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The Effect of Mild Hypothermia on Insulin-like Growth Factors After Severe Asphyxia in the Preterm Fetal Sheep

Vincent Roelfsema, MD, BSc, Alistair Jan Gunn, MBChB, PhD, Bernhard H. Breier, PhD, Josine S. Quaedackers, MD, BSc, and Laura Bennet, PhD

OBJECTIVES: Persistent reductions in insulin-like growth factor I (IGF-I) levels in the preterm infant are strongly associated with increased risk of retinopathy and other complications, and may result from exposure to severe hypoxia. The effects of a potential new treatment for hypoxic-ischemic encephalopathy, cerebral hypothermia, on the responses of the IGF axis to hypoxia are unknown. The aim of this study was to examine the effects of prenatal asphyxia and cerebral hypothermia on changes in IGF-I and -II, IGF-binding protein 1 (IGFBP-1), and insulin levels.

METHODS: Fetal sheep at 0.7 of gestation underwent either sham asphyxia and sham cooling ($n = 7$), asphyxia induced by 25 minutes of complete umbilical cord occlusion alone ($n = 8$), or asphyxia and head cooling ($n = 8$, extradural temperature $29.5 \pm 2.6^\circ\text{C}$ [vs $39.4 \pm 0.3^\circ\text{C}$; $P < .05$]). Fetuses were studied for 3 days post-insult, during which time fetal blood samples were taken for endocrine measurements.

RESULTS: There were no IGF axis changes during occlusion. Plasma IGF-I levels significantly decreased between 6 hours and 48 hours after asphyxia and IGF-II levels by 10 hours, in both asphyxia groups. IGFBP-1 rose from 4 hours, reaching a peak at 10 hours and returning to control values by 48 hours in the normothermia group, and by 24 hours in the hypothermia group. Insulin levels decreased between 2 hours and 10 hours after asphyxia in both asphyxia groups, and subsequently normalized.

CONCLUSIONS: This study demonstrates for the first time that transient asphyxia in the preterm sheep fetus is associated with a significant decrease in IGF-II as well as IGF-I during recovery, and that these responses are not altered by mild systemic hypothermia. (*J Soc Gynecol Investig* 2005;12: 232–7) Copyright © 2005 by the Society for Gynecologic Investigation.

KEY WORDS: Asphyxia, prematurity, hypothermia, insulin-like growth factors.

There is now strong experimental evidence that cerebral hypothermia initiated during the early hours after perinatal hypoxia-ischemia can prevent delayed cell death and improve neural recovery.¹ While clinical trials are currently underway in the term infant,² hypothermia has not been evaluated in the preterm infant, despite their much higher burden of neurologic impairment and systemic complications.³ It has been suggested from studies at term that hypothermia can cause adverse endocrine and metabolic effects that may reduce its neuroprotective effects and impair recovery⁴; however, there are no data from preterm paradigms.

In recent years, there has been increasing interest in the key role insulin-like growth factors (IGFs) may play in metabolic regulation, growth, and organ function and development in preterm infants.^{5–8} Preterm birth is associated with a rapid fall in serum

IGF-I levels.^{9,10} Persistent reductions in IGF-I levels are associated with an increased risk for later retinopathy, and other complications of prematurity, particularly in infants born around the critical organ development period of 30–33 weeks.^{11,12} Although the etiology of preterm injury is highly complex and multifactorial,¹³ hypoxia-ischemia is one of the many factors that may complicate the courses of preterm infants,¹³ and it may contribute to adverse outcomes. Hypoxia-ischemia can occur before, during, or after birth^{14,15} and it may exacerbate the low levels of IGF-I in premature infants.¹⁶

It was the purpose of the current study, therefore, to determine the IGF, insulin, and IGF-binding protein 1 (IGFBP-1) responses of the preterm fetal sheep to severe acute hypoxia in utero and to evaluate whether these responses are modified by cerebral hypothermia at an age corresponding to human neuronal maturation of 30–33 weeks.¹⁷

MATERIALS AND METHODS

Experimental Preparation

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

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singleton Romney/Suffolk fetal sheep were operated on at 98–99 days of gestation (term = 147 days). Food, but not water, was withdrawn 18 hours before surgery. Ewes were given 5 mL of Streptopen (Procaine Penicillin [250,000 IU] and Dihydrostreptomycin [250 mg · mL⁻¹; Stockguard Labs Ltd, Hamilton, New Zealand]) intramuscularly for prophylaxis 30 minutes prior to the start of surgery. Anesthesia was induced by intravenous (IV) injection of Alfaxan (3 mg · kg⁻¹; Alphaxalone, Jurox, Rutherford, NSW, Australia), and general anesthesia maintained using 2–3% halothane in O₂. Ewes were not ventilated and the depth of anesthesia, maternal heart rate, and respiration were constantly monitored by trained anesthetic staff.

