# Differential Signaling of Insulin and IGF-1 Receptors to Glycogen Synthesis in Murine Hepatocytes†

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ABSTRACT: We have used SV40-transformed hepatocytes from insulin receptor-deficient mice (−/−) and normal mice (WT) to investigate the different abilities of insulin and IGF-1 receptors to stimulate glycogen synthesis. We report that insulin receptors are more potent than IGF-1 receptors in stimulating glycogen synthesis. Both receptors stimulate glycogen synthesis in a PI 3-kinase-dependent manner, but only the effect of insulin receptors is partially rapamycin-dependent. Insulin and IGF-1 receptors activate Akt to a similar extent, whereas GSK-3 inactivation in response to IGF-1 is considerably lower in both −/− and WT cells, compared to the effect of insulin in WT cells. The findings indicate that (i) the potency of insulin and IGF-1 receptors in stimulating glycogen synthesis correlates with their ability to inactivate GSK-3, (ii) the extent of GSK-3 inactivation does not correlate with the extent of Akt activation mediated by insulin or IGF-1 receptors, indicating that the effect of insulin on GSK-3 requires additional kinases, and (iii) the pathways required for insulin stimulation of glycogen synthesis in mouse hepatocytes are PI 3-kinase-dependent and rapamycin-sensitive.

Insulin and IGF-1[1] receptors mediate metabolism, cell growth, and differentiation (*1*, *2*). Targeted mutagenesis in mice has shown that the actions of the two receptors are quite distinct, with insulin receptors mediating metabolic responses and IGF-1 receptors mediating growth (*3*, *4*). However, the two receptors share significant homology, especially in their tyrosine kinase domain, and utilize similar signaling mechanisms, based on IRS-mediated activation of PI-3 kinase, or shc-mediated activation of the ras-MAP kinase pathway (*5*, *6*). Since the biological response to each ligand is quite different, these observations raise the question of how the specificity of insulin versus IGF-1 signaling is achieved (*7*).

To address this question, we have used cultures of SV40-transformed hepatocytes derived from mice lacking insulin receptors. These cells possess a sizable amount of IGF-1 receptors ($\sim 10^5$ per cell), and yet fail to mediate typical insulin responses, such as stimulation of glycogen synthase activity and inhibition of glucose production (*8*). Thus, comparisons of these cells with normal cells, which possess both insulin and IGF-1 receptors, provide a unique model for dissecting the specificity of insulin and IGF-1 signaling.

In this study, we focused on the signaling pathway that mediates insulin-dependent glycogen synthesis. The stimulation of glycogen synthesis by insulin is complex, in that it involves activation of numerous kinases and phosphatases (*9*). GSK-3 is one of the important kinases that phosphorylate and inactivate glycogen synthase. Recently, it has been suggested that GSK-3 is a direct substrate of the serine/threonine kinase Akt. Phosphorylation of GSK-3 in an insulin-dependent manner leads to its inactivation, and to a decreased level of phosphorylation and increased enzymatic activity of glycogen synthase (*10*). Whether the same mechanism operates in all tissues and whether this is the only or even the main mechanism by which insulin stimulates glycogen synthesis remain to be determined. Interestingly, it has been shown that, in primary cultures of rat hepatocytes, both insulin and EGF inhibit GSK-3 activity, but only insulin stimulates glycogen synthesis, thus raising the question of whether GSK-3 phosphorylation is indeed sufficient for insulin-dependent glycogen synthesis (*11*).

## EXPERIMENTAL PROCEDURES

*Antibodies and Western Blotting.* The following antibodies were used: rabbit polyclonal anti-Akt and anti-phospho Akt (Ser 473) antisera (New England Biolabs, Boston, MA) and sheep polyclonal anti-GSK3α and anti-phospho GSK3α (Ser 21) (Upstate Biotechnologies, Lake Placid, NY). Western blotting was performed according to standard techniques, followed by detection with enhanced chemiluminescence (ECL) (Amersham, Buckinghamshire, U.K.), and quantitation using NIH Image 1.60 software.

