Insulin signaling during perinatal liver development in the rat

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Insulin signaling during perinatal liver development in the rat. Am J Physiol Endocrinol Metab 283: E844–E852, 2002. First published June 25, 2002; 10.1152/ajpendo.00111.2002.—Insulin has long been assigned a key role in the regulation of growth and metabolism during fetal life. Our prior observations indicated that hepatic insulin signaling is attenuated in the late-gestation fetal rat. Therefore, we studied the perinatal ontogeny of hepatic insulin signaling extending from phosphatidylinositol 3-kinase (PI3K) to the ribosome. Initial studies demonstrated markedly decreased insulin-mediated activation of ribosomal protein S6 kinase 1 (S6K1) in the fetus. We found a similar pattern in the regulation of Akt, a kinase upstream from S6K1. Insulin produced minimal activation of insulin receptor substrate (IRS)-1-associated PI3K activity in fetal liver. A modest IRS-2-associated response was seen in the fetus. However, levels of both IRS-1 and IRS-2 were very low in fetal liver relative to adult liver. IRS-1 content and insulin responsiveness of PI3K, Akt, and S6K1 showed a transition to the adult phenotype during the first several postnatal weeks. Examination of downstream insulin signaling to the translational apparatus showed marked attenuation, relative to the adult, of fetal hepatic insulin-mediated phosphorylation of 4E-BP1, the regulatory protein for the eukaryotic initiation factor eIF4E, and ribosomal protein S6. The mammalian target of rapamycin (mTOR), a key integrator of nutritional and metabolic regulation of translation, was present in low amounts, was hypophosphorylated, and was not insulin sensitive in the fetus. Our results indicate that protein synthesis during late-gestation liver development may be mTOR and insulin independent. Reexamination of the role of insulin in fetal liver physiology may be warranted.

hepatocyte; fetus; signal transduction; ribosome; translation

THE ROLE OF INSULIN IN FETAL DEVELOPMENT has to a large degree been deduced from clinical and experimental observations on conditions associated with fetal hypo- or hyperinsulinemia. In general, fetal insulin production is directly related to fetal size (19). The hyperinsulinemic human infant of a diabetic mother has macrosomia and selective organomegaly (13). As demonstrated in a primate model, the well characterized fetopathy associated with diabetes in pregnancy can be reproduced by the direct administration of insulin to the fetus (42). Similarly, in situ insulin administration to late-gestation fetal rats has been shown by a number of investigators to augment fetal growth (12, 37). Conversely, interruption of normal insulin production (16, 21) or signaling (30) results in intrauterine growth retardation.

The role of insulin in hepatic growth and metabolism during the postnatal period is well established. Insulin contributes to the recovery of liver mass that occurs after partial hepatectomy in the rat (33). Recently characterized mechanisms for insulin-mediated transcriptional regulation support a direct role for insulin in the control of hepatic glucose production (47). A mouse model in which hepatic insulin signaling is abolished through tissue-specific, targeted deletion of hepatocyte insulin receptors (32) provides perhaps the most compelling evidence for the direct hepatic effects of insulin. Overexpression of the primary hepatocyte insulin receptor substrate (IRS)-1 in hepatocellular carcinoma, as observed by Tanaka et al. (43), may reflect a role for the insulin-signaling network in the process of hepatic carcinogenesis. These investigators pursued this observation by developing a mouse model in which IRS-1 is selectively overexpressed in hepatocytes (43). These animals show augmented liver growth beyond the neonatal period.

With regard to liver growth in the fetus, the role of insulin is less clear. Although exogenous hyperinsulinemia in the primate fetus results in increased liver mass, this increase is proportional to the generalized fetal macrosomia (42). This is in contrast to myocardium, adipose tissue, and skeletal muscle, all of which show disproportionate growth augmentation in this model. Similarly, intrauterine growth retardation associated with fetal hypoinsulinemia is associated with minimal effects on liver mass despite a generalized effect on somatic growth (21).

