IGF-I and insulin regulate eIF4F formation by different mechanisms in muscle and liver in the ovine fetus

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Shen, Weihua, Daniel Mallon, David W. Boyle, and Edward A. Liechty. IGF-I and insulin regulate eIF4F formation by different mechanisms in muscle and liver in the ovine fetus. Am J Physiol Endocrinol Metab 283: E593–E603, 2002. First published May 15, 2002; 10.1152/ajpendo.00570.2001.—The mechanisms by which insulin-like growth factor I (IGF-I) and insulin regulate eukaryotic initiation factor (eIF)4F formation were examined in the ovine fetus. Insulin infusion increased phosphorylation of eIF4E-binding protein (4E-BP1) in muscle and liver. IGF-I infusion did not alter 4E-BP1 phosphorylation in liver. In muscle, IGF-I increased 4E-BP1 phosphorylation by 27%; the percentage in the γ-form in the IGF-I group was significantly lower than that in the insulin group. In liver, only IGF-I increased eIF4G. Both IGF-I and insulin increased eIF4E-eIF4G binding in muscle, but only insulin decreased the amount of 4E-BP1 associated with eIF4E. In liver, only IGF-I increased eIF4E-eIF4G binding. Insulin increased the phosphorylation of p70 S6 kinase (p70S6k) in both muscle and liver and protein kinase B (PKB/Akt) in muscle, two indicative signal proteins in the phosphatidylinositol (PI) 3-kinase pathway. IGF-I increased PKB/Akt phosphorylation in muscle but had no effect on p70S6k phosphorylation in muscle or liver. We conclude that insulin and IGF-I modulate eIF4F formation; however, the two hormones have different regulatory mechanisms. Insulin increases phosphorylation of 4E-BP1 and eIF4E-eIF4G binding in muscle, whereas IGF-I regulates eIF4F formation by increasing total eIF4G. Insulin, but not IGF-I, decreased 4E-BP1 content associated with eIF4E. Insulin regulates translation initiation via the PI 3-kinase-p70S6k pathway, whereas IGF-I does so mainly via mechanisms independent of the PI 3-kinase-p70S6k pathway. Insulin; insulin-like growth factor I; fetus; eukaryotic initiation factors; p70 S6 kinase

INSULIN HAS LONG BEEN REGARDED as the primary fetal growth factor. Hyperinsulinemic fetuses of mothers with diabetes or the Beckwith-Weideman syndrome are usually large for gestational age. In contrast, the infant born with pancreatic agenesis and hypoinsulinemia is uniformly small for gestational age. Insulin administration to the fetus results in accelerated growth (1, 43, 52, 60) and increased protein utilization (54).

Insulin-like growth factor I (IGF-I) is one of the potent metabolic, mitogenic, and differentiative factors and is considered to be one of the important regulators of fetal growth (12, 22, 29). IGF-I is widely expressed in fetal tissues (19, 47). The circulating concentration of IGF-I has been shown to correlate directly with fetal weight in humans, and decreased concentrations of IGF-I have been found in association with fetal growth retardation (28, 41). Deletion of the IGF-I gene in mice results in high mortality, reduced birth weight, and retarded rate of postnatal growth (35).

Both insulin and IGF-I have been found to regulate protein anabolism in fetal and postnatal life (3, 13, 14, 21, 23, 48, 56). However, the cellular mechanisms by which IGF-I and insulin regulate fetal protein anabolism in vivo are largely unknown. One possible mechanism for IGF-I and insulin-induced increases in fetal protein synthesis is via alterations in the amount and/or activity of eukaryotic initiation factors (eIF). The initiation of mRNA translation is a complex process requiring several steps and more than a dozen eIFs (42, 46, 51). It is believed that the binding of mRNA to the 43S preinitiation complex is one of the rate-limiting steps in protein synthesis. The binding of mRNA to the 43S preinitiation complex is regulated by a multisubunit complex, called the eIF4F complex (2). The complex consists of three proteins: 1) eIF4A, an RNA helicase that functions to unwind secondary structure in the 5' untranslated region of the mRNA; 2) eIF4E, a protein that binds the 7-methyl-GTP (m7GTP) cap present at the 5' end of eukaryotic mRNAs; and 3) eIF4G, a 220-kDa polypeptide that functions as a scaffold for eIF4E, eIF4A, the mRNA, and the ribosome. Presumably, increased formation of the eIF4F complex will lead to an increase of the cap-dependent mRNA translation.