All surgical procedures were performed using sterile techniques.¹⁸ Polyvinyl catheters were placed in the left fetal femoral artery and vein, right brachial artery, and the amniotic sac. A stainless steel electrode (Cooner Wire, Chatsworth, CA) was placed across the fetal chest to measure the fetal electrocardiogram (ECG). A thermistor (Incu-Temp-1, Mallinckrodt Medical Inc, St Louis, MO) was placed over the parasagittal dura 20 mm anterior to the bregma; the burr holes were sealed, and the skin over the fetal skull was closed using cyanoacrylate glue. A cooling cap made from a coil of silicone tubing (external diameter, 7.9 mm; internal diameter, 4.8 mm; Degania Silicone, Degania, Bet, Emek Hayarden, 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. An inflatable silicone occluder was placed around the umbilical cord of all fetuses (In Vivo Metric, Healdsburg, CA). A second thermistor (to measure fetal core body temperature) was placed in the fetal esophagus at the level of the right atrium. All fetal leads were exteriorized through the maternal flank and a maternal long saphenous vein was catheterized to provide access for postoperative care and euthanasia. Antibiotics (80 mg Gentamicin; Pharmacia and Upjohn, Rydalmere, NSW, Australia) were administered into the amniotic sac prior to closure of the uterus.

Postoperatively, all sheep were housed together in separate metabolic cages with access to water and food ad libitum. They were kept in a temperature-controlled room (16 ± 1°C, humidity 50% ± 10%), on a 12-hour light/dark cycle. A period of 4–6 days postoperative recovery was allowed before experiments commenced, during which time antibiotics were administered IV to the ewe daily for 4 days (600 mg Benzylpenicillin Sodium; Novartis Ltd, Auckland, New Zealand, and 80 mg Gentamicin). Fetal catheters were maintained patent by continuous infusion of heparinized saline (20 U/mL at 0.15 mL/h) and the maternal catheter maintained by daily flushing.

Experimental Design

Experiments were conducted at 103–104 days gestation (0.7 of gestation). Mean arterial pressure (MAP), corrected for maternal movement by subtraction of amniotic fluid pressure, and the fetal ECG were recorded continuously from 12 hours before occlusion to 72 hours after occlusion. Data were stored

Figure 1. Time course of changes in fetal core body temperature (squares) and extradural temperature (circles) in the normothermic (open symbols) and hypothermic asphyxia (closed symbols) groups. Data are mean ± SEM.

to disk by custom software for off-line analysis (Labview for Windows; National Instruments Ltd, Austin, TX). Fetuses were randomly assigned to one of three groups: sham controls (sham asphyxia and sham cooling, $n = 7$), asphyxia group (asphyxia with sham cooling, $n = 8$), and hypothermia group (asphyxia with induced hypothermia, $n = 8$). In the asphyxia groups, fetal asphyxia was induced by rapid inflation of the umbilical cord occluder for 25 minutes with sterile saline of a defined volume known to completely inflate the occluder as determined in pilot experiments with a Transonic flow probe placed around an umbilical vein.¹⁹ The duration of occlusion was chosen as one that we have previously reported to represent an acute, severe, near-terminal insult but which could be survived without post-asphyxial cardiac support.^{18,20} Successful occlusion was confirmed by observation of a rapid onset of bradycardia with a rise in MAP, and by pH and blood gas measurements.

In the hypothermia group, intrauterine cooling was initiated 90 minutes after the end of asphyxia and continued until 2 hours before the end of the study (72 hours after asphyxia or sham asphyxia). Cooling was induced by circulating cold water (10°C) through the silicone coil around the fetal head. Cooling was titrated in the first 2 hours to reduce fetal extradural temperature from $39.4 \pm 0.1^\circ\text{C}$ to between 29 and 33°C (Figure 1).