The signaling mechanisms that mediate insulin’s effects on cell metabolism and growth have been elucidated to a significant degree over the last two decades. Upon insulin binding, the insulin receptor tyrosine kinase undergoes self-phosphorylation, which activates its ability to catalyze the phosphorylation of...
other cellular proteins. Key among these are members of the IRS family (46). These docking proteins interact with signaling molecules through SH2 homology domains, resulting in the activation of a diverse series of signaling pathways. These include the pathway involving phosphatidylinositol 3-kinase (PI3K). The pivotal role of PI3K in insulin-mediated metabolic regulation is perhaps best established in 3T3-L1 adipocytes. In these cells, inhibitors of PI3K, as well as transfection with dominant negative constructs of the enzyme, block most of the metabolic actions of insulin, including stimulation of glucose transport, glycogen, and lipid synthesis (41).

Catalytic activation of PI3K is promoted by the binding of the 85-kDa PI3K regulatory subunit to IRS proteins (34). The phosphoinositides produced by PI3K activate the phosphoinositide-dependent kinases 1 and 2. These kinases phosphorylate and activate the serine/threonine kinase Akt (1). The mammalian target of rapamycin (mTOR), a 290-kDa serine/threonine kinase and amino acid sensor, is a direct target for Akt (2, 35, 40). mTOR can phosphorylate and activate the prototypical p70 S6 kinase, which along with an alternative product from the same gene, p85 S6 kinase, is termed S6K1. mTOR-mediated activation of S6K1 is blocked by the mTOR inhibitor, rapamycin (39). S6K1 is involved in the phosphorylation of ribosomal protein S6, a constituent of the 40S ribosomal subunit. S6 is believed to mediate the translation of a subgroup of mRNAs with a 5'-oligopyrimidine tract at their transcriptional start sites (15). These mRNAs may number as few as 100–200, but they can account for 20–30% of total cellular mRNA. They encode many of the components of the translational apparatus, including ribosomal proteins and elongation factors that are necessary for cell cycle progression.

mTOR is also known to regulate mRNA translation by activation of the translation initiation factor eIF4E (26). This is mediated through the phosphorylation of an eIF4E inhibitory binding protein, 4E-BP1. Phosphorylation of 4E-BP1 results in the dissociation of the eIF4E-4E-BP1 complex, which in turn permits eIF4E to interact with the other translation initiation proteins, including eIF4G, eIF4A, and eIF3. This multi-protein complex forms at the 5' cap of mammalian mRNAs and promotes the initiation of translation (26).

Late gestation is a time of rapid growth in the rat. During the last 3 days of gestation, fetal body weight triples, with a proportionate increase in liver weight (31). Our prior studies on mitogenic signaling during liver development in the rat have suggested that the well characterized pathways that mediate growth factor-induced mitogenesis in adult rat hepatocytes are not operative in the late-gestation fetus (3, 8, 28). The present studies examine the ontogeny of the hepatic insulin-signaling pathway involving PI3K, Akt, mTOR, and downstream signaling to the ribosome.

**METHODS**

**Materials.** Insulin (pork) was obtained from Elanco (Indianapolis, IN). S6 kinase substrate peptide (RRRLSSRLA) and Akt substrate peptide (RPRAATF) were purchased from Upstate Biotechnology (Lake Placid, NY), as were antibodies directed toward Akt, the carboxy terminus of rat IRS-1, IRS-2, phosphotyrosine (4G10), and mTOR. Antibodies to S6K1, 4E-BP1, and actin, as well as protein kinase A inhibitor and protein kinase C inhibitor, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to ribosomal protein S6, phospho-S6 (Ser235/Ser236), eIF4E, and phospho-mTOR (Ser2448) were obtained from Cell Signaling Technology (Beverly, MA). Protein A-Sepharose CL-4B, protein G-Sepharose 4FF, and 7-methyl (7m) GTP-Sepharose 4B were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). [γ-32P]ATP (3,000 Ci/mmol) was obtained from Perkin-Elmer Life Sciences (Boston, MA).

