Regulation of Hepatic Insulin-Like Growth Factor-Binding Protein-1 Gene Expression by Insulin: Central Role for Mammalian Target of Rapamycin Independent of Forkhead Box O Proteins

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The expression of IGF-binding protein-1 (IGFBP-1) is induced in rat liver by dexamethasone and glucagon and is completely inhibited by 100 nM insulin. Various studies have implicated phosphatidylinositol 3-kinase, protein kinase B (Akt), phosphorylation of the transcription factors forkhead in rhabdomyosarcoma 1 (Foxo1)/Foxo3, and the mammalian target of rapamycin (mTOR) in insulin’s effect. In this study, we examined insulin regulation of IGFBP-1 in both subconfluent and confluent hepatocytes. In subconfluent hepatocytes, insulin inhibition of IGFBP-1 mRNA levels was blocked by inhibiting PI3 kinase activation, and there was a corresponding inhibition of Foxo1/Foxo3 phosphorylation. In these same cells, inhibition of the insulin effect by rapamycin occurred in the presence of insulin-induced Foxo1/Foxo3 phosphorylation. In confluent hepatocytes, insulin could not activate the phosphatidylinositol 3-kinase (PI3 kinase)-Akt-Foxo1/Foxo3 pathway, but still inhibited IGFBP-1 gene expression in an mTOR-dependent manner. In subconfluent hepatocytes, the serine/threonine phosphatase inhibitor okadaic acid (100 nM) partially inhibited IGFBP-1 gene expression by 40%, but did not produce phosphorylation of either Akt or Foxo proteins. In contrast, 1 nM insulin inhibited the IGFBP-1 mRNA level by 40% and correspondingly activated Akt and Foxo1/Foxo3 phosphorylation to a level comparable to that observed with 100 nM insulin. These results suggest a potential role for a serine/threonine phosphatase(s) in the regulation of IGFBP-1 gene transcription, which is not downstream of mTOR and is independent of Akt. In conclusion, we have found that in rat liver, insulin inhibition of IGFBP-1 mRNA levels can occur in the absence of the phosphorylation of Foxo1/Foxo3, whereas activation of the mTOR pathway is both necessary and sufficient. (Endocrinology 147: 2383–2391, 2006)

The binding of insulin to the insulin receptor (IR) tyrosine kinase is followed by IR tyrosine kinase autophosphorylation, activation, and the phosphorylation of specific substrates (viz. IRS1 and -2) on tyrosine residues (1). The phosphotyrosine motifs in the IRSs bind adaptor proteins, such as the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3 kinase). The consequent activation of PI3 kinase results in the phosphorylation and activation of downstream protein kinases, especially protein kinase B/Akt (2). In turn, Akt phosphorylates and alters the behavior of various substrates (3).

The mammalian target of rapamycin (mTOR), a large protein (289 kDa) with a catalytic domain homologous to that of PI3 kinase (4), has been implicated in insulin-induced protein synthesis (5) and mitogenesis (6). Its activation has been attributed to the activation of PI3 kinase (7–9). The serine/threonine (Ser/Thr) phosphatase 2A (PP2A) regulates various processes, including aspects of insulin signaling (10–13). The effects of insulin can be mediated by the inhibition of PP2A and structurally related enzymes, leading to increased protein phosphorylation (11–13). The mTOR pathway (14) is regulated by several studies have implicated PP2A in the control of mTOR pathway (14). Thus, Peterson and co-workers (15) provided evidence that mTOR phosphorylates and inactivates PP2A, preventing it from dephosphorylating and inactivating downstream effectors, such as 4E-BP1 and S6 kinase. In addition, evidence has been provided (16) that Akt is a direct target of PP2A, preventing Akt from activating downstream effectors, such as mTOR.

