Consequences of maternal undernutrition for fetal and postnatal hepatic insulin-like growth factor-I, growth hormone receptor and growth hormone binding protein gene regulation in the rat

S M Woodall, N S Bassett, P D Gluckman and B H Breier

Research Centre for Developmental Medicine and Biology, Faculty of Medicine and Health Science, University of Auckland, Private Bag 92019, Auckland, New Zealand

(Requests for offprints should be addressed to N S Bassett)

ABSTRACT

The mechanisms that contribute to postnatal growth failure following intrauterine growth retardation (IUGR) are poorly understood. We demonstrated previously that nutritional deprivation in the pregnant rat leads to IUGR in offspring, postnatal growth failure and to changes in endocrine parameters of the somatotrophic axis. The present study examines the effects of maternal undernutrition (30% of the ad libitum available diet; IUGR group) throughout pregnancy on hepatic insulin-like growth factor-I (IGF-I), growth hormone receptor (GHR) and GH-binding protein (GHBP) gene expression using solution hybridisation/RNase protection assays (RPAs). Animals were killed at fetal (E22, term=23 days) and postnatal (birth, days 5, 9, 15, 21) ages, livers were collected and RNA extracted for RPAs. Results demonstrate the presence of all IGF-I mRNAs resulting from transcription start sites (ss) in exon 1 (ss1/2, ss3, ss2 spliced), exon 2, the two IGF-I E-domain variants (Ea and Eb) as well as GHR and GHBP mRNAs in hepatic tissue at E22 in both the ad libitum fed and IUGR offspring. In the postnatal liver, IGF-I ss1/2, ss3, ss2 spliced, Ea and Eb IGF-I variants as well as GHR and GHBP mRNA transcripts increased in abundance from birth to day 21. IGF-I exon 2 transcripts were relatively constant from E22 until postnatal day 15, then increased at postnatal day 21 in both the ad libitum fed and IUGR offspring. The expressions of all hepatic IGF-I leader exon ss and Ea domain variants were significantly reduced in IUGR offspring (P<0·05) from E22 to postnatal day 9. In contrast, relative abundance of hepatic IGF-I Eb variants, GHR and GHBP mRNAs were unaltered in IUGR offspring compared with the ad libitum fed animals. Whether these postnatal effects of undernutrition are a direct consequence of IUGR or whether they are related, in part, to differences in postnatal food intake remains to be investigated.

In summary, we have demonstrated that hepatic IGF-I ss within exon 1 and exon 2 are coordinately regulated. Use of exon 1 ss increased during normal development and decreased with IUGR without changes in GHR or GHBP gene expression. Eb transcripts, thought to represent GH-dependent endocrine regulation of IGF-I, were unchanged in IUGR. These results suggest a possible post-receptor defect in GH action as a consequence of IUGR.

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INTRODUCTION

Understanding of the molecular basis of IGF-I biosynthesis has been vastly improved by characterisation of the IGF-I gene structure and function (Shemer et al. 1992). The rat IGF-I gene spans nearly 100 kilobases, comprising six exons with multiple transcription initiation start sites (Shemer et al. 1992). The leader exon and E domain variants are expressed in a tissue-specific pattern, Ea and exon 1 transcripts are present in all tissues while exon 2 and Eb mRNAs are relatively liver-specific (Shemer et al. 1992). Based on the differing patterns of expression between tissues, during development (Adamo et al. 1991, Shemer et al. 1992), and the response to growth hormone (GH) therapy and hypophysectomy (Foyt et al. 1992) it has been proposed that exon 1 and Ea mRNAs encode autocrine/paracrine forms of IGF-I while exon 2 and Eb mRNAs encode endocrine IGF-I (LeRoith & Roberts 1991). Evidence from studies in fasted and diabetic rats (Adamo et al. 1991) and in sheep (Pell et al. 1993) suggests that the different IGF mRNAs encoding these variants are also differentially regulated. However, the biological significance of these variants remains to be elucidated.