*Cell Lines.* Hepatocytes derived from normal mice (WT) and from insulin receptor-deficient mice (−/−) have been described in a previous publication (*8*). Cells were maintained

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[1] Abbreviations: IGF-1, insulin-like growth factor type 1; IRS, insulin receptor substrate; MAP kinase, mitogen-activated protein kinase; PI 3-kinase, phosphatidylinositol 3′-kinase; Shc, SH2 and collagen homology-containing protein; GSK-3, glycogen synthase kinase-3; EGF, epidermal growth factor; Akt, serine/threonine kinase, the cellular product of the viral oncogene v-akt.

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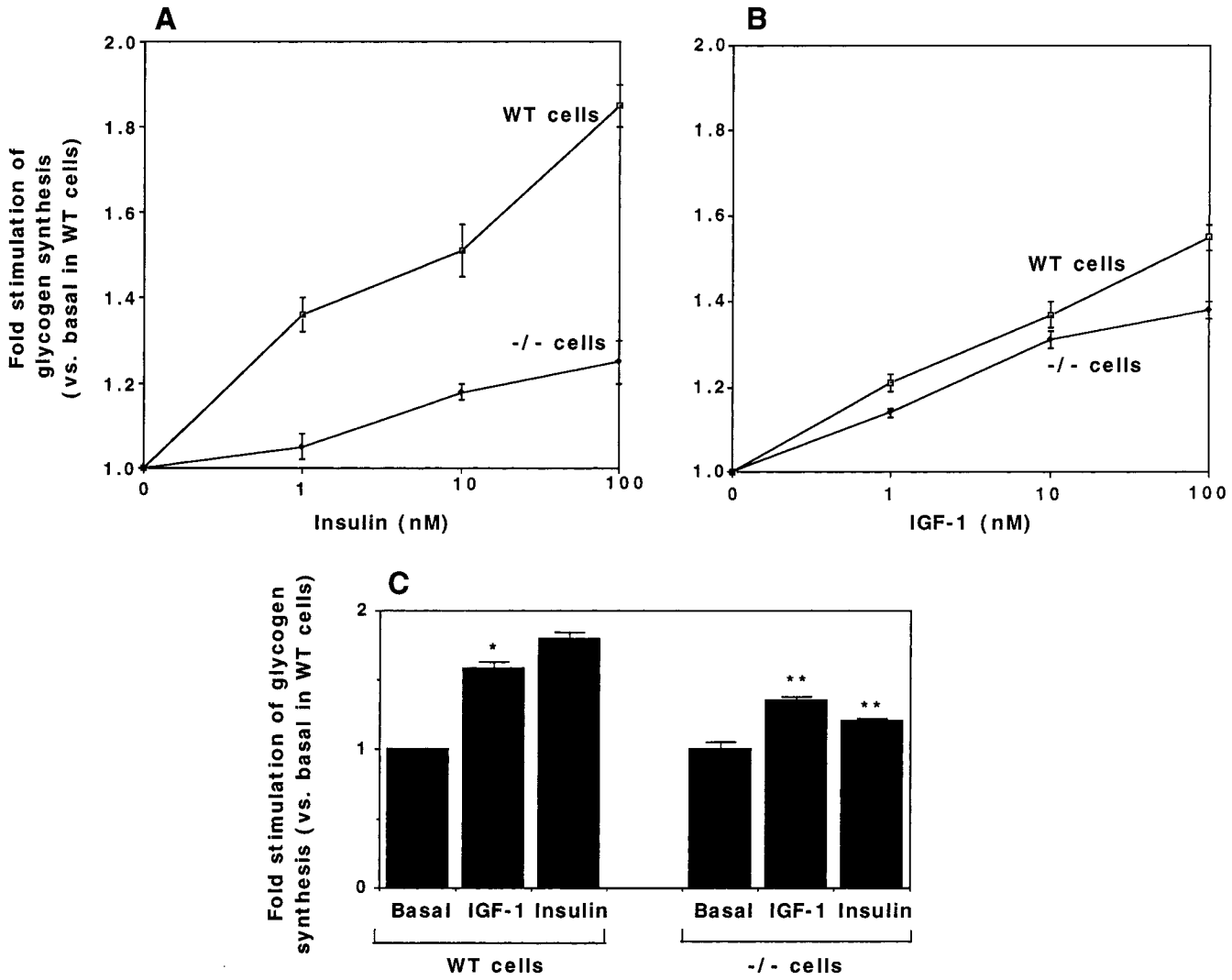


