Insulin Regulation of Glucose-6-phosphate Dehydrogenase Gene Expression Is Rapamycin-sensitive and Requires Phosphatidylinositol 3-Kinase*

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Glucose-6-phosphate dehydrogenase (G6PDH) controls the flow of carbon through the pentose phosphate pathway and also produces NADPH needed for maintenance of reduced glutathione and reductive biosynthesis. Hepatic expression of G6PDH is known to respond to several dietary and hormonal factors, but the mechanism behind regulation of this expression has not been characterized. We show that insulin similarly induces expression of endogenous hepatic G6PDH and a reporter construct containing 935 base pairs of the G6PDH promoter linked to luciferase in transient transfection assays. Using well tested and structurally distinct inhibitors of Ras farnesylation, lovastatin and B581, and a specific inhibitor of mitogen-activated protein kinase kinase activation, PD 98059, we show that the Ras/Raf/mitogen-activated protein kinase kinase activity is necessary for the induction of G6PDH expression by insulin. Rapamycin, an inhibitor of FRAP protein, which is involved in the activation of pp70 S6 kinase, blocks the insulin induction of G6PDH, suggesting that S6 kinase is also necessary for the insulin induction of G6PDH expression.

Insulin elegantly regulates a variety of metabolic responses. The actions of insulin at the cellular level are initiated by insulin binding to its plasma membrane receptor. Following ligand-induced autophosphorylation of the receptor, phosphorylation of endogenous substrates occurs to mediate the transmission of an insulin signaling pathway (1). The information available to date clearly indicates that under a number of different circumstances a variety of phosphoproteins are activated by insulin (1, 2). Members of the IRS family, which includes IRS-1, IRS-2, and the recently reported IRS-3 (p60), are thought to be essential for many of insulin’s biological responses (2, 3). IRS-1 and IRS-2 contain common functional units but are thought to regulate unique signaling pathways in part to their distinct cellular distribution. IRS-1 and IRS-2 appear in all tissue; however, IRS-2 predominates in cells of myeloid lineage.

A great deal of study has concentrated on IRS-1, and it has been found that activated IRS-1 recognizes and binds to Src homology 2 domains of various signal transduction proteins such as PI 3-kinase, SH-PTP-2, Grb2, and Nck (4). The critical role of PI 3-kinase in diverse actions of insulin has been established in a number of independent studies. One report demonstrates that PI 3-kinase in 3T3-L1 cells is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation (5), whereas another shows that PI 3-kinase, while functioning upstream of Ras and Raf, is necessary for mediating insulin stimulation of c-fos transcription (6). Other reports using the PI 3-kinase inhibitor wortmannin, have implicated the involvement of PI 3-kinase in insulin regulation of glycogen synthase (7, 8), glycogen synthase kinase-3 (9, 10), Glut-4-mediated glucose transport (5, 11), membrane ruffling (12), and PEPPCK expression (13, 14).

Wortmannin also blocks the insulin activation of pp70 S6 kinase, the protein responsible for the hormonal and growth factor-stimulated in vivo phosphorylation of ribosomal protein S6 kinase (15, 16). Rapamycin is a potent inhibitor of insulin-stimulated phosphorylation of the ribosomal protein S6 kinase but has no effect on the stimulation of PI 3-kinase by insulin. Regulation of PEPPCK expression by insulin has recently been shown to be rapamycin-insensitive (4, 17) as has the insulin regulation of glycogen synthase (7), glycogen synthase kinase-3 (9, 10), gene 33 (17), and 6-phosphofructo-2-kinase (18), suggesting that an insensitive pathway predominates in the insulin regulation of genes for enzymes in metabolism.

In addition to the IRS family and the PI 3-kinase pathway, another set of proteins has been identified as a proximal target for several growth factor tyrosine kinases including the insulin receptor (19, 20). Once activated by phosphorylation, the Shc proteins associate with various downstream effector molecules including Grb2 and the guanine nucleotide-releasing factor (21). Grb2/SOS interaction plays a role in the insulin activation of Ras, since SOS facilitates the exchange of GDP for GTP on Ras proteins (22).

