Nutrient–hormone interaction in the ovine liver: methionine supply selectively modulates growth hormone-induced IGF-I gene expression

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Abstract

This study tested the hypothesis that specific amino acids are responsible for modulating the insulin-like growth factor-I (IGF-I) response to growth hormone (GH) in ovine hepatocytes. Cells were grown in media of defined amino acid composition, based on physiological concentrations (P.C.) of amino acids in sheep plasma. Relative to culture in $5 \times$ P.C., amino acid limitation to $0.2 \times$ P.C. had inhibitory effects on IGF-I RNA expression, peptide release and p70 S6 kinase phosphorylation ($P < 0.01$ in each case). Limitation of methionine levels to $0.2 \times$ P.C. against a background of $5 \times$ P.C. for the other amino acids blocked GH-stimulated IGF-I peptide release and RNA expression, although basal expression was unaffected. In contrast, limitation of the other amino acids present in the culture medium had no effect on basal or GH-stimulated IGF-I expression. Selective methionine limitation to $0.2 \times$ P.C. levels had no effect on cellular or secretory protein synthesis rates relative to cells grown in complete

$5 \times$ P.C. medium but did cause a partial reduction in p70 S6 kinase phosphorylation, which was also observed when medium was selectively limited for other essential amino acids. The addition of rapamycin (5 ng/ml) to cells grown in $5 \times$ P.C. media completely abolished p70 S6 kinase phosphorylation ($P < 0.001$), implicating mTOR in the response of S6 kinase phosphorylation to changing amino acid supply. By contrast, inclusion of rapamycin (100 ng/ml) had no effect on levels of IGF-I gene expression. These results indicate that methionine is the key limiting amino acid involved in the modulation of IGF-I expression in the ovine liver. This nutrient–hormone interaction is a highly selective phenomenon, occurring against a background of modest effects on general protein synthetic control. The partial inhibitory effects of methionine on mTOR activity are not sufficient to account for this selectivity of action.

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Introduction

The endocrine control of growth is driven by the pituitary hormone, somatotrophin/growth hormone (GH). Many of the peripheral actions of GH are indirect, and mediated by insulin-like growth factor-I (IGF-I) (Gluckman *et al.* 1987). The GH/IGF-I axis has been proposed as a mediator of the effects of nutrient supply on growth (Breier *et al.* 1988). IGF-I gene expression has a wide tissue distribution, including the liver and skeletal muscle, but the bulk of circulatory IGF-I is of hepatic origin (Schwander *et al.* 1983). Studies in transgenic animals indicate that normal growth and development depends on both local (paracrine) and circulatory (endocrine) IGF-I production (Sjögren *et al.* 1999, Yakar *et al.* 1999, Silha *et al.* 2001).

Low protein diets reduce circulating titres of IGF-I (Wyn *et al.* 1991), and cause a loss of hepatic responsiveness to GH (Kriel *et al.* 1982, Breier *et al.* 1988, Ketelslegers *et al.* 1995). Depressed IGF-I titres caused by protein restriction are associated with reduced levels of IGF-I mRNA in the liver (Van de Haar *et al.* 1991, Pell *et al.* 1993), and these effects are mirrored by those of amino acid limitation on cultured hepatocytes, with basal and GH-stimulated IGF-I mRNA levels and peptide production being reduced *in vitro* (Harp *et al.* 1991, Thissen *et al.* 1994, Wheelhouse *et al.* 1999). Expression of IGF-I RNA or peptide in amino acid-restricted hepatocytes is reduced, and these cells become unresponsive to GH (Harp *et al.* 1991, Thissen *et al.* 1994, Wheelhouse *et al.* 1999).

It remains unclear how changes in amino acid supply are initially transduced into changes in gene expression, or whether specific amino acids are of greater importance than others in modulating GH-induced IGF-I gene expression. The mammalian orthologue of yeast TOR (for Target of Rapamycin), mTOR, relays signals through the protein kinase B (PKB)/Akt pathway (Campbell *et al.* 1999), and is highly sensitive to amino acid supply in mammalian cells (Hara *et al.* 1998, Wang *et al.* 1998, Iiboshi *et al.* 1999, Shigemitsu *et al.* 1999). This pathway modulates translation initiation through effects on the activity of eukaryotic initiation factor-4E (eIF-4E), and ribosomal p70 S6 kinase (Jefferies *et al.* 1997, Proud & Denton 1997). Additionally, the phosphorylation of the transcription factors STAT3 and CCAAT/enhancer binding protein α (C/EBP α), is mTOR-dependent (Hemati *et al.* 1997, Yokogami *et al.* 2000), suggesting that effects on mTOR activity could mediate effects of amino acid supply on mammalian gene expression.

