Effect of Cerebral Hypothermia on Cortisol and Adrenocorticotrophic Hormone Responses after Umbilical Cord Occlusion in Preterm Fetal Sheep

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ABSTRACT: The hypothalamic-pituitary-adrenal (HPA) axis is essential for adaptation to stress. In the present study, we examined the hypothesis that head cooling with mild systemic hypothermia would adversely affect fetal adrenocorticotrophic hormone (ACTH) and cortisol responses to an asphyxial insult. Chronically instrumented preterm fetal sheep (104 d of gestation, term is 147 d) were allocated to sham occlusion ($n = 7$), 25 min of complete umbilical cord occlusion ($n = 7$), or occlusion and head cooling with mild systemic hypothermia ($n = 7$, mean $\pm$ SEM esophageal temperature $37.6 \pm 0.3^\circ\text{C}$ vs $39.0 \pm 0.2^\circ\text{C}$; $p < 0.05$) from 90 min to 70 h after occlusion, followed by spontaneous rewarming. During umbilical cord occlusion, there was a rapid rise in ACTH and cortisol levels, with further increases after release of cord occlusion. ACTH levels returned to sham control values after 10 h in both occlusion groups. In contrast, plasma cortisol levels remained elevated after 48 h in both occlusion groups and were still significantly elevated in the hypothermia-occlusion group 2 h after rewarming, at 72 h, compared with the normothermia-occlusion and sham groups. In conclusion, hypothermia does not affect the overall HPA responses to severe asphyxia in the preterm fetus but does prolong the cortisol response. (*Pediatr Res* 63: 51–55, 2008)

There is now strong clinical and experimental evidence that a prolonged period of moderate cerebral hypothermia initiated within a few hours after severe hypoxia-ischaemia can reduce subsequent neuronal loss and improve behavioral recovery in adults and term newborn infants (1). Preterm infants have a much higher burden of neurologic impairment (2). Although the etiology of preterm injury is multifactorial, exposure to hypoxia as shown by metabolic acidosis, active labor, abnormal heart rate traces in labor, and subsequent low Apgar scores is an important factor associated with adverse outcomes (3–6), raising the possibility that some preterm infants might be candidates for treatment (7). However, despite the remarkable safety record of hypothermia so far in term infants, older clinical evidence suggests increased mortality in preterm infants (<1500 g) exposed to even mild systemic hypothermia (8–10).

In the first few days after birth, premature infants commonly have unstable arterial blood pressure, and, in turn, exposure to severe hypotension is associated with adverse outcomes (11,12). The hypothalamic-pituitary-adrenal (HPA)-mediated increase in cortisol during severe stress is essential to maintain blood pressure, and there is evidence that abnormal HPA function in some preterm infants is associated with cardiovascular compromise (13,14). The effect of hypothermia on the HPA responses of preterm infants is unknown. Mild hypothermia during cerebral aneurysm surgery has been associated with a significant decrease in plasma levels of cortisol and adrenocorticotrophic hormone (ACTH) at extubation (15) that potentially might compromise cardiovascular adaptation. Conversely, hypothermia might cause an excessive HPA response; for example, data in term piglets suggest that induced hypothermia during recovery from severe hypoxia-ischemia without sedation was associated with an excessive rise in plasma cortisol levels that may reduce its neuroprotective effects (16). However, there are no data from preterm paradigms. We have recently reported that, consistent with the data at older ages, cerebral cooling with mild systemic hypothermia markedly reduces loss of immature oligodendroglia and neurons after a period of asphyxia in the preterm fetal sheep (17,18), with a mild bradycardia compared with normothermia but without hypotension (17). Central cooling was sustained for 72 h but was milder than typically used during clinical cooling in term infants (19). In this paradigm, asphyxia has been associated with prolonged elevation of plasma cortisol levels, sustained for 48 h after occlusion (20). The effect of hypothermia on this response is unknown. We therefore examined the hypothesis that hypothermia after profound hypoxia induced by umbilical cord occlusion in unanesthetized preterm fetal sheep at 0.7 of gestation (term 147 d gestation) would be associated with an impaired HPA response.