Fetal arterial blood samples were taken 15 minutes prior to asphyxia or sham asphyxia, 5 and 20 minutes during asphyxia or sham asphyxia, and 2, 4, 6, 10, 24, 48, and 72 hours post-asphyxia or sham asphyxia for blood gas, acid-base balance (Ciba-Corning Diagnostics 845 blood gas analyzer and co-oximeter; Boston, MA) and for glucose and lactate determination (YSI model 2300; Yellow Springs, OH). In the hypothermia group, the 72-hour blood sample was taken after rewarming for 2 hours. Blood was transferred immediately upon collection to chilled test tubes and centrifuged at 4°C (3000 rpm) for 15 minutes. Plasma was stored at -20°C for subsequent hormone analysis (IGF-I, IGF-II, IGFBP-1, insu-

Table 1. Fetal Arterial pH, Blood Gas, Glucose, and Lactate Values for Sham Control, Asphyxia Plus Normothermia, and Asphyxia Plus Hypothermia Groups Before Umbilical Cord Occlusion (baseline), After 20 Minutes of Occlusion or Sham Occlusion (asphyxia), and 2, 4, 6, 24, 48, and 72 Hours After Release of Occlusion

	Baseline	Asphyxia	2 hours	4 hours	6 hours	24 hours	48 hours	72 hours
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
A	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 ± 0.02*	7.39 ± 0.00	7.46 ± 0.01*	7.44 ± 0.00*	7.43 ± 0.01*	7.41 ± 0.00*	7.43 ± 0.00*
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
A	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	48.5 ± 1.6	139.0 ± 9.0*	38.0 ± 1.0*	40.0 ± 1.0*	40.0 ± 1.1*	39.0 ± 3.0*	42.5 ± 1.0*	42.9 ± 2.0
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
A	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 ± 1.1	9.1 ± 1.6*	24.7 ± 1.3	25.1 ± 1.4	24.4 ± 1.1	23.6 ± 0.9	24.5 ± 1.5	27.0 ± 2.7
Glucose (mM)								
S	1.03 ± 0.10	1.03 ± 0.11	1.01 ± 0.12	1.10 ± 0.06	1.11 ± 0.10	1.20 ± 0.12	1.20 ± 0.10	1.20 ± 0.10
A	1.02 ± 0.10	0.67 ± 0.10*	1.24 ± 0.10	1.30 ± 0.10*	1.40 ± 0.10*	1.50 ± 0.14*	1.30 ± 0.10	1.23 ± 0.10
H	0.95 ± 0.10	0.53 ± 0.14*	1.30 ± 0.10	1.40 ± 0.12*	1.52 ± 0.10*	1.43 ± 0.13	1.30 ± 0.13	1.42 ± 0.10
Lactate (mM)								
S	0.73 ± 0.10	0.73 ± 0.11	0.66 ± 0.12	0.75 ± 0.04	0.75 ± 0.15	0.64 ± 0.10	0.83 ± 0.11	0.90 ± 0.12
A	0.72 ± 0.03	6.46 ± 0.17*	3.6 ± 0.52*	2.48 ± 0.44*	1.86 ± 0.24*	1.00 ± 0.12*	0.78 ± 0.10	0.88 ± 0.10
H	0.70 ± 0.03	6.11 ± 0.52*	2.90 ± 6.0*	1.40 ± 0.32*	1.21 ± 0.16*	0.92 ± 0.12	0.73 ± 0.10	0.80 ± 0.10

Values are mean ± SEM.

S = sham control; A = asphyxia plus normothermia; H = hypothermia.

* $P < .05$ compared to sham control.

lin). After the last blood sample ewes and fetuses were killed by an IV overdose of pentobarbitone sodium (9 g) to the ewe (Pentobarb 300; Chemstock International, Christchurch, New Zealand).

Data Analysis and Statistics

Fetal plasma IGF-I, IGF-II, IGFBP-1, and insulin levels were measured using specific ovine radioimmunoassays (RIA) established and validated in this laboratory.¹⁶ Statistical analyses were performed using SPSS version 10 (SPSS Inc, Chicago, IL). Differences between groups were determined by analysis of variance, adjusted using baseline levels as a covariate (ANCOVA); the endocrine time series data were treated as repeated measures to allow for repeated sampling. Where a significant effect of treatment group or an interaction with group was found, post hoc comparisons were undertaken using the least significant difference test. Statistical significance was accepted at $P < .05$.