**Animals.** Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were used for all studies. Fetal and newborn rats were derived from dams of known gestational age (term being 21 days), which were allowed to deliver spontaneously and remain with their offspring. Adult animals were males weighing between 150 and 175 g. All animals were fed standard laboratory chow ad libitum. Gestation was confirmed at the time of mating and newborns under 3 days of age, the entire hindlimb was used for the aforementioned protease inhibitors. After homogenization, Triton X-100 was added to a final concentration of 1%, and the samples were incubated on ice for 30 min. The detergent-extracted homogenates were centrifuged at 1,000 × g for 15 min. The resulting supernatant was centrifuged at 40,000 × g for 20 min. The final supernatant was stored at −70°C pending analyses.

**Preparation of homogenates and cell extracts.** Except where noted, liver homogenates were prepared using 10 volumes of “S6K lysis buffer” (50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM sodium pyrophosphate, 100 mM NaF, 1.5 mM MgCl2, 1 mM EGTA, 200 μM NaVO3, 1 μM microcystin, and 10% glycerol) containing protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotonin, and 34.4 μg/ml 4-[2-a minoethy1]benzenesulfonyl fluoride). After homogenization, Triton X-100 was added to a final concentration of 1%, and the samples were incubated on ice for 30 min. The detergent-extracted homogenates were centrifuged at 1,000 × g for 15 min. The resulting supernatant was centrifuged at 40,000 × g for 20 min. The final supernatant was stored at −70°C pending analyses.

**Samples for PI3K assay were prepared using 10 volumes of a homogenization buffer containing (in mM) 50 HEPES, pH 7.5, 137 NaCl, 1 MgCl2, 1 CaCl2, 2 NaNbO4, 10 sodium pyrophosphate, 10 NaF, and 2 EDTA, plus 10% glycerol and the aforementioned protease inhibitors.** After homogenization, NP-40 was added to a final concentration of 1%, and the samples were incubated on ice for 30 min. The detergent-extracted homogenates were centrifuged at 1,000 × g for 15 min. The resulting supernatant was centrifuged at 40,000 × g for 20 min. The final supernatant was stored at −70°C pending analyses.

**Preparation of homogenates and cell extracts.** Except where noted, liver homogenates were prepared using 10 volumes of “S6K lysis buffer” (50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM sodium pyrophosphate, 100 mM NaF, 1.5 mM MgCl2, 1 mM EGTA, 200 μM NaVO3, 1 μM microcystin, and 10% glycerol) containing protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotonin, and 34.4 μg/ml 4-[2-aminoethy1]benzenesulfonyl fluoride). After homogenization, Triton X-100 was added to a final concentration of 1%, and the samples were incubated on ice for 30 min. The detergent-extracted homogenates were centrifuged at 1,000 × g for 15 min. The resulting supernatant was centrifuged at 40,000 × g for 20 min. The final supernatant was stored at −70°C pending analyses.

Skeletal muscle was processed using the same methods as for the preparation of liver homogenates. Protein determinations on all samples were made using the bicinchoninic acid method (Pierce), with bovine serum albumin as the standard.

**Immune complex kinase assays.** S6K1 and Akt activities were determined by measuring kinase activity after immunoprecipitation. For both assays, antibodies were cross-
linked to protein A-Sepharose CL-4B by use of dimethyl pimelimidate (7). After immunoprecipitation, kinase assays were performed as described previously for the S6K1 assay (6). The Akt assay differed only in the kinase substrate that was used.