Insulin regulates the expression of numerous genes (17), including IGF-binding protein-1 (IGFBP-1), which is induced by dexamethasone and glucagon and inhibited by insulin (18) in a PI3 kinase-dependent manner (19). Two potential pathways, downstream of PI3 kinase, have been proposed to regulate IGFBP-1 mRNA levels. One involves activation of Akt (20), which directly affects the transcription factors forkhead in rhabdomyosarcoma 1 (Foxo1) (21), Foxa2 (22), and Foxo3 (3), each of which has been shown to mediate the transcription of insulin-responsive genes. In particular, Akt-dependent phosphorylation of Foxo1 and Foxo3 leads to their nuclear exclusion (3, 23, 24) and the inhibition of their binding to the insulin response element in the promoter of the IGFBP-1 gene (3, 21, 25, 26), thus negating its contribution...
to the induction of IGFBP1. Alternatively, mTOR, activated downstream of PI3 kinase in rat hepatocytes (19) and H4IE hepatoma cells (27), may affect IGFBP-1 mRNA levels via a mechanism independent of the modulation of Foxo1 (27). Furthermore, it has been observed in H4IE hepatoma cells that the binding of Foxo3 to the PEPCK and IGFBP-1 insulin response elements did not correlate with the effect of insulin on gene transcription (26).

In previous work we compared insulin and epidermal growth factor action in confluent (C) and subconfluent (SC) cultured hepatocytes (6). In this study we extended this comparison to the mechanism by which insulin regulates IGFBP-1 mRNA downstream of PI3 kinase. The work demonstrates that in confluent hepatocytes, insulin suppresses the induction of IGFBP-1 mRNA by activating mTOR independently of the activation of PI3 kinase and Akt and the phosphorylation of Foxo1/Foxo3. We provide evidence for a possible role of a Ser/Thr phosphatase(s) in this process, which does not appear to operate downstream of mTOR and seems to be independent of Akt.

Materials and Methods

Porcine insulin was a gift from Eli Lilly Research Laboratories (Indianapolis, IN). Glucagon, desamethasone, wortmannin, okadaic acid (OA), and calf intestinal alkaline phosphatase were purchased from Sigma-Aldrich Corp. (St. Louis, MO); rapamycin and Ly290042 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Akt, antiphospho-Akt (Ser473), Foxo1, and antiphospho-Foxo1 (Thr24)/Foxo3 antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). The PI3 kinase p85 and PP2A antibodies as well as the Ser/Thr phosphatase assay kit 1 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Protein A-Sepharose was obtained from Amersham Biosciences (Baie d’Urfe, Quebec, Canada). Collagenase was obtained from Worthington Biochemical Corp. (Lake-wood, NJ). Cell culture medium and antibiotics were purchased from Invitrogen Life Technologies Canada, Inc. (Burlington, Canada), and Vitrogen-100 was obtained from Collagen Biomaterials (Toronto, Canada). [*-32P]ATP was purchased from PerkinElmer (Wellesley, MA). [α-32P]Deoxy-CTP and [*32P]Irritantib IgG were provided by ICN Biomedicals Canada Ltd., Mississauga, Canada. Most other reagents and chemicals were obtained from Sigma-Aldrich Corp. and were of the highest grade available.

Cell culture and hormone treatments

Primary hepatocytes, isolated from 120- to 140-g male Sprague Dawley rats (Charles River Laboratories, St. Constant, Canada) by in situ liver perfusion with collagenase (protocol 4110, approved by McGill University), were plated on a collagen matrix (Vitrogen-100; Collagen Corp., Toronto, Ontario, Canada). SC and C cultures were prepared by seeding 1 × 10⁸ and 3 × 10⁸ cells onto 9.6-cm² six-well plates, respectively (Corning, Costar, Cambridge, MA) or 5 × 10⁸ and 1.5 × 10⁸ cells, respectively, onto 78-cm² culture dishes (Starstedt Canada, St. Laurent, Quebec, Canada). Cells were bathed for 24 h in seeding medium (DMEM/Ham’s F-12 containing 10% fetal bovine serum, 10 mM HEPES, 20 mM NaHCO₃, 500 IU/ml penicillin, and 500 µg/ml streptomycin) and then for 48 h in serum-free medium that differed from the seeding medium in that it lacked fetal bovine serum and contained 1.25 µg/ml fungizone, 0.4 mM ornithine, 2.25 µg/ml l-tartrate acid, 2.5 ± 0.8 mM selenium, and 1 ± 0.4 mM methionine. Hormone and drug treatments, carried out in serum-free medium, were initiated 72 h after plating for the times and at the concentrations indicated in the figure legends. Cells overexpressing dominant-negative p85 were infected with stocks of recombinant (AdΔp85) adenovirus containing a DNA encoding the p85 regulatory subunit, whose p110 binding region was deleted (Δp85) (28). After viral exposure, Δp85-infected cells were serum starved for 20 h in serum-free medium and treated with the hormones indicated in the figure legends.

