The effect of intermittent umbilical cord occlusion on insulin-like growth factors and their binding proteins in preterm and near-term ovine fetuses

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Abstract

Intermittent umbilical cord compression with resultant fetal hypoxia can have a negative impact on fetal growth and development. Insulin-like growth factors (IGFs) and their binding proteins (IGFBPs) are the most important regulators of fetal growth. In preterm (107–108 days of gestation) and near-term (128–131 days of gestation) ovine fetuses, we have determined the effect of intermittent umbilical cord occlusion (UCO) over a period of 4 days on the profile and expression of IGFs and IGFBPs. In experimental group animals (preterm $n=7$; near term $n=7$) UCOs were carried out by complete inflation of an occluder cuff (duration 90 s) every 30 min for 3–5 h each day, while control fetuses (preterm $n=7$; near term $n=7$) received no UCOs. Ewes were euthanized at the end of day 4, and fetal heart, lung, kidney, liver, skeletal muscle and placenta were collected. During UCOs, PO_2 fell (by ~ 13 mmHg), pH fell (by ~ 0.05) and PCO_2 increased (by ~ 7 mmHg), and changed to a similar extent in both preterm and near-term groups. In both preterm and near-term groups, there was no difference in fetal body or organ weight between UCO and control fetuses. No significant changes were observed in plasma IGF-I and -II

concentrations or IGFBP-1, -2, -3 or -4 levels throughout the 4-day study at either gestational age. In the preterm group UCO fetuses, IGF-II mRNA (1.2–6.0 kb) levels were lower in fetal lung (33%, $P<0.05$), heart (54%, $P<0.01$) and skeletal muscle (29%, $P<0.05$), but there were no differences in IGF-I mRNA levels (7.3 kb); IGFBP-2 mRNA (1.5 kb) levels were lower in the right lobe of the liver (42%, $P<0.05$) and kidney (22%, $P<0.01$), but higher in the heart (72%, $P<0.01$), while IGFBP-4 (2.4 kb) levels were lower in skeletal muscle (21%, $P<0.01$). In the near-term group UCO fetuses, IGFBP-2 mRNA levels were greater in the placenta (39%, $P<0.05$). Thus, intermittent UCO as studied has a greater effect on the expression of genes encoding certain peptides of the fetal IGF system in selected tissues in preterm fetuses than that in near-term fetuses. Altered IGFBP-2 mRNA levels with reduced IGF-II mRNA levels in selected tissues may mediate changes in growth and/or differentiation that might become apparent if the length of the UCO study were extended.

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Introduction

In human pregnancies, near-term 'variable type' fetal heart rate (FHR) decelerations, indicative of umbilical cord compression and resultant fetal hypoxaemia, are seen in 2–10% of FHR tracings (Anyagbunam *et al.* 1986, Dawes *et al.* 1993) and can be associated with an increased

incidence of neonatal acidosis, low Apgar (see Jennett *et al.* 1981) scores and nuchal cord complications at the time of delivery. We have recently reported that infants with the umbilical cord around the neck at birth are smaller than those without cord complications, and also that their placentas are larger (Osak *et al.* 1997). Nuchal cord encirclements are detected several weeks prior to labour

Figure 1 Diagrammatic representation of the experimental procedure on days 1–4 of the study of preterm and near-term groups. Each segment represents 1 h and the shaded region denotes the time period over which UCOs (see crosses) were carried out every 30 min. The arrows denote the times at which blood was sampled: small arrows, blood-gas analysis only (1 ml); large arrows, blood-gas analysis combined with hormone analysis (3–4 ml). RIA (radioimmunoassay) and WLB (Western ligand blot) denote the time points at which analysis of plasma IGF-I and -II and plasma IGFBPs respectively was carried out. Cardiovascular variables were monitored continuously. No UCOs were carried out in control-group fetuses but the same blood sampling and monitoring regime was followed.

and delivery (Collins *et al.* 1995) and therefore the mechanisms underlying the decreased birth weight are likely to involve a reduction in umbilical blood flow and thus altered nutrient and/or oxygen delivery. The increased placental weight may represent an adaptive overgrowth to sustain nutrient supply, as seen in humans with mild hypoxaemia at high altitude (see Barker 1994) and in some instances of maternal undernutrition in sheep (McCrabb *et al.* 1991).

