (McPhee & Maxwell, 1987; Morii *et al.* 1987). However, as mentioned previously, 8-SPT is relatively impermeable to the plasma membrane and has been shown to not cross the blood brain barrier (Baumgold *et al.* 1992; Coney & Marshall, 1998; Meno *et al.* 2001). In the present study, 8-SPT infusion resulted in an inhibition of much of the characteristic increase of cortical blood flow



provoked by hypoxia, thus supporting previous work indicating the importance of adenosine receptor activation in mediating increased cortical blood flow during hypoxia and suggesting that these receptors are located outside the blood brain barrier. Such findings are in agreement with studies in the adult rat, in which topical application of 8-SPT to the exposed cortex had no effect on hypoxic cerebral vasodilatation, also suggesting that the receptors mediating cerebral vasodilatation are located outside the blood brain barrier (Coney & Marshall, 1998).

Unlike 8-SPT, DPCPX infusion had no effect on the hypoxic increases in cortical blood flow, suggesting that adenosine A<sub>1</sub> receptors are not involved in the cortical blood flow response. These findings are in agreement with work by others studying changes in arteriole diameter following application of selective adenosine receptor agonists to the exposed cortex of the preterm fetal sheep. These previous studies found that the adenosine A<sub>2</sub> receptor plays a greater role in cerebral vasodilatation than does the A<sub>1</sub> receptor (Kurth & Wagerle, 1992). Similar results have also been observed in the adult rat where adenosine A<sub>2</sub> agonists resulted in greater cerebral vasodilatation than A<sub>1</sub> agonists (Meno *et al.* 1993). Likewise, selective adenosine A<sub>2A</sub> antagonists have been shown to effectively block increases in cerebral arteriole diameter, while A<sub>1</sub> antagonists had little or no effect (Coney & Marshall, 1998).

Although there is growing evidence that adenosine is a strong mediator of cerebral blood flow during hypoxia in the fetus, the possible mechanisms by which it may act are many and complex. Adenosine may lower cerebrovascular resistance during hypoxia by direct activation of adenosine receptors on the cerebrovascular smooth muscle, resulting in cerebrovascular vasodilatation. It is also possible that adenosine acts by the regulation of other mediators of cerebrovascular tone. For example, vasopressin decreases cerebral vascular resistance and infusion of vasopressin receptor antagonists to the fetus during hypoxia attenuates increases in cerebral blood flow (Eisenach *et al.* 1992). During hypoxia, fetal plasma vasopressin concentrations increase, a response that is blocked by infusion of 8-SPT (Koos *et al.* 1994b). Adenosine's vasodilatory actions may also be exerted by stimulation of prostaglandin and nitric oxide production in the cerebral vasculature, pathways that have been observed in skeletal muscle of the adult rat (Marshall, 2002). However, in contrast to the findings of the present study, hypoxic vasodilatation in skeletal muscle appears to be mediated primarily by the A<sub>1</sub> receptor, indicating that considerable tissue-specific differences may exist (Marshall, 2002). We have recently observed a partial attenuation of hypoxic increases in cortical blood flow during nitric oxide synthase blockade with L-NAME, indicating that nitric oxide may indeed play

a role in addition to or as a result of adenosine receptor activation (Hunter *et al.* 2003c). Clearly, the cascade of events leading to vasodilatation is multifactorial and further work is necessary to determine which of several possible pathways are involved in the adenosine-mediated hypoxic increases in fetal cerebral blood flow.

The control of adenosine on cerebral blood flow during hypoxia may also include systemic regulation of arterial blood pressure. Autoregulation of cerebral blood flow is intact in the fetal sheep by 0.6 gestation, such that cerebral perfusion remains relatively constant over a range of perfusion pressures from 45 to 85 mmHg (Papile *et al.* 1985). However, during hypoxic stress, fetal autoregulation is lost and cerebral blood flow becomes pressure passive (Tweed *et al.* 1983). The findings of the present study support this previous work, since fetal arterial blood pressure increased gradually over the first 20 min to a plateau of 10–15 mmHg above baseline, while cerebral vascular resistance remained unchanged. Therefore, at least part of the hypoxic increase in cerebral blood flow is due to the combined increase in driving pressure and decreased autoregulatory response. It is possible that the decrease in arterial P<sub>CO<sub>2</sub></sub> noted for the control and 8-SPT groups may have played a role in preventing the fall in cerebral vascular resistance during hypoxia. However, this possibility seems unlikely since P<sub>CO<sub>2</sub></sub> is a relatively poor regulator of fetal cerebral blood flow compared to the adult (Ashwal *et al.* 1984; Yamashita *et al.* 1991). The fact that there was no significant attenuation of the hypoxic hypertension during 8-SPT or DPCPX infusion, and no clear difference between the hypoxic cerebral vascular resistance of control and 8-SPT experiments in the present study makes it impossible to discern the relative contribution of systemic and local effects of adenosine. It seems likely that the hypoxic increase in cerebral blood flow is due to a combination of both increased perfusion pressure and local vasodilatation, both of which have been shown to be mediated, at least in part, by adenosine (Winn *et al.* 1981a; Koos *et al.* 1995; Giussani *et al.* 2001).

Following restoration of normoxia, cerebral blood flow decreased to 75–80% of baseline values in those fetuses receiving 8-SPT, a response that was associated with pronounced increases of cerebral vascular resistance persisting for an hour or more. In contrast, blood flow did not decline below initial baseline values in control experiments. These results are consistent with previous studies in the fetal sheep using theophylline (Blood *et al.* 2002) and may indicate a role for adenosine in inhibiting the effect of vasoconstricting factors. As these post-hypoxic increases of resistance were not observed in fetuses receiving DPCPX, they are probably attributable to the action of A<sub>2</sub> receptors.

## Summary

The present findings add to a growing body of evidence that adenosine is an important modulator of adaptive responses to hypoxic stress in the fetal brain. Cortical blood flow is modulated during hypoxia by activation of adenosine receptors that are probably located outside the blood brain barrier. Adenosine also maintains cortical blood flow in the post-hypoxia period. Finally, the data suggest an active suppression of cortical metabolic rate, mediated by adenosine A<sub>1</sub> receptors located on the inside of the blood brain barrier. Together these mechanisms are likely to be an important part of the remarkable tolerance of the fetal brain to hypoxic stress and provide strong evidence for adaptive hypometabolism mediated by adenosine.

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