Ras activation is required for initiation of the downstream events leading to MAP kinase activation. Studies have indicated a reasonable correlation between the activation of MAP kinase and events leading to cellular proliferation (23, 24). MAP kinases have also been implicated in phosphorylation of some transcription factors that could be involved in insulin-mediated gene expression. In vitro, MAP kinase phosphorylates c-Jun and TCF/ELK as well as ATF-2 to restore DNA
binding activity (25). The role of MAP kinase in metabolic events has not been demonstrated, since no obligatory role has been established in insulin stimulation of glycolysis synthesis (8, 26–28), lipogenesis (26, 29), gluconeogenesis (13, 14), or glucose uptake (5, 11).

As recently reviewed by O’Brien and Granner (30), insulin regulates the transcription of the genes for several metabolic enzymes. Glycogen synthase (31), glucokinase (32, 33), PEPCCK (34, 35), and fatty acid synthase (36, 37), key enzymes in glycogen synthesis, glycolysis, gluconeogenesis, and fatty acid biosynthesis, respectively, have been targets of study with regard to insulin action. In the liver, the pentose phosphate pathway is a primary pathway responsible for the insulin-influenced fate of carbohydrate, since up to 50% of the glucose 6-phosphate can be shuttled through. Glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), the key rate-limiting enzyme in the pentose phosphate pathway, controls the flow of carbon through this pathway and also produces NADPH needed for maintenance of reduced glutathione and reductive biosynthesis. G6PDH has not been extensively characterized with regard to insulin action, since in most tissues and organisms it has been considered to have a “housekeeping” role (38). While the housekeeping role of G6PDH is not disputed, the insulin induction of G6PDH activity and mRNA (38–40) has been studied both in vivo and in vitro.

Recently, we have cloned the promoter region of the G6PDH gene (41). In transient expression studies in primary rat hepatocytes, we show that a chimeric construct containing 935 base pairs of the G6PDH promoter region responds to insulin similarly to endogenous G6PDH. Few studies have utilized primary cells in culture for elucidation of signal pathways, since they are not easily experimentally manipulated or conducive to dominant negative or overexpression approaches. They are, however, an ideal model for elucidating mechanisms utilized for gene expression, since they can be maintained in a chemically defined, serum-free medium and since the metabolic responses to hormones or nutrients generally mimic those observed in vivo. For these reasons, we have utilized primary hepatocytes in culture, a representative cell population from a primary target tissue of insulin, to investigate the role of insulin signal proteins in the induction of G6PDH expression. To determine which signal proteins are necessary for transmission of the message from the insulin receptor to the G6PDH gene, we have completed studies using well characterized inhibitors of insulin signal proteins. We show, using inhibitors of Ras farnesyltransferase (B581 and lovastatin) as well as an inhibitor of MEK (PD 98059), that the Ras/Raf/MAPK pathway is not utilized in the insulin induction of G6PDH. Wortmannin and LY 294002, two mechanistically distinct inhibitors of PI 3-kinase, however, completely block the insulin-induced activation of G6PDH expression, demonstrating a role for PI 3-kinase in the regulation of this gene. In rat hepatocytes in culture, we find that S6 kinase is activated by insulin in a rapamycin-sensitive and -insensitive way. Results from double immunoblot analysis suggest that the rapamycin-insensitive pathway for insulin activation of S6 kinase is mediated via Akt. Unlike previous studies that have found genes of glycolysis and gluconeogenesis be rapamycin-insensitive and possibly mediated downstream of PI 3-kinase via Akt, we find the insulin induction of G6PDH to be rapamycin-sensitive.

**EXPERIMENTAL PROCEDURES**

**Materials**—PD 98059 was generously provided by Dr. Alan Saltiel (Parke-Davis). Lovastatin, was a gift from Merck. B581 and LY 294002 were purchased from Biomol Research Laboratories. Wortmannin was purchased from Sigma. Rapamycin was obtained from Calbiochem. Insulin was a gift from Lilly.

