Amniotic IGF-I supplementation of growth-restricted fetal sheep alters IGF-I and IGF receptor type 1 mRNA and protein levels in placental and fetal tissues

S Shaikh, F H Bloomfield, M K Bauer, H H Phua, R S Gilmour and J E Harding

Liggins Institute, University of Auckland, Private Bag 92019, Auckland, New Zealand

(Requests for offprints should be addressed to F H Bloomfield; Email: f.bloomfield@auckland.ac.nz)

Abstract

We have previously reported that chronic intra-amniotic supplementation of the late gestation growth-restricted (IUGR) ovine fetus with IGF-I (20 µg/day) increased gut growth but reduced liver weight and circulating IGF-I concentrations. Here we report mRNA and protein levels of IGF-I, the type 1 IGF receptor (IGF-1R) and IGF-binding proteins (IGFBP)-1, -2 and -3 in fetal gut, liver, muscle and placenta from fetuses in that earlier study in an attempt to explain these contrasting results. mRNA and protein were extracted from tissues obtained at post mortem at 131 days of gestation (term, 145 days) from three groups of fetuses (control, IUGR+saline and IUGR+IGF-I, $n=9$ per group). Control fetuses were unembolised and untreated. In the IUGR groups, growth restriction was induced from 113 to 120 days by placental embolisation; from 120 to 130 days fetuses were treated with daily intra-amniotic injections of either saline or 20 µg IGF-I. mRNA was measured by RT-PCR or

real-time RT-PCR, and protein by Western blot. In liver, muscle and placenta, IGF-I mRNA and protein levels were reduced by between 8 and 30% in IGF-I-treated fetuses compared with saline-treated fetuses and controls with no change in IGF-1R mRNA or protein levels. In contrast, in the gut, IGF-I mRNA and protein levels were not significantly altered with IGF-I treatment, but IGF-1R levels were increased, especially in the jejunum. Immunolocalisation demonstrated that IGF-1R expression was confined to the luminal aspect of the gut. mRNA levels of all three IGFBPs were reduced in the gut of IGF-I-treated fetuses, but hepatic expression was significantly increased. These data demonstrated tissue-specific regulation of IGF-I, IGF-1R and IGFBPs-1, -2 and -3 in response to intra-amniotic IGF-I supplementation, though the underlying mechanisms remain obscure.

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Introduction

Intra-uterine growth restriction (IUGR) remains a major cause of perinatal morbidity and mortality (Resnik 2002). In addition, recent epidemiological and experimental data suggest that being born small carries an increased risk of diseases in later life, such as the metabolic syndrome, cardiovascular disease and stroke (Barker 1994, Osmond & Barker 2000, Ozanne 2001, Kajantie *et al.* 2004). This increased risk is thought to be consequent upon a permanent resetting of fetal homeostatic and metabolic mechanisms to a level appropriate for a poor intrauterine nutritional environment. However, this altered physiology may be inappropriate for postnatal life. Indeed, recent data suggest that postnatal catch-up growth, previously regarded as a positive outcome, may in fact increase the risk of later disease (Eriksson *et al.* 2001, 2002). Therefore, we and others have proposed that the optimum time to

attempt to improve the growth of IUGR fetuses is during intrauterine life (Harding & Bloomfield 2004).

Placental insufficiency is a major contributing factor to many cases of IUGR in the developed world, limiting delivery of nutrients and growth factors to the fetus. We have therefore utilised amniotic fluid, a fetal compartment accessed regularly by clinicians, as a route for administering potential therapies to the IUGR fetus. Insulin-like growth factor (IGF)-I is a major fetal growth factor in late gestation (Oliver *et al.* 1999), is present in amniotic fluid (Merimee *et al.* 1984), is nutritionally regulated (Oliver *et al.* 1996) and circulating levels are correlated with birthweight (Verhaeghe *et al.* 1993). We have recently reported the effects of chronic, 10-day supplementation of amniotic fluid with a low dose of IGF-I (20 µg/day) in late gestation growth-restricted fetal sheep (Bloomfield *et al.* 2002a). We found a significant increase in gut weight, with histological evidence of increased gut growth and

increased numbers of mitotic bodies in the intestinal crypts. However, there was no increase in fetal body weight or fetal growth rate. In contrast, fetal liver weight was actually 11% lower than that of saline-treated IUGR fetuses, and circulating concentrations of IGF-I were reduced by 30–40% (Bloomfield *et al.* 2002a). This was despite the fact that amniotic fluid IGF-I levels were increased up to fivefold, and that ^{125}I -labelled IGF-I given into amniotic fluid is swallowed by the fetus and is taken up intact across the gut and into the portal venous system and thence into the systemic circulation (Bloomfield *et al.* 2002b).