RESULTS

Blood Composition Measurements

Data for fetal arterial pH, blood gas, and glucose levels are presented in Table 1.

Effect of Cooling on Brain and Body Temperature

Head cooling caused a significant fall in extradural temperature starting within 1 hour after the onset of cooling, reaching a nadir of $29.5 \pm 2.6^\circ\text{C}$ (vs $39.4 \pm 0.3^\circ\text{C}$; $P < .0001$, ANOVA). Extradural temperature gradually increased during the second

and third day of cooling to a mean of $32.3 \pm 2.2^\circ\text{C}$ by 72 hours. During cooling, core body temperature dropped to a nadir of $37.2 \pm 0.8^\circ\text{C}$ (vs $39.3 \pm 0.1^\circ\text{C}$; $P < .05$) and averaged $37.5 \pm 0.7^\circ\text{C}$ over the whole cooling period (Figure 1).

Endocrine Data

There was no significant difference between groups for any endocrine measure during the baseline period. There were no significant changes during asphyxia. IGF-1 levels showed a significant effect of treatment group ($P = .006$, repeated measures ANOVA), with a significant interaction between time and group ($P = .001$). There was a significant reduction in circulating plasma concentrations of IGF-I after asphyxia from 6 to 48 hours in the asphyxia and hypothermia groups ($P < .05$ for both groups compared to sham controls, Figure 2), with no significant difference between the asphyxia and hypothermia groups. There was no overall effect of group on IGF-II levels, but an interaction between asphyxia and time ($P < .05$), with a fall in IGF-II levels at 10 hours in the asphyxia-normothermia group and at 6 and 10 hours in the hypothermia group ($P < .05$ compared to sham control, Figure 2). However, there was no significant difference between the normothermia and hypothermia groups. Changes in IGFBP-1 concentrations showed a significant effect of treatment group ($P = .001$) with a significant interaction between time and treatment group ($P = .003$), such that concentrations of IGFBP-1 increased dramatically from 4 hours in both asphyxia groups, and remained significantly elevated until 24 hours in the asphyxia group and until 10 hours in the hypothermia group ($P < .05$).

Figure 2. Fetal plasma concentration changes in IGF-I and IGF-II before, during (sample taken at 20 minutes of a 25-minute occlusion insult) and after (2, 4, 6, 10, 24, 48, and 72 hours), in sham-controls (open bars), asphyxia with normothermia (dark bars) or asphyxia with hypothermia started 90 minutes after the insult and continued until 2 hours before the 72-hour sample (hatched bars). Data are mean $\pm$ SEM. * P < .05 compared to sham controls.

.05, Figure 3). Plasma insulin levels showed a significant overall effect of treatment group (P < .05) but no interaction between treatment and time (P = .3). There was a significant fall in plasma concentrations of insulin between 2 and 10 hours after asphyxia in both asphyxia groups (P < .05, Figure 3), with no significant difference between the normothermic and hypothermic asphyxia groups.

DISCUSSION

The present study demonstrated that prenatal exposure to asphyxia is associated with a rapid fall in circulating levels of insulin, IGF-I and IGF-II in unanesthetized preterm fetal sheep. These changes were associated with a dramatic rise in IGFBP-1 levels, which would help to further reduce bioavailable circulating IGF-I. The data demonstrate that cerebral cooling with mild systemic hypothermia did not significantly alter the pattern of changes in circulating IGF-I and IGF-II, IGFBP-1, and insulin after an acute severe asphyxia. Further, the current study demonstrates for the first time, in contrast

with previous data,¹⁶ that exposure to asphyxia can be associated with a transient reduction in IGF-II levels.