Formation of an eIF4F complex may be regulated by alterations in either the phosphorylation state or the availability of eIF4E. Phosphorylation of eIF4E is suggested to stimulate translation rates through increased association with eIF4G and eIF4A (5) and/or increased mRNA cap-binding affinity (39). The availability of eIF4E appears to be regulated by a group of
small acid and heat-stable proteins termed eIF4E-binding proteins (4E-BP1, 4E-BP2, and 4E-BP3). Hypophosphorylated 4E-BP1 binds to eIF4E to form an eIF4E-4E-BP1 complex. When eIF4E is bound to 4E-BP1, eIF4E binds to mRNA but cannot form an eIF4E:eIF4G complex (18), therefore blocking the binding of mRNA to the ribosome. The binding of eIF4E to 4E-BP1 is, in turn, regulated by phosphorylation of 4E-BP1. Phosphorylation of 4E-BP1 releases eIF4E from the eIF4E:4E-BP1 complex and allows the eIF4E-mRNA complex to bind to eIF4G and through eIF4G to the 40S ribosome (16, 49, 51).

The intracellular signal transduction pathways leading to translation initiation are beginning to be elucidated in cell culture systems. It has been demonstrated that the phosphatidylinositol (PI) 3-kinase pathway is responsible for the phosphorylation of 4E-BP1 and p70 S6 kinase (p70S6K) (45, 49). The signaling pathway that leads to phosphorylation of 4E-BP1 and p70S6K appears to bifurcate immediately upstream of the two proteins, likely at the mammalian target of rapamycin (mTOR) (57). Protein kinase B (PKB/Akt) has been implicated in the activation of the mTOR pathway and in the regulation of 4E-BP1 and p70S6K (15). However, in vivo data from intact animals are sparse, and essentially nothing is known from the fetal model regarding the signal pathways leading to activation of eIFs.

The present study was designed to test the hypotheses that IGF-I and insulin increase the formation of the eIF4F complex. In addition, the possible pathways involved in insulin- and IGF-I-induced alterations in eIFs were explored by measuring the phosphorylation of the indicative signal proteins of specific signaling pathways. The results show that both insulin and IGF-I alter the eIF4F complex formation in the fetus but that the response of component initiation factors to insulin infusion is organ specific. The results also show that the signal pathway of insulin-induced alterations in initiation factors is different from that of IGF-I.

 METHODS

Animals and surgical procedures. Thirty-three ewes of 115–120 days of gestation were utilized for this study. Animal care was in strict compliance with National Institutes of Health guidelines within an American Association for Laboratory Animal Care-certified facility, and the study protocols were approved by the Institutional Animal Care and Use Committee.

Surgical procedures were performed aseptically under general anesthesia. Anesthesia was induced with intravenous ketamine and maintained with isoflurane inhalation. Fetal catheters were placed in the inferior vena cava and abdominal aorta. Catheters were exteriorized and irrigated daily with 0.9% saline containing 50 U heparin/ml. All ewes consumed ad libitum a diet consisting of hay and pelletized alfalfa and had constant access to water and a salt lick.

Study design. The animals were allowed a minimum of 5 days of recovery from operative stress before the study. Complete recovery and fetal health were assessed by monitoring maternal food intake, fetal and maternal glucose concentrations, and acid-base status. The animals were fed ad libitum before and throughout the study.

During the hormone infusion, fetal whole blood glucose was clamped at the baseline level by frequent glucose concentration measurement with a glucose analyzer (YSI 2300, Yellow Springs Instrument, Yellow Springs, OH) and a variable infusion of 20% glucose. Likewise, the total fetal plasma branched-chain amino acid (BCAA) concentrations were measured at 15-min intervals (58), and a balanced amino acid solution (Aminosyn II, Abbott Laboratories, Abbott Park, IL) was infused at a variable rate to maintain plasma BCAA at the baseline level. Seven hours after initiation of infusion, the ewe was anesthetized, and fetal biopsy samples (muscle and liver) were taken. Finally, the ewe underwent euthanasia, and fetal size and fetal weight were recorded. All catheter placements were confirmed at autopsy, and fetal autopsy samples were taken. Tissue samples were snap-frozen in liquid nitrogen and stored at −70°C before extraction for initiation factors.

Measurements of leucine, IGF-I, and insulin. During the hormone infusion, total BCAA concentrations were monitored by spectrophotometry as described in the previous paragraph. The fetal leucine concentrations in plasma and whole blood from representative samples were measured with an automated amino acid analyzer (Beckman 6300, Beckman-Coulter, Palo Alto, CA).

Insulin concentrations in fetal plasma were determined in duplicate by a double-antibody RIA using ovine insulin to construct the standard curve (kit no. SRI-13K, Linco Research, St. Charles, MO). Total IGF-I in fetal plasma was determined by a validated competitive RIA that employed ovine insulin to construct the standard curve (kit no. SRI-13K, Linco Research, St. Louis, MO), and 1% protease inhibitor cocktail (Sigma, St. Louis, MO), and 1% protease inhibitor cocktail (Sigma, St. Louis, MO) were added immediately before use. The homogenate was centrifuged at 13,000 g at 4°C for 30 min. The protein concentration of the supernatant was measured by the Lowry method (36). The supernatant was stored at −70°C until further analysis. Our preliminary study showed that, in the 13,000-g pellets, less than 5% of eIF4E was found, and eIF4G and 4E-BP1 were undetectable, suggesting that the eIF extraction protocol works well.

eIF4G was extremely unstable during sample preparation. Our preliminary studies found that the protease inhibitor cocktail from Sigma is effective in preventing eIF4G degradation during tissue homogenization, centrifugation, and m7GTP resin purification; so this inhibitor cocktail was used.
for all tissue homogenate preparations. In addition, comparison of fresh tissue homogenates with tissues stored at −70°C for 6 mo showed no loss of eIF4G content.