The aims of this study were to investigate whether these apparently paradoxical results could be explained by tissue-specific changes in mRNA and protein levels of IGF-I and the type 1 IGF receptor (IGF-1R) in fetal gut tissues, and also in liver, muscle and placenta, which are the sources of circulating IGF-I and of IGF-I clearance in the ovine fetus (Iwamoto *et al.* 1992, Kind *et al.* 1995), and of mRNA levels of the IGF-binding proteins (IGFBP)-1, -2 and -3, as the IGFBPs influence IGF-I bioavailability and clearance (Clemmons 1998, Baxter 2000).

Materials and Methods

Animals

Approval for animal studies was obtained from the institutional animal ethics committee. The experimental protocol for the animal studies has been reported previously in detail (Bloomfield *et al.* 2002a). In brief, 29 ewes carrying singleton fetuses underwent surgery at 110 days of gestation (term 145 days) for implantation of chronic indwelling vascular and amniotic catheters. After surgery, ewes were housed in individual cages with free access to water and *ad libitum* quantities of a standard laboratory diet of chaff and pelleted stock feed. Ewes were randomly assigned to one of three groups: a control group ($n=9$) and two growth-restricted groups. Growth restriction was induced by uteroplacental embolisation using microspheres from 113 to 120 days of gestation, and growth-restricted fetuses were then treated with once daily intra-amniotic injections of 2 ml saline ($n=9$) or 20 μg human recombinant IGF-I (Pharmacia and Upjohn, Peapack, NJ, USA) mixed with 2 ml fresh amniotic fluid ($n=11$) for 10 days, from 120 to 130 days of gestation. We calculated that this dose would increase amniotic fluid IGF-I levels tenfold, but the actual increase was approximately fivefold (Bloomfield *et al.* 2002a). Control fetuses were unembolised and untreated. At 131 days of gestation ewes were killed with an overdose of pentobarbitone and the uterus and contents were rapidly removed. The fetus and placenta were weighed, and samples of fetal liver, muscle (biceps femoris), placenta (type B placentome) and gut (samples collected as follows: stomach, half way along the length of the abomasum; duodenum, first 20 cm of small intestine;

jejunum, 20 cm distal to duodeno-jejunal junction; ileum, 20 cm proximal to ileo-caecal valve; colon 20 cm distal to ileo-caecal valve) were snap frozen in liquid nitrogen and stored at -80°C until analysis. Tissue suitable for mRNA and protein extraction was obtained from nine fetuses in each group.

Measurement of IGF-I and IGF-1R mRNA levels by RT-PCR

Total RNA was extracted by the Trizol method (Gibco BRL, Life Technologies, Auckland, New Zealand). The final concentration of RNA was quantified using spectrophotometric OD_{260} measurements and purity was assessed by $\text{OD}_{260}/\text{OD}_{280}$ ratio, with values >1.9 being of acceptable purity. RNA integrity was electrophoretically verified on 1.2% formaldehyde agarose gels followed by ethidium bromide staining. RNA were stored at -80°C until analysis. Following cDNA synthesis from 2.5 μg total RNA, IGF-I and IGF-1R mRNA expression was analysed by relative quantitative RT-PCR. Three RT reactions were performed for each sample and the product pooled, and amplification of each target was then performed in duplicate. Averages of the duplicates were used for analysis. 18s ribosomal RNA was used as an internal control with over-expression curbed by using Ambion's quantum Universal 18s rRNA primers and competitors (Ambion Inc., Austin, TX, USA). The optimum ratio of 18s primer to competitor for each gene was determined by serial multiplex PCR reactions containing different 18s rRNA primer:competitor ratios and either the IGF-I or IGF-1R primer. The ratio for which the 18s band was of equal intensity to the target band on subsequent electrophoresis was taken as the optimum ratio. To determine the linear range for amplification of primers, serial multiplex PCR reactions from 18 to 38 cycles were performed for each gene and 18s rRNA. Following gel electrophoresis as described below, individual gene expression was normalised to 18s RNA expression and the arbitrary densitometric units plotted against the number of cycles. The mid-point of the linear part of the curve was selected as the optimum number of cycles; this was 30 for IGF-I and 32 for IGF-1R. Optimum annealing temperatures for both primers were determined by serial PCR reactions at different temperatures keeping other parameters constant, and was 58°C for both primers. For the 18s primer, the manufacturers recommend an annealing temperature of between 55 and 68°C . In all reactions, both optimisation and actual experiments, the digoxigenin-labelled nucleotide, dig dUTP (Roche Diagnostics NZ Ltd, Auckland, New Zealand), was incorporated during the PCR reaction. PCR products were electrophoresed through 2% agarose gel, transferred onto a membrane (Roche Diagnostics NZ Ltd) and detected using an alkaline phosphatase (ALP) conjugated anti-digoxigenin antibody (Roche Diagnostics NZ Ltd). CPD Star (Roche Diagnostics NZ Ltd) was used

as a chemiluminescent substrate for ALP. Chemiluminescent signals were captured with LabWorks software (UVP BioImaging Systems, Upland, CA, USA) and bands were analysed densitometrically (Scion Image; Scion Corporation, Frederick, MD, USA). All signals are expressed as optical densities relative to the signal for 18s rRNA.