Hence the present study determined the effect of selectively limiting individual amino acids on GH-induced IGF-I expression in cultured ovine hepatocytes, and assessed the role of mTOR in this response to amino acid supply.

Materials and Methods

Preparation of sheep hepatocytes

Ovine hepatocytes were prepared from sheep killed at a local abattoir as previously described (Wheelhouse *et al.* 1999). In all cell preparations approximately 90% of the cells were viable as determined by ability to exclude Trypan blue. Cells were plated in collagen-coated tissue culture plates at 1×10^6 viable cells/ml of recovery medium (Eagle's Minimal Essential Medium supplemented with 10% newborn calf serum, Insulin Transferrin Selenite media supplement (Sigma Chemicals Company, Poole, Dorset, UK), 0.4 nM dexamethasone, 0.15 nM tri-iodothyronine, antibiotic/antimycotic (Gibco BRL, Paisley, Strathclyde, UK), 4.2 mM sodium acetate, 0.33 mM sodium butyrate, 2.0 mM sodium propionate and 1.15 mM calcium lactate). The cells were incubated at 37 °C in 5% CO₂ for four hours before transfer to incubation media.

Incubation media

Test media were made using constituents of the RPMI-1640 Select-Amine kit (Gibco BRL). Hormone and volatile fatty acid (VFA) concentrations were as described for recovery media. Free amino acid concentrations were $5 \times$, $1 \times$, and $0.2 \times$ physiological concentrations (P.C.) based upon *in vivo* ovine portal venous blood data in *ad libitum* fed sheep (Lobley *et al.* 1995).

IGF-I RIA

Analyses of IGF-I levels in acid ethanol extracts of lyophilized media samples were performed as described previously (Wheelhouse *et al.* 1999).

Measurement of phosphorylation of p70 S6 kinase

The phosphorylation of p70 S6 kinase was determined by Western blotting. Cells were lysed in a preheated (95 °C) buffer composed of 12.5 mM Tris HCl (pH 7.5), 1% SDS, 10% glycerol, 2.5% dithiothreitol and 0.04% bromophenol blue and stored at -80 °C until electrophoresis. Proteins were resolved by SDS-PAGE, with a gel acrylamide content of 10%. Resolved proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Poly-screen). Following transfer, membranes were washed briefly in 20 mM Tris HCl, 150 mM NaCl (pH 7.5) (TBS), containing 0.1% v/v Tween 20 (TBST), and then incubated for 1 h at room temperature in TBST containing 3% non-fat milk powder. Membranes were incubated overnight at 4 °C in primary antibody solution containing anti-phospho-(Thr 389)-p70 S6 kinase or anti p70 S6 kinase (New England Biolabs, Hitchin, Hertfordshire, UK) diluted 1:1000 in TBST containing 5% w/v bovine serum albumin. The next day membranes were washed five times in TBST, and incubated in secondary antibody solution containing anti-rabbit IgG-horseradish peroxidase conjugate (Transduction Laboratories) diluted 1:2000 in TBST containing 5% w/v bovine serum albumin for 1 h at room temperature. After the second antibody incubation, membranes were washed as before, and labelled proteins were visualised using the ECL chemiluminescence detection system (Amersham). The intensity of labelled bands was quantified using a LASER densitometer (Amersham) and Phoretix I-D Advanced image analysis software.

Measurement of IGF-I gene expression

Measurement of IGF-I gene expression was conducted by RNase protection assay (RPA) as described previously (Wheelhouse *et al.* 1999).

Cellular protein synthesis rates

Cellular protein synthesis rates were measured by metabolic labelling over a 30-h period, starting 18 h after transfer to defined media. These experiments were conducted essentially as described previously (Wheelhouse *et al.* 1999), except that the tracer was [³H]leucine, instead of [³⁵S]methionine.

Replication and statistical analyses

Data were analysed by factorial ANOVA and comparison of individual sample means was made by Fisher's least

significant difference test. All analyses were conducted using Minitab version 11 (Minitab Inc. Pennsylvania, PA, USA). Positively skewed data were normalised by $\log_{10}$ transformation prior to analysis.