Wild-type HTC rat hepatoma cells were transfected with expression plasmids containing the human IR cDNA (29). These HTC-IR cells were used to measure PP2A activity. Cells were grown to 75% confluence in DMEM with 10% FCS and were serum starved for 48 h before stimulation with hormones and drugs for the times and at the concentrations indicated in the figure legends.

RNA extraction and dot-blot hybridization analysis

Total RNA was extracted from primary rat hepatocytes using a previously described procedure (30). Previous work compared Northern blotting with dot blots and established that the latter were as quantitatively accurate in assessing IGFBP-1 mRNA levels (19, 31). As in previous work, dot-blot analyses of 20 μg total RNA were performed on Hybond-N nylon membranes (GE Healthcare Life Sciences, Baie d’Urfe, Quebec, Canada) in a dot-blot manifold (Bio-Rad Laboratories, Inc., Hercules, CA), according to the manufacturer’s protocol. RNA was fixed to the membranes by UV cross-linking and hybridized sequentially, with intermittent stripping, with IGFBP-1 and 18S cDNA probes.

Western blot analysis

To prepare the Triton X-100-soluble (TS) fraction, rat primary hepatocytes were rinsed twice with ice-cold PBS (pH 7.4) and solubilized with lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 1.5 mM MgCl₂, 1 mM EGTA, 200 µM sodium orthovanadate, 1 mM phenylmethylsulfonylfluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10% glyceral, and 1% Triton X-100]. Cell lysates were centrifuged at 10,000 × g for 10 min at 4°C, and protein concentrations in the resulting supernatants were determined using the Bio-Rad protein assay (32). To determine the presence and the phosphorylation state of the Foxo1/Foxo3 transcription factors in C cells, total lysates were prepared as previously described for the TS fraction, except that after solubilization in Laemmli buffer [2% sodium dodecyl sulfate, 2% β-mercaptoethanol, 10% (vol/vol) glycerol, and 50 mg/liter bromophenol blue in 0.1 M Tris-HCl buffer (pH 6.8)], cell lysates were sonicated for 5 sec using the Vivarrac sonicator (Sonics & Materials, Newton, CT) at an amplitude of 21%. To prepare the Triton-insoluble (TI) fraction, cells were rinsed twice with ice-cold PBS (pH 7.4) and directly solubilized in hot Laemmli buffer. To fragment DNA after lysis of the nuclei, the TI fraction of cell lysates was passed through a syringe before loading onto a gel. To evaluate the total phosphorylation state of Foxo proteins, the TS fraction prepared using the Ser/Thr protein was incubated for 5 min at 30°C with 500 U cell intestinal alkaline phosphatase, then boiled for 5 min in Laemmli buffer. After separation on SDS-PAGE, extracted proteins were transferred to Immobilon-P membranes (Millipore Ltd., Mississauga, Canada) and probed with the indicated first antibody for 90 min, followed by a 1-h incubation with [*32P]Irritantib IgG. Immunoreactive proteins were detected by autoradiography, and quantification of densitometry signals was performed using a Bio-Rad densitometer (model GS-700).