Insulin-like growth factors (IGF-I and IGF-II) are polypeptide growth factors with potent mitogenic, metabolic and differentiating actions, and play an important role in the regulation of fetal growth (Han & Hill 1994). Insulin-like growth factors are synthesized by different fetal tissues and are present in all extracellular fluids bound to one or more of a family of IGF-binding proteins (IGFBPs). These binding proteins act as carrier/storage proteins of IGFs in the circulation, as modulators of the biological actions of IGFs, and may even have cellular functions independent of IGFs (Rosenfeld 1997). The ubiquity and the spatial and temporal pattern of expression of IGF and IGFBP genes in fetal tissues are appropriate for their principle role in the regulation of fetal growth. Clinical studies of intrauterine growth restriction (IUGR) suggest that changes in IGF and IGFBPs are important biochemical markers of the degree of growth restriction and are causally related to IUGR (Wang *et al.* 1991, Verhaeghe *et al.* 1993, Giudice *et al.* 1995). Experimental studies with induced IUGR in rats (Vileisis & D'Ercole 1986, Straus & Takemoto 1990) and sheep

(Owens *et al.* 1994) demonstrate that fetal growth restriction is variably associated with a decrease in circulating and/or tissue IGF-I and -II, and an increase in IGFBP-1. In ovine fetuses subjected to sustained hypoxaemia over hours to days, plasma IGF-I is reduced (Iwamoto *et al.* 1992), whereas plasma IGFBP-1 is increased and IGFBP-2 is reduced with corresponding alterations in tissue IGFBP mRNA levels (McLellan *et al.* 1992). These changes are associated with a reduction in the rate of DNA synthesis in specific organs and tissues (Hooper *et al.* 1991, Asano *et al.* 1997). A reduction in fetal growth, with associated changes in IGFs and IGFBPs, is likely to constitute tissue-specific energy expenditure adaptations in the face of an altered substrate and/or oxygen supply and could be mediated, in part, by endocrine changes involving catecholamines (Hooper *et al.* 1994) or cortisol (Braems *et al.* 1998), for example.

In the present study, we determined the effect of intermittent umbilical cord occlusion (UCO) with severe but reversible hypoxia and no cumulative acidosis (to ensure longer term survival) on circulating levels of IGFs and IGFBPs and on tissue IGF and IGFBP mRNA abundance. We hypothesized that changes in IGFs and IGFBPs are biochemical markers that precede changes in organ and tissue weights. Chronically catheterized ovine fetuses were studied both at preterm and near term to determine whether there was any developmental change in the fetal IGF and IGFBP responses to intermittent hypoxic insults.

Table 1 Morphometric data, age at post-mortem and gender in preterm and near-term group fetuses. Organ weights are expressed as a percentage of body weight. All values are means $\pm$ S.E.M. Within preterm and near-term groups, the control and UCO fetuses were well matched in terms of age and gender. In addition, there were no differences between control and occluded fetuses in terms of body weight or brain, liver and heart weights expressed as a percentage of body weight

	Control		UCO	
	Preterm	Near term	Preterm	Near term
Age (days)	114	136	114	136
Sex	3F, 4M	3F, 4M	4F, 3M	4F, 3M
Body weight (BW, kg)	2.0 $\pm$ 0.2	3.9 $\pm$ 0.2	2.0 $\pm$ 0.2	4.5 $\pm$ 0.2
Brain (% BW)	1.8 $\pm$ 0.1	1.3 $\pm$ 0.1	1.9 $\pm$ 0.2	1.2 $\pm$ 0.1
Liver (% BW)	4.4 $\pm$ 0.2	2.2 $\pm$ 0.2	4.3 $\pm$ 0.3	2.5 $\pm$ 0.1
Heart (% BW)	0.8 $\pm$ 0.0	0.7 $\pm$ 0.0	0.7 $\pm$ 0.1	0.6 $\pm$ 0.0