**Hepatocyte Isolation and Maintenance**—Hepatocytes were isolated using collagenase, hyaluronidase perfusion as described by Stapleton et al. (42). Male Sprague-Dawley rats (Harlan, Kalamazoo, MI) weighing 200–300 g were food-deprived 48 h prior to the isolation procedure. After collagenase perfusion, the liver was excised and forced through four layers of sterile gauze. The cells were washed twice with Waymouth’s MB 752/1 medium (Life Technologies, Inc.) and pelleted for 3 min at 4 °C and 50 × g. The pellet was gently resuspended in medium, and an aliquot of cells was counted with a hemocytometer. Cell viability was determined by trypan blue dye exclusion. Collagenase-coated 60-mm tissue culture dishes were plated with 3.0 × 10⁶ cells/plate. The cells were incubated in serum-free Waymouth’s MB 752/1 medium supplemented with gentamicin (10 μg/ml) under a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. After about 4 h of incubation, the cells were washed, and fresh medium was applied. When used in the subsequent studies, the inhibitors PD 98059, B581, wortmannin, and LY 294002 were added 1 h prior to the addition of insulin. Lovastatin, however, was added 24 h prior to insulin addition. It has previously been shown that short preincubation times with lovastatin had little to no effect on the insulin-stimulated MAP kinase activity (22, 43).

**RNA Isolation and Northern Analysis**—Total RNA was extracted using the guanidinium isothiocyanate method described by Chomczynski and Sacchi (45). Total RNA (25 μg) was treated with formaldehyde and subjected to electrophoresis in 1.0% agarose gels (46). The separated RNAs were transferred to GeneScreen (NEN Life Science Products, Boston, MA) prehybridized with high stringency (3 × SSC, 2× Denhardt’s solution, 0.1% bovine myelin basic protein in gel) and hybridized with [32P]dCTP random-primed (Amersham Pharmacia Biotech) labeled cDNA for G6PDH (47). The membranes were hybridized, washed (42), and subjected to autoradiography at −70 °C with Kodak XAR-5 film and intensifying screens. The exposed films were quantitated by densitometric scanning. The amount of G6PDH mRNA was normalized to the control probe, β-actin (48).

**Immunoprecipitation and Western Analysis**—After 5 min of insulin treatment (80 nM), cells were washed twice with ice-cold PBS. Cells were flash frozen and then scraped and processed in a buffer as described previously (49). The total cell extracts were used to determine the association of PI 3-kinase with Akt using the dual immunoprecipitation method. Akt was immunoprecipitated from the cell extracts using the Akt C-terminal antibody (Upstate Biotechnology, Inc.). The immunocomplex was washed with PBS and suspended in Laemmli sample buffer and subsequently subjected to SDS-polyacrylamide gel electrophoresis in a 8% acrylamide gel. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane using the directions provided by the manufacturer of the apparatus, NOVEX. The membrane was blocked overnight in blocking buffer with 5% milk in PBS containing 1% Tween 20 (PBST). It was then incubated with the primary antibody, anti-PI 3-kinase (N-terminal) (Upstate Biotechnology, Inc.) as described previously (49). After the denaturation step, the gel was washed with 0.04% Triton X-100 in buffer B and then in 40 mM HEPES, pH 8.0, 2 mM dithiothreitol, and 10 mM MgCl₂. Finally, the gel was incubated with 40 μl [γ-32P]ATP, rinsed in 5% trichloroacetic acid and 1% sodium pyrophosphate, dried, and exposed to x-ray film. Results were quantitated by a scanning densitometer.