PP2A activity

After treatment with the test agents for the times and concentrations indicated in the figure legends, HTC-IR cells were rinsed twice with 20 mM Tris (pH 7.4) and 150 mM NaCl and solubilized in IPTase lysis buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 1% (vol/vol) Nonidet P-40, 2 µM phenylmethylsulfonylfluoride, 2 µM leupeptin, and 10 µU/ml aprotinin]. Cell lysates were clarified by centrifugation at 10,000 × g for 10 min at 4°C, and protein concentrations in the resulting supernatants were determined using the Bio-Rad protein assay (32). Cell lysates were then incubated for 2 h at 4°C with an anti-PP2A antibody in the presence of protein A-Sepharose. The beads were collected by centrifugation and washed three times in IPTase lysis buffer, and PP2A activity was measured using the Ser/Thr protein as substrate for 1 h according to the manufacturer’s protocol, except that the reaction was incubated for 10 min and not 1 h as recommended in the standard protocol. The PP2A activity was then quantified using an Emax precision microplate reader (Molecular Devices, Sunnyvale, CA).
PI3 kinase activity

TS cell lysates (500 µg protein) from insulin-treated (100 nM insulin for 2 min) or nontreated SC and C cells were immunoprecipitated in the presence of protein A-Sepharose using anti-PY antibody. Immunoprecipitated pellets were resuspended in 50 µl kinase assay buffer [20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.5 mM EGTA] containing 0.5 mg/ml l-α-phosphatidylserine (Avanti Polar Lipids, Inc., Alabaster, AL) and assayed for PI3 kinase activity as described previously (33).

Results

Inhibition of IGFBP-1 gene expression by insulin in SC and C hepatocyte cultures: roles of PI3 kinase and mTOR

In previous work we showed that in primary rat hepatocytes seeded at low density (SC), but not in cells seeded at high density (C), insulin induced [3H]thymidine incorporation into DNA by a mechanism involving insulin-induced activation of both PI3 kinase and mTOR (6). In the present work we explored this difference further by examining the effect of insulin on IGFBP-1 mRNA levels in both SC and C cells. As previously observed the incubation of serum-deprived rat hepatocytes for 6 h in the presence of dexamethasone and glucagon (Dex/Gluc) markedly increased IGFBP-1 mRNA levels (19, 31), and insulin completely prevented this stimulation in both SC and C hepatocytes (Fig. 1). Interestingly the PI3 kinase inhibitors, wortmannin and LY294002 (34–36), prevented insulin from suppressing IGFBP-1 mRNA expression in SC, but not in C, cells (Fig. 1).

We evaluated the efficacy of overexpressing Δp85, a dominant-negative construct of the p85 regulatory subunit of PI3 kinase (28), on insulin suppression of IGFBP-1 gene expres-
duced suppression of IGFBP-1 gene expression requires the activation of PI3 kinase in SC, but not in C, cells. In contrast, rapamycin inhibited the effect of insulin in both SC and C hepatocytes (Fig. 1A), indicating that this effect is mediated by mTOR (33, 37) in both culture conditions. These studies indicate that independently of PI3 kinase activation, mTOR can mediate insulin-induced suppression of IGFBP-1 gene expression in rat hepatocytes.

Phosphorylation and activation of PI3 kinase, Akt, and Foxo1/Foxo3 in SC vs. C cells in response to insulin

Previous studies have implicated Akt and Foxo1/Foxo3, downstream of PI3 kinase, in the regulation by insulin of IGFBP-1 gene expression (3, 20, 21, 23, 26, 38). We, therefore, analyzed the expression and activation of key signaling molecules leading to Akt activation and Foxo1/Foxo3 phosphorylation in both SC and C hepatocyte cultures. In preliminary studies we found that in C hepatocytes there was a redistribution of cytosolic proteins, such as IRS-1, IRS-2, and p85, from the TS to the TI fraction. Therefore, in studying events in these cells, we examined both the TS and TI fractions prepared as described in Materials and Methods (Fig. 3). Western blot analysis of protein extracted from SC cells showed that p85 was almost exclusively present in the TS fraction, whereas in C hepatocytes, it was only detected in the TI fraction (Fig. 3A, top panels). In agreement with the Western blot analysis, PI3 kinase activity, measured in the TS fraction, was only detected in SC cells (Fig. 3A, bottom panels). Western blot analysis also showed that Akt is present in the TS fraction from SC and the TI fraction from C hepatocytes (Fig. 3B, left panels). Using antiphospho-Akt (Ser473) antibody, we demonstrated that insulin produced phosphorylation of Akt in the TS fraction of SC cells, but had no effect on Akt phosphorylation in the TI fraction from C hepatocytes (Fig. 3B, right panels).