FIGURE 1: Insulin and IGF-1 stimulation of glucose incorporation into glycogen. Glycogen synthesis was assayed by measuring the extent of [U-$^{14}$C]-D-glucose incorporation into glycogen carrier (1 mg/mL). Cells in six-well plates were incubated for 3 h in serum-free medium containing 5.5 mmol/L glucose and 0.33 $\mu$Ci/mL of [U-$^{14}$C]-D-glucose in the absence or presence of insulin (100 nM) and IGF-1 (100 nM). Glycogen was isolated by repeated precipitation with ethanol. Data are the average of three independent determinations. Each determination is the average of four replicates. Results are expressed as fold stimulation from basal incorporation of [U-$^{14}$C]-D-glucose into glycogen in WT cells. (A) Dose−response curve for insulin stimulation of glycogen synthesis in WT and −/− cells. (B) Dose−response curve for IGF-1 stimulation of glycogen synthesis in WT and −/− cells. (C) Comparison of the effects of maximally stimulating doses of insulin (100 nM) and IGF-1 (100 nM) on glycogen synthesis in WT and −/− cells. Asterisks indicate a statistically significant difference as determined by ANOVA. One asterisk indicates $P < 0.05$ between insulin and IGF-1 stimulation in WT cells. Two asterisks indicate $P < 0.01$ between insulin stimulation in WT cells and insulin or IGF-1 stimulation in −/− cells.

in AMEM supplemented with 1 mM L-glutamine, 200 nM dexamethasone, and 4% fetal calf serum at 33 °C.

*Glycogen Synthesis*. The determination of glycogen synthesis was carried out by the incorporation of [U-$^{14}$C]-D-glucose into glycogen. Confluent monolayers of SV40-transformed hepatocytes in six-well plates were incubated overnight in medium containing 1% dialyzed serum, and then for 3 h in serum-free AMEM containing 5.5 mM glucose and 0.33 $\mu$Ci/mL [U-$^{14}$C]-D-glucose in the absence or presence of 100 nM insulin and 100 nM IGF-1 with or without inhibitors (100 nM wortmannin and 100 nM rapamycin). The inhibitors were added 5 min prior to incubation with insulin or IGF-1. The incubation was terminated by removing the medium and rinsing the cells five times in ice-cold PBS. Proteins and lipids were extracted in 20% KOH for 2 h, followed by an additional protein extraction in 8% TCA (w/v), and neutralization in 2 M HCl. Cell extracts were transferred to 15 mL conical tubes, and the wells were rinsed once with 0.3 mL of distilled water to collect the remaining material. The cell extracts were boiled for 5 min, and 1 mg of glycogen was added as a carrier to each sample. Total glycogen was isolated by precipitating twice with 80% ethanol (final concentration) for 2 h at −20 °C followed by centrifugation at 1100*g* for 10 min. After the pellets had been redissolved in distilled water, the samples were precipitated again as described above. The amount of radioactivity incorporated was determined by liquid scintillation counting. Results were expressed as the fold stimulation over basal incorporation of [U-$^{14}$C]-D-glucose into glycogen in WT cells.

*Akt Activity*. Confluent monolayers of SV40-transformed hepatocytes in 6 cm dishes were washed once with PBS and incubated for 18 h at 37 °C in AMEM supplemented with 1% dialyzed fetal calf serum. Thereafter, cells were incubated