**MAP Kinase Assay**—Analysis of MAP kinase activity was carried out using a myelin basic protein in-gel activity assay (50). The samples were electrophoresed in a 12% polyacrylamide gel containing 0.53 mg/ml bovine myelin basic protein (Life Technologies, Inc.). After electrophoresis, the gel was washed sequentially in solutions containing 20% isopropanol alcohol in buffer B (50 mM Tris, pH 8.0, and 5 mM β-mercaptoethanol), buffer B, and 8 M guanidine HCl in buffer B. After the denaturation step, the gel was washed with 0.04% Triton X-100 in buffer B and then in 40 mM HEPES, pH 8.0, 2 mM dithiothreitol, and 10 mM MgCl₂. Finally, the gel was incubated with 40 μl [γ-32P]ATP, rinsed in 5% trichloroacetic acid and 1% sodium pyrophosphate, dried, and exposed to x-ray film. Results were quantitated by a scanning densitometer.

**3-Kinase Assay**—PI 3-kinase was immunoprecipitated from cellular lysates with anti-PI 3-kinase N-Src homology 2 (Upstate Biotechnology) as described by the supplier. The immunoprecipitate was trapped with 50% Sepharose in PBS. The precipitate was washed first with a PBS solution containing 1% Tween 40 and 100 μM vanadate. Subsequent washes contained 100 mM Tris, pH 7.5, and 500 mM LiCl₂ and finally 20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 100 μM vanadate. The assay for PI 3-kinase activity was then carried out as described previously (51). The phosphorylated product was extracted with a 1:1 chloroform/methanol mixture. The sample was then applied to a silica gel thin layer chromatography plate coated with 1% potassium oxalate. After development, the plates were exposed to x-ray film. The radioactivity incorporated during the PI 3-kinase reaction was quantitated using a liquid scintillation counter.
S6 Kinase Assay—S6 kinase assay was carried out following the directions supplied by the manufacturer of the S6 kinase kit (Upstate Biotechnology). Basically, S6 Kinase antibody was incubated with the protein A-agarose slurry for 30 min at 4 °C. The beads were washed, and cell lysate containing 200–300 μg of total protein was added to the beads and incubated for 2 h at 4 °C. Kinase reaction using [32P]ATP was carried out on the beads for 10 min at room temperature. Product was purified on phosphocellulose paper, and the amount of radioactivity was quantitated using a scintillation counter.

Statistical Analysis—The results are expressed as the mean ± S.E. Significance was evaluated by one-tailed Student’s t test. Significance was tested at p < 0.05.

RESULTS

Role of Ras and MEK in Insulin Regulation of G6PDH Gene Expression—The role of Ras in the insulin regulation of G6PDH gene expression was studied by transiently transfecting the 935G6PDH-LUC into hepatocytes and then assessing luciferase activity in the presence of insulin plus inhibitors of Ras farnesylation. The effect of the Ras farnesylation inhibitors was also tested on the insulin induction of endogenous G6PDH mRNA. Two structurally different farnesylation inhibitors, lovastatin and B581, have been proven to be effective in a variety of cell types (18, 52, 53). To test for their effectiveness in the primary hepatocytes, MAP kinase activation by insulin was measured. Cells pretreated with varying concentrations of either lovastatin or B581 showed concentration-dependent decreases in insulin-stimulated MAPK activity. Preincubation with either 25 μM lovastatin or 50 μM B581 completely blocked the 2–3-fold insulin stimulation of MAP kinase activity (Fig. 1A). Neither inhibitor at concentrations effective at blocking the insulin-stimulated MAPK activity, however, had an effect on the insulin-induced expression of 935G6PDH-LUC (Fig. 1B), suggesting that Ras is not utilized in the insulin-induced expression of G6PDH. These same results were also observed when the inhibitors were tested for their ability to block the insulin induction of endogenous G6PDH mRNA (Fig. 2).
Ras activation leads to the subsequent activation of MEK. A specific inhibitor of MEK activation, PD 98059, has recently been described (54). PD 98059, also in a concentration-dependent manner, blocks MAP kinase activation by insulin in the hepatocytes (Fig. 1A). Similar to the inhibitors of Ras farnesylation, inhibition of MEK has no effect on the insulin-induced expression of either the 935G6PDH-LUC (Fig. 1B) or endogenous G6PDH mRNA (Fig. 2). These results indicate that the Ras/Raf/MAP kinase pathway is not utilized for the insulin-induced stimulation of G6PDH gene expression.