Materials and Methods

Surgical preparation

Surgery and experiments were conducted in accordance with the Canadian Council on Animal Care regulations. Pregnant mixed western ewes (term = 147 d) were instrumented, using sterile technique, under general anaesthesia (1 g thiopental sodium in solution intravenously for induction (Abbott Laboratories Ltd, Montreal, Canada), followed by 1–1.5% halothane in O₂ for maintenance) between 107 and 108 days of gestation (preterm group: control $n=7$; UCO $n=7$) and between 128 and 131 days of gestation (near-term group: control $n=7$; UCO $n=7$). Polyvinyl catheters filled with heparinized saline were placed in left and right brachial arteries, a brachial vein and the amniotic cavity. Stainless-steel electrodes were sewn onto the fetal chest for monitoring using an electrocardiograph. In UCO fetuses, an inflatable occluder cuff (OC14, *In Vivo* Metric, Healdsburg, California, USA) was positioned around the proximal portion of the umbilical cord and was secured to the abdominal skin. The volume that was required for complete occluder cuff inflation was determined at surgery. A catheter was placed in a maternal femoral vein for administration of fluids and transfusion of maternal blood to the fetus. Ewes received a broad-spectrum, long-acting antibiotic immediately prior to surgery (1.2 g oxytetracycline intramuscularly; Rogar, STB Inc., London, Canada). Following surgery, ewes were given an analgesic (75 mg flunixin intramuscularly; Schering, Pointe-Claire, Canada) and fluids (500–1000 ml saline intravenously).

A 3-day postoperative recovery period was allowed prior to experimentation, during which daily antibiotic treatment was given to the fetus (1 million IU penicillin G sodium intravenously; Novopharm Ltd, Toronto, Canada) and introduced into the amniotic cavity (1 million IU penicillin G sodium); fetal arterial blood was collected for blood-gas analysis.

Experimental procedure and tissue collection

Both the preterm (111–116 days of gestation) and the near-term (132–138 days of gestation) groups were studied on four successive days (Fig. 1). In experimental fetuses, following a 1 h control period (0900 to 1000 h each day), either 7 (days 1 and 4) or 11 (days 2 and 3) UCOs were carried out by complete inflation of the occluder cuff (~ 3 ml saline) for 90 s every 30 min (Fig. 1). The time of day for study, and the duration, frequency and intensity of the UCO insult administered to the preterm and near-term group fetuses were similar. In control fetuses no UCOs were carried out.

In control and experimental fetuses, blood was sampled at predetermined intervals throughout the 4 day protocol (arrows, Fig. 1). 'Pre-UCO' samples were taken 5 min before inflation of the occluder cuff and 'post-UCO' samples were taken 5 min after release of the occluder cuff. Blood samples during UCOs (days 1 and 4 only) were always taken 60 s into the 90 s occlusion period (blood-gas analysis only). 'Recovery' blood samples were taken at the end of a 1 h recovery period (days 1 and 4 only). Blood-gas composition and pH were analysed at all fetal blood-sampling time points. Plasma for subsequent hormonal analysis was collected at a selection of time points only (3–4 ml; larger arrows in Fig. 1); Fig. 1 shows where this included analysis of plasma IGFs by radioimmunoassay (RIA) and of IGFBPs by Western ligand blotting. The volume of fetal blood taken was replaced with an equal volume of maternal venous blood at the end of study each day. Fetal arterial blood samples were collected on ice and analysed for blood-gas composition (ABL 500 blood-gas analyser and OSM2 haemoximeter, Radiometer, Copenhagen, Denmark) or spun at 4 °C (10 min at $\times 2000$ g, TJ-6; Beckman, Fullerton, CA, USA) and the plasma stored in separate aliquots at -20 °C for subsequent IGF and IGFBP analysis.

After the 1 h recovery period on day 4, ewes were killed with an overdose of barbiturate (30 mg sodium

Figure 2 Percentage change in plasma IGF-I and IGF-II from pre-1st UCO (crosses) day 1 in preterm and near-term control (□) and UCO (■) fetuses. Values are means $\pm$ S.E.M. values. Intermittent UCO did not alter plasma IGFs over the 4-day study.

pentobarbital intravenously; MTC Pharmaceuticals, Cambridge, Canada) and the fetus was delivered immediately. At this time, the location and function of the umbilical occluder cuff was confirmed. Fetal heart, lung, liver, kidney, skeletal muscle and cotyledons were harvested, weighed, frozen rapidly in liquid nitrogen and stored at -70°C until analysis.