MATERIALS AND METHODS

Experimental preparation. All procedures were approved by the Animal Ethics Committee of The University of Auckland. Twenty-one time-mated

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Abbreviations: HPA, hypothalamic-pituitary-adrenal; PGE₂, prostaglandin E₂

singleton Romney/Suffolk fetal sheep were instrumented at 97–99 d of gestation (term = 147 d) under general anesthesia (2% halothane in O₂). Although the sheep is relatively precocial compared with humans, this age is neurodevelopmentally equivalent to the human fetus of approximately 30–32 wk of gestation, before the onset of cortical myelination (21,22), and the adrenal gland is immature (23). We have previously reported changes in white and grey matter cell loss, cardiovascular variables, arterial pH, blood gases, and plasma levels of glucose and lactate in these fetuses (17,24). Details of anesthetic and fluid management, the sterile surgical preparation, intraoperative and postoperative antibiotic prophylaxis, and management have been described in detail previously (17).

Fetal catheters were placed in the left femoral artery and vein, right brachial artery and vein, and the amniotic sac. Two pairs of electroencephalogram electrodes (AS633-5SSF, Cooner Wire Co., Chatsworth, CA) were placed on the dura over the parasagittal parietal cortex (5 mm and 15 mm anterior to bregma and 10 mm lateral), with a reference electrode sewn over the occiput. A thermistor (Incutech-1, Mallinckrodt Medical, Inc., St. Louis, MO) was placed over the parasagittal dura 20 mm anterior to bregma to measure extradural temperature, and the burr holes were sealed and the skin over the fetal skull secured with cyanoacrylate glue. Electrocardiogram electrodes were sewn across the chest to record the fetal heart rate (FHR). A second thermistor (to measure fetal core body temperature) was placed in the fetal esophagus at the level of the right atrium. An inflatable silicone occluder was placed around the umbilical cord of all fetuses (In Vivo Metric, Healdsburg, CA). A cooling coil made from silicone tubing (external diameter 7.9 mm, internal diameter 4.8 mm; Silclear, Degania Silicone, Degania Bet, Israel) was attached over the dorsal surface of the scalp and extended over the lateral surface of the cranium down to the level of the external auditory meatus. All fetal catheters and leads were exteriorized through the maternal flank, and the maternal skin incision was infiltrated with the long-acting local anesthetic Marcaine (bupivacaine hydrochloride 0.25% with adrenaline 1:400,000; Astra Zeneca, North Ryde, NSW, Australia).

After surgery, sheep were housed together in separate metabolic cages with access to water and food as desired. A period of 4–5 d of postoperative recovery was allowed before starting experiments. The health and welfare of all animals was closely monitored by the researchers and supervised by the university veterinarian. Fetal brachial arterial blood was taken daily for gas analysis. Catheters were maintained patent by continuous infusion of heparinized isotonic saline (20 U mL⁻¹, 0.2 mL h⁻¹).

Experimental design and recordings. Experiments were conducted at 103–104 d gestation. Fetuses were randomly assigned to either normothermia-occlusion ($n = 7$), hypothermia-occlusion ($n = 7$), or normothermia-sham occlusion (sham control, $n = 7$) groups. Fetal asphyxia was induced in both occlusion groups by rapid inflation of the umbilical cord occluder for 25 min with sterile saline of a defined volume known to completely inflate the occluder (17). Successful occlusion was confirmed by observation of a rapid onset of bradycardia with a rise in mean arterial blood pressure (MAP) and by pH and blood gas measurements. In all groups, fetal arterial blood was taken at 15 min before occlusion, 20 min during occlusion, and 30 min, 2 h, 6 h, 24 h, 48 h, and 72 h postocclusion for blood gas, acid-base balance (Ciba-Corning Diagnostics 845 blood gas analyzer and co-oximeter, East Walpole, MA), glucose, and lactate determination (YSI model 2300, Yellow Springs, OH), and ACTH and cortisol measurements. Intrauterine cooling was performed from 90 min to 70 h after the end of occlusion. In the hypothermia group, the 72 h blood sample was taken after rewarming for 2 h. Cooling was induced by circulating cold water (10°C) through a coil around the fetal head. Cooling was titrated in the first 2 h to reduce fetal extradural temperature from $39.4 \pm 0.1^\circ\text{C}$ to less than 34°C . At 72 h after the end of occlusion, the ewes and fetuses were killed by an overdose of sodium pentobarbitone (9 g intravenously to the ewe, Pentobarb 300, Chemstock International, Christchurch, New Zealand), and fetal adrenal weights were measured postmortem.