Role of PI 3-Kinase in Insulin Regulation of G6PDH Gene Expression—Wortmannin inhibits PI 3-kinase by covalently binding to the p110 subunit. Wortmannin is relatively unstable after approximately 2 h of incubation at pH 7. Studies that require longer incubation times have successfully utilized wortmannin by re-adding it every 2 h to the incubation medium (13). Insulin significantly induces PI 3-kinase in primary rat hepatocytes, and this induction is blocked in a concentration-dependent manner by wortmannin (Fig. 3A). Complete inhibition of insulin-stimulated PI 3-kinase was observed when 100 nM wortmannin was included in the media (Fig. 3A). The insulin-induced expression of 935G6PDH-LUC transiently transfected into hepatocytes was inhibited by wortmannin in a concentration-dependent manner (data not shown), and complete inhibition was observed with 100 nM wortmannin (Fig. 3C). A mechanistically different inhibitor of PI 3-kinase, LY 294002, competes with ATP for binding to the p110 subunit. LY 294002, in a concentration-dependent manner, also decreased the insulin induction of PI 3-kinase (Fig. 3B). Complete inhibition of insulin-stimulated 935G6PDH-LUC was observed with 50 μM LY 294002 (Fig. 3C). Parallel results were observed when LY 294002 was tested on insulin-induced mRNA levels for endogenous G6PDH (Fig. 2). Various concentrations of LY 294002 were tested for the ability to inhibit insulin-induced endogenous G6PDH mRNA. Fifty percent inhibition was observed at approximately 10 μM LY 294002, and complete inhibition was obtained with 50 μM LY 294002. These results therefore indicate that PI 3-kinase activity is necessary for the induction of G6PDH expression by insulin.

Role of S6 Kinase in Insulin Regulation of G6PDH Gene Expression—S6 kinase has been shown to be a downstream target of PI 3-kinase. Rapamycin, an immunosuppressant, blocks the stimulation of S6 kinase by insulin in a variety of cell types. We analyzed the activity of S6 kinase in response to insulin in the presence and absence of rapamycin (Fig. 4A). The addition of concentrations of rapamycin up to 500 μM to hepatocytes did not completely block the insulin induction of S6 kinase, suggesting a rapamycin-insensitive pathway can also mediate insulin induction of this kinase in these cells. Maximal inhibition was observed at 100 μM rapamycin (Fig. 4A). Insulin-induced expression of G6PDH in the presence of rapamycin was also tested, and it was found that rapamycin significantly inhibited the insulin-induced expression of both the 935G6PDH-LUC (Fig. 4B) and endogenous G6PDH mRNA (Fig. 2).

as described under “Experimental Procedures” for their ability to inhibit insulin-induced PI 3-kinase activity. A and B, after 5 min of insulin (80 nM) incubation, the cells were processed for PI 3-kinase activity as indicated under “Experimental Procedures.” Results of -fold decreases in PI 3-kinase activity are expressed (n = 4 ± S.E.). C, cells were transiently transfected with 935G6PDH-LUC. After inhibitor pre-incubation of either wortmannin (100 nM) or LY 294002 (50 μM), insulin was added, and cells were processed for luciferase activity after 24 h. Since wortmannin is unstable in media, it was added every 2 h during insulin incubation. Results of -fold increases in luciferase activity are expressed (n = 5 ± S.E.).
Akt has been shown to be a candidate for mediating the effects of PI 3-kinase on downstream processes including the activation of S6 kinase. Insulin stimulation of Akt requires its association with PI 3-kinase. Densitometric quantitation of the double immunoblot Western analysis of hepatocytes treated with and without insulin show that activated PI 3-kinase does associate with Akt, and this association is prevented in the presence of inhibitors of PI 3-kinase activity (Fig. 5). Thus, in primary rat hepatocytes, insulin may activate S6 kinase in a rapamycin-insensitive pathway that involves Akt.