Western immunoblotting. Where noted, Western immunoblotting was preceded by immunoprecipitation, performed as described in the previous paragraph. For these analyses, immunoprecipitated protein was eluted from the protein A-Sepharose beads with 60 μl of Laemmli sample buffer, separated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinyldene difluoride membranes, and subjected to immunoblotting with antibodies directed toward S6K1 or Akt. Direct immunoblotting for ribosomal protein S6 and phospho-S6 was performed as described previously (6). Immunoblotting for mTOR and phosphorylated mTOR employed the same procedure. For all Western immunoblotting, detection employed an enhanced chemiluminescence method (Amersham Pharmacia Biotech). Densitometric quantification of immunoblots was performed as described previously (3).

IRS-1, IRS-2, and PI3K analyses. For these studies, immunoprecipitations were performed without antibody cross-linking. Homogenate protein (1 mg) was combined with 2 μg of IRS-1 or IRS-2 antibody. After overnight incubation at 4°C, immunocomplexes were recovered using protein A-Sepharose beads. The immunoprecipitates were analyzed by Western immunoblotting, with the aforementioned antibodies directed toward IRS-1 or IRS-2 and phosphotyrosine.

PI3K activity was measured in IRS-1 and IRS-2 immunoprecipitates. Immune complexes were washed twice with wash buffer 1 (100 mM Tris-HCl, pH 7.5, and 500 mM LiCl), one time with wash buffer 2 (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 1 mM EDTA), and one time with reaction buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 mM EGTA, 10 mM MgCl₂, and 300 μM adenosine). Sepharose-bound immune complexes were then incubated for 10 min at 30°C with 50 μl of reaction buffer containing 5 μg of phosphatidylinositol, 5 μg of phosphatidylinerine, and 10 μM [γ-32P]ATP (8 Ci/mmol). Reactions were quenched with HCl and extracted with methanol-chloroform (1:1). Labeled phospholipids were spotted onto AL SIL G plates (Whatman; Maidstone, Kent, UK) to separate phosphatidylinositol monophosphates.

eIF4E-4E-BP1 analyses. The association of 4E-BP1 with eIF4E was evaluated by determining the ability of 4E-BP1 to bind to 7m GTP-Sepharose beads through its interaction with eIF4E. Forty microliters of 7m GTP-Sepharose beads were washed once with freshly prepared binding assay buffer (50 mM MOPS, pH 7.2, 0.5 mM EDTA, 0.5 mM EGTA, 100 mM KCl, 1 mM dithiothreitol, 50 mM NaF, 80 mM β-glycerophosphate, 100 μM GTP, and 1 μM microcystin) containing protease inhibitors (same as for S6K lysis buffer). Liver homogenate containing 0.8 mg total protein was added to the washed beads, and the volume was brought up to 500 μl with binding assay buffer. This mixture was incubated overnight at 4°C on a rotator, after which the beads were washed three times with binding assay buffer containing protease inhibitors. Forty microliters of Laemmli sample buffer were added to the washed beads, and the samples were boiled for 10 min. These samples were analyzed by sequential Western immunoblotting with antibodies directed toward 4E-BP1 and eIF4E.

Statistical analyses. Multiple comparisons between fetal and adult control and insulin-treated samples were performed using one-way analysis of variance with a Tukey post hoc test.

RESULTS

The perinatal ontogeny of hepatic S6K1 activity. Our initial experiments were aimed at measuring the level of activity of S6K1, a critical downstream component of the PI3K-mTOR pathway. We also measured the insulin responsiveness of this kinase in fetal and postnatal rat livers. Results (Fig. 1) showed that basal levels of S6K1 activity were very low in the late-gestation fetus, a developmental stage at which there is a high rate of hepatocyte proliferation in vivo (20) and high circulating levels of insulin (5, 17). Insulin administration produced minimal S6K1 activation in late-gestation fetuses. In fact, data combined from multiple experiments revealed no significant effect of insulin on S6K1 activity in E19 fetuses. Postnatal animals showed maximal basal and insulin-stimulated activity in adult animals. The transition from fetal to adult levels was gradual, occurring during the first two postnatal weeks. It should be pointed out that all animals were studied in a fed state so as to allow a comparison with the fetal animals. Additional studies (not shown) indicate that a period of 24 h of food withdrawal lowers basal S6K1 activity without decreasing insulin-stimulated activity.