Hormone analysis. Fetal plasma ACTH and cortisol levels were measured using specific radioimmunoassays (RIA) established and validated for ovine plasma (25). Total immunoreactive cortisol concentrations were determined in triplicate after extraction with diethyl ether by an in-house RIA validated for use with maternal and fetal ovine plasma. The antiserum to cortisol was raised in rabbits against Cortisol 3-CMO:bovine serum albumin and was used at a final dilution of 1:19200. The cross-reactivity of the antiserum at 50% binding with other relevant steroid-related compounds was 3.3% 11-deoxycortisol, 0.18% cortisone, 0.5% corticosterone, 0.015% progesterone, and 0.002% 11 α -hydroxyprogesterone. No detectable cross-reactivity was observed for 17 α -hydroxypregnenolone, pregnanediol, 21-deoxycortisone, aldosterone, cholesterol, or dexamethasone. The lower limit of detection was 10 pg/tube (0.13 ng/mL); samples containing less than 0.13 ng/mL were given this value for the purposes of analysis. The intra- and interassay coefficients

of variations were 3.68% and 3.97%, respectively, at the cortisol concentrations determined in plasma.

Immunoreactive concentrations of ACTH were measured in duplicate using a commercially available ¹²⁵I RIA kit (24130, DiaSorin, Stillwater, MN) previously validated for use with both fetal and maternal ovine plasma. The intra-assay and interassay coefficient of variation were 9.7% and 12.8%, respectively. The mean sensitivity of the ACTH assay was 9.7 pg/mL; samples containing less than 9.7 pg/mL were given this value for analysis.

Data analysis and statistics. Offline analysis of the physiologic data were performed using customized Labview programs (Labview, National Instruments, Austin, TX). The effect of occlusion was evaluated by analysis of variance (ANOVA; SPSS v12, SPSS, Inc., Chicago, IL). The baseline period was taken as the mean of the samples before occlusion. Differences between groups were determined by ANOVA adjusted using baseline levels as a covariate. Where a significant effect of treatment group or an interaction between time and group was found, post hoc comparisons were undertaken using the least significant difference test. Statistical significance was accepted at $p < 0.05$. Data are mean $\pm$ SEM.

RESULTS

Data for arterial pH, blood gases, glucose, lactate, FHR, and MAP for all groups are presented in Table 1. Occlusion of the umbilical cord was associated with marked fetal hypoxia and acidosis, bradycardia, and hypotension (Table 1) ($p < 0.001$), as previously reported (17). After reperfusion, there was rapid recovery of FHR and MAP. MAP was significantly higher at 2–6 h after occlusion in the normothermia-occlusion group compared with sham controls (Table 1) ($p < 0.005$, ANOVA) and returned to sham control values by 24 h. In the hypothermia-occlusion group, MAP was intermediate between the sham controls and the normothermia-occlusion group and not significantly different from normothermia occlusion. FHR was significantly lower in the normothermia-occlusion group than sham controls from 24 to 72 h. FHR was transiently lower in the hypothermia-occlusion group than the normothermia-occlusion group from 4 h to 6 h and lower than in sham controls from 4 to 48 h after occlusion but returned to sham control values by 72 h.

Brain and body temperature. Head cooling was initiated 90 min after the end of the umbilical cord occlusion and was associated with a significant fall in extradural and esophageal temperatures starting within an hour after the onset of cooling (17). The mean extradural temperature during cooling was $31.0 \pm 0.8^\circ\text{C}$ (versus $39.4 \pm 0.2^\circ\text{C}$ normothermia occlusion, $p < 0.001$), and the mean esophageal temperature was $37.6 \pm 0.3^\circ\text{C}$ (versus $39.0 \pm 0.2^\circ\text{C}$, $p < 0.001$).