Measurement of IGFBP -1, -2 and -3 mRNA levels by real-time RT-PCR

After performing the above RT-PCR, we acquired a real-time PCR machine and therefore performed analysis of IGFBP mRNA levels using real-time PCR. RNA was extracted as described above. Expression levels were quantitated using a one-step PCR reaction. Briefly, RNA samples were treated with RNase-free DNase I (Invitrogen NZ Ltd, Auckland, New Zealand) before RT-PCR to eliminate any potential genomic DNA. First-strand cDNA was synthesised using SuperScript First-Strand Synthesis System (Invitrogen NZ Ltd) according to the manufacturer's instructions. All reactions were treated with RNase H before real-time PCR. PCR amplification was performed in triplicate on an ABI Prism 7900HT Sequence Detector (Applied Biosystems, Foster City, CA, USA) using the standard cycling conditions recommended by the manufacturer (50 °C for 2 min, 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min). Singleplex amplification was carried out in 384-well plates with a total reaction volume of 20 µl, containing 10 µl of TaqMan Universal PCR Master Mix (Applied Biosystem), 1 µl cDNA template, 200 nM probe and 900 nM forward and reverse primers. A standard curve of the target gene and 18s were included in each plate, consisting of twofold serial dilutions of cDNA synthesised from stock fetal muscle of identical gestational age. Real-time PCR efficiencies (E) were calculated from the slopes of the standard curves for each target gene ($E = 10^{-1/\text{slope}}$). Samples from the control group were selected as a calibrator. Gene expression in the saline- and IGF-I-treated groups were expressed relative to the calibrator and as a ratio to 18s rRNA using the formula (Pfaffl 2001):

$$\text{Relative expression ratio} = \frac{(E_{\text{target}})^{\Delta\text{CP}_{\text{target}}(\text{control}-\text{treated})}}{(E_{18\text{s}})^{\Delta\text{CP}_{18\text{s}}(\text{control}-\text{treated})}}$$

Where E_{target} is the real-time PCR amplification efficiency of target gene transcript; $E_{18\text{s}}$ is the real-time PCR amplification efficiency of 18s; $\Delta\text{CP}_{\text{target}}(\text{control} - \text{treated})$ and $\Delta\text{CP}_{18\text{s}}(\text{control} - \text{treated})$ are the cycle threshold differences between the calibrator (the control group) and the treated group (calculated separately for the IGF-I- and saline-treated groups) for the target gene and 18s rRNA respectively.

Data for real-time PCR are therefore expressed as relative expression ratios to the control group with 95% confidence intervals (CI).

Oligonucleotide primers and probe design

For RT-PCR of IGF-I and IGF-1R, sequences of oligonucleotide primers for IGF-I were selected to cover the end of the 2nd exon and the entire 3rd exon and were designed to include an intron to discriminate products from genomic DNA and cDNA (forward GT GCA TGC TCT CCA GTT CGT and reverse CTT CTG AGC CTT GGG CAT GT (228 bp)). Primers for IGF-1R were specific for the β -subunit of the IGF-1R gene (forward GCC TCG AAC TTT GTC TTT GC and reverse GCT CAA ATA CTC CGG GTT CA (498 bp)). For real-time RT-PCR, the available ovine sequences for IGFBP-1, -2 and -3 in GenBank were analysed using the Primer Express software (Applied Biosystems) to determine optimum primer and probe locations. A BLAST search was conducted to ensure that primers and probes were not constructed from any homologous regions that would encode for other proteins, and the specificity of the PCR product was confirmed by high-resolution gel electrophoresis to verify that the transcripts were of the predicted molecular size. Each probe was labelled at the 5' end with fluorescein (for the IGFBPs) or VIC (for ribosomal 18s RNA) and at the 3' end with a minor groove-binder non-fluorescent quencher. Primers and probes for target genes were manufactured by Invitrogen NZ Ltd and Applied Biosystems respectively. The probe and primer for ribosomal 18s RNA (18s rRNA) were obtained from Applied Biosystems (Assay-on-Demand). IGFBP-1 (285 bp; accession AF327650) forward and reverse primers were constructed from bp 210–221 with a sequence of GCCAAACTGCAACAAGAATGG and from bp 242–261 with a sequence of TCCGTCCAGCGAAGTCTCA respectively. The probe for IGFBP-1 was located between bp 223 and 241 with a sequence of TTCTATCACAG CAAACAGT. IGFBP-2 (1396 bp; accession S44612) forward and reverse primers were constructed from bp 816–836 with a sequence of TGACAAGCATGGCCT GTACAA and from bp 858–874 with a sequence of CACGCTGCCCGTTTCAGA respectively. The probe for IGFBP-2 was located between bp 840 and 856 with a sequence of CAAACAGTGCAAGATGT. IGFBP-3 (360 bp; accession AF327651) forward and reverse primers were constructed from bp 59–78 with a sequence of GCCAGCGCTACAAGGTTGAC and from bp 104–126 with a sequence of CTTGGACTCGGAGGAGAAG TTCT respectively. The probe for IGFBP-1 was located between bp 80 and 95 with a sequence of ACGAGTCT CAGAGCAC.