The present study suggests that following acute severe hypoxia, IGFs and binding proteins play a key role in metabolic regulation in the preterm sheep fetus. Previous studies in the fetal sheep have shown that an infusion of IGF-I with insulin is highly anabolic.²¹ Thus, the reductions of both circulating IGF-I and insulin levels in the present study may facilitate peripheral protein catabolism. The present study suggests that IGF-II may also contribute to acute metabolic regulation, with circulating IGF-II levels falling at 6 hours and significantly suppressed by 10 hours after occlusion, albeit more modestly than IGF-I. In the fetus, IGF-II has been classically seen as constitutive, and IGF-I more labile. Large environmental changes, such as severe starvation or very prolonged hypoxia, were required before circulating concentrations of IGF-II changed significantly.^{22,23} Recent studies, however, suggest an important role for IGF-II in modulating both the placental supply and fetal demand for nutrition.^{24,25} Thus, the present finding of relatively acute changes in IGF-II levels may reflect

Figure 3. Fetal plasma concentration changes in IGFBP-1 and insulin levels before, during (sample taken at 20 minutes of a 25-minute occlusion insult) and after (2, 4, 6, 10, 24, 48, and 72 hours), in sham-controls (open bars), asphyxia with normothermia (dark bars), or asphyxia with hypothermia started 90 minutes after the insult and continued until 2 hours before the 72-hour sample (hatched bars). Data are mean $\pm$ SEM. * P < .05 compared to sham controls.

the dynamic metabolic adaptations required after severe asphyxia.

The bioavailability and activity of the IGFs is further regulated by changes in the IGFBPs and the IGF receptors. For example, although there are limited data, hypoxia can modulate IGF-I receptor levels in some tissues.²⁶ The increase in IGFBP-1 levels after asphyxia may augment the impact of reduced circulating IGF-1 levels by reducing its bioavailability. Several mechanisms are likely to have contributed to the increase in IGFBP-1. In vitro, expression of IGFBP-1 in the liver has been shown to be stimulated by hypoxia.²⁷ The intron 1 region of the IGFBP-1 gene contains an hypoxia response element (HRE), which can be induced either directly by hypoxia or indirectly through release of hypoxia-inducible factor-1 (HIF-1).²⁸ In the present study, IGFBP-1 started to rise from 4 hours after umbilical cord occlusion, at a time when the hypoxia was resolved completely, consistent with the observed delay in vitro between the onset of hypoxia and the subsequent rise in IGFBP-1.²⁸

In addition, insulin is an important modulator of IGFBP-1 levels,²⁹ such that fetal IGFBP-1 levels are inversely related to insulin concentrations.³⁰ Consistent with this, our current findings show that reduced insulin levels after asphyxia preceded the rise in IGFBP-1 levels. We have recently demonstrated in the 0.7 gestation fetus that sympathetic tone is significantly elevated after asphyxia.²⁰ This increase in sympathetic activity may also be important because the hypoxic inhibition of insulin secretion is mediated by an α_2 -adrenergic mechanism,³¹ which in turn stimulates IGFBP-1 production.³² In adults, IGFBP-1 is cleared from the circulation by the kidney and impaired renal function results in high circulating IGFBP-1 levels³³; however, this is unlikely to be important in the fetus as at this gestation there is only a small, transient reduction in glomerular filtration rate following asphyxia.¹⁸

The present study demonstrates for the first time that induced head cooling with mild systemic hypothermia did not affect the changes in plasma IGF-I levels or IGF-II after asphyxia. IGFBP-1 levels resolved by 24 hours in the hypothermic asphyxia group but remained elevated in the normothermic asphyxia group, even though insulin levels had returned to normal in both groups. This was associated with higher blood glucose levels in the normothermia asphyxia group, and may reflect the hyperglycemic effects of IGFBP-1.³⁴ We did observe a mild metabolic alkalosis during cooling, which may be related to stimulation of maternal breathing by cooling.³⁵

Compared with a previous report in younger fetuses (0.6 of gestation) the present study demonstrated a less pronounced fall in IGF-I (50% vs 80%) and an acute fall in IGF-II levels in contrast with a small secondary rise in the days following asphyxia in the younger fetuses.¹⁸ The insults at each gestation are comparable and near terminal. The extent to which these differences reflect differences in metabolic demand and degree of injury between the two ages, maturational differences in sympathetic tone,²⁰ or renin-angiotensin system activity³⁶ remains to be determined.

In conclusion, this study demonstrates that therapeutic head-cooling in the preterm fetus is not associated with major changes in the responses of circulating concentrations of IGFs after asphyxia. The overall profile of the changes in IGFs and insulin is consistent with facilitation of catabolism. Intriguingly, this study suggests for the first time that by 0.7 of gestation changes in IGF-II may also contribute, in addition to IGF-I, to regulation of fetal metabolism during the acute phase of post-asphyxial recovery in utero.

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