We then analyzed the effect of insulin on Foxo1 and Foxo3 phosphorylation in SC rat hepatocytes. Using an antibody that specifically recognized Foxo1, we found that in non-treated cells, Foxo1 migrated at 77 kDa (Fig. 4A, top panel). It would thus appear that in SC cells the bulk of Foxo1 is not phosphorylated in the absence of exogenous stimulation by insulin. Upon insulin treatment of SC cells, the band migrating at 77 kDa disappeared, and a band migrating at approximately 80 kDa appeared on the Western blot. This band corresponds to the band observed on immunoblotting with antiphospho-Foxo1 (Thr24; Fig. 4A, lower panel). The antiphospho-Foxo1 (Thr24) also recognized phosphorylated Foxo3 (Thr32), whose phosphorylation paralleled that of Foxo1 (Fig. 4A, bottom panel). Total phosphorylation of the Foxo proteins in SC cells was reduced by phosphatase treatment, validating the observations made with the phosphospecific antibodies (Fig. 4B). The phosphorylation of both Foxo1 and Foxo3 was markedly reduced by treatment with the potent PI3 kinase inhibitor, Ly290042, but not by the mTOR inhibitor, rapamycin (Fig. 4A). Because Foxo1/Foxo3 phosphorylation persists in the presence of rapamycin, we conclude that such phosphorylation is independent of mTOR and is not sufficient for the insulin effect on IGFBP-1 gene expression (Fig. 1) (19).

TI and TS fractions were also prepared to analyze insulin’s effect on Foxo1 and Foxo3 in C hepatocytes. Surprisingly, we were unable to detect the presence of Foxo1 or Foxo3 in the two fractions, perhaps indicating a sequestration of these factors in a particular cellular compartment (data not shown). The cells were extracted using a more stringent method involving sonication, as described in Materials and Methods. In such fractions we observed Foxo1. However, insulin did not appear to induce phosphorylation of Foxo1, as reflected by the absence of shift on the Western blot depicted in Fig. 4C (top panel) and our failure to identify phosphorylated forms of either Foxo1 or Foxo3 using specific antibodies to the phosphorylated forms of Foxo1/Foxo3 (Fig. 4C, bottom panel). The failure of insulin to effect Foxo1 and Foxo3 phosphorylation in C hepatocytes is compatible with its inability to activate PI3 kinase and Akt in these cells (Fig. 3). These observations indicate that insulin-induced Foxo1/Foxo3 phosphorylation is not essential for insulin action on IGFBP-1 gene expression.

Effect of insulin and OA on Ser/Thr phosphatase activity, IGFBP-1 gene expression, and upstream signaling molecules

Various studies have implicated Ser/Thr phosphatases in insulin action (10, 39–42). Furthermore, the activation of mTOR has been linked to the inhibition of Ser/Thr phosphatases, such as PP2A, PP4, and PP6 (43–46). It was also
demonstrated that Akt is a direct target of PP2A, which could induce dephosphorylation and down-regulation of Akt activity (16, 47, 48). OA is a potent tumor promoter (49) that binds to and inhibits PPIc and PP2Ac with dissociation constants (K<sub>i</sub>) of 147 and 0.032 nM, respectively (50). We assessed the effects of both insulin and OA on hepatic phosphatase activities in HTC-IR cells, a hepatoma cell line overexpressing the IR that was previously used in our laboratory to study insulin signaling (33). This cell line was employed because phosphatase activity could be reproducibly measured in immunoprecipitates from lysates of these cells as described in Materials and Methods. As shown in Table 1, there was significant basal PP2A activity detected in HTC-IR cells. In agreement with previous reports (11–13), insulin (100 nM) alone inhibited PP2A activity by about 20%, whereas 100 nM OA reduced basal PP2A activity by approximately 85%.