IGF-I gene expression in liver and other tissues is GH dependent, reduced by hypophysectomy and partially restored with GH replacement (Roberts et al. 1986, 1987). Thus changes in IGF-I gene expression may also reflect changes in GH synthesis and action. Regulation of the GH receptor (GHR) is also an important mechanism of control within the somatotrophic axis. The GH binding protein (GHBP), a second product of the GHR gene corresponding to the extracellular domain of the GHR, may also regulate GH action both at an endocrine and at a local level (Baumann 1994). In rodents, the liver is the primary source of GHBP in plasma, although most non-hepatic tissues also express GHBP mRNA (Tiong & Herington 1991). GHBP mRNA expression in rat liver increases with age (Tiong & Herington 1992), similar to the increase observed in hepatic GHR mRNA (Tiong & Herington 1992, Ymer & Herington 1992). In diabetic and protein-restricted rats, the expression of GHR and IGF-I mRNA are both reduced (Bornfeldt et al. 1989), suggesting that these may reflect altered GH action.

In the present study, we have examined the effects of maternal undernutrition throughout gestation on the expression of IGF-I, GHR and GHBP genes in fetal and neonatal liver in order to determine whether altered gene expression may be associated with IUGR and whether these effects persist postnatally. These studies may provide evidence for a potential mechanism that contributes to the growth retardation observed at birth and during the neonatal period.

MATERIALS AND METHODS

Animal model

The experimental protocol was approved by the Animal Ethics Committee of the University of Auckland. The experimental approach to induce IUGR in this study has been documented previously (Woodall et al. 1996). In brief, following confirmation of mating, Wistar dams aged between 80 and 100 days of age were randomly assigned to one of two feeding regimes: with food available ad libitum or with food restricted. Control dams (n=6) had pelleted rat chow available ad libitum throughout pregnancy (ad libitum group). The restricted fed group (n=6) were fed 30% of the ad libitum group intake from day 1 and then throughout pregnancy (IUGR group).

At birth (postnatal day 1, term=23 days, see Woodall et al. 1996), the size of each litter was reduced and standardised to 8 pups per dam (mean litter size for both groups at birth=12±1 pups). Fetuses (E22) and neonates (birth, postnatal days 5, 9, 15 and 21, n=6 per age) were weighed and then killed by decapitation under halothane anaesthesia. Tissue samples collected were rinsed in saline, snap frozen in liquid N2 and stored at −80 °C until RNA extraction.

Preparation of RNA

Total RNA was extracted with Trizol (Gibco BRL, Life Technologies, Inc, Grand Island, NY, USA) according to the manufacturer’s instructions. The concentration of RNA, and the degree of protein contamination was assessed by spectrophotometric analysis at 260 and 280 nm in duplicate. Samples (10 µg) were electrophoresed through 1% agarose gels containing 0·66 M formaldehyde, transferred by capillary action onto nylon membrane (Hybond N+, Amersham Life Sciences, New Zealand), baked at 80 °C and stained with methylene blue to check RNA integrity of each extract.

Antisense RNA constructs

The cDNA templates for IGF-I were kindly donated by Dr D LeRoith and Dr C Roberts (National Institutes of Health, Bethesda, MD, USA). Two probes were used to detect IGF-I mRNAs and transcript variants by solution hybridisation/RNase protection assay (RPA) as
described previously (Butler et al. 1994). The first (5'-specific) was used in RPAs to distinguish between alternate transcription start sites. The cRNA probe was generated following linearisation of the cDNA/plasmid vector using EcoRI and T7 RNA polymerase. Hybridisation with this probe yielded the following expected protected bands: 530 bp for start sites (ss) 1 and 2, 428 bp for ss 3, 260 bp for alternately spliced ss1 and 2 mRNAs, 197 bp for ss 4 (all of which are derived from exon 1), and 168 bp for exon 2 mRNAs. A second IGF-I probe (3'-specific probe) distinguishes between alternatively spliced E domains (Lowe et al. 1988). This construct was linearised with HindIII and T7 RNA polymerase used to generate the cRNA probe. Use of this probe resulted in three major bands, a full-length band of 376 bp representing Eβ transcripts and bands at 224 bp and 100 bp representing Eα transcripts lacking the 52 bp exon 5 sequence.