ACTH and cortisol. There were no significant baseline differences in ACTH or cortisol levels between the groups. After 20 min of occlusion, there was a significant rise in plasma concentrations of both ACTH ($p < 0.05$, ANOVA) and cortisol ($p < 0.05$) in both occlusion groups compared with sham controls. After reperfusion, plasma ACTH levels remained significantly elevated for the first 24 h after occlusion, peaking between 2 and 4 h in both the normothermia and hypothermia groups (Fig. 1). There were no significant differences in the ACTH responses of the normothermia-occlusion and hypothermia-occlusion groups (ANOVA). Plasma cortisol levels peaked at 6 h after occlusion in both the normothermia and hypothermia groups ($p < 0.05$) (Fig. 1) and remained significantly elevated compared with sham controls until 48 h after occlusion. Mild systemic hypothermia did not significantly affect the response to occlusion in the first 48 h;

Table 1. Fetal arterial pH, blood gas, glucose and lactate values for sham control (S), normothermia-occlusion (N), and hypothermia-occlusion (H) groups before a 25 min period of umbilical cord occlusion (baseline), at 20 min of occlusion or sham occlusion (occlusion), and 2, 4, 6, 24, 48, and 72 h after occlusion

	Baseline	Occlusion	2 h	4 h	6 h	24 h	48 h	72 h
pH								
S	7.39 ± 0.00	7.38 ± 0.00	7.39 ± 0.00	7.39 ± 0.00	7.38 ± 0.00	7.38 ± 0.00	7.37 ± 0.00	7.37 ± 0.01
N	7.37 ± 0.00	6.82 ± 0.03*	7.34 ± 0.01	7.40 ± 0.00	7.41 ± 0.00	7.37 ± 0.00	7.38 ± 0.00	7.38 ± 0.00
H	7.39 ± 0.00	6.85 ± 0.03*	7.39 ± 0.01	7.46 ± 0.01	7.46 ± 0.02	7.43 ± 0.02	7.41 ± 0.01	7.43 ± 0.01
PaCO ₂ mm Hg								
S	48.8 ± 1.3	48.3 ± 1.5	46.8 ± 1.0	47.3 ± 0.8	47.8 ± 1.2	45.0 ± 1.7	48.0 ± 1.1	46.3 ± 1.6
N	48.2 ± 1.0	142.2 ± 6.0*	46.8 ± 1.0	47.8 ± 1.2	45.6 ± 1.4	47.4 ± 1.1	47.2 ± 0.9	46.5 ± 1.2
H	47.8 ± 4.3	138.4 ± 24.0	37.9 ± 2.9*†	39.8 ± 4.0*†	39.5 ± 3.0*†	38.8 ± 7.3*†	41.5 ± 2.9*†	42.9 ± 5.1
PaO ₂ mm Hg								
S	22.5 ± 0.9	22.7 ± 0.9	22.1 ± 1.0	22.6 ± 1.0	22.3 ± 1.0	22.2 ± 2.1	23.4 ± 1.5	23.5 ± 1.4
N	23.0 ± 0.9	8.7 ± 0.8*	26.2 ± 1.3*	24.8 ± 1.0	24.8 ± 0.9	26.6 ± 1.3	28.0 ± 1.3*	28.3 ± 3.6*
H	24.7 ± 3.0	9.1 ± 4.3*	24.7 ± 3.7	25.1 ± 3.7	24.4 ± 3.2	23.6 ± 2.6	24.5 ± 4.3	27.0 ± 7.1
Glucose mmol/L								
S	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
N	1.0 ± 0.1	0.7 ± 0.1*	1.2 ± 0.1	1.3 ± 0.1*	1.4 ± 0.1*	1.5 ± 0.1*	1.3 ± 0.1	1.2 ± 0.1
H	0.9 ± 0.2	0.5 ± 0.4*	1.3 ± 0.3	1.4 ± 0.3*	1.5 ± 0.3*	1.4 ± 0.4	1.3 ± 0.4	1.4 ± 0.3
Lactate mmol/L								
S	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.0	0.8 ± 0.2	0.6 ± 0.1	0.8 ± 0.1	0.9 ± 0.1
N	0.7 ± 0.0	6.5 ± 0.2*	3.6 ± 0.5*	2.5 ± 0.4*	1.9 ± 0.2*	1.0 ± 0.1*	0.8 ± 0.1	0.9 ± 0.1
H	0.7 ± 0.1	5.8 ± 1.4*	2.9 ± 1.6*	1.4 ± 0.8†	1.2 ± 0.5*†	0.9 ± 0.3	0.7 ± 0.2	0.8 ± 0.2
MAP mm Hg								
S	36.2 ± 0.7	36.0 ± 0.6	35.6 ± 0.8	35.9 ± 0.9	35.8 ± 0.8	35.0 ± 0.8	36.3 ± 1.2	36.6 ± 1.2
N	37.4 ± 1.0	14.2 ± 1.1*	43.0 ± 1.4*	40.0 ± 2.1*	41.9 ± 2.2*	37.6 ± 1.6	36.7 ± 1.0	35.6 ± 0.9
H	35.8 ± 1.0	13.8 ± 1.3*	41.5 ± 1.6*	37.5 ± 0.9	38.6 ± 0.7	38.3 ± 1.3*	37.3 ± 1.2	38.1 ± 1.2
FHR bpm								
S	189.0 ± 3.3	176.0 ± 2.4	188.6 ± 3.3	190.7 ± 1.7	195.9 ± 2.7	189.0 ± 5.0	187.6 ± 3.6	187.5 ± 3.9
N	183.7 ± 3.7	73.0 ± 4.9*	182.4 ± 5.4	207.2 ± 10.8	202.2 ± 6.0	176.5 ± 3.8*	174.4 ± 7.8*	176.2 ± 5.2*
H	182.7 ± 2.6	71 ± 4.2*	184.1 ± 8.7	188.6 ± 11.0*†	174.7 ± 8.1*†	164.1 ± 3.3*	162.8 ± 4.1*	186.9 ± 4.2†