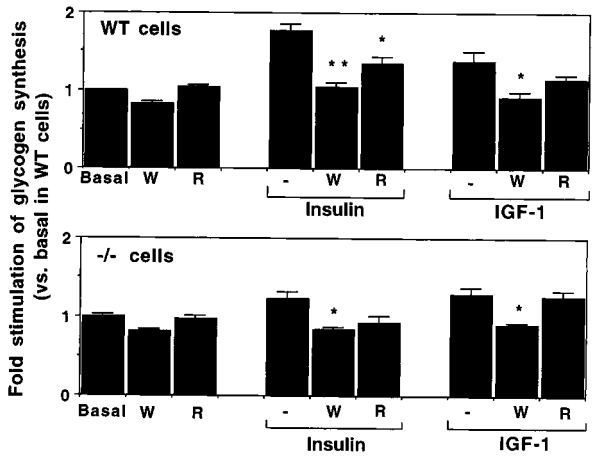


FIGURE 2: Effects of wortmannin and rapamycin on insulin- and IGF1-stimulated glycogen synthesis in WT and −/− cells. Cell monolayers in six-well plates were incubated in serum-free medium overnight and then incubated in the presence of insulin (100 nM) or IGF-1 (100 nM) in the absence or presence of wortmannin (W, 100 nM) or rapamycin (R, 100 nM) for 3 h. Control buffers were supplemented with the same final concentration of DMSO as buffers containing the inhibitors. Each inhibitor was added 5 min prior to incubation with insulin or IGF-1. The extent of glycogen synthesis was measured as described in Experimental Procedures. Asterisks indicate a statistically significant difference between the values observed in insulin- or IGF-1-treated WT and −/− cells in the absence or presence of inhibitors. One asterisk indicates $P < 0.05$; two asterisks indicate $P < 0.01$ as determined by ANOVA.

in the absence or presence of 100 nM insulin or 100 nM IGF-1 for the indicated time periods. At the end of the incubation, cells were washed once with PBS and frozen in a dry ice/ethanol bath. Monolayers were lysed in 500 $\mu$L of ice-cold lysis buffer containing 50 mM HEPES (pH 7.6), 1% Triton X-100, 1 mg/mL bacitracin, 1 mM PMSF, 1 mM $Na_3VO_4$, 10 mM NaF, 30 mM $NaPP_i$, 150 mM NaCl, and 1 mM EDTA. The lysates were clarified by ultracentrifugation and subjected to immunoprecipitation with polyclonal anti-Akt antibodies (*12*). This antiserum has been shown to recognize all three isoforms of Akt (Akt 1−3). Immunoprecipitates were washed three times in 0.1% Triton buffer and once in kinase buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 1 mM DTT and then incubated for 30 min at 30 °C in 30 $\mu$L of kinase mixture containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 25 $\mu$M ATP, 1 $\mu$M PKAI, 3 $\mu$Ci of [$\gamma$-$^{32}$P]ATP, and 6 $\mu$g of histone 2B as a substrate. The reaction was terminated by the addition of SDS sample buffer, followed by boiling for 5 min. The samples were electrophoresed on 14% SDS−polyacrylamide gels. The radioactive bands corresponding to histone 2B were detected and quantitated with an image analyzer (Molecular Dynamics Storm 860). Nonspecific activity was determined by incubating extracts with normal rabbit serum. The Akt activity in the normal rabbit immunoglobulin precipitates was ∼10% of basal activity.

In parallel experiments, Akt phosphorylation was assessed using a phosphospecific antibody directed against Ser 473 in the amino acid sequence of murine Akt. Immunoprecipitations were performed in duplicate with either an anti-akt or anti-phospho Akt (Ser 473) antibody, followed by immunoblotting with the anti-Akt antibody. All Akt antibodies employed have been reported to react with all Akt isoforms.

*GSK-3 Phosphorylation.* Confluent monolayers of SV40-transformed hepatocytes in 6 cm dishes were treated as described above for the measurement of Akt activity, except that the lysis buffer contained 50 mM Tris-HCl (pH 7.5), 1% NP, 10% glycerol, 137 mM NaCl, 1 mM $Na_3VO_4$, and 1 mM PMSF. Lysates were immunoprecipitated with 1 $\mu$g of GSK-3$\alpha$ (Upstate Biotechnology) and GSK-3$\beta$ antibodies (Transduction Laboratories), and immunocomplexes were captured on protein G beads (50:50 slurry). Immunoprecipitates were washed with 100 mM Tris-HCl (pH 7.5) and 0.5 M LiCl, then with 10 mM Tris-HCl (pH 7.5), and finally twice with 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 1 mM DTT. The precipitates were assayed by incubating for 10 min at 30 °C in a final volume of 30 $\mu$L of a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 50 $\mu$M ATP, the glycogen synthase peptide substrate (100 $\mu$M), and 3 $\mu$Ci of [$\gamma$-$^{32}$P]ATP. The reactions were terminated by spotting on P81 phosphocellulose paper squares (2 cm × 2 cm), followed by washing once in 3% $H_3PO_4$ for 10 min, and three times in 1% $H_3PO_4$ for each 10 min. All squares were dried, and the amount of radioactivity was measured by liquid scintillation counting. Results were expressed as a percentage decrease from the basal activity.