Measurements of IGF-I and IGF-1R protein by Western blotting

Tissue was homogenised in the presence of Complete Mini EDTA-free protease inhibitors (Roche Diagnostics NZ Ltd), 100 µM sodium orthovanadate and radio

immunoprecipitation assay lysis buffer (Sigma Chemical Co.). After centrifugation at 8 000 g at 4 °C for 15 min, protein content was assayed in duplicate by the Bradford protein assay (BioRad New Zealand, Auckland, New Zealand). Protein standards (Invitrogen NZ Ltd) and 30 µg protein were separated by electrophoresis on 15% (IGF-I) or 6% (IGF-1R) SDS polyacrylamide gels at 80 volts for 5 min, then 200 volts until the dye front reached the bottom of the gel. As there were too many samples to fit on one gel, a control pool of protein extracted from fetal liver was included in each gel. Proteins were transferred to nitrocellulose membranes (BioRad New Zealand) at 350 mA for 1.5 h at 4 °C. Equal loading and transfer were visually confirmed by staining with S-Ponceau (Sigma Chemical Co., St Louis, MO, USA). Non-specific binding was blocked by overnight incubation at 4 °C with 5% (w/v) skimmed milk and 0.1% Tween-20 in phosphate-buffered saline (PBS). Blots were then incubated with rabbit anti-human primary antibodies, diluted in blocking solution, for 1 h at room temperature (IGF-I polyclonal, dilution 1:1000 (Abcam Ltd, Cambridge, Cambs, UK), IGF-1R polyclonal to the α -subunit, dilution 1:500 (Chemicon International Inc., Boronia, Australia)). Following PBS/0.1% Tween-20 washes, blots were incubated with secondary antibody (mouse monoclonal anti-rabbit horseradish peroxidase (HRP) conjugate (Sigma Chemical Co.)) at a 1:10 000 dilution in blocking solution for 1 h at room temperature. Specific protein bands were detected by SuperSignal chemiluminescence using SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, Rockford, IL, USA) and the signal detected with LabWorks software (UVP Bio-Imaging Systems). Membranes were then stripped and processed for detection of β -actin in a similar way (primary mouse anti-human monoclonal antibody, dilution 1:2500 (Abcam Ltd), secondary antibody polyclonal rabbit anti-mouse HRP conjugate, dilution 1:10 000 (Abcam Ltd)). Images were analysed using Scion Image (Scion Corporation). All signals are expressed as optical densities relative to the signal for β -actin. The relative optical densities (ROD) for the control pool were compared between gels of the same tissue to ensure comparable loading and transfer and to enable comparison between gels.

Localisation of IGF-1R protein by immunohistochemistry

Longitudinal sections of jejunum were fixed in 10% buffered formalin, paraffin embedded and mounted onto poly-L-lysine-coated glass slides. Slides were deparaffinised in xylene and rehydrated through a series of ethanol solutions with a final rinse in PBS. All sections were blocked for non-specific reactivity for 2 h at room temperature with 2% normal goat serum in PBS containing 0.5% bovine serum albumin. Sections were then incubated with the primary antibody (mouse monoclonal to IGF-1R (Chemicon International Inc.)) diluted 1:300 in the block-

ing solution and incubated for 1 h at room temperature. After three washes in PBS, slides were incubated for 1 h at room temperature with the secondary antibody (anti-mouse alkaline phosphatase (ALP) conjugated; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA; 1:300 dilution). After washes with PBS and then with 0.1 M Tris-HCl buffer (pH 9.5), the slides were incubated in the dark for 20–30 min with ALP substrate until colour developed. Endogenous ALP was blocked with 0.02% levamisole (Sigma Chemical Co.). The reaction was stopped by incubating the slides in 0.1 M Tris EDTA (pH 8.0) buffer for 5 min. Slides were then rinsed in water and mounted in aqua mount (glycerol 9 ml, 1,4-diazabicyclo(2,2,2)octane 233 g (Sigma Chemical Co.), Tris buffer (pH 8) 200 µl and distilled water 800 µl).