Values are mean ± SEM.

MAP and FHR values before and after occlusion or sham occlusion are hourly averages; values at 20 min of occlusion are 1 min averages.

* $p < 0.05$ compared with sham control; † $p < 0.05$ compared with normothermia occlusion.

however, cortisol levels were significantly greater at 72 h than in either the sham control or normothermia-occlusion groups ($p < 0.05$, ANOVA).

Adrenal weight. There was no overall effect of group on mean adrenal weight at postmortem ($p = 0.1$, one way ANOVA): sham controls 0.10 ± 0.01 mg, normothermia occlusion 0.11 ± 0.01 mg, hypothermia occlusion 0.13 ± 0.01 mg.

DISCUSSION

The HPA responses to stress are critical for newborn adaptation. In preterm infants, there is evidence that cortisol levels are significantly correlated with the lowest blood pressure in the immediate postnatal period (14), and at least some infants with refractory hypotension have adrenocortical insufficiency that is responsive to exogenous steroid therapy (13,14). The present study demonstrates that cerebral hypothermia with mild systemic cooling after severe hypoxia neither suppressed the HPA response nor triggered an exaggerated rise in cortisol. Consistent with our previous report (20), occlusion led to a robust, prolonged ACTH and cortisol response that continued well after the end of occlusion. Hypothermia started after the occlusion period did not affect either the ACTH or cortisol response in the first 48 h after occlusion but was associated with a small increase in cortisol, but not ACTH levels, 72 h after asphyxia compared with normothermia.