*Statistical Analysis.* Data were analyzed by ANOVA followed by Duncan's test for multiple comparisons using the Statsview software (Abacus Concepts, Inc.).

## RESULTS

We have previously shown that hepatocytes lacking insulin receptors are unable to stimulate glycogen synthase activity, despite the expression of IGF-1 receptors (*8*). Since activation of glycogen synthase is the main mechanism by which insulin increases the level of glycogen synthesis, we used insulin receptor-deficient hepatocytes (−/− cells) and control hepatocytes (WT cells) to examine whether insulin and IGF-1 receptors differ in their ability to stimulate glycogen synthesis, and to activate elements in the signaling pathway to glycogen synthase. As shown in previous publications (*8*, *13*), both cell types express similar amounts of IGF-1 receptors (∼ $10^5$ per cell). In addition, WT cells express ∼$10^5$ insulin receptors per cell.

In WT cells, insulin stimulated glycogen synthesis in a dose-dependent manner, as assessed by [U-$^{14}$C]-D-glucose incorporation into glycogen (Figure 1A). At the highest dose that was employed (100 nM), insulin stimulated glycogen synthesis by ∼2-fold in WT cells, and by 1.2-fold in −/− cells ($P < 0.01$ by ANOVA; see Figure 1C). IGF-1 also stimulated glycogen synthesis in a dose-dependent manner. The maximal effect of IGF-1 was ∼1.5-fold in WT cells and 1.35-fold in −/− cells ($P$ = ns). At maximally stimulating doses (100 nM), the effect of IGF-1 was significantly smaller than the effect of insulin in both WT cells (25% decrease, $P < 0.05$ by ANOVA) and −/− cells (60% decrease, $P < 0.01$ by ANOVA). These data are summarized in Figure 1C and suggest that IGF-1 is less potent than insulin in stimulating glycogen synthesis, and that part of the effect of IGF-1 in WT cells is due to cross-activation of insulin receptors, or to IGF-1 binding to hybrid insulin−IGF-1 receptors (*14*).

To evaluate whether both insulin and IGF-1 receptors utilize the same signaling pathways leading to glycogen