The supernatant was diluted with SDS buffer and subjected to reduced discontinuous SDS-PAGE on a 4.0% stacking gel and 6% (eIF4G), 10% (eIF4E), or 15% (4E-BP1) acrylamide separating gel at 90 V at room temperature. An equal amount of protein (50 µg/lane) was loaded into each well. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane with a Bio-Rad transfer unit at 90 V for 90 min and blocked with blocking buffer (PBS containing 0.1% Tween 20 and 5% Carnation nonfat milk) at 4°C overnight. The membrane was incubated with a goat polyclonal antibody to eIF4G (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal antibodies to eIF4E (1:1,500; Santa Cruz Biotechnology), or 4E-BP1 (1:500; Santa Cruz Biotechnology) for 1 h at room temperature. The blots were then developed using an enhanced chemiluminescence Western blotting kit (Amersham Pharmacia Biotech, Piscataway, NJ). Films were scanned with a scanner, and images were quantitated using Scion image software (Scion, Frederick, MD). The membrane was stripped and reprobed with a polyclonal antibody to actin and/or stained with Coomassie blue R-250 to confirm equal protein loading per sample.

Quantification of 4E-BP1 phosphorylation and eIF4E phosphorylation. Previous experiments had established that phosphorylation of 4E-BP1 retards the protein migration rate on SDS-polyacrylamide gels (25, 32, 33). Consequently, when the tissue or cell extract is subjected to SDS-PAGE, multiple electrophoretic forms may be resolved. These forms have been identified as α (least phosphorylated and fastest migrating), β (intermediate), and γ (most phosphorylated and slowest migrating). The phosphorylation of 4E-BP1 was expressed as the percentage of the γ-form in the total content (α + β + γ).

eIF4E phosphorylation at Ser209 was determined by immunoblotting with the use of a phosphospecific rabbit polyclonal antibody (1:1,000; Cell Signaling Technology, Beverly, MA).

Determination of eIF4G and 4E-BP1 associated with eIF4E. The association of 4E-BP1 or eIF4G with eIF4E was assessed by determining how much eIF4G or 4E-BP1 was recovered when eIF4E was extracted with m7GTP-Sepharose 4B (Amersham Pharmacia Biotech). In addition to free eIF4E, eIF4E associated with eIF4G, or eIF4E associated with 4E-BP1, is presumably to bind the m7GTP-Sepharose 4B as well (25, 33). One hundred twenty microtiter wells of pre-washed and pre-equilibrated (with buffer A) suspension of m7GTP-Sepharose 4B were added in a minicolumn. Tissue extract of equal protein concentration (4 mg of muscle protein or 10 mg of liver protein) was subsequently added and mixed for 1 h at 4°C. After incubation, the resin was washed three times with ice-cold buffer A (1.5 ml/wash) with the use of a Vac-Man vacuum manifold to pull the buffer through the column. The resin was then resuspended in 300 µl of 100 µM m7GTP (Sigma) in buffer A and incubated for 5 min on ice. The eluate was then collected. The eluate was reapplied to the column, and the final eluate was collected. The eluate was diluted with an equal volume of the SDS sample buffer and subjected to electrophoresis. Proteins were then electrophoretically transferred to PVDF membranes, and eIF4G and 4E-BP1 were quantified as described. The amount of eIF4G and 4E-BP1 detected in the eluate represents eIF4G or 4E-BP1 associated with eIF4E in the muscle and liver. Then, the membranes were stripped and reprobed for total eIF4E. The results were normalized to the amount of eIF4E.

Determination of p70S6k and PKB/Akt phosphorylation. Muscle or liver homogenates were combined with an equal volume of SDS sample buffer, and the diluted samples were subjected to electrophoresis on a 7.5% polyacrylamide gel. The samples were then analyzed by protein immunoblot analysis by use of rabbit anti-rat p70S6k (1:1,500; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal antibodies to eIF4E (1:500; Santa Cruz Biotechnology), or 4E-BP1 (1:500; Santa Cruz Biotechnology) for 1 h at room temperature. The blots were then developed using an enhanced chemiluminescence Western blotting kit (Amersham Pharmacia Biotech, Piscataway, NJ). Films were scanned with a scanner, and images were quantitated using Scion image software (Scion, Frederick, MD). The membrane was stripped and reprobed with a polyclonal antibody to actin and/or stained with Coomassie blue R-250 to confirm equal protein loading per sample.