As we have noted, the term S6K1 refers to two isoforms derived from the same gene, p70 S6K and p85 S6K. The latter possesses a nuclear localization signal that accounts for its activity as a nuclear S6 kinase (18). Because the p70 and p85 S6Ks share most of their primary structure, the S6K1 antibodies we used identify both isoforms. Western immunoblotting for S6K1 content (Fig. 1) showed consistent levels of immuno-

![Fig. 1. Ontogeny of hepatic ribosomal protein S6 kinase 1 (S6K1).](http://ajpendo.physiology.org/Downloadedfrom)
active p70 S6K1 in fetal and postnatal samples. Longer exposures of S6K1 Western blots (Fig. 1) showed low levels of p85 S6K1. These data were interpreted as indicating that our activity measurements reflect a p70 S6K1 predominance, and that differences in S6K1 activity were a function of the activation state of the kinase and not its abundance. The results shown in Fig. 1 are representative of those obtained in multiple experiments.

The perinatal ontogeny of hepatic Akt activity. The low activity of S6K1 and data indicating the presence of functional insulin receptors led us to examine intermediate steps in this cascade. Analysis of basal and insulin-stimulated Akt activity in fetal and postnatal samples (Fig. 2) showed a pattern similar to that seen for S6K1. Basal Akt activity was low in fetal compared with postnatal liver, and insulin produced marginally higher levels of Akt activity in fetal animals. Combined data from three experiments revealed no significant effect of insulin injection on Akt activity in E19 fetuses, whereas adult animals showed a significant approximately eightfold activation. As was the case for S6K1, a transition from fetal to adult activity levels occurred during the first two postnatal weeks, consistent with coordinated regulation of both kinases. Western immunoblotting for Akt (Fig. 2) showed similar amounts of Akt in all samples, indicating that activation state rather than the amount of Akt was responsible for the changing activity. The results shown in Fig. 2 are representative of data obtained in several experiments.

IRS-1 and IRS-2 abundance and activity during liver development. Given the results shown in Figs. 1 and 2, we moved on to study more proximal insulin-mediated signaling events. We examined the abundance and tyrosine phosphorylation of immunoprecipitated IRS-1 and IRS-2 (Fig. 3). Levels of total and tyrosine-phosphorylated IRS-1 and IRS-2 were extremely low in E19 and E21 fetal samples. Levels of both total and tyrosine-phosphorylated IRS-1 and IRS-2 rose by postnatal day 7.

To pursue the functional significance of these findings, we measured PI3K activity in IRS-1 and IRS-2 immunoprecipitates. Results (Fig. 4) showed low basal and insulin-stimulated levels of IRS-1-associated PI3K.
activity in fetal and neonatal animals. A marked increase was seen between postnatal days 3 and 14. In contrast, basal IRS-2-associated PI3K activity was consistent at all developmental ages studied. However, insulin-mediated activation was greatest in E19 and E21 animals.

To confirm the potency of the injected insulin, skeletal muscle homogenates were processed from selected animals (including those used for the experiment shown in Fig. 4). IRS-1-associated PI3K activity was stimulated ≥10-fold above control levels in all animals receiving insulin, regardless of the stage of development (data not shown). We therefore interpreted our results to indicate that hepatic insulin signaling through IRS-1 is markedly attenuated in the developing fetus. The ability of insulin to activate IRS-2-associated PI3K from E19 to P7 may be an indication that hepatic insulin signaling through IRS-1 is markedly attenuated in the developing fetus. The ability of insulin to activate IRS-2-associated PI3K from E19 to P7 may be an indication that hepatic insulin signaling through IRS-1 is markedly attenuated in the developing fetus.