For negative controls, sections of each tissue were subjected to identical procedures with the exception of either (a) no primary antibody incubation or (b) replacement of the primary antibody with non-specific mouse IgG (Zymed Laboratories, Gympie, NSW, Australia) at the same dilution (1:300).

Statistical analyses

Data were analysed using one-way ANOVA. If there was a significant effect ($P < 0.05$), differences between groups were compared with the Bonferroni-Dunn post-hoc test (Statview; SAS Institute, Cary, NC, USA). All data are expressed as mean (S.D.).

Results

The effects of embolisation and IGF-I treatment on fetal growth, gut histology, fetal metabolism, IGF-I concentrations, blood and amniotic fluid amino acid concentrations and fetal gut uptake of amino acids have been previously reported (Bloomfield *et al.* 2002a,d).

IGF-I and IGF-1R mRNA and protein expression in fetal and placental tissues

For gut tissues, we present data from the stomach, jejunum and colon. Data from duodenum and ileum were similar to stomach and colon and are not shown. Placental embolisation did not alter IGF-I mRNA or protein levels in liver, placenta, muscle or most regions of the gut, although in the jejunum both mRNA and protein levels for IGF-I were reduced (Figs 1 and 2). Intra-amniotic IGF-I treatment reduced IGF-I mRNA and protein levels in liver, muscle and placenta compared with both control and saline-treated fetuses by between 8 and 30% (Fig. 1). However, IGF-I mRNA and protein levels in the gut were not altered by IGF-I treatment (Fig. 2).

In contrast, IGF-1R mRNA levels were not altered in liver, placenta or muscle either by growth restriction or by IGF-I treatment (Fig. 3). Protein levels for IGF-1R

Figure 1 (A) IGF-I mRNA and (B) protein levels in liver, muscle and placenta. mRNA and representative Western blots are shown above histograms representing the ratio of IGF-I mRNA or protein ROD to 18s or β -actin ROD respectively. Solid bars, control; hatched bars, saline-treated fetuses; open bars, IGF-I-treated fetuses. Values are mean (S.D.) for an $n=9$ per group. * $P<0.05$ vs saline, # $P<0.05$ vs control.

followed a similar pattern, although IGF-1R protein was significantly reduced in the placenta with intrauterine growth restriction (Fig. 3). In the gut, IGF-1R mRNA levels were significantly reduced in regions distal to the stomach with growth restriction. However, intra-amniotic IGF-I treatment reversed this, so that IGF-1R mRNA levels in all regions of the gut were significantly higher than in saline-treated IUGR fetuses (Fig. 4). In the stomach, the increase was 30%, and IGF-1R mRNA levels here was also higher than in control fetuses (Fig. 4). Protein levels for the IGF-1R were also twofold higher in the jejunum after IGF-I treatment, but were not significantly increased in other regions of the gut (Fig. 4).

Immunohistochemical localisation of IGF-1R protein

We performed immunohistochemistry of IGF-1R in the gut to localise the receptor to the luminal or basal aspect of the enterocytes. In sections of jejunum, IGF-1R signal was only detected on the luminal surface of the enterocytes (Fig. 5).

IGFBP-1, -2 and -3 mRNA expression in fetal liver, jejunum and placenta

Placental embolisation did not significantly alter mRNA levels of IGFBPs 1–3 in the liver (Table 1). In the

placenta, IGFBP-3 mRNA levels were increased twofold but there was no significant change in mRNA levels of IGFBP-1 or -2. In the jejunum, IGFBP-1 mRNA levels were decreased by approximately 60%; mRNA levels of IGFBP-2 and -3 were unaltered (Table 1). Intra-amniotic IGF-I treatment increased mRNA levels of all three IGFBPs in the liver compared with control fetuses (Table 1). In the placenta, IGFBP-3 mRNA levels were increased and IGFBP-1 mRNA levels were decreased compared with control fetuses. In the jejunum, a similar decrease to that in saline-treated fetuses was seen in IGFBP-1 mRNA levels, but IGFBP-2 and -3 mRNA levels were also decreased by 30% compared with controls. There were no statistically significant differences in mRNA levels of any of the IGFBPs compared with saline-treated fetuses (Table 1).

Discussion

Our findings have provided clear evidence that mRNA and protein levels of IGF-I and the IGF-1R, and mRNA levels of IGFBP-1, -2 and -3, are regulated differently in different tissues following chronic intra-amniotic supplementation with IGF-I.