cDNA probes

Ovine-specific cDNAs encoding IGF-I, IGF-II, IGFBP-1, IGFBP-2, IGFBP-3 and IGFBP-4 were cloned in our laboratory. The IGF-I cDNA was the 540 bp SacI-BamHI fragment encoding the sheep prepro-IGF-I (McLellan *et al.* 1992), the IGF-II cDNA was the 863 bp EcoRI fragment encoding the sheep prepro-IGF-II (Delhanty & Han 1993), the IGFBP-1 cDNA was the 870 bp EcoRI-BamHI fragment encoding the mature peptide of the sheep IGFBP-1 (McLellan *et al.* 1992), the IGFBP-2 cDNA was the 784 bp SmaI-SacI fragment encoding the midregion of the sheep IGFBP-2 (Delhanty & Han 1992), the IGFBP-3 cDNA was the 1.6 kb XbaI-HindIII fragment encoding prepro-IGFBP-3 (Hayatsu *et al.* 1994),

and the IGFBP-4 cDNA was the 784 bp BamHI-EcoRI fragment (our unpublished observations).

Northern blotting

Total RNA was isolated using a modification of the single-step acid guanidium thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi 1987). The integrity of RNA was checked on non-denaturing 1% agarose gels and the sample concentration was determined by spectrophotometric absorbance at 260 nm. Individual total RNA samples (20 μg) and an RNA ladder (3 μg , 0.24–9.5 kb; Gibco-BRL, Life Technologies, Burlington, Ontario, Canada) were subjected to Northern blot analysis as described previously (Delhanty & Han 1993). Blots were hybridized with ^{32}P -labelled ovine cDNA probe (1×10^6 c.p.m./ml) at 42°C and washed at a maximum stringency of $0.1 \times \text{SSC}/0.1\%$ SDS (2×30 min) at 42°C . The blots were exposed to X-ray film (XAR or BMR film with intensifying screens; Eastman Kodak, Rochester, NY, USA). Prior to hybridization with a different probe, the blots were stripped (3×20 min) in $0.01 \times \text{SSC}/0.1\%$ SDS at 80 – 90°C . All blots were probed with ^{32}P -labelled mouse

Figure 3 Northern blot hybridization (images on the left-hand side) of total RNA (20 µg per lane) from preterm lung (A) and skeletal muscle (B) of control and UCO fetuses with 32 P-labelled ovine IGF-II (upper panel) and 18S rRNA (lower panel) cDNA. A number of IGF-II mRNA transcripts were observed between ~1.2 and 6.0 kb in both tissue types. Graphs show that the abundance of IGF-II mRNA (all transcripts) expressed as a ratio to 18S rRNA was lower in UCO fetuses than in control fetuses ($*P<0.05$), according to densitometric analysis (means $\pm$ S.E.M. values). Numbers above lanes identify individual animals. Note: certain total RNA samples from the lung and skeletal muscle of the UCO group (two and one, respectively) were consistently found to be degraded and therefore were not included in the Northern blot.

18S rRNA cDNA to determine the consistency of sample loading and transfer.

Western ligand blotting

The profiles and relative levels of IGFFBPs in fetal sheep plasma were determined by the Western ligand blot method, as previously described (McLellan *et al.* 1992). Analysis was carried out on 10 µl plasma sampled pre-1st UCO, pre-7th UCO and at the end of the recovery hour on days 1 and 4, and pre-1st UCO on days 2 and 3. Blots were hybridized overnight at 4 °C with 125 I-labelled IGF-II (200 000 c.p.m./blot), washed and exposed to X-ray film (XAR and BMR with intensifying screens) at -70 °C.

IGF-I and IGF-II radioimmunoassay

The IGF-I and IGF-II concentrations were determined in plasma by the modification of a previously described RIA method validated for fetal plasma (McLellan *et al.* 1992). Plasma samples (100 µl) from pre-1st-UCO days 1 and 4 and from the end of the recovery hour on day 4 were analysed. The IGFs in plasma samples were separated from IGFFBPs using acid extraction followed by Sephadex G50 gel chromatography (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The absence of IGFFBPs from IGF-containing fractions was confirmed by Western ligand blot analysis of the column eluent from a non-experimental fetal plasma sample of an equivalent gestational age group.