When incubating serum-deprived rat SC hepatocytes for 6 h in the presence of Dex/Glu and 100 nM OA, we found a suppression of Dex/Glu-induced IGFBP-1 mRNA expression by about 40%. A similar level of inhibition was observed in the presence of 1 nM insulin (Fig. 5A). As expected, increasing insulin concentrations increased the inhibition of IGFBP-1 mRNA levels, with a maximal inhibition of 93% observed in the presence of 100 nM insulin. Despite considerable differences in the extent of suppression of IGFBP1 mRNA by insulin at low vs. high doses, there was a substantial degree of Akt phosphorylation on Ser473 at 1 and 3 h insulin compared with that at 100 nM insulin (Fig. 6). Interestingly, 100 nM OA, which inhibited the expression of IGFBP-1 mRNA to a similar level as that in the presence of 1 nM insulin (Fig. 5A), did not induce Akt phosphorylation at Ser473 (Fig. 6). Similar results were obtained for the phosphorylation of Akt at Thr308 (data not shown). Parallel with our observations for Akt, 100 nM OA did not effect phosphorylation of Foxo1, as indicated by the lack of shift in Foxo1 migration, whereas 1 nM insulin did effect a shift of Foxo1 mobility not markedly different from that observed with 100 nM insulin (Fig. 6). The same observation was made using the phosphospecific antibody that recognizes phosphorylated forms of Foxo1 and Foxo3 (Fig. 6, bottom panel). Incubating the cells with a combination of OA and rapamycin revealed that the OA effect on Dex/Glu-induced IGFBP-1 mRNA level was lost in the presence of the mTOR inhibitor (Fig. 5B). Taken together, these data indicate that OA, a Ser/Thr phosphatase inhibitor, can induce a significant decrease in the level of expression of the IGFBP-1 mRNA independently of the activation of Akt and phosphorylation of Foxo1/Foxo3. Thus, in SC cells, the phosphorylation of Foxo1/Foxo3 is not required to observe the suppression of IGFBP-1 gene transcription.

In addition, the results demonstrate that the phosphatase does not operate downstream of mTOR, because its inhibitory effect is prevented by coincubation with rapamycin. However, though 100 nM OA inhibited PP2A by 85% (Table 1), it only induced 40% inhibition of the level of expression of the IGFBP-1 mRNA.

**TABLE 1. PP2A activity in HTC-IR cells**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>PP2A activity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no treatment)</td>
<td>100</td>
</tr>
<tr>
<td>Insulin (100 nM)</td>
<td>79.7 ± 4.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>OA (100 nM)</td>
<td>15.9 ± 6.1</td>
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HTC-IR cells (serum starved for 48 h) were cultured in the absence of hormone or with 100 nM insulin for 10 min. Serum-starved cells were treated for 45 min with vehicle or 100 nM OA. Thereafter, the cells were pelleted and resuspended in lysis buffer without phosphatase inhibitors. PP2A activity was measured as described in Materials and Methods.

<sup>a</sup> P < 0.05 insulin vs. control.
mRNA (Fig. 5A). Therefore, the role of inhibiting PP2A in the regulation of IGFBP-1 mRNA levels must be regarded as limited at best and functioning in concert with other processes yet to be fully defined. In conclusion, the present study emphasizes the importance of an mTOR-dependent pathway that can be activated in a PI3 kinase-independent manner and can regulate IGFBP-1 mRNA levels in the absence of Foxo1/Foxo3 phosphorylation.