Table 1. Fetal weights at study conclusion and glucose, leucine, IGF-I, and insulin plasma concentrations in the fetal artery

<table>
<thead>
<tr>
<th>Control (n = 10)</th>
<th>IGF-1 (n = 7)</th>
<th>Insulin (n = 10)</th>
<th>IGF/Ins (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal weights, kg</td>
<td>3.19 ± 0.07</td>
<td>3.30 ± 0.09</td>
<td>4.21 ± 0.08</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>16.37 ± 0.54</td>
<td>17.90 ± 0.64</td>
<td>15.15 ± 0.57</td>
</tr>
<tr>
<td>Leucine, µM</td>
<td>123 ± 4.81</td>
<td>133 ± 5.68</td>
<td>120 ± 5.11</td>
</tr>
<tr>
<td>IGF-I, ng/ml</td>
<td>89.64 ± 7.37</td>
<td>359.81 ± 8.8</td>
<td>122.44 ± 8.17</td>
</tr>
<tr>
<td>Insulin, µU/ml</td>
<td>22.00 ± 21.15</td>
<td>38.81 ± 25.99</td>
<td>254.68 ± 22.19</td>
</tr>
<tr>
<td>Glucose infused, µmol·kg⁻¹·min⁻¹</td>
<td>2.71 ± 1.23a,c</td>
<td>14.12 ± 1.54a,b,c</td>
<td>23.6 ± 1.44b,d</td>
</tr>
<tr>
<td>Leucine infused, µmol·kg⁻¹·min⁻¹</td>
<td>0.08 ± 0.06a,c</td>
<td>0.47 ± 0.11a,b,c</td>
<td>0.81 ± 0.11a,b,c</td>
</tr>
</tbody>
</table>

Results are given as means ± SE. Data were analyzed by 2-way ANOVA and Tukey’s Honestly Significant Difference post hoc test to determine group differences. Letters a-d indicate significant differences (P < 0.05) between groups. IGF-I, insulin-like growth factor I; IGF/Ins, group infused with IGF-I and insulin simultaneously.
ations in fetal weight. All metabolic data were normalized to fetal weight to minimize the effect of variability in fetal weight. The baseline levels of glucose and leucine varied among animals; however, all of the measurements were within normal ranges.

Fetal plasma concentrations of IGF-I and insulin are also given in Table 1. Infusion of IGF-I resulted in a more than threefold increase of IGF-I in the fetal circulation. As expected, insulin concentrations in the fetal circulation were significantly increased after a 7-h infusion of insulin. However, there was no significant difference in insulin concentrations between the control group and the IGF-I group, which received insulin at a replacement dosage to counteract the somatostatin-like effect of IGF-I on inhibition of insulin secretion. There was a significantly higher insulin concentration in the combined infusion group (IGF/Ins) despite identical absolute rates of insulin infusion. This was likely due to the lesser weight of the fetuses in this group, resulting in a greater weight-normalized insulin infusion rate.

Glucose and amino acid infusion rates for clamp. During the experimental infusions, arterial glucose and BCAA were held constant by infusing a variable amount of glucose and a mixture of amino acids into the fetal inferior vena cava (Table 1). In the IGF-I group, an average glucose infusion rate of 14 μmol·kg⁻¹·min⁻¹ was needed to keep glucose concentration constant. In the insulin infusion group, 24 μmol·kg⁻¹·min⁻¹ of exogenous glucose was needed to keep fetal glucose concentrations constant. As expected, more exogenous glucose (33 μmol·kg⁻¹·min⁻¹) was needed for the purpose of the “clamping” when insulin and IGF-I were infused in combination. To keep fetal arterial amino acids constant, exogenous amino acids were infused in the IGF/Ins group. When both hormones were used in combination, a significantly higher amino acid infusion rate was required to maintain amino acid concentrations.

Initiation factors. eIF4E, one of the subunits of the eIF4F complex, plays a crucial role in the binding of mRNA to the 43S preinitiation complex. The total amount of eIF4E was not affected by insulin or IGF-I infusion in ovine skeletal muscle (Fig. 1A), and only IGF-I altered eIF4E phosphorylation (Fig. 1B). Phospho-eIF4E varied little among individuals in the control group. In the IGF, Insulin, or IGF/Ins groups, however, phospho-eIF4E varied greatly among individuals, from a level similar to that of the controls to a level more than threefold that of the controls. This can be seen by the large increase in the SE bars in the IGF, Insulin, and IGF/Ins groups.