Concern that hypothermia after hypoxic-ischemic injury might adversely affect HPA responses was raised by a study in which moderate whole-body cooling was not protective in the unanesthetized piglets after an hypoxic-ischemic event, and cooling was associated with increased cortisol levels (16). There is evidence that blockade of the glucocorticoid receptor after ischemia in adult rodents can at least transiently improve neuronal survival (26), and thus an exaggerated cortisol response might well impair hypothermic protection. In contrast, the present study suggests that a known protective interval of hypothermia (17) had only a very small effect on the HPA axis. Partly, this may be because the degree of central cooling in the present study was relatively mild, with less than a 2°C fall in central temperature compared with 4°C in the piglets (16); this is also somewhat less than currently used during clinical head cooling (a mean reduction of 2.5°C) (19). Potentially, it might reflect higher levels of circulating inhibitory neuromodulators such as adenosine, which could attenuate stress experienced by the fetus (27). Against this possibility, the HPA response to asphyxia in the present study was very marked, with a relative rise greater than that reported after a severe hypoxic insult in the piglet (16).

In part, it likely also reflects the global asphyxial insult used in the present study and correspondingly much more severe cardiovascular impact (28) than seen in the neonatal piglet

Figure 1. Time sequence of changes in fetal plasma ACTH (top) and cortisol levels (bottom) before, during, and after sham occlusion (open bars) or 25 min of occlusion of the umbilical cord followed by normothermia (black bars, normothermia occlusion) or head cooling with mild systemic hypothermia from 90 min to 70 h after occlusion (grey bars, hypothermia occlusion). Occ, 20 min of occlusion; 30 m, 30 min after occlusion. Data are mean $\pm$ SEM. * p < 0.05 vs sham control. † p < 0.05 vs normothermia occlusion.

model of controlled hypoxia and hypotension (29). We have previously reported that peripheral vascular resistance was increased after umbilical cord occlusion in preterm fetal sheep and that cortisol levels during this time were correlated positively with MAP (20), suggesting that the cortisol response was at least partly contributing to maintenance of blood pressure at a time when cardiac function was impaired after hypoxia. Thus, we may speculate that the effect of hypothermia on the HPA axis may have been masked in the first 48 h by much stronger stimuli consequent to profound asphyxia.

The early rises in ACTH and cortisol in the present study suggest an initial ACTH driven response. In contrast, at 24 and 48 h after hypoxia, cortisol levels were dissociated from those of ACTH, remaining significantly elevated well after ACTH levels had returned to sham control levels (20). In the hypothermia group, cortisol levels were still elevated 2 h after rewarming at 72 h compared with sham controls. Although there was a trend for slower resolution of the postasphyxial rise in ACTH during cooling, by 72 h after the end of occlusion, ACTH values were not different from sham controls. A potential limitation is that we do not know whether ACTH was elevated at 70 h, before rewarming. However, because cortisol levels were still elevated, this is suggestive of an ACTH-independent adrenal response, as reported during chronic hypoxia (30,31). These data suggest for the first time

that hypothermia may also trigger such a response in the preterm fetus.

Potential mechanisms might include increased sympathetic activity (32), with activation of the splanchnic nerve (33), or increased levels of other steroidogenic factors, including the neuropeptides and prostaglandin E2 (PGE2) (31). Although endogenous and exogenous PGE2 substantially increase cortisol levels, this effect is primarily mediated through hypothalamic ACTH release (34). However, there is some *in vitro* evidence of a paracrine effect of PGE2 to sensitize adrenal cells to ACTH stimulation in preterm fetal sheep (35). Alternatively, it might potentially reflect slower clearance of cortisol during cooling; however, postnatal data suggest that clearance is only delayed by significantly greater falls in rectal temperature than in the present study (36). Finally, there is evidence that chronic hypoxia can increase the size and enzymatic activity of the adrenal gland (31,37). Thus, potentially, the delayed fall in cortisol levels could reflect local adrenal up-regulation. However, although there was a trend to increased adrenal weight after hypothermia compared with sham controls in the present study, with intermediate values in the normothermia-occlusion group, there was no significant overall effect. In conclusion, the present study suggests that hypothermia does not adversely affect the prolonged up-regulation of HPA activity seen after profound reversible asphyxia in preterm fetal sheep.

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