Discussion

We previously found that in primary rat hepatocytes, insulin inhibited the induction of IGFBP-1 mRNA levels by Dex/Gluc via the activation of both PI3 kinase- and mTOR-dependent pathways (19). Various studies of the regulation of IGFBP-1 mRNA expression have identified a role for Akt and Foxo1/Foxo3 downstream of PI3 kinase (21, 25, 26). The involvement of mTOR in the regulation of this process has also been noted in the hepatoma cell line H4EI (27).

Most studies of the roles of Foxo1 and Foxo3 in the regulation of IGFBP-1 transcription have used systems overexpressing Foxo proteins (3, 21, 26, 27, 38, 51) and have not sought to establish the relative roles of these specific isoforms in the regulation of IGFBP-1 gene expression. In the present study we tried to evaluate the roles of the endogenous factors in cultured primary hepatocytes as a possibly more accurate reflection of physiological processes.

In SC hepatocytes, the inhibition of PI3 kinase activity by both specific inhibitors (wortmannin and LY294002) and the transfection of a dominant-negative construct of p85 (Δp85) abrogated insulin suppression of IGFBP-1 gene expression and Foxo1/Foxo3 phosphorylation. Rapamycin also inhibited the effect of insulin without affecting Foxo1/Foxo3 phosphorylation. This latter observation of a dissociation between phosphorylation of Foxo1/Foxo3 and insulin action on gene expression.

Fig. 5. Comparison between the effects of insulin and OA on IGFBP-1 mRNA levels. A, SC hepatocytes (serum starved for 48 h) were incubated for 6 h in the absence of hormone (H), in 100 nM dexamethasone and 100 nM glucagon (G) with or without 100 nM OA, or in 100 nM dexamethasone, 100 nM glucagon, and insulin (I) at the indicated doses (*, P = 0.006, OA- vs. DG-treated cells). B, SC hepatocytes (serum starved for 48 h) were incubated for 6 h in the absence of hormone with or without 100 nM OA (H) or in 100 nM dexamethasone and 100 nM glucagon with 100 nM OA in the presence and absence of 200 nM rapamycin (R). IGFBP-1 mRNA levels were quantified as described in Fig. 1. Results are expressed as the mean of three separate measurements; bars indicate the SD.

Fig. 6. Comparison between the effects of insulin and OA on Akt and Foxo1/Foxo3 phosphorylation. Total cell lysates were obtained from SC hepatocytes after treatment with vehicle, the noted doses of insulin, or 100 nM OA for 15 min. Proteins were then resolved on 8% SDS-PAGE and immunoblotted with an antiphospho-Akt (Ser473) antibody (top panels), an anti-Akt antibody (second panels), an antifoxo1 antibody (third panels), or an antibody that recognized the phosphorylated isoforms of Foxo1 and Foxo3 [antiphospho-Foxo1 (Thr24)/Foxo3 (Thr32); bottom panels]. The autoradiographs are representative of two different experiments.
transcription has been clearly established in other studies. Thus, phorbol esters induced activation of Akt and phosphorylation of Foxo1 without suppressing IGFBP-1 gene expression (52), and H2O2 attenuated the insulin effect on IGFBP-1 expression without affecting insulin-induced phosphorylation of Akt and Foxo1 (37). From these data, it seems clear that the phosphorylation of Foxo1/Foxo3 is not sufficient to mediate the effect of insulin on IGFBP-1 gene transcription.