4E-BP1. The availability of eIF4E can be regulated through changes in the amount of eIF4E bound to 4E-BP1. The association of 4E-BP1 with eIF4E is regulated by the phosphorylation of 4E-BP1. In skeletal muscle, both insulin and IGF-I infusion increased the phosphorylation of 4E-BP1, but the insulin effect was much more pronounced (Fig. 2A). In the control group, three phosphorylation forms of 4E-BP1 were typically detected, and the percentage of the γ-form was 41%. In contrast, only β- and γ-forms were usually detected in the insulin group, and 85% of 4E-BP1 was in the γ-form. In the IGF group, 4E-BP1 phosphorylation was increased by 27% (P < 0.05), but the percentage of the highly phosphorylated form (γ) in the IGF-I group was lower than that in the insulin group (P < 0.05). When insulin and IGF-I were infused simultaneously, a substantial increase in the hyperphosphorylated form, similar to that seen in the insulin infusion alone, was observed.

In contrast to the findings in skeletal muscle, in liver extracts only insulin infusion resulted in a significant change in the amount of 4E-BP1 phosphorylation (Fig. 2B). 4E-BP1 was present in a very small amount. 4E-BP1 amount in liver extract was only ~0.05% of that in skeletal muscle.

eIF4G. As illustrated in Fig. 3A, IGF-I and insulin infusion increased muscle total eIF4G by 21 and 9%, respectively, but this was not a significant difference.
when compared with the control group. When IGF-I and insulin were infused simultaneously, eIF4G in skeletal muscle increased significantly (P < 0.05). In contrast, liver eIF4G content was markedly increased by IGF-I infusion (Fig. 3B). Insulin infusion did not alter eIF4G content and in fact appeared to have an inhibitory effect on IGF-induced increase in the eIF4G content when both hormones were infused simultaneously.

Association of eIF4G or 4E-BP1 with eIF4E. In skeletal muscle, insulin decreased the association of eIF4E with the repressor protein 4E-BP1 as well as the expected reciprocal increase in eIF4G associated with eIF4E (Fig. 4). The degree of eIF4E binding to eIF4G is reflective of eIF4F formation. IGF-I had no effect on the association of eIF4E with 4E-BP1, consistent with its moderate effect on 4E-BP1 phosphorylation. However, it did result in an increased association of eIF4E with eIF4G (P < 0.01). In addition, there was a statistically significant interaction effect between insulin and IGF-I, and the degree of association of the eIF4E and eIF4G was no greater when the hormones were given simultaneously than when either was given alone. Thus the significant interaction effect may indicate that one or both hormones have a negative impact on the other’s ability to stimulate eIF4E association with eIF4G.

The association of eIF4G with eIF4E in hepatic tissue is given in Fig. 5. Insulin alone or the combination of the two peptides had no significant effect on eIF4E·eIF4G binding in liver. However, IGF-I alone increased eIF4G associated with eIF4E in liver.

Phosphorylation of p70S6K. p70S6K is resolved into several bands on SDS-polyacrylamide gels. Incubation

Fig. 2. Effect of IGF-I and insulin on phosphorylation of eIF4E-binding protein-1 (4E-BP1) in skeletal muscle (A) and liver (B) in the ovine fetus. Phosphorylation of 4E-BP1 was determined by protein immunoblot after SDS-PAGE from extracts of skeletal muscle and liver of ovine fetuses. Amount of 4E-BP1 in phosphorylated form (γ) is expressed as a percentage of the sum of phosphorylated and nonphosphorylated forms. P values for main effects (IGF and Insulin) and interactions (Int) between main effects are given at top left. Letters a-d indicate significant differences among groups (P < 0.05). Representative immunoblots from Control (lane 1), IGF-I (lane 2), Insulin (lane 3), and IGF/Ins (lane 4) groups are given above the graph.

Fig. 3. Effect of insulin and IGF-I on eIF4G in skeletal muscle (A) and liver (B) in the ovine fetus. P values for main effects (IGF and Insulin) and interactions (Int) between main effects are given at top left. Letters a-d indicate significant differences among groups (P < 0.05). Representative immunoblots from Control (lane 1), IGF-I (lane 2), Insulin (lane 3), and IGF/Ins (lane 4) groups are given above the graph.
of muscle or liver extracts with calf alkaline phosphatase at 37°C for 45 min converted the slow migration species to fast migration species, confirming that increased phosphorylation corresponds to decreased electrophoretic mobility. The results showed that insulin significantly stimulated p70S6 kinase (p70S6K) phosphorylation in ovine fetal skeletal muscle (Fig. 6). However, IGF-I did not exhibit effects on p70S6K phosphorylation. Similar results were found in fetal liver (data not shown).

Phosphorylation of PKB/Akt. Neither IGF-I nor insulin had any effect on total PKB/Akt content in muscle (Fig. 7A). Insulin significantly increased the phosphorylation of PKB/Akt in the muscle (Fig. 7B). The two-way ANOVA P value for the IGF-I effect was 0.16, but there was a significant difference between the control group and the IGF-I group by post hoc analysis. There was also a significant interaction effect between insulin and IGF-I, which suggests that IGF-I may inhibit the insulin effect on PKB/Akt phosphorylation.