Results

Effects of limitation of individual amino acids on GH-induced IGF-I release

In ovine hepatocytes grown in $5 \times \text{P.C.}$ levels of amino acids, GH (10 nM) caused an approximately twofold induction of IGF-I peptide release over a 48-h incubation period (Fig. 1a). By comparison, cells grown in $0.2 \times \text{P.C.}$ medium had lower basal levels of IGF-I peptide release ($P < 0.01$) and were unresponsive to GH either at 10 nM (Fig. 1) or at any concentrations between 1 and 100 nM (data not shown). Limitation of individual amino acids to $0.2 \times \text{P.C.}$ against a background of $5 \times \text{P.C.}$ levels had differential effects on this response to GH. In the case of methionine the GH response was abolished, although basal IGF-I release was similar to that seen in $5 \times \text{P.C.}$ medium (Fig. 1a). In contrast, when levels of leucine, isoleucine, valine, cysteine, histidine, lysine, or tryptophan were individually reduced, GH-stimulated IGF-I release was not inhibited relative to levels in $5 \times \text{P.C.}$ medium (Fig. 1b).

Effect of amino acid supply on cellular and secretory protein synthesis rates

Consistent with our earlier work (Wheelhouse *et al.* 1999), cellular protein synthesis was approximately linear up to 30 h in culture in $5 \times \text{P.C.}$ media (Fig. 2a). General limitation of amino acid supply to $0.2 \times \text{P.C.}$ caused a pronounced reduction in cellular synthesis rates ($P < 0.001$); by contrast selective limitation of methionine to $0.2 \times \text{P.C.}$ did not inhibit cellular protein synthesis over the 30-h period. A similar picture was seen for secretory protein synthesis, except that a lag was apparent in the leucine incorporation into the secretory fraction, reflecting the post-translational processing of secretory proteins (Fig. 2b).

Effect of methionine limitation on IGF-I RNA expression

Since GH induction of hepatic IGF-I expression occurs through transcriptional activation, we tested the hypothesis that the effects of methionine limitation on IGF-I peptide release stemmed from a reduction of GH-induced IGF-I RNA expression (Fig. 3). In $5 \times \text{P.C.}$ medium, GH (10 nM) induced a twofold activation of IGF-I RNA expression ($P < 0.01$), and this response was almost completely abolished by general amino acid depletion to

Figure 1 Effect of selective amino acid limitation on IGF-I production. (a) Cells were incubated in complete $5 \times \text{P.C.}$ or $0.2 \times \text{P.C.}$ media, or in $5 \times \text{P.C.}$ media selectively depleted of methionine to $0.2 \times \text{P.C.}$ level ($0.2 \times \text{Met}$), and in the absence or presence of GH (10 nM). Media for the period 24–48 h in culture were assayed for IGF-I release. Data are normalised against unstimulated IGF-I release in $5 \times \text{P.C.}$ medium, and are expressed as means $\pm$ S.E.M. of three separate cultures. ** $P < 0.01$, significantly greater production relative to values in the absence of GH in the same culture medium. (b) Responsiveness of cells grown in medium selectively limited to $0.2 \times \text{P.C.}$ for the amino acids indicated (Leu, leucine; Isoleu, isoleucine; Val, valine; Cys, cysteine; His, histidine; Lys, lysine; Tryp, tryptophan; Met, methionine) or for all amino acids. Data are GH-induced IGF-I release expressed as a fraction of the release seen in GH-stimulated cells grown in $5 \times \text{P.C.}$ media. ** $P < 0.01$, significantly reduced response to GH. Data are means $\pm$ S.E.M. of three separate cultures.

$0.2 \times \text{P.C.}$ or by selective limitation of methionine. As was the case for IGF-I peptide release (Fig. 1) general amino acid limitation, but not selective methionine limitation, also reduced basal IGF-I RNA expression.

Figure 2 Effect of methionine limitation on cellular and secretory protein synthesis. Cells were grown in $0.2 \times$ P.C. or $5 \times$ P.C. medium, or in medium selectively depleted of methionine ($0.2 \times$ Met), and labelled with [3 H]leucine. Leucine incorporation into cellular (a) or secretory (b) protein was determined and normalised against total cellular protein. Data are means $\pm$ S.E.M. from 3 separate experiments.