A specific GHR cDNA was used to assess GHR and GHBP gene expression and was kindly provided by Dr W R Baumbach (American Cyanamid Co., Princeton, NJ, USA). This cDNA was used as the template to generate a 445 bp cRNA probe corresponding to extracellular transmembrane and intracellular regions of the GHR and has been described elsewhere (Butler et al. 1996). The construct was linearised with BamHI and T7 RNA polymerase used to generate the cRNA probe. RNase digestion resulted in protected fragments of 439 bp (GHR mRNA) and 290 bp (GHBP mRNA).

A 300 bp cyclophilin cRNA probe was generated using SP6 RNA polymerase after linearisation of the plasmid construct (in pSP65) with HindIII (donated by J Douglas, Vollum Institute, Portland, Oregon, USA). This probe served as a sample loading control and was used to facilitate quantification.

Solution hybridisation/RNase protection assay (RPA)

RPAs were performed as described previously (Butler et al. 1994) using standard riboprobe generation procedures according to the manufacturer’s instructions (Riboprobe Gemini System II kit, Promega, Madison, WI, USA). Briefly, 20 µg freeze-dried RNA sample were hybridised with an excess of 32P-labelled antisense RNA probe. 32P was used for labelling the cyclophilin loading control in order to reduce the signal intensity. Non-hybridised RNA was digested by treatment with RNaseA and T1 and protected RNA–RNA hybrids resolved on denaturing 8% acrylamide gels. Autoradiograms were exposed to X-ray film (Eastman Kodak, Rochester, NY, USA) at −80 °C. Autoradiograms were quantified using Image Quant (Molecular Dynamics, Sky Valley, CA, USA).

All RPAs investigating ontogeny of hepatic IGF-I, GHR and GHBP mRNAs and those comparing expression between ad libitum and IUGR groups were performed in duplicate. To control for differences in loading, all measurements were calculated as a ratio of probe mRNAs to cyclophilin mRNA.

Statistical analyses

Statistical analyses were carried out using the Sigma Stat Statistical package (Jandel Scientific, San Rafael, CA, USA). Differences between gestational ages and between ad libitum and IUGR groups were assessed by one-way ANOVA to compare the basal expression of liver IGF-I mRNA variants and GHR, GHBP mRNA. In addition, two-way ANOVA was utilised for age and nutrition comparisons with a post-hoc Bonferroni t-test. All data are expressed as percentage changes from a given time point and are presented as means ± s.e.m. P values of less than 0.05 were considered statistically significant.

RESULTS

The fetal and neonatal effects of nutritional restriction have been described previously (Woodall et al. 1996). In brief, maternal undernutrition resulted in IUGR which was reflected by a significant decrease in fetal weight (P<0.001) and placental weight (P<0.01) in the restricted-fed group at E22 of gestation. In offspring from undernourished dams, body weights were significantly reduced by 25% at birth (P<0.05) and remained reduced at all subsequent postnatal ages. There was no evidence of catch-up growth at weaning (day 21) or during the following postnatal period (see Woodall et al. 1996 for details).

Ontogeny of IGF-I mRNA transcripts in ad libitum and IUGR rats

In both nutritional groups, transcripts resulting from start sites in exon 1 and exon 2 were found to be expressed (Figs 1 and 2). The developmental regulation of the start sites in exon 1 and exon 2 were the same for both ad libitum and IUGR offspring with a peak in expression occurring between postnatal days 15–21 (Figs 1b, 2b). Relative abundance of IGF-I mRNA in start sites
1/2, 3 and 2 spliced was low in ad libitum and IUGR offspring at E22 and at birth. After birth these transcripts showed a steady increase ($P<0.05$) in expression until postnatal day 21. IGF-I mRNA expression in exon 2 was low until postnatal day 15 before increasing markedly ($P<0.05$) at 21 days of age (Figs 1b, 2b).