DISCUSSION

The roles of insulin and IGF-I in promoting mRNA translation initiation have not been previously explored in the fetus. Our present results demonstrate that, although both hormones increase eIF4F complex formation, only insulin does so by altering phosphorylation of 4E-BP1. Conversely, only IGF-I infusion increases the amount of eIF4G. Thus insulin and IGF-I,
of insulin secretion by IGF-I during the IGF-I infusion. The replacement dose of insulin used in the IGF group was optimal in counteracting the inhibition of insulin secretion during IGF-I infusion. The amino acid clamping procedure was likewise successful at preventing the usual 30–50% decline in amino acid concentrations during insulin or IGF-I infusion (30, 31). There were some fluctuations in leucine concentrations due to individual variations in their response to exogenous amino acid infusion. However, individual data analysis within a group demonstrated minor fluctuations (within 10% of the baseline level) in amino acid concentrations. We do not believe that these fluctuations would cause the alterations in eIF4F formation observed in this study. Rather, the alterations in initiation factors in the study were directly due to the insulin or IGF-I infusion.

Insulin stimulates eIF4F formation with a reciprocal decrease of 4E-BP1 associated with eIF4E. The association of eIF4E with eIF4G is reflective of eIF4F formation. Our results show that insulin infusion increases 4E-BP1 phosphorylation (Fig. 2) and decreases eIF4E-4E-BP1 binding while increasing the eIF4E-eIF4G binding significantly in fetal skeletal muscle (Fig. 4).

Stimulation of 4E-BP1 phosphorylation and eIF4E-eIF4G binding by insulin has been previously demonstrated in vitro and in postnatal animals. Insulin increased 4E-BP1 phosphorylation and decreased association of eIF4E to 4E-BP1 in 3T3-L1 adipocytes in DMEM medium (33). In a hindlimb model, insulin perfusion (1 mM) increased protein synthesis, increased release of eIF4E from the eIF4E-4E-BP1 complex secondary to increased 4E-BP1 phosphorylation, and increased eIF4E-eIF4G binding (26). In diabetic animals, insulin treatment increased 4E-BP1 phosphorylation and released eIF4E from the eIF4E-4E-BP1 complex (25).

Our present results suggest that insulin may regulate protein translation initiation factors in fetal skeletal muscle by the same mechanism as that in vitro or in postnatal life.

Our results also suggest that the turnover of eIF4E phosphorylation is altered after insulin infusion in both skeletal muscle (Fig. 1B) and liver (results not shown). The physiological significance of eIF4E phosphorylation is still controversial (5, 11, 16, 26, 37, 39). When it is considered that translation initiation on the cap structure is a cyclic event, the turnover rate of eIF4E phosphorylation, rather than its static phosphorylation state measured at a single time point, may be most important in regulating the rate of protein synthesis. This principle may well explain the disparity of previous studies of eIF4E phosphorylation.

These insulin-induced alterations in the translation initiation factors are organ specific in the fetus. After insulin infusion, eIF4E-eIF4G binding increased significantly in skeletal muscle (Fig. 4) but not in liver (Fig. 5). There are several possible explanations for this apparent organ-specific function of insulin. First, there could be differences in the abundance and/or distribution of insulin receptors among different tissues. However, insulin receptors have been found in both fetal anabolic hormones, appear to modulate the eukaryotic initiation factors by distinct cellular mechanisms.

Amino acid and glucose concentrations decrease with insulin or IGF-I infusion because of inhibitory effects of the peptides on protein breakdown (6, 13, 21, 38, 48). It has been well documented that amino acids, specifically leucine, and glucose can regulate protein translation initiation (2, 55, 59). To minimize this uncertainty and assess an independent effect of the insulin or IGF-I infusion on protein translation initiation, a replacement dose of insulin was administered to counteract the inhibition...
the fetal liver of humans and many animal species (40). Also, we found that insulin stimulated the phosphorylation of eIF4E and 4E-BP1/p70S6K, the downstream effectors of extracellular signal-regulated kinase-1/2-mitogen-activated protein kinase (MAPK) and PI 3-kinase, respectively, in fetal liver. Presumably, the activation of both cascades is initiated by interaction of insulin with insulin receptors.

Alternatively, uncharacterized component(s) required for eIF4E-eIF4G binding might not be expressed in fetal liver. However, this is also unlikely, as IGF-I increased eIF4E-eIF4G binding in fetal liver in similar experiments (Fig. 5). Increased 4E-BP1 phosphorylation is the best characterized regulatory mechanism for releasing eIF4E. Therefore, our results run counter to the prevailing hypothesis that increases in eIF4E without alteration in the amount of 4E-BP1 bound to eIF4E. However, the 4E-BP1 amount in the liver was only ~0.05% of that in skeletal muscle. Although more than one mechanism may be involved in the regulation of eIF4E-eIF4G binding after insulin treatment, we think that the low expression of 4E-BP1 in the liver may be responsible. Even though insulin stimulated 4E-BP1 phosphorylation in fetal liver, it would be less likely for 4E-BP1 to play an important role in releasing eIF4E in the liver due to the low overall expression level. Furthermore, the fact that hepatic eIF4E-eIF4G binding increased after IGF-I treatment alone, but not when IGF-I was administered with insulin, suggests that IGF-I may be acting by a mechanism which is antagonized by insulin.