Table 2 Summary of changes in IGF and IGFBP mRNA abundance in preterm and near-term fetuses following repetitive UCO

	mRNA abundance					
	IGF-I	IGF-II	IGFBP-1 ²	IGFBP-2	IGFBP-3	IGFBP-4
Preterm						
Right ventricle	=	↓*	ND	↑**	=	=
Lung	=	↓*	ND	=	=	=
Kidney	=	=	ND	↓**	=	=
Right liver	=	=	=	↓*	=	=
Skeletal muscle	=	↓*	ND	=	=	↓**
Placenta	=	=	ND	=	=	=
Near term¹						
Right ventricle	=	=	ND	=	=	=
Kidney	=	=	ND	=	=	=
Right liver	=	=	=	=	=	=
Skeletal muscle	=	=	ND	=	=	=
Placenta	=	=	ND	↑*	=	=

¹Lung tissue was not investigated in the near-term group.²ND, not determined.

=, No difference; ↑, a rise compared to control; ↓, a fall compared to control.

P*<0.05 and *P*<0.01, significant difference.

Data analysis

In preterm and near-term groups, the blood gas and pH responses during periods of UCO on days 1 and 4, and in pre-1st UCO values over the course of the 4-day study, were analysed using a repeated measures ANOVA followed by a Student's *t*-test with the Bonferroni method of correction for multiple comparisons made at a given point in time.

Plasma IGFBPs on Western ligand blots, and IGF and IGFBP mRNAs on Northern blots, were quantified by scanning laser densitometry (IP Lab Gel, Signal Analytics Corp., Vienna, VA, USA). For Northern blots, the relative density of bands (arbitrary units per mm) were expressed as a ratio to the relative density of the corresponding 18S band and statistical analysis was carried out between control and UCO fetuses using the Student's unpaired *t*-test. For Western ligand blots, the relative densities of IGFBPs were expressed as a ratio to the relative density of the IGF-II receptor, which was known not to change over the period of gestation in the present study or to be altered during 24 h of continuous hypoxia (McLellan *et al.* 1992, Delhanty & Han 1993); a statistical analysis was conducted on values over the course of the 4-day protocol, using two-way ANOVA for repeated measures. Plasma levels of IGF-I and IGF-II were analysed between groups and over time using two-way ANOVA for repeated measures. Values are expressed as means ± s.e.m. values throughout. Statistical significance was accepted at *P*<0.05.

Results

Fetal blood gases and pH

In both preterm and near-term groups, UCO caused a large decline in fetal arterial PO₂ (preterm: 24.1 ± 0.5 to

6.7 ± 0.8 mmHg, *P*<0.01; near term: 19.9 ± 0.7 to 7.6 ± 0.8 mmHg *P*<0.01), O₂ saturation (preterm: 68.7 ± 1.6 to 12.4 ± 2.5%, *P*<0.01; near term: 53.4 ± 2.2 to 11.0 ± 1.8%, *P*<0.01) and pH (preterm: 7.36 ± 0.00 to 7.30 ± 0.01, *P*<0.01; near term: 7.35 ± 0.01 to 7.30 ± 0.01, *P*<0.01), and a rise in PCO₂ (preterm: 52.3 ± 0.6 to 60.2 ± 1.0 mmHg, *P*<0.01; near term: 53.5 ± 0.7 to 61.1 ± 1.1 mmHg, *P*<0.01) as studied on days 1 and 4; a return to control values occurred after occluder release.

Baseline (pre-1st UCO) measurements of blood gases and pH were unaltered over the course of the 4-day study in control and UCO fetuses, apart from a small fall in pH in preterm (day 1 versus day 4: control, 7.37 ± 0.01 to 7.32 ± 0.01, *P*<0.01; UCO, 7.38 ± 0.01 to 7.34 ± 0.02, *P*<0.01) and near term (day 1 versus day 4: control, 7.36 ± 0.01 to 7.34 ± 0.00, *P*<0.05; UCO, 7.39 ± 0.02 to 7.35 ± 0.01, *P*<0.05) groups, and in O₂ saturation in the UCO near-term fetuses only (day 1 versus day 4: 58.4 ± 3.5 to 49.4 ± 4.2%, *P*<0.01).

Fetal organ and body weights

In preterm and near-term groups, we measured fetal body weight, and brain, liver and heart weights at the time of post-mortem. In both groups, there were no differences between control and UCO fetuses in terms of body weight, or brain, liver or heart weight expressed as a percentage of fetal body weight (Table 1).