Hepatic ribosomal protein S6 phosphorylation in fetal and adult rat liver. Our prior studies showed that fetal hepatocyte proliferation in vivo is not dependent on hyperphosphorylation of ribosomal protein S6 (6). However, we did find that hepatic S6 is hyperphosphorylated in the late-gestation fetus under basal conditions. We therefore considered the S6 phosphorylation state to be an indicator of hepatic insulin responsiveness. Such responsiveness is unlikely to be mediated by S6K1 (see Fig. 1). However, it is possible that insulin could promote S6 phosphorylation via activation of an alternative kinase or through changes in protein phosphatase activity. Direct examination of S6 phosphorylation using phosphospecific antibodies (Fig. 5) confirmed that S6 in late-gestation fetal liver is hyperphosphorylated under basal conditions. Insulin administration to E19 fetal rats did not cause an increase in S6 phosphorylation. In contrast, the ratio of phosphorylated to total S6 in adult animals was low under basal conditions and markedly increased in response to insulin (Fig. 5).

An incidental finding in these experiments deserves comment. We consistently observed marked variability in hepatic S6 content. Although equal amounts of protein were loaded onto each lane, S6 content varied as much as fivefold between samples. Such variability was not seen when the Western blots were stripped and reprobed for β-actin (not shown). Our ability to obtain a consistent yield of S6 was confirmed by triplicate analysis of a single sample. We concluded that there exists considerable biological variability in the hepatic content of S6 in the late-gestation fetus. Because S6 is a stoichiometric constituent of the ribosome, variability in S6 content implies either marked variability of fetal liver ribosomal content (something we have not observed in ribosomal preparations) or variability in a nonribosomal pool of S6. Although the marked variation in fetal hepatic S6 content is unexplained, it does not invalidate our observation that S6 phosphorylation is insulin sensitive after birth but not in the fetus.

Regulation of 4E-BP1 phosphorylation. Translation of most RNA species is regulated at the initiation phase when a ribosome is recruited to the mRNA 5’ end. As discussed below, eIF4E is a key component of this process, whose function is regulated by the phosphorylation state of a family of eIF4E-binding proteins (4E-BPs). We examined the effect of insulin on 4E-BP1 phosphorylation and release from eIF4E in fetal and adult liver through the use of 7m GTP-Sepharose beads. These beads mimic the cap structure of mRNAs and therefore bind eIF4E. When 4E-BP1 is hypophosphorylated, it associates with the 7m GTP beads by binding eIF4E. This behavior of eIF4E and 4E-BP1 was used to assess the activation state of hepatic eIF4E and the role of insulin in its regulation (Fig. 6). Samples from both saline-injected control and insulin-injected E19 fetal rats showed consistent eIF4E-associated 4E-BP1. The level of eIF4E-4E-BP1 association in fetal liver was lower than that seen under fed conditions in adult liver, consistent with a higher basal level of translational activation. In contrast to findings in the fetal animals, liver homogenates from insulin-injected adult animals showed a marked decrease in eIF4E-associated 4E-BP1 relative to control animals. As expected, 4E-BP1 migrated as a doublet at 18 and 20 kDa. Fetal samples showed additional lower molecular weight species in the 4E-BP1 immunoblot that were not detected in the adult samples. Their identity is unknown. The results of these experiments on 4E-BP1 regulation indicate that, as was the case for S6K1, Akt, and S6 phosphorylation, hepatic 4E-BP1 activity is not regulated by insulin in the late-gestation fetal rat.
Insulin-mediated regulation of mTOR. Given the central role of mTOR in insulin signaling to the ribosome, we investigated the effect of insulin on hepatic mTOR activation in fetal vs. adult rats. This was accomplished using phosphospecific antibodies that react with a site that is required for mTOR activity. As was the case for S6 and 4E-BP1, insulin induced the phosphorylation of mTOR in adult rats but not in fetal rats (Fig. 7). Our results also showed a lower level of both phosphorylated (activated) and total mTOR in fetal vs. adult rats. This was accomplished using phosphospecific antibodies that react with a site that is required for mTOR activity. As was the case for S6 and 4E-BP1, insulin induced the phosphorylation of mTOR in adult rats but not in fetal rats (Fig. 7). Our results also showed a lower level of both phosphorylated (activated) and total mTOR in fetal liver relative to adult liver.