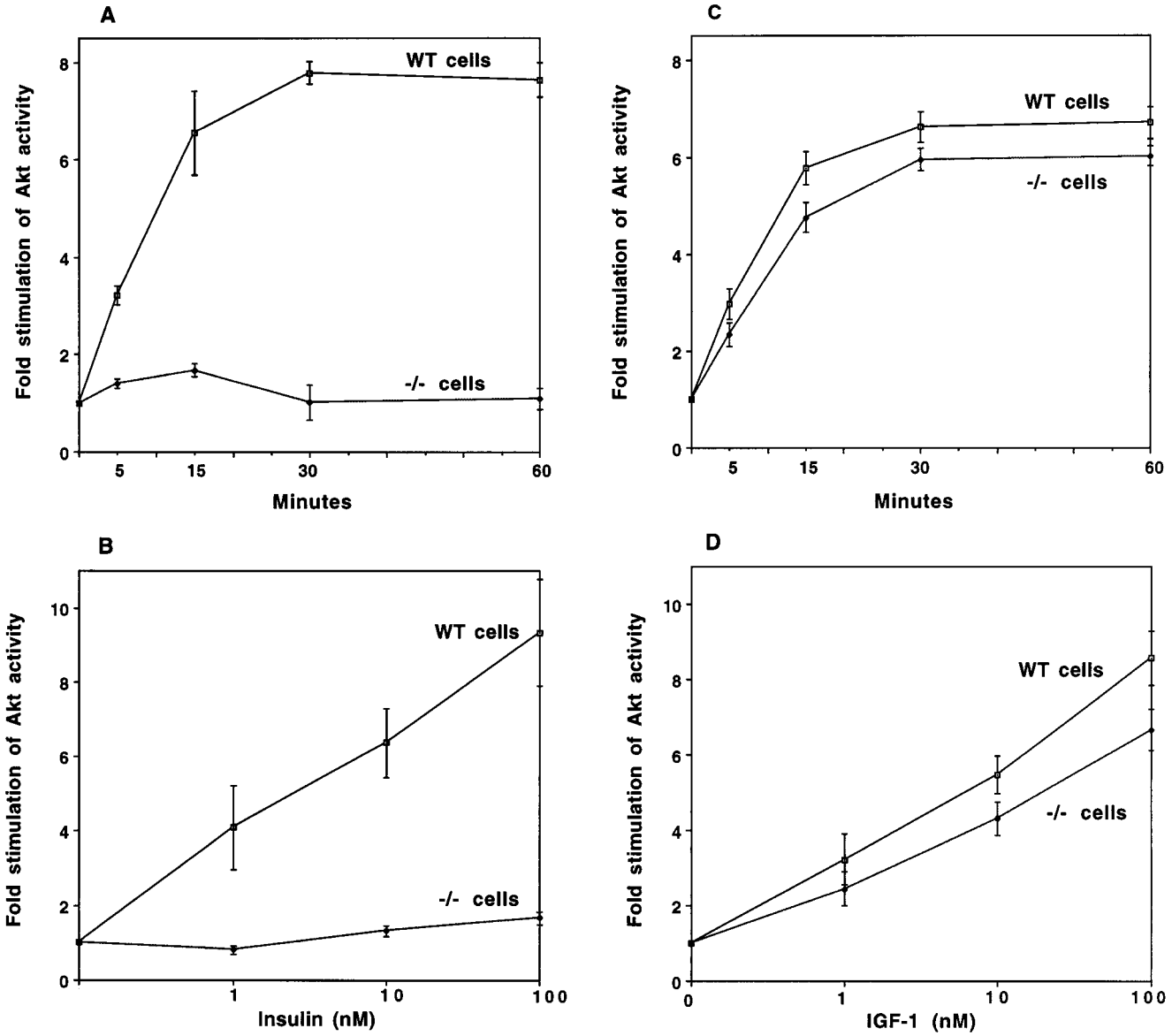


FIGURE 3: Time course and dose−response curves of insulin- and IGF1-induced Akt stimulation in WT and −/− cells. Confluent monolayers of SV40-transformed hepatocytes were incubated for 18 h in serum-free medium and then with 100 nM insulin for the indicated time periods at 37 °C. Akt was isolated by immunoprecipitation, and its kinase activity was measured using histone 2B as a substrate. (A) Time course of insulin stimulation of Akt activity in WT and −/− cells. (B) Dose−response curve of insulin stimulation of Akt activity in WT and −/− cells. (C) Time course of IGF-1 stimulation of Akt activity in WT and −/− cells. (D) Dose−response curve of IGF-1 stimulation of Akt activity in WT and −/− cells. The antibody used in these experiments has been shown to cross-react with all three Akt isoforms (*12*). The data represent the average of three independent determinations. Each experiment was performed in triplicate.

synthesis, we studied the effects of the PI 3-kinase inhibitor wortmannin and of the p70 S6 kinase inhibitor rapamycin on glycogen synthesis (Figure 2). In both WT and −/− cells, the PI 3-kinase inhibitor wortmannin completely abolished the response to 100 nM insulin and IGF-1, consistent with the notion that PI 3-kinase-dependent activity is required to stimulate glycogen synthesis. Interestingly, the effect of rapamycin was different in WT and −/− cells. In WT cells, rapamycin treatment blocked 61% of the effect of insulin on glycogen synthesis and 54% of the effect of IGF-1. In −/− cells, rapamycin had virtually no effect on IGF-1-stimulated glycogen synthesis. The conclusion of these experiments is that PI 3-kinase plays a permissive role in insulin and IGF-1 stimulation of glycogen synthesis, and that rapamycin-sensitive pathways play a role in insulin- but not in IGF1-stimulated glycogen synthesis.

Activation of the serine/threonine kinase Akt is thought to play a pivotal role in insulin's ability to stimulate glycogen synthesis (*10*, *12*, *15*, *16*). In WT cells, insulin stimulated Akt kinase to phosphorylate the exogenous substrate histone 2B ~8-fold in a time-dependent manner. In contrast, insulin stimulated Akt in −/− cells 2-fold and in a transient manner (Figure 3A). The effect of insulin was dose-dependent, and reached its peak at 100 nM in WT cells, but did not differ from the basal effect in −/− cells (Figure 3B) ($P < 0.05$ by ANOVA). IGF-1 stimulated Akt 6.5-fold in WT cells and 5.8-fold in −/− cells (Figure 3C) ($P < 0.05$ by ANOVA). The effect of IGF-1 was also dose-dependent and reached its maximum at 100 nM (Figure 3D).