Fetal plasma IGFs

There was no significant effect of 4 days of intermittent UCO on plasma IGF-I or IGF-II in either preterm or near-term groups (Fig. 2).

Figure 4 Preterm-group plasma IGFBP levels throughout the 4-day study. (A) Western ligand blot from a representative preterm fetus showing samples taken on days 1–4 (d1–d4) during pre-UCO (crosses) and recovery periods. (B) Values from densitometric analysis of ligand blots expressed as a ratio to plasma IGF-II receptor and as a percentage change from day 1 pre-1st (crosses) values in control (○) and UCO (●) fetuses. Values are means $\pm$ S.E.M. values. Plasma IGFBPs were unaltered over the 4-day study period in preterm and near-term groups (data not shown).

Tissue IGF mRNAs

IGF-I mRNA was detected in all preterm and near-term tissues examined by Northern blotting (heart, lung, kidney, liver, skeletal muscle and placenta). On the basis of autoradiographic exposure times, IGF-I mRNA was of lower abundance than IGF-II mRNA in all tissues examined. There were no differences in IGF-I mRNA levels between control and UCO fetuses in preterm or near-term groups.

IGF-II mRNA was detected in all preterm and near-term tissues (heart, lung, kidney, liver, skeletal muscle and placenta). Five IGF-II mRNA transcripts (between 1.2 and 6.0 kb) were easily identified and densitometric analysis of these transcripts as a whole demonstrated significantly lower levels in the right ventricle (54%), lung (33%; Fig. 3A) and skeletal muscle (29%; Fig. 3B) of the UCO compared with control fetuses from the preterm, but not the near-term, group (Table 2).

Fetal plasma IGFBPs

In Western ligand blots from both preterm and near-term fetuses, we identified six protein species with the ability to

bind to 125 I-IGF-II (Fig. 4A). The profiles were similar to those observed previously in the fetal sheep (McLellan *et al.* 1992, Delhanty & Han 1993), in which it was noted that these bands correspond to the IGF-II receptor ($\sim$ 220 kDa), IGFBP-3 (glycosylated ($\sim$ 41 kDa) and non-glycosylated ($\sim$ 38 kDa)), IGFBP-2 ($\sim$ 34 kDa), IGFBP-1 ($\sim$ 30 kDa) and IGFBP-4 ($\sim$ 23 kDa).

In preterm and near-term groups, plasma IGFBP levels over the course of the 4-day protocol, expressed as a percentage change from the level measured pre-1st UCO on day 1 and as a ratio to the corresponding IGF-II receptor band showed no significant changes either temporally or between control and UCO fetuses (Fig. 4B).

Tissue IGFBP mRNAs

IGFBP-2, -3 and -4 mRNAs were detected in all of the tissues examined (heart, lung, kidney, liver, skeletal muscle and placenta). Previous studies have shown that IGFBP-1 mRNA is detected only in the liver (McLellan *et al.* 1992); in the present study, therefore, we restricted our measurement of IGFBP-1 mRNA levels to that in liver tissue.

A. Right Heart

B. Kidney

Figure 5 A and B.

C. Right Liver

D. Placenta

Figure 5 C and D.

Figure 5 Northern blot hybridization of total RNA (20 µg per lane) from preterm right heart (A), preterm kidney (B), preterm right liver (C), and near-term placenta (D) of control and UCO fetuses with ³²P-labelled ovine IGFBP-2 (upper panels) and 18S rRNA (lower panels) cDNA. A single IGFBP-2 mRNA transcript (~1.5 kb) was observed in (B) and (C), whereas two transcripts were detected in (A) and (D) (1.6 and 1.8 kb). Graphs show the results of densitometric analysis (means ± S.E.M. values). The abundance of IGFBP-2 mRNA (expressed as a ratio to 18S rRNA) was lower in preterm kidneys (***P*<0.01) and right livers (**P*<0.05), but higher in right hearts (preterm, ***P*<0.01) and placentas (near term, **P*<0.05) of UCO fetuses relative to control fetuses. Numbers above lanes identify individual animals. Note: total RNA samples from the right liver of one UCO-group fetus was consistently found to be degraded and therefore was not included in the Northern blot.