DISCUSSION

Given the clinical data showing a correlation between insulinemia and somatic growth in mammalian fetuses, it is generally accepted that insulin is a key regulator of fetal growth. Studies on liver regeneration after partial hepatectomy established the ability of the liver to regulate its mass relative to body size (33). Thus it is possible that changes in liver size seen in situations in which fetal growth is perturbed in concert with altered insulinemia may represent a secondary effect on hepatic growth rather than primary regulation by insulin. This conclusion is supported by results of the present studies.

Such a conclusion would suggest a major difference in the growth regulation between fetal and adult hepatocytes in vivo. Our prior studies have demonstrated other marked differences in the regulation of hepatoctye growth when the late-gestation fetal rat was compared with the adult. These include markedly attenuated signaling through the extracellular signal-regulated kinase (ERK) pathway in fetal liver and regulation of hepatic c-myc expression at the level of RNA stability in the fetus but not the adult (28). Most recently, we found that hepatocyte proliferation in the late-gestation fetal rat is resistant to the mTOR inhibitor rapamycin, whereas the proliferation of adult hepatocytes in vivo shows high sensitivity to its effects (6). This was an unexpected finding in the face of results indicating that intact S6 function and activation are required for cell cycle progression after partial hepatectomy in the adult rat (44). This extraordinary difference between fetal and adult hepatocytes in the intact rat led us to the present studies, which were undertaken to characterize differences in the signaling pathway leading from insulin to the ribosome.

Like other receptor tyrosine kinases, the insulin receptor can mediate the activation of a broad repertoire of signaling cascades. Primary among those that stimulate cell proliferation is the MAPK cascade involving the extracellular signal-regulated kinases ERK1 and ERK2. Blocking ERK activation with dominant negative mutants or pharmacological inhibitors prevents the stimulation of cell growth by insulin but has no effect on the metabolic actions of the hormone (27). However, our own studies indicate that insulin is not a potent activator of this pathway in rodent hepatocytes.
(9) and that the pathway is uncoupled at a distal step in fetal liver (8). Therefore, we decided to focus on fetal hepatic insulin signaling downstream from PI3K.

We first confirmed our prior observation (6) that S6K1 activation by insulin is attenuated in fetal liver. A transition to adult-type insulin responsiveness was found to occur during the first two postnatal weeks. This same pattern was reiterated in the ontogeny of IRS-1 content, IRS-1-associated PI3K activity, and Akt activation. This transition period coincides with that during which other changes in liver signal transduction occur, including the declining expression of a number of genes that are overexpressed in fetal liver (22, 28). It also coincides with the developmental period, during which coupling of ERK signaling occurs (8). Although the ERK pathway may contribute to signaling through S6Ks, the contribution of ERK is not uniform (15). In some systems, ERK activation is neither required nor sufficient for S6 phosphorylation. Nonetheless, attenuated ERK activation could contribute to attenuated S6K activity in fetal liver at the end of gestation. Thus it appears that hepatocytes undergo a transition from a fetal to an adult mitogenic signaling phenotype during the 2 wk after birth, and that the low level of S6K activity in fetal liver can be attributed to attenuated signaling at the level of both Akt and mTOR. This is particularly surprising given that activation of both Akt and mTOR is generally associated with cell growth and proliferation.