**DISCUSSION**

Regulation of gene expression by insulin has been an intense and complex area of study for many years. Gaps still remain between understanding the upstream signaling molecules and downstream trans-acting factors that are important in this regulation of gene expression by insulin (30). Genes involved in hepatic glucose homeostasis that have been shown to be directly regulated by insulin include glucokinase and PEPCK, key enzymes in glycolysis and gluconeogenesis, respectively. Limited studies to elucidate insulin regulation, however, have been done in regard to the pentose phosphate pathway, an intermediary metabolism pathway essential to carbohydrate and fatty acid metabolism. G6PDH is the key enzyme in this pathway and is the focus of this paper.

Recently, the use of inhibitors of specific steps of insulin signaling pathways has permitted definition of the importance of that pathway to metabolic responses. Most of these studies have not focused on the liver, a major target organ of insulin. When liver cells have been used, they are generally transformed cells and thus may not always represent the metabolic responses observed in vivo. Inhibitors of farnesylation, a critical step in Ras processing, have been successfully used to show the role of the Ras/MAP kinase pathway in insulin signal transduction (52, 53). Lovastatin, a compound that has been shown to disrupt the early events of insulin mitogenic signaling (53), blocks posttranslational modification of Ras (52). Unfortunately, lovastatin has been shown to have other sites of action including inhibition of hydroxymethyl-glutaryl coenzyme A reductase, the rate-limiting step of cholesterol biosynthesis. It has also been shown to block cell transformation by Raf, a signal protein downstream of Ras (57), as well as inhibit both the platelet-derived growth factor and insulin activation of PI 3-kinase (56). For these reasons, we also chose to use a new specific inhibitor of Ras farnesylation, B581, to substantiate our studies. B581 mimics the CAA binding site of farnesyltransferase and blocks Ras transformation and subsequent MAP kinase activation (57). We show for the first time in primary hepatocytes in culture that both of these inhibitors efficiently block MAP kinase activation; however, neither inhibitor prevents insulin induction of G6PDH expression.
In light of evidence that suggests that farnesylated Ras-GTP interacts with the catalytic subunit of PI 3-kinase and increases its activity, we tested whether or not the farnesylation inhibitors had any effect on PI 3-kinase activation by insulin (58). Neither lovastatin nor B581 inhibited the induction of PI 3-kinase, suggesting that in hepatocytes, Ras is not an upstream mediator of PI 3-kinase (data not shown).

Mechanisms have been proposed for Ras-independent activation of MAP kinase (59, 60). MAP kinase has a number of substrates including transcription factors that may be involved in regulation of gene expression (54, 61, 62). MEK or MAP kinase, a dual specific kinase catalyzes the phosphorylation of MAP kinase on threonine and tyrosine residues causing activation. Recently, an inhibitor of MEK, PD 98059, has been described (54). This inhibitor has been used to show the role of MEK and MAP kinase in the regulation of c-fos transcription (54). Inhibition of MEK, however, had no effect on insulin-stimulated glucose uptake in 3T3-L1 cells; glycogen synthase in 3T3-L1 cells and M6 myocytes (54); or inhibition of PEPCK expression in H4IIE cells (13). Consistent with these results, treatment of hepatocytes with PD 98059 had no effect on the insulin stimulation of G6PDH expression.