Effect of general or selective amino acid limitation on p70 S6 kinase phosphorylation in ovine hepatocytes

Immunostaining of Western blots with antisera to phosphorylated (Thr 389) p70 S6 kinase revealed a major band of 70 kilodaltons (kDa), corresponding to p70 S6 kinase (Fig. 4a). Additionally, the antibody recognised a higher molecular weight species of approximately 85 kDa, which probably represents p85 S6 kinase, and a lower weight species of below 57 kDa which was not identified. The intensity of staining of phosphorylated p70 S6 kinase was highly sensitive to amino acid supply, such that exposure to $5 \times$ P.C. media for 2 h resulted in phosphorylation levels an order of magnitude higher than those exposed to $0.2 \times$ P.C. amino acids for the same period (Fig. 4a,c; $P < 0.001$). The stimulatory effect of $5 \times$ P.C. amino acids on p70 S6 kinase phosphorylation was completely blocked by co-addition of the mTOR inhibitor, rapamycin (5 ng/ml), and at this concentration inhibition was maintained for at least 4 h (Fig. 4b and data not shown). Selective limitation for methionine, or for any of the branched chain amino acids, resulted in

Figure 3 Effect of methionine limitation on IGF-I RNA expression. Cells were incubated in complete $5 \times$ P.C. or $0.2 \times$ P.C. media, or in $5 \times$ P.C. media selectively depleted of methionine ($0.2 \times$ Met), and in the absence or presence of GH (10 nM) as indicated. After 24 h, RNA was extracted and IGF-I RNA expression was determined by RNase protection assay. Data are normalised against unstimulated IGF-I expression in $5 \times$ P.C. medium, and are means $\pm$ S.E.M. of three separate cultures. ** $P < 0.01$, significantly greater expression relative to values in the absence of GH in the same culture medium.

intermediate levels of p70 S6 kinase phosphorylation (Fig. 4a,c).

Effect of rapamycin on IGF-I expression

Since both amino acid limitation and rapamycin inhibit phosphorylation of p70 S6 kinase, we tested the possibility that rapamycin would mimic the inhibitory effects of amino acid limitation on IGF-I gene expression. As before, IGF-I RNA expression was highly sensitive to amino acid supply and GH (Fig. 5), with levels in cells grown in $0.2 \times$ P.C. medium being 60–80% lower than in $5 \times$ P.C. media ($P < 0.001$ in both cases). In contrast, rapamycin (5 ng/ml) completely failed to inhibit basal or GH-stimulated IGF-I gene expression. To exclude the possibility that the lack of effect observed in these initial experiments was due to loss of rapamycin bioactivity, we also tested the effects of 100 ng/ml rapamycin and even under these circumstances no inhibitory effect of rapamycin on IGF-I gene expression was observed (Fig. 5).

Discussion

The aim of the present study was to investigate the mechanisms through which changes in amino acid supply affect basal and GH-stimulated IGF-I expression in ovine hepatocytes. In previous studies we have shown that general limitation of amino acids to $0.2 \times$ P.C. reduces basal IGF-I expression and blocks the stimulatory effect of GH thereon (Wheelhouse *et al.* 1999). Here, we used

Figure 4 The effect of amino acid availability and rapamycin on p70 S6 kinase phosphorylation. (a) Cells were incubated in 0.2 x P.C. or 5 x P.C. media, or in media selectively limited for methionine (0.2 x Met), and in the absence or presence of GH (10 nM), as indicated, for a period of 2 h. Extracts were resolved by SDS-PAGE, blotted and probed with antiserum to phosphorylated p70 S6 kinase. The arrow indicates the band corresponding to phospho-p70 S6 kinase. (b) Cells were incubated in 5 x P.C. media and in the absence or presence of rapamycin (5 ng/ml) for 2 h prior to determination of p70 S6 kinase phosphorylation. (c) Relative optical density measurements of p70 S6 kinase phosphorylation under the conditions described in (a), and in cells selectively limited for the branched chain amino acids: leucine (0.2 x Leu), isoleucine (0.2 x Isoleu) or valine (0.2 x Val). Data are means $\pm$ S.E.M. of 3 experiments, expressed relative to phosphorylation levels in cells grown in 5 x P.C. medium.

selective limitation of individual amino acids to 0.2 x P.C., against a background of 5 x P.C. levels, to determine whether specific amino acids exert more potent effects than others. Using this approach we found that of those amino acids tested, methionine limitation was uniquely able to block GH-stimulated IGF-I peptide

Figure 5 The effect of rapamycin on IGF-I mRNA expression. Cells were grown in 0.2 x P.C. or 5 x P.C. amino acids for a total of 24 h, in the absence or presence of rapamycin (100 ng/ml) and GH (10 nM) as indicated, and IGF-I RNA abundance was determined by RNase protection assay. Data are normalised relative to values in 5 x P.C. media with no additions, and are means $\pm$ S.E.M. for 3 experiments. * P < 0.05, significantly greater expression relative to values in the absence of GH in the same culture medium.

release, although unlike general amino acid limitation, no effect was seen on basal IGF-I peptide release.