IGF-I mRNA and protein levels in the gut were not altered, despite direct exposure of the luminal aspect of the

Figure 2 (A) IGF-I mRNA and (B) protein levels in stomach, jejunum and colon. mRNA and representative Western blots are shown above histograms representing the ratio of IGF-I mRNA or protein ROD to 18s or β -actin ROD respectively. Solid bars, control; hatched bars, saline-treated fetuses; open bars, IGF-I-treated fetuses. Values are mean (s.d.) for an $n=9$ per group. $##P<0.01$ vs control.

gut to swallowed IGF-I, which we have demonstrated is present in an intact form along the length of the gut (Bloomfield *et al.* 2002b). As the gut is exposed to both luminal and systemic influences, it may be that the unchanged IGF-I mRNA and protein levels in the gut reflect a balance of the effects of reduced circulating, but elevated local, levels of IGF-I. The developing gut also expresses IGFBP mRNA. In the fetal rat, levels of expression of the IGFBPs increase with advancing gestation (Shoubridge *et al.* 2001). Although IGF-I treatment enhanced gut growth in our study, morphological development of the small intestine appeared to be delayed with persistence of the apical endocytic complex (Bloomfield *et al.* 2002a). The reduced mRNA levels of IGFBP-1, -2 and -3 in the gut that we report here may be consistent with delayed functional maturation. IGFBP-2 produced by intestinal cells *in vitro* has been reported to attenuate the stimulatory effects of IGF-I on DNA synthesis in a dose-dependent manner (Park *et al.* 1992); the reduced levels of IGFBP-2 in the gut in the IGF-I-treated group would be consistent with enhanced action of the lumenally delivered IGF-I and increased mitosis and growth in the gut.

Our finding of up-regulation of mRNA for the IGF-1R in the gut in the IGF-I-treated fetuses, confirmed by

increased protein expression in the jejunum, may be consistent with the reduced circulating IGF-I concentrations in these fetuses, as IGF-1R mRNA levels are reported to be inversely regulated by IGF-I (Werner *et al.* 1989, Eshet *et al.* 1993, Kind *et al.* 1996, Hernandez-Sanchez *et al.* 1997). Since we found IGF-1R expression to be confined to the luminal aspect of the gut, where IGF-I concentrations were high, this might suggest that IGF-1R levels here are regulated by systemic factors rather than local factors. However, we did not find an increase in IGF-1R mRNA levels in liver, muscle or placenta after IGF-I treatment, despite the reduced circulating IGF-I concentrations. These results are therefore difficult to explain on the basis of a simple relationship between systemic IGF-I concentrations and tissue IGF-1R mRNA levels, although a concomitant increase in IGF-I and IGF-1R mRNA in the jejunum of fasted rats at the time of refeeding has been reported (Ziegler *et al.* 1995). IGF-I can act in a paracrine and autocrine fashion in peripheral tissues, including the gut (Jux *et al.* 1998, Simmons *et al.* 1999), and both IGF-I and IGF-1R mRNA expression is differentially regulated in a tissue-specific manner (Werner *et al.* 1989, Butler & LeRoith 2001, Fowden 2003). Our data suggested that, in fetal life, local IGF-I may have different effects on IGF-1R mRNA expression

Figure 3 (A) IGF-1R mRNA and (B) protein levels in liver, muscle and placenta. mRNA and representative Western blots are shown above histograms representing the ratio of IGF-1R mRNA or protein ROD to 18s or β-actin ROD respectively. Solid bars, control; hatched bars, saline-treated fetuses; open bars, IGF-I-treated fetuses. Values are mean (s.d.) for an $n=9$ per group. # $P<0.05$ vs control.

in different tissues, and also different effects from those seen in postnatal life.

We have previously reported reduced liver size and reduced circulating concentrations of IGF-I in these animals (Bloomfield *et al.* 2002a), and the current findings of reduced mRNA and protein levels of IGF-I in fetal liver, muscle and placenta are consistent with this. In fetal sheep, IGF-I mRNA levels are highest in liver and muscle, suggesting that these organs are the predominant source of circulating IGF-I (Kind *et al.* 1995). Levels of IGF-I mRNA in these tissues correlate positively with circulating IGF-I concentrations (Kind *et al.* 1995), and fetal sheep with growth restriction secondary to placental restriction (carunclectomy) have reduced levels of IGF-I mRNA in muscle, but not liver (Kind *et al.* 1995). It may therefore be surprising that we did not see greater effects on IGF-I mRNA levels in the tissues of embolised fetuses that were treated with saline. We did find a non-significant increase in hepatic IGFBP-1 and a significant increase in placental IGFBP-3 mRNA levels in embolised fetuses, but these changes were small. Circulating IGF-I concentrations fell acutely with embolisation, but had recovered in the saline-treated fetuses by the time of post mortem 10 days later (Bloomfield *et al.* 2002a). Therefore, any changes in IGF-I or IGFBP mRNA levels associated with a growth-restricting insult, either mediated by increased circulating fetal cortisol concentrations secondary

to embolisation (Gagnon *et al.* 1994) or other mechanisms, may have resolved. Exogenous glucocorticoids have been reported to reduce both circulating IGF-I concentrations and tissue IGF-I mRNA levels (Luo & Murphy 1989, Jensen *et al.* 2002). However, other studies have reported no effect of glucocorticoids on circulating IGF-I concentrations or tissue IGF-I mRNA levels, but an increase in IGFBP expression, suggesting that IGF bioavailability may be decreased (Mosier *et al.* 1987, Price *et al.* 1992).