**Expression of IGF-I mRNA transcripts between ad libitum and IUGR rats**

Transcripts resulting from start sites in exon 1 and exon 2 were coordinately reduced in IUGR offspring from E22 to postnatal day 5 (Fig. 3b) and at postnatal days 9 and 15 in selected start sites of...
exon 1 and exon 2 (Fig. 4b). Exon 1 start sites and exon 2 IGFBP-I mRNA variants were significantly \((P<0.05)\) decreased in both ad libitum and IUGR offspring at E22 and at birth compared with postnatal day 5 (Fig. 3b). The relative abundances of all exon 1 start site variants were reduced \((P<0.05)\) in IUGR rats at postnatal day 9 (Fig. 4b). Start sites 3 and 2 spliced were also reduced at postnatal day 15 and exon 2 at postnatal days 9 and 15 (Fig. 4b). However, in ad libitum offspring, only exon 2 IGFBP-I mRNA expression was decreased \((P<0.05)\) at day 9 compared with postnatal day 21 (Fig. 4b).

**Ontogeny of IGFBP-I, GHR and GHBP mRNA expression between ad libitum and IUGR rats**

Total expressions of IGFBP-I, GHR and GHBP mRNAs were measured simultaneously using the E domain and GHR antisense RNA probes. Eb and

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**FIGURE 2.** (a) Representative solution hybridisation/RNase protection assay (RPA) and (b) densitometric analysis of RPAs of total RNA from IUGR offspring hepatic tissue hybridised with a cRNA probe to detect IGFBP-I mRNAs derived from transcription start sites (ss) as described for Fig. 1, i.e. ss1/2, ss3, ss2 spliced, ss4 from exon 1 and exon 2 from E22 to postnatal (P) day 21 \((n=4\) per time point). CP, cyclophilin loading control. C1 and C2, control lanes using probe alone with (C1) and without (C2) RNase treatment. M, maternal liver. Values are expressed as means \(\pm\) s.e.m. \(^{abcde}P<0.05\), same letter denotes a significant difference by one-way ANOVA followed by Bonferroni t-test.
Ea IGF-I mRNA expression was developmentally regulated in both ad libitum and IUGR offspring (Figs 5b, 6b) where a gradual increase was observed from birth to postnatal day 21.

The expression of Eb IGF-I mRNA was not affected by maternal undernutrition in utero from E22 to postnatal day 5 (Fig. 7b). Although there appeared to be a developmental increase during this period, it was not significant (P=0.1). Relative abundance of Eb IGF-I mRNA expression was significantly (P<0.05) reduced at postnatal day 9 in both ad libitum and IUGR offspring compared with postnatal day 21. In contrast, the expression of Ea IGF-I mRNA was reduced in IUGR rats compared with ad libitum rats at E22 and postnatal day 5. A developmental increase (P<0.05) was observed in both...
groups (Fig. 7b). At the later time points (i.e. postnatal days 9, 15 and 21), there were no significant differences observed between ad libitum and IUGR offspring in the expression of Eb IGF-I mRNA. Ea IGF-I mRNA expression was reduced ($P<0.05$) in both ad libitum and IUGR offspring at postnatal day 9 compared with postnatal day 21 (Fig. 8b).

GHR and GHBP mRNAs were also developmentally regulated in both ad libitum and IUGR offspring (Figs 5b, 6b) and followed a similar pattern to that observed for Ea and Eb IGF-I mRNAs.

There was no significant difference in the pattern of developmental GHR and GHBP expression.
between \textit{ad libitum} and IUGR offspring at any age investigated. In addition, GHR and GHBP gene expressions were not changed by maternal under-nutrition throughout gestation at either the earlier (Fig. 7b) or later (Fig. 8b) time points. At E22 and postnatal day 9, a decrease ($P<0.05$) in GHR and GHBP mRNAs was observed in both \textit{ad libitum} and IUGR offspring compared with postnatal days 5 and 21 respectively (Figs 7b, 8b). In addition, \textit{ad libitum} offspring exhibited reduced ($P<0.05$) hepatic GHR gene expression at birth compared with postnatal day 5 (Fig. 7b).