Thus, the evidence available to date suggests that the MAP kinase pathway is not required for insulin mediation of metabolic pathways in any cell type. Similarly, evidence is accumulating to show a role for PI 3-kinase in insulin regulation of metabolic processes. Wortmannin and more recently LY 294002 have been used to indicate the importance of PI 3-kinase in insulin-stimulated glucose uptake in adipocytes (5, 10, 63). The effects of insulin on glycogen synthase (7, 8) or glycogen synthase kinase-3 (9, 10) are also blocked by inhibiting PI 3-kinase activity with wortmannin. In hepatoma cells, wortmannin has also been effectively used to block the actions of insulin on gene 33 expression (17), yet in this same study, wortmannin was ineffective in altering the response of PEPCK mRNA to insulin. Other recent studies, however, using wortmannin or LY 294002 show that PEPCK expression is sensitive to inhibition of PI 3-kinase activity (13, 14). Our studies substantiate the role of PI 3-kinase in the regulation of metabolic processes, since insulin-induced expression of G6PDH is sensitive to both wortmannin and LY 294002.

The signal transduction pathways that lie between PI 3-kinase and potential IRE sequence-binding proteins remain to be identified. One protein that lies downstream of PI 3-kinase is pp70 S6 kinase. This protein has been shown to be responsible for insulin-stimulated phosphorylation of ribosomal protein S6 in vivo (15, 16). Wortmannin, a potent inhibitor of PI 3-kinase, also blocks the activation of pp70 S6 kinase. Rapamycin, an immunosuppressant, blocks pp70 S6 kinase activation without affecting PI 3-kinase activity. In 3T3-L1 cells, glycogen synthase is inhibited by rapamycin (8); however, insulin regulation of glycogen synthase in PC-12 cells is rapamycin-insensitive (7). The insulin regulation of glycogen synthase kinase-3 (9, 10), gene 33 (17), PEPCK (14), and most recently 6-phosphofructo-2-kinase (18) have all also been shown to be rapamycin-insensitive. The accumulation of these data to date suggest that a rapamycin-insensitive pathway may be responsible for the regulation of metabolic genes. We show that in hepatocytes, a rapamycin-insensitive pathway for activation of S6 kinase does exist through Akt; however, the insulin regulation of G6PDH gene expression is rapamycin-sensitive. Akt is a serine/threonine kinase that functions downstream of PI 3-kinase and may regulate pp70 S6 kinase (64, 65). Controversy exists over the exact mechanism by which Akt is activated. The generation of the lipid products phosphoinositide 3,4-bisphosphate and phosphoinositide 3,4,5-trisphosphate by PI 3-kinase has been suggested to play a role in not only the translocation of Akt to the membrane but also in modulation of its activity (66, 67). Another report, however, suggests that the lipid products of PI 3-kinase are required for interaction of Akt with a specific kinase, 3-phosphoinositide-dependent protein kinase 1, that regulates its activity (44). Whatever the mechanism, it does appear clear that the activation of PI 3-kinase is necessary for Akt activation.

Our data tend to support the hypothesis that PI 3-kinase is a point of divergence in insulin signaling (5) and that the direction of divergence may be tissue-specific. This divergence results in rapamycin-sensitive (pp70 S6 kinase) and -insensitive (Akt) regulation of metabolic gene expression. With respect to G6PDH regulation by insulin, the rapamycin-sensitive pathway is utilized.

In conclusion, based on the inhibitor data, the activation of PI 3-kinase is required for regulation of G6PDH expression by insulin. Unlike studies on many other insulin-regulated metabolic genes, this regulation is also rapamycin-sensitive. The Ras/MAP kinase pathway does not appear to be involved in insulin regulation of the expression of G6PDH in liver. Conversely does exist as to the specificity of inhibitors and the validity of results obtained with inhibitors. In our studies, however, two structurally distinct inhibitors of Ras and PI 3-kinase were utilized as well as inhibitors of other signal proteins in the pathways to alleviate a concern over nonspecific interactions of these compounds. The concentration-dependent effect of these inhibitors on their respective target proteins in this system was also shown, attesting to the specificity of the inhibitor. Much work is still needed in the identification of downstream signal proteins of PI 3-kinase including transcription factors that bind to the insulin regulatory region of G6PDH and other genes. This will undoubtedly aid in the further delineation of the signal pathway(s) utilized in the regulation of metabolic processes.

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