The decreased hepatic IGF-I mRNA level that we found in the IGF-I-treated fetuses was consistent with the effects of exogenous IGF-I treatment on hepatic IGF-I mRNA levels in malnourished rats (Schalch *et al.* 1989), growth hormone-deficient dwarf rats (Butler *et al.* 1994) and hypophysectomised rats (Gosteli-Peter *et al.* 1994), and also in healthy fetal sheep (Kind *et al.* 1996). However, we used a very low dose of IGF-I compared with these studies (20 µg/day, approximately 100-fold lower than that used in the study by Lok and colleagues (Kind *et al.* 1996, Lok *et al.* 1996)). Although this resulted in a fivefold increase in amniotic fluid IGF-I concentrations (Bloomfield *et al.* 2002a), portal venous concentrations of IGF-I were not increased, despite demonstrable portal venous uptake of ^{125}I -IGF-I from amniotic fluid in another study (Bloomfield *et al.* 2002b). However, even if all the IGF-I administered into the amniotic fluid in this study was swallowed, and all the swallowed IGF-I was

Figure 4 (A) IGF-1R mRNA and (B) protein levels in stomach, jejunum and colon. mRNA and representative Western blots are shown above histograms representing the ratio of IGF-1R mRNA or protein ROD to 18s or β-actin ROD respectively. Solid bars, control; hatched bars, saline-treated fetuses; open bars, IGF-I-treated fetuses. Values are mean (s.d.) for an *n*=9 per group. **P*<0.05 vs saline, #*P*<0.05 vs control.

taken up into the portal vein, we calculate that any change in portal venous concentrations would be below the sensitivity of our assay to detect. Nevertheless, we presume that some exogenous IGF-I was delivered to the liver via the portal circulation. Whether these very small amounts of exogenous IGF-I are sufficient to explain the

down-regulation of IGF-I mRNA and protein in the liver is less clear. Furthermore, it seems unlikely that this could explain their down-regulation in muscle, which is a more

Table 1 IGFBP-1–3 mRNA levels in liver, placenta and jejunum relative to control fetuses (*n*=9). Data are fold change with 95% CIs. Where the CIs do not cross 1, values are different from control fetuses. All CIs for saline and IGF-treated groups overlap, demonstrating no statistically significant differences between groups

	Saline-treated (<i>n</i> =9)	IGF-I-treated (<i>n</i> =9)
Liver		
IGFBP-1	1.7 (0.6–4.7)	7.2 (1.6–31.9)
IGFBP-2	1.5 (0.8–5.7)	4.1 (1.6–10.4)
IGFBP-3	1.0 (0.7–1.5)	2.2 (1.1–4.3)
Placenta		
IGFBP-1	0.5 (0.1–1.9)	0.5 (0.4–0.6)
IGFBP-2	1.0 (0.1–9.5)	1.2 (0.3–4.6)
IGFBP-3	2.0 (1.8–2.2)	1.6 (1.4–1.8)
Jejunum		
IGFBP-1	0.4 (0.2–0.9)	0.4 (0.2–0.9)
IGFBP-2	1.2 (0.6–2.4)	0.71 (0.68–0.73)
IGFBP-3	1.0 (0.7–1.4)	0.71 (0.51–0.99)

Figure 5 Representative light micrographs (20 × magnification) of immunohistochemistry for the α-subunit of IGF-1R in jejunum. (A) Control, (B) saline-treated, (C) IGF-I-treated and (D) negative control with non-specific mouse IgG instead of primary antibody. The bars represent 200 μm.

distal organ. Changes in placental mRNA and protein expression could conceivably occur through local feedback on the placenta of the elevated intra-amniotic IGF-I concentrations. There are some circumstantial data suggesting that the placenta may contribute to the regulation of circulating fetal IGF-I concentrations, taking up IGF-I from the placenta when levels are high (Bassett *et al.* 1990) and releasing it into the circulation when they are low (Iwamoto *et al.* 1992). Although the low circulating concentrations in this study may therefore have been expected to result in an increase in placental IGF-I expression, the very high amniotic levels may have had the opposite effect, acting through local receptors on membranes and/or placenta (Ryan *et al.* 1991).

An alternative explanation for the down-regulation of IGF-I mRNA and protein in distal organs following enteral exposure to IGF-I could be the release of a second messenger from the gut. Possibilities include the IGFBPs, other gut-derived hormones, or nutrient signalling. IGF-I acting at IGF-R1s in the gut can stimulate local release of IGF-I, IGF-II and IGFBPs (Park *et al.* 1992, Simmons *et al.* 1999). However, as IGFBP mRNA expression was reduced in the gut, this seems an unlikely signal to the liver.