Organ-specific regulation of eIF4F formation parallels organ-specific stimulation of protein synthesis by insulin, which has been previously implicated in mice, lambs, and pigs. Intravenous infusion of insulin (4 mIU·kg−1·min−1) increased the protein fractional synthetic rate in the gastrocnemius muscle in mice by 52%; however, there was no significant effect of insulin on the fractional synthetic rate in liver (3). In fetal lambs, when amino acids were infused, hyperinsulinemia enhanced protein synthesis in skeletal muscle, but no effect was observed in liver (21). In 7-day-old piglets, insulin alone was able to stimulate protein synthesis in muscle but not liver (8–10). These findings suggest that the protein synthetic system in skeletal muscle is more sensitive to insulin than that of other tissues. Although the underlying mechanism remains speculative, tissue-specific sensitivity of eIF4F formation to insulin is a distinct possibility.

IGF-I regulates eIF4F formation without altering 4E-BP1 bound to eIF4E. Our results showed that IGF-I increased eIF4G associated with eIF4E despite not altering 4E-BP1 phosphorylation in liver (Figs. 2 and 5). In skeletal muscle, IGF-I increased eIF4G bound to eIF4E without alteration in the amount of 4E-BP1 associated with eIF4E (Fig. 4). Our findings are consistent with those of Vary et al. (56) and Svanberg et al. (53). Using a perfused hind limb model, Vary et al. (56) found that IGF-I increased binding of eIF4G to eIF4E in adult rats but had no effect on eIF4E-4E-BP1 binding. The mechanism by which IGF-I increases eIF4E·eIF4G binding in the fetus is unclear. It has been speculated that both eIF4E availability (51) and eIF4E phosphorylation (5) can regulate eIF4E·eIF4G binding. Our data suggest that increased availability of eIF4E is an unlikely explanation. The eIF4E amount was constant after IGF-I treatment in skeletal muscle and liver (Fig. 1). Although IGF-I increased 4E-BP1 phosphorylation in skeletal muscle (Fig. 2), the response was moderate compared with insulin, and IGF-I-induced alterations in 4E-BP1 phosphorylation did not result in a decrease in eIF4E·4E-BP1 binding in skeletal muscle (Fig. 4). Therefore, it is less likely that the release of eIF4E from the eIF4E·4E-BP1 complex increased after IGF-I infusion.

Our data also suggest that the net amount of phosphorylated eIF4E cannot totally explain our results. Although the overall level of eIF4E phosphorylation increased after IGF-I infusion, eIF4E phosphorylation was extremely variable from one animal to another, whereas eIF4E associated with eIF4G was consistently twofold higher than that in the controls. Furthermore, we could not find a correlation between eIF4E phosphorylation and eIF4E-eIF4G binding. Similar results have been reported in other studies. In rat skeletal muscle, eIF4E-eIF4G binding has been shown to increase without alterations in eIF4E phosphorylation after IGF-I treatment (56). In cell culture, Knauf et al. (27) have demonstrated that eIF4E phosphorylation is not crucial to the formation of the eIF4F complex. However, a poor correlation between the net amount of eIF4E phosphorylation and eIF4F formations at the sampling time does not rule out the possibility of involvement of eIF4E phosphorylation in the eIF4F formation. As we discussed earlier, it is more important to determine the turnover of eIF4E phosphorylation. Unfortunately, we were unable to measure the turnover in the present study.

The finding that the amount of eIF4G bound to eIF4E changes in response to IGF-I infusion in the absence of changes in the amount of 4E-BP1 bound to eIF4E has raised several possibilities regarding the mechanisms of eIF4E-eIF4G binding. First, the eIF4E amount may not be a limiting factor in eIF4E·eIF4G binding. Second, the other two components of the eIF4F complex, eIF4G and eIF4A, could regulate the formation of the eIF4F complex. In the present study, we found that IGF-I increased the total amount of eIF4G with a magnitude similar to that of the increase in eIF4E·eIF4G binding in fetal liver (Figs. 3B and 5). In skeletal muscle, although no significant difference was detected in the total amount of eIF4G, mean eIF4G was 21% higher in the IGF-I group than that in the control group (Fig. 3A). We speculate that the increased eIF4G amount could contribute to increased eIF4E·eIF4G binding in both skeletal muscle and liver after IGF-I infusion. In addition, it has been suggested that alterations in the phosphorylation state of eIF4G might modulate the interaction of eIF4G and eIF4E (44). Third, the other components of 48S preinitiation complex, such as eIF3, the poly(A)-binding protein,
may directly or indirectly modulate eIF4E/eIF4G binding. Finally, although the amount of eIF4E associated with 4E-BP1 did not change, the other two eIF4E-binding proteins, 4E-BP2 and 4E-BP3, might regulate eIF4E availability independently. This is especially possible in liver, where 4E-BP2 is highly expressed (34).