DISCUSSION

We have demonstrated previously that maternal undernutrition in the rat, sufficient to induce IUGR, results in offspring which are 25% growth retarded at birth and which do not demonstrate significant catch-up growth in the postnatal period (Woodall et al. 1996). The current study extends these original studies and demonstrates that IGF-I mRNA transcripts in liver are reduced during the neonatal period by maternal undernutrition throughout gestation. These data are consistent with previously reported reductions in circulating IGF-I during neonatal development as a consequence of altered maternal nutrition (Maes et al. 1984, Woodall et al. 1996). The current study demonstrates that reduced IGF-I gene expression persists into the neonatal period despite returning

**FIGURE 6.** (a) Representative solution hybridisation/RNase protection assay (RPA) and (b) densitometric analysis of RPAs hybridised with the specific cRNA probes described in legend to Fig. 5 to detect expression of IGF-I Ea and Eb mRNAs, GHR and GHBP mRNA from IUGR offspring hepatic tissue from E22 to postnatal (P) day 21 (n=4 per time point, except birth where n=3). CP, cyclophilin loading control. C1 and C2, control lanes using probe alone with (C1) and without (C2) RNase treatment. M, maternal liver. Values are expressed as means ± s.e.m. abcdP<0.05, same letter denotes a significant difference by one-way ANOVA followed by Bonferroni t-test.
the dam to normal nutrient intake. We have
previously shown, in an unrelated study, that
IUGR neonates cross-fostered at birth onto
*ad libitum* fed mothers, continue to demonstrate a fail-
ure to show catch-up growth (Woodall et al. 1997).
Although in the present study we did not cross-foster
offspring from nutritionally restricted dams onto *ad libitum*
fed mothers after birth, we consider it un-
likely that undernutrition during pregnancy would
have contributed significantly to the observed lack of
postnatal catch-up growth in our study. The possi-
bility that maternal undernutrition may have a post-
natal effect on appetite in the growth retarded pups
clearly warrants further investigation. Although we
did not investigate this possibility in the current
study, studies in piglets have demonstrated that this
may well be the case (Dauncey et al. 1994). These
studies demonstrated that when appropriate-for-
gestational age piglets are pair-fed to the intake of their small-for-gestational age littermates, plasma IGF-I concentrations are similar by day 14 post-natally and suggest that reductions in plasma IGF-I may be a consequence of reduced food intake.

In the present study, we observed low expression of both exons 1 and 2 transcripts from E22 of gestation which then increased in abundance from birth. Exons 1 and 2 IGF-I transcripts have been reported to show independent ontogenic development. An increase in the expression of hepatic exon 2 transcripts occurs from 20 days after birth, whereas exon 1 transcripts increase in a linear manner after birth (Adamo et al. 1991). Studies in the rat have shown that hypophysectomy reduces all IGF-I mRNA transcripts. However, those initiated at ss 3 in exon 1 and the major ss in exon 2 appear to be preferentially reduced in the adult (Foyt et al. 1992) and juvenile hypophysectomized rats (Domene et al. 1993), suggesting GH dependent regulation of these transcripts. The IGF-I response
during postnatal development and to insulinopenic diabetes and fasting appears to result from the coordinated regulation of transcription ss within exon 1 in the liver (Adamo et al. 1991). Transcription initiation at the single major site in exon 2 was also reduced in diabetes and fasting. In the GH deficient dwarf rat, hepatic IGF-I mRNAs are reduced by 50% compared with normal rats (Butler et al. 1996). This reduction was coordinated for all of the exon 1 start sites and exon 2 IGF-I mRNA variants.