Gut-derived hormones other than the IGFBPs could include ghrelin and the incretins such as the glucagon-like peptides (Kojima *et al.* 1999, Burrin *et al.* 2001). We have demonstrated the presence of ghrelin protein in the stomach of the fetal sheep in this study by immunohistochemistry (V Roelfsema, unpublished observation), but do not have quantitative measures of either protein levels in the gut or of circulating concentrations. Ghrelin and the incretins are nutritionally regulated intestinal peptides that are expressed in fetal life and have effects on the somatotropic and glucose/insulin axes (Roberge & Brubaker 1993, Sjöholm *et al.* 2000, Petersen *et al.* 2001, Liu *et al.* 2002). Investigation into the role of these hormones in future studies of amniotic supplementation of IUGR fetuses with hormones and/or nutrients may provide mechanistic insights.

It is also possible that the effects of enteral IGF-I exposure on distal organs is mediated via effects on specific nutrients. Both IGF-I and the IGFBPs are nutritionally regulated in many species including the fetal sheep (Estivariz & Ziegler 1997, Lee *et al.* 1997, Clemmons 1991, Oliver *et al.* 1993, 1996). Furthermore, IGF-I treatment in fetal sheep alters placental nutrient transfer (Harding *et al.* 1994, Liu *et al.* 1994, Bloomfield *et al.* 2002c), although the same effects may not be present in IUGR sheep (Jensen *et al.* 1999). Furthermore, IGF-1R mRNA was reduced in the placenta following embolisation in this study and, in the IGF-I-treated fetuses, circulating IGF-I concentrations were reduced, suggesting that an effect of IGF-I on placental nutrient transfer is unlikely.

In contrast, local IGF-I concentrations in the lumen of the gut were presumably increased, and IGF-1R mRNA

and protein levels were also increased in the jejunum following intra-amniotic IGF-I treatment. There may therefore have been a local effect on nutrient uptake by the gut, which may have had both local and systemic effects. There were no changes in circulating fetal amino acid concentrations, either in arterial or portal venous blood, or in amniotic fluid amino acid concentrations, with IGF-I treatment (Bloomfield *et al.* 2002d). However, we did find evidence of altered gastrointestinal glutamine metabolism with IGF-I treatment (Bloomfield *et al.* 2002d). Glutamine and IGF-I have been reported to have a synergistic effect on gut growth. Furthermore, in rats with short bowel syndrome (SBS) secondary to massive gastrointestinal resection, oral glutamine, but not subcutaneous IGF-I, supplementation prevented the reduction in hepatic IGF-I mRNA expression seen with SBS (Ziegler *et al.* 1996). Thus altered amino acid metabolism by the gut in response to swallowed IGF-I may have influenced hepatic gene expression of components of the IGF axis.

Several studies of growth restriction induced by different means in different species have reported a 1.7- to 10-fold upregulation of hepatic IGFBP-1 and/or BP-2 expression; for example, prolonged hypoxia in sheep (McLellan *et al.* 1992), maternal fasting (Straus *et al.* 1991), dexamethasone treatment (Price *et al.* 1992) and being small for gestational age in the rat (Unterman *et al.* 1990) and uterine artery ligation in the guinea pig (Carter *et al.* 2005). We found a 1.7-fold increase in IGFBP-1 expression in liver in the saline-treated fetuses but this was not statistically significant. However, in IGF-I-treated fetuses the increase was sevenfold, with lesser, but significant, increases in IGFBP-2 and IGFBP-3 mRNA levels also. Exogenous IGF administration to the ovine fetus has been reported to increase hepatic IGFBP-1 mRNA levels (Shen *et al.* 2001), and this is a possibility here also. Alternatively, the altered amino acid metabolism by the gut, as described above, may have contributed to altered nutritional regulation of hepatic IGFBP production.

In summary, we have demonstrated tissue-specific regulation of IGF-I and IGF-1R mRNA and protein levels with intra-amniotic IGF-I supplementation. Despite a fivefold increase in amniotic IGF-I concentrations, hepatic, muscle and placental IGF-I mRNA and protein levels were reduced, consistent with reduced circulating IGF-I concentrations. IGF-1R mRNA and protein levels were not altered by IGF-I treatment in these tissues, despite the reduced circulating IGF-I concentrations. In the gut, IGF-1R mRNA levels were increased, and were localised to the luminal aspect of the intestine where IGF-I concentrations were presumably increased. Furthermore, the reduced IGFBP mRNA levels in the gut in IGF-I-treated fetuses may also have contributed to the increased gut mitosis and growth. The mechanisms and physiological significance of this tissue-specific regulation of IGF-I and IGF-1R mRNA and protein remain uncertain.

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