Surprisingly, we found that insulin failed to activate PI3 kinase and Akt or phosphorylate Foxo1/Foxo3 in C hepatocytes. Nevertheless, insulin inhibited Dex/Gluc-induced IGFBP-1 mRNA production. This latter effect of insulin in C hepatocytes was fully reversed by rapamycin, indicating that mTOR mediated insulin’s effect on IGFBP-1 gene transcription independently of PI3 kinase activation. The existence of a pathway bypassing PI3 kinase-Akt-Foxo1/Foxo3 was also demonstrable in SC hepatocytes, because we could demonstrate that the Ser/Thr phosphatase inhibitor OA significantly decreased the IGFBP-1 mRNA level by 40% without affecting the activation and phosphorylation state of Akt and Foxo1/Foxo3. Taken together, these observations indicate that the phosphorylation of Foxo1/Foxo3 is neither necessary nor sufficient for the insulin effect on IGFBP-1 gene transcription, but may play a role in augmenting and consolidating the effect of insulin in SC hepatocytes. In the latter, the activation of PI3 kinase by insulin appears necessary to activate mTOR. However, in C hepatocytes, where there are important differences in the cellular distribution of molecules, mTOR activation appears to have become independent of the requirement for PI3 kinase activation. Perhaps this occurs as a consequence of a change in cellular localization of mTOR, which has been suggested as one possible mechanism by which its activation is accomplished (53–55). The implication of mTOR in insulin-regulated gene expression seems to be specific for IGFBP-1, because the regulation by insulin of the transcription of genes for glucose-6-phosphatase and phosphoenolpyruvate carboxykinase was not antagonized by rapamycin (56, 57).

Patel et al. (37) used the expression of active S6 kinase to demonstrate that this enzyme was not the key element downstream of mTOR leading to the insulin-dependent suppression of IGFBP-1 gene expression. A role for Ser/Thr phosphatases in insulin action has been previously established (10–13). In this study we found that the Ser/Thr phosphatase inhibitor OA partially mimicked insulin by inhibiting the induction of IGFBP-1 gene expression by Dex/Gluc. Furthermore, this occurred in the absence of activation of the Akt-Foxo1/Foxo3 pathway. Other work has implicated phosphatase inhibition in the mTOR pathway (14). Thus, genetic screening has identified several phosphatases and a phosphatase-associated protein as part of a rapamycin-sensitive signaling pathway in Saccharomyces cerevisiae (44, 58). However, several studies implicating PP2A, PP4, and PP6 in mTOR signaling have located these enzymes downstream of mTOR itself (43–46, 59, 60). In the present study, it appears that the phosphatase targeted by OA does not operate downstream of mTOR, because the effect of OA on IGFBP-1 mRNA levels was reversed by rapamycin. Both the identity of the putative phosphatase as well as its precise target in response to insulin remain to be clarified.

C hepatocytes represent a cellular model in which insulin suppressed the induction of IGFBP-1 mRNA levels by a mechanism involving mTOR in a manner completely independent of activation of the PI3 kinase-Akt-Foxo1/Foxo3 signaling pathway. Indeed, in contrast to SC cells, we found that insulin did not activate the PI3 kinase pathway at all in C cells. Although most work involving primary rat hepatocytes has been performed in SC cells, there are studies that have raised the possibility that SC hepatocytes may not accurately reflect the actual cellular environment within whole liver. For example, it is known that the expression of several liver-specific functions is inversely related to the degree of cell proliferation (61, 62), a feature readily observed in SC, but not C, primary rat hepatocytes (6). Furthermore, evaluation of urea, albumin, and glucose synthesis as well as the expression of specific hepatic genes demonstrated a lower level of liver-specific function in SC than in C hepatocytes (63). Indeed, C and not SC cells were noted to retain the adult phenotype, thus reflecting the in vivo state of fully differentiated hepatocytes (64). Therefore, our observations made in C primary hepatocytes may be relevant to in vivo physiology. It is possible that there are different cell populations in intact liver, some of which behave like C hepatocytes and others, perhaps located differently in the liver lobule, which behave like SC hepatocytes.

In summary, the present work compared the regulation of IGFBP-1 gene expression in SC and C hepatocytes. In the latter, we showed that insulin suppressed Dex/Gluc-induced IGFBP-1 mRNA levels by an mTOR-dependent mechanism and in the absence of activating the PI3 kinase-Akt-Foxo1/Foxo3 pathway. The phosphorylation of Foxo1/Foxo3 appears to be neither necessary nor sufficient for this insulin effect.

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