Figure 6 Northern blot hybridization of total RNA (20 µg per lane) from preterm skeletal muscle of control and UCO fetuses with ^{32}P -labelled ovine IGFBP-4 (upper panel) and 18S rRNA (lower panel) cDNA. A single IGFBP-4 mRNA transcript was observed at ~2.4 kb. The graph shows that the abundance of IGFBP-4 mRNA, expressed as a ratio to 18S rRNA, was lower in UCO fetuses than in control fetuses (** $P < 0.01$), according to densitometric analysis (means $\pm$ S.E.M. values). Numbers above lanes identify individual animals. Note: total RNA samples from one of the UCO-group fetuses was consistently found to be degraded and therefore was not included in the Northern blot.

Densitometric analysis of IGFBP-1 and IGFBP-3 mRNA transcripts showed no differences between control and UCO fetuses in any of the tissues examined from both the preterm and the near-term group fetuses (Table 2). The kidney and the liver expressed a single IGFBP-2 mRNA transcript (~1.5 kb, Fig. 5B and C respectively), whereas preterm right ventricle and near-term placenta expressed two transcripts (~1.4 and 1.7 kb, Fig. 5A and D respectively). IGFB-2 mRNA was most abundant in the liver and kidney of both preterm and near-term groups. In UCO fetuses of the preterm group, IGFBP-2 mRNA levels were greater in the right ventricle (72%), but lower in the liver (42%) and the kidney (22%). In UCO fetuses of the near-term group, IGFBP-2 mRNA levels were greater in placental tissue (39%) (Fig. 5 and Table 2). In UCO fetuses of the preterm group, skeletal muscle was the only tissue that showed a decrease in IGFBP-4 mRNA (21%; Fig. 6 and Table 2).

Discussion

In the present study, we found no significant differences in the plasma levels of IGF-I, IGF-II or IGFBPs between control and UCO fetuses. IGFs are paracrine/autocrine, rather than endocrine, factors (Han *et al.* 1988, Delhanty & Han 1993, Yakar *et al.* 1999) and therefore plasma concentrations reflect synthesis by all tissues of the fetus. Previous studies have demonstrated that 3 h of fetal hypoxia with acidosis produced by reduction of maternal

inspired oxygen (Iwamoto *et al.* 1992), but not 24 h of continuous fetal hypoxia induced by reduced uterine blood flow (McLellan *et al.* 1992), causes a fall in plasma IGF-I levels with no change in IGF-II levels. However, both Iwamoto *et al.* (1992) and McLellan *et al.* (1992) observed a dramatic elevation in plasma IGFBP-1. Differences between these studies and the present work include the intensity and duration of the stimulus.

IGF-I and -II mRNA and IGFBP mRNAs were detectable in all tissues examined, with the exception of IGFBP-1 (which is only expressed in the liver). As we have reported previously, the abundance of IGF-II was greater than that of IGF-I, and IGFBP2 mRNA levels were highest in the liver and the kidney (Delhanty & Han 1993). UCO reduced the abundance of IGF-II mRNA, but not of IGF-I mRNA, in selected tissues from the preterm group (111–116 days of gestation). In contrast, 24 h of restricted uterine blood flow (RUBF) in ovine fetuses aged between 115 and 120 days of gestation resulted in a significant decline in liver IGF-I mRNA and only a tendency for IGF-II mRNA to decrease (McLellan *et al.* 1992). We observed no changes in tissue IGF-I or -II mRNA levels in the near-term group in response to UCO, although previous work in late-gestation (126–130 and 134–136 days of gestation) ovine fetuses has shown that 48 h of acute isocapnic hypoxia reduced IGF-II mRNA expression in the adrenal gland (Braems *et al.* 1998). Our results suggest a greater susceptibility of IGF-II gene expression to UCO in younger fetuses, in which IGF-II mRNA levels are higher (Delhanty & Han 1993).

We found no differences in hepatic IGFBP-1 mRNA abundance between control and UCO fetuses from pre-term or near-term groups, in contrast to our previous finding of a 3–7-fold elevation of hepatic IGFBP-1 mRNA levels following 24 h of continuous fetal hypoxia (McLellan *et al.* 1992). Recently, it was demonstrated that the human IGFBP-1 gene contains HRE sequences in the intron 1 region, which can be induced by either hypoxia or hypoxia-inducible factor-1 (HIF-1) (Tazuke *et al.* 1998) and thus it is possible that either the ovine IGFBP-1 gene does not contain HREs or that the sequences are induced by sustained, and not acute, hypoxia.