Akt has an established role in the regulation of diverse cellular processes, including glucose metabolism, proliferation, apoptosis, transcription, and cell migration (10). Its downstream targets include mTOR, the S6Ks, and glycogen synthase kinase (GSK)-3 (10). mTOR, through its effects on S6 kinase, is considered crucial for ribosomal biogenesis (39), a process that is required for hepatocyte proliferation. Akt-mediated phosphorylation of GSK-3 promotes glycogen synthesis. Our analyses demonstrated nearly equivalent ratios of phosphorylated (active) mTOR to total mTOR in late-gestation fetal liver and adult liver. Thus mTOR activation state and hepatocyte proliferation are dissociated during normal liver development. In addition, insulin did not stimulate phosphorylation of hepatic mTOR in the fetus, a response that was readily demonstrated in the intact adult rat. Given these known actions of Akt and mTOR, our results bring into question the role of insulin as an anabolic factor for the developing liver.

In yeast, two mTOR proteins with significant homology, TOR1 and TOR2, have been identified (23). Mammalian cells are known to contain a single, well-characterized form of mTOR. The existence of an alternative mTOR is a possible but unprecedented explanation for our findings. Another potential mechanism for hepatocyte proliferation in the face of low mTOR activity is a lower level of activity of phosphatases that act on mTOR substrates. Finally, alternative growth regulators expressed in fetal hepatocytes may bypass the need for mTOR-mediated signaling.

Our studies to date do not allow us to distinguish among these possibilities.

We found that hepatocyte proliferation during late gestation is associated with basal activation of eIF4E, as evidenced by a low level of 4E-BP1 binding. The mechanism of cap-dependent initiation of translation involving eIF4E is particularly important for mRNAs that have highly structured 5′ untranslated regions, such as c-myc and cyclin D1. Activation of PI3K or Akt results in 4E-BP1 hyperphosphorylation (45). This hyperphosphorylation is reversed by rapamycin, presumably through the inhibition of mTOR (4, 29). We found that insulin promotes dissociation of 4E-BP1 from eIF4E in adult liver but not in fetal liver, indicating that 4E-BP1 regulation may be independent of signaling mechanisms that are independent of signaling through mTOR. This conclusion is consistent with the previous observation by Jiang et al. (25) that the regulation of 4E-BP1 during liver regeneration is rapamycin resistant.

In determining the mechanism for attenuated insulin signaling in the fetus, we made another unexpected observation. Fetal hepatocytes have very low levels of the primary hepatic insulin receptor substrates IRS-1 and IRS-2. However, the low level of IRS-1 and -2 in fetal liver does not explain the attenuation of downstream signaling, since we observed insulin-stimulatable IRS-associated PI3K activity. One hypothetical mechanism for uncoupling of mTOR from PI3K might involve the activity of a phosphatase located between PI3K and Akt activation. The enzyme PTEN serves such a function (11). It is known that cellular oxidative stress can block the activity of PTEN (38). We speculate that PTEN might be highly active in fetal liver, owing to the reductive environment that is associated with the relative hypoxia of the in utero environment. At the time of birth, increasing atmospheric oxygen and cardiovascular changes are associated with an abrupt and marked increase in tissue oxygenation (14). This is associated with cellular oxidative stress (36), which could inactivate PTEN, thus “coupling” the PI3K-mTOR pathway after birth.

Whatever the mechanisms for attenuated signaling through Akt, mTOR, and S6Ks in late-gestation fetal liver, our findings lead to several conclusions. The first is that the mechanisms regulating hepatocyte protein synthesis during late gestation are independent of pathways involving mTOR. This may account for the rapamycin resistance of fetal hepatocyte proliferation, a characteristic that is shared with numerous cancer cell types (24). Thus elucidation of the mechanisms that support protein synthesis during liver development may provide insight into mechanisms of carcinogenesis. Second, it appears that insulin is not a regulator of hepatic protein synthesis in the late-gestation fetal rat. Our results suggest the presence of pathways independent of insulin signaling that can promote anabolism during a phase of rapid liver growth. Thus a reexamination of the role of insulin in fetal liver physiology may be warranted.
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REFERENCES