The current data demonstrate that exons 1 and 2 transcripts are coordinately reduced in IUGR offspring from E22 until postnatal day 9 and selected transcripts at postnatal day 15. This is consistent with our previous report that circulating IGF-I levels were reduced in IUGR rats until day 9 postnatal but were normalised by about day 15 (Woodall et al. 1996). Parallel decreases in liver IGF-I mRNA and serum IGF-I levels have also been observed in growth retarded fetuses from fasted pregnant dams (Straus et al. 1991), protein restricted dams (Muaku et al. 1995) and during acute protein restriction of neonatal rats (Moats-Staats et al. 1989). The reductions in serum IGF-I and IGF-I mRNA suggests that these responses are mediated by decreased gene expression at the level of transcription or RNA stabilisation (Moats-Staats et al. 1989). Whether these may also be related to reduced appetite or reduced food intake is unclear.

We have demonstrated that hepatic GHR and GHBP mRNAs are developmentally regulated in both ad libitum and IUGR offspring and are not influenced by maternal nutrition during pregnancy. The expression of GHBP mRNAs in liver is regulated by the pattern of GHR stimulation, with high basal levels of GH in the circulation stimulating GHBP mRNA expression (Carmignac et al. 1992, Maiter et al. 1992). GH secretion is generally believed to be markedly reduced in catabolic states. Fasting and a protein-reduced diet decreased specific GH binding and hepatic GH receptor mRNA in young rats (Maes et al. 1984, 1991, Bornfeldt et al. 1989) which return to normal with refeeding (Maes et al. 1984, 1991). Whether the lack of alteration in GHR/GHBP gene expression in our current study reflects the development of GH resistance or is secondary to altered GH secretion is unclear and warrants further investigation.

Transcripts for both GHR and GHBP are found in several different tissues in the rat and appear to be differentially regulated (Carlsson et al. 1990, Frick et al. 1990). In situ hybridisation studies demonstrate a distinct pattern of cellular distribution of GHR and GHBP in the developing rat fetus (Edmondson et al. 1995). These data suggest a role for GH, or a GH-like peptide, perhaps acting both directly and indirectly via IGF-I to promote fetal growth and development. Variation in abundance and developmental profiles of the GHR and GHBP mRNA in fetal and postnatal tissues by Northern analysis, suggests that GHR and GHBP may mediate differences in GH responsiveness (Walker et al. 1992). The increases with age in liver and kidney GHR mRNA abundance is consistent with the increased metabolic demands of maturing animals (Walker et al. 1992). Tissue specific regulation of GHR and GHBP mRNA in diabetic rats (Menon et al. 1994) suggests insulin may also be a regulator of GHR and/or GHBP gene expression.

Several reports demonstrate that fasted animals or those subjected to prolonged protein restriction after a period of fasting show a close correlation between the fall in plasma IGF-I concentration and the reduction in liver somatogenic binding sites (Maiter et al. 1988). This has led to the development of the hypothesis that down-regulation of hepatic GHR may contribute to the development of an insensitivity to GH as a consequence of malnutrition (Maes et al. 1984, 1991). This hypothesis is supported by observations that young growing rats with adequate energy intake subjected to short-term protein deprivation, exhibit a prompt and marked decrease in serum concentrations of IGF-I while hepatic GH binding was only slightly reduced (Maiter et al. 1988). The dissociation between these two parameters suggests that a post-receptor defect in GH action might contribute to the development of GH resistance as a consequence of protein malnutrition (Maiter et al. 1988). A transient lack in GH responsiveness has recently been observed in neonatal rats growth retarded at birth, while juvenile IUGR rats exhibited a normal growth response to GH administration (Woodall et al. 1997). Reduced circulating IGF-I levels in IUGR offspring (Woodall et al. 1996) in conjunction with the currently reported reduction in IGF-I gene expression with little change in GHR gene expression, supports our hypothesis of a post-receptor defect in GH action in IUGR offspring.

In summary, we postulate that reduced hepatic IGF-I mRNA in the face of unaltered GHR synthesis or binding may reflect a change in GH sensitivity similar to that demonstrated in other states of GH resistance, such as nutritional deprivation and diabetes (Straus & Takeimoto 1990, Adamo et al. 1991). The reduction in hepatic IGF-I mRNA and plasma IGF-I observed in our current studies appears to be transient and occurs prior to
weaning. Any possible long term changes that may persist within the somatotropic axis as a consequence of IUGR remain to be elucidated.

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