We detected two IGFBP-2 mRNA transcripts in the preterm right heart and near-term placenta which appear to be differentially regulated, with an increase in the 1.7 kb transcript and no change in the 1.4 kb transcript in UCO fetuses. The cause of the two transcripts is unknown, but could be the result of a post-transcriptional modification or variations in polyadenylation. Others have observed two transcripts in the brains of 21-day-old rats made hypoxic by carotid artery ligation (Klempt *et al.* 1993). The abundance of IGFBP-2 mRNA decreased in the preterm right liver and kidney as in previous studies using 24 h hypoxia (McLellan *et al.* 1992), and increased in the preterm right ventricle of the heart and near-term placenta of UCO fetuses. The alteration in IGFBP-2 gene expression with UCO in preterm compared with near-term fetuses may be due to changes in gene transcription.

A number of factors could be responsible for the alterations in IGF and IGFBP gene expression in the present study. Already, we have demonstrated that UCO produces a reversible fetal asphyxia, bradycardia and a rise in blood pressure in these fetuses (Green *et al.* 1999, Kawagoe *et al.* 1999). In addition, previous work has shown a redistribution of blood flow, favouring the brain and heart at the expense of, for example, the skeletal muscle during UCOs of different intensities and duration (Richardson *et al.* 1996, Giussani *et al.* 1997). Therefore it is possible that differences in oxygen/nutrient delivery to specific organs could have differentially affected IGF/IGFBP gene expression.

In addition, UCO produces changes in a number of hormone systems that could impact on the control of the IGF-axis. Plasma catecholamines are elevated by UCO in the near-term fetal sheep (Smith *et al.* 1991) and are implicated in the increased synthesis and release of IGFBP-1 during prolonged fetal hypoxia (Hooper *et al.* 1994). Cortisol has been shown to suppress IGF-II gene expression in the liver (Li *et al.* 1993) and the adrenal gland (Li *et al.* 1994). We have reported previously that plasma cortisol is increased to a variable extent by UCO in the fetuses involved in the present study (Green *et al.* 1997), and therefore it is possible that this altered circulating cortisol contributed to the tissue-specific decline in IGF-II.

IGFBP-2 is a membrane-bound and -soluble binding protein that normally functions to facilitate the action of IGFs (Gluckman 1995). Therefore, a decline in IGFBP-2 mRNA abundance in the liver and the kidney could lead to a condition whereby IGFBP-2 is reduced at local tissue level to reduce growth in these tissues in response to tissue-specific decreases in blood flow/oxygen uptake during hypoxia. Similarly, the increase in IGFBP-2 mRNA in the heart might indicate a potentiation of growth when combined with the tendency for blood flow to increase as a result of UCO (Richardson *et al.* 1996). IGFBP-2 mRNA is expressed in the dense caruncular-like stroma lining the luminal epithelium and the placental capsule (Reynolds *et al.* 1997), and therefore the increase in placental IGFBP-2 mRNA in the near-term fetuses of the present study may have been a form of compensation in the placenta when blood flow was compromised acutely by UCO. We have also observed a decline in IGFBP-4 in the skeletal muscle of preterm UCO fetuses. The control of ovine fetal IGFBP-4 has been linked to IGF-I (Carr *et al.* 1995), and IGFBP-4 may be involved in modulating the action of IGF-II produced by the invading trophoblast in the guinea-pig placenta (Han *et al.* 1999). In the lung and skeletal muscle, the observed decrease in IGF-II mRNA levels suggests a move towards reduced growth in tissues; it is interesting that these tissues are some of those in which blood flow and growth are sacrificed during hypoxic challenges.

In conclusion, we have demonstrated that repetitive UCO over a period of days does not alter fetal body or organ weight but does produce tissue-specific alterations in components of the IGF system which were not reflected in circulating plasma levels and which were confined primarily to the preterm group. We speculate that altered IGFBP-2 mRNA levels and reduced IGF-II mRNA levels in selected tissues may be early indicators of changes in growth.

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