This effect of methionine was not due to effects on overall cellular or secretory protein synthesis rates although general amino acid limitation clearly affected these parameters. This led us to explore the possibility that a selective effect on IGF-I RNA expression might be involved. Our results are consistent with this hypothesis: methionine limitation selectively ablated the IGF-I RNA response to GH stimulation whilst leaving basal IGF-I RNA levels similar to those seen in cells grown in 5 x P.C. media.

Injection of GH increases nuclear IGF-I transcript levels in hypophysectomised rats, indicating that GH regulates IGF-I expression through transcriptional activation (Gronowski & Rotwein 1995) and, consonant with this, we have found that the transcription inhibitor, actinomycin-D, blocks GH effects on IGF-I RNA abundance in ovine hepatocytes (A K Stubbs & D G Hazlerigg, unpublished observations). The present results are therefore consistent with the view that methionine limitation selectively blocks the transcriptional response to GH, leading to reduced IGF-I expression.

Many recent studies highlight effects on the mTOR pathway as a primary response to amino acid supply in mammalian cells. The most firmly established consequences of altered mTOR activity are changes in the phosphorylation state and activity of elements controlling ribosomal activity – in particular p70 S6 kinase and

eukaryotic initiation factor 4 binding protein-1 (Hara *et al.* 1998). Since changing activity of these factors has differential consequences for translation of different classes of RNA species – with those encoding regulatory proteins often being particularly sensitive (Jefferies *et al.* 1997), altered mTOR activity potentially has multiple downstream consequences beyond affecting overall cellular protein synthesis rates. This is highlighted by the recent demonstration that rapamycin treatment affects the phosphorylation and activity of the transcription factors STAT3 and C/EBP α (Hemati *et al.* 1997, Yokogami *et al.* 2000).

We therefore examined the effects of general and selective amino acid limitation on p70 S6 kinase phosphorylation in ovine hepatocytes. As predicted, p70 S6 kinase phosphorylation was sensitive to amino acid availability in ovine hepatocytes, and selective limitation of methionine caused a partial reduction in phosphorylation of this enzyme. Although these data are consistent with the idea that the mTOR pathway may contribute to the overall response to methionine limitation, two observations argue that it is not involved in the selective effects on GH-stimulated IGF-I expression. First, limitation of the branched chain amino acids – which modulate insulin sensitivity in muscle (Garlick & Grant 1988), had a similar effect on p70 phosphorylation, but had no effect on IGF-I expression. Secondly, the pharmacological inhibitor of mTOR, rapamycin, had no measurable effect on GH-stimulated IGF-I RNA expression, despite causing the predicted sustained inactivation of another mTOR output – p70 S6 kinase phosphorylation. Together, these data suggest that although amino acids regulate mTOR-dependent signalling in ovine hepatocytes, as in other cell types (e.g. Hara *et al.* 1998, Patti *et al.* 1998, Wang *et al.* 1998, Iiboshi *et al.* 1999, Shigemitsu *et al.* 1999), this effect is not sufficient to account for the blockade of GH-induced IGF-I RNA expression seen in methionine-limited cells.

Interestingly, studies of two other amino acid sensitive genes, asparagine synthetase and CHOP (C/EBP-homologous protein), also suggest that a specific, but as yet unidentified, mechanism exists allowing certain genes to be particularly sensitive to methionine supply (Fafournoux *et al.* 2000). The physiological significance of this remains unclear, but it is worth noting that, for ruminants, methionine has been highlighted as the first amino acid to become limiting in some forage and silage-based diets (Richardson & Hatfield 1978). This provides a possible rationale for the selective sensitivity of the GH/IGF-I system to methionine reported here, since this phenomenon ensures that the drive to grow is modulated not by general amino acid availability, but by the amino acid least available in the nutrient supply. Studies in rats and pigs indicate that IGF-I expression is sensitive to lysine and tryptophan supply (Harp *et al.* 1991, Brameld *et al.* 1999), but neither of these amino acids had any effect on IGF-I release during the present study.

Hence it is possible that the relative importance of specific amino acids in determining hepatic IGF-I expression varies between species in relation to their adaptation to different food sources.

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