Interaction of insulin and IGF-I in regulation of initiation factors. The overall effect of simultaneous infusion of insulin and IGF seems to be the same as that of insulin alone in regulating eIF4F assembly. Our previous studies suggest that IGF-I does not act simply through the insulin receptor in the ovine fetus (4, 31). We initially hypothesized that insulin and IGF-I act via distinct mechanisms to promote protein accretion in the fetus, possibly resulting in synergistic activity. The present studies show that insulin and IGF-I regulate translation initiation via different mechanisms, therefore supporting the initial hypothesis. However, the effects of the two peptides in regulating the initiation factors seem in many cases to be antagonistic rather than synergistic. It appears that, in the fetus, the insulin effect predominates when both hormones are administered simultaneously. It is possible that there is an overlap interaction between insulin/IGF-I and insulin receptors/IGF type 1 receptors when insulin and IGF-I are present in pharmacological concentrations. It has been speculated that the effects of both insulin and IGF-I are biphasic (4, 13, 20). At low concentrations, insulin specifically binds to insulin receptors and IGF-I binds to IGF type 1 receptors. However, at high concentrations, insulin cross-reacts with IGF type 1 receptors and IGF-I cross-reacts with insulin receptors. We speculate that, in the IGF/Ins group, pharmacological concentrations of insulin may occupy IGF type 1 receptors and block the function of IGF-I.

Insulin, but not IGF-I, activates translation initiation via the PI 3-kinase-p70S6 kinase pathway. Our results show that insulin stimulates the phosphorylation of PKB/Akt, p70S6 kinase, and 4E-BP1 in the skeletal muscle and liver, suggesting that insulin stimulates protein translation initiation via the PI 3-kinase → PKB/Akt → mTOR → p70S6 kinase pathway, which is consistent with the in vitro model (24) and in postnatal life.

However, our findings do not necessarily mean that the rapamycin-sensitive pathway is the only mechanism by which insulin stimulates 4E-BP1 phosphorylation in vivo. It has been implicated in cell culture that MAPK could also play a role in phosphorylating 4E-BP1 at Ser64 (32). Even in the presence of rapamycin, insulin still increased 4E-BP1 phosphorylation three- to fourfold in cells in culture (33).

The signal transduction pathway leading to IGF-I-stimulated initiation factor activation is less clear. Investigations in vitro have shown that, as observed after insulin treatment, IGF-I increases 4E-BP1 phosphorylation and p70S6 kinase phosphorylation in a parallel manner through a rapamycin-sensitive pathway (17). However, our results showed that IGF-I did not stimulate p70S6 kinase phosphorylation. It could be argued that p70S6 kinase phosphorylation was returned to basal levels after a rapid-responsive period in the IGF infusion study. However, we think this is unlikely, because we were able to demonstrate insulin stimulation of p70S6 kinase phosphorylation under the same experimental protocol. In skeletal muscle, IGF-I increased PKB phosphorylation at Ser473 and 4E-BP1 phosphorylation, but the response was moderate compared with insulin stimulation. The two-way ANOVA P value for the IGF-I effect on PKB phosphorylation at Ser473 was 0.16, although there was a significant difference between the control group and the IGF-I group by post hoc analysis. In addition, the moderate IGF-I-induced alterations in 4E-BP1 phosphorylation did not lead to a detectable decrease in eIF4E/4E-BP1 binding. Our results suggest that IGF-I is not a potent stimulus of the PI 3-kinase pathway in the ovine fetus. The reasons for the disparity between those studies in vitro and ours of IGF-I-induced signal pathways in vivo are unknown, but free IGF-I concentration might be one of the important factors. IGF-I added to cell culture medium is generally free IGF-I, whereas that administered into the circulation in our study is rapidly captured by the IGF-binding proteins (50). In vivo, the majority (~99%) of the circulating IGF-I is associated with IGF-binding proteins, which act to block the bioactivity of IGF-I. On the basis of our data, mTOR-insensitive mechanisms may be more important in the regulation of IGF-I-stimulated mRNA translation initiation in vivo.

In summary, these data are the first to show that intravenous infusions of IGF-I and insulin modulate fetal mRNA translation initiation factors. IGF-I enhances eIF4F complex formation in liver and skeletal muscle partly by increasing the amount of eIF4G protein. On the other hand, insulin increases eIF4F complex formation in skeletal muscle by increasing 4E-BP1 phosphorylation and subsequently releasing eIF4E from the eIF4F/4E-BP1 complex. The upstream pathways of the two peptides in stimulating translation initiation are different. Insulin does via the PI 3-kinase pathway, but IGF-I does so mainly via a pathway independent of the PI 3-kinase-p70S6 kinase pathway.

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