The effect of insulin in stimulating Akt activity was compared to that of IGF-1 in both cell types. In WT cells, insulin (100 nM) stimulated Akt by ~10-fold and IGF-1 (100

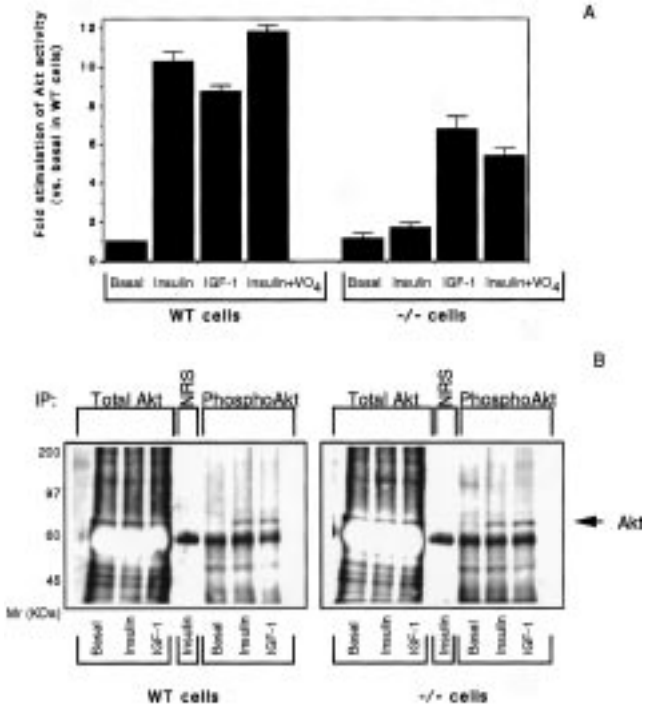


FIGURE 4: (A) Comparison of the effects of insulin, IGF-1, and vanadate on the stimulation of Akt kinase in SV40-transformed hepatocytes. Cells were treated as indicated in Experimental Procedures with either 100 nM insulin, 100 nM IGF-1, or 100 nM insulin and 0.1 mM vanadate, and Akt activity was measured in immunopellets. The Akt activity detected in immunoprecipitates with normal rabbit immunoglobulin was subtracted as background. This activity was ~10% of the basal activity. Results are expressed as fold stimulation over basal values in WT cells. (B) Immunoprecipitation of Akt in insulin- and IGF-1-treated cells using a phosphospecific antibody. Cells were treated as described with 100 nM insulin or IGF-1. Thereafter, Akt was immunoprecipitated using either a polyclonal rabbit antiserum (total Akt) or a phosphospecific antibody that recognizes phosphoserine 473 (New England Biolabs). NRS is normal rabbit serum.

nM) by ~8-fold (Figure 4A). Incubation of cells with vanadate (0.1 mM) potentiated insulin's effect. In −/− cells, insulin had no effect on Akt, but IGF-1 stimulated Akt activity ~7-fold, or ~70% of the values observed in insulin-treated WT cells, and ~80% of the values in IGF1-treated WT cells. Vanadate was as potent as IGF-1 in −/− cells. These results are consistent with the notion that both receptors can activate Akt in SV40-transformed murine hepatocytes. The failure of insulin to stimulate Akt in −/− cells can be explained by the fact that insulin binds poorly to the IGF-1 receptor.

The extent of activation of the Akt kinase was correlated with the extent of Akt phosphorylation using a phosphospecific antibody that recognizes the carboxy-terminal phosphorylation site of Akt (Ser 473). In WT cells, insulin increased the extent of Akt phosphorylation ~10-fold, and IGF-1 increased the extent of Akt phosphorylation ~9-fold. In contrast, in −/− cells, insulin increased the extent of Akt phosphorylation ~7-fold and IGF-1 11-fold (Figure 4B). Thus, both the extent of Akt phosphorylation and Akt activity were impaired in −/− cells. However, Akt activity in −/− cells appeared to be decreased more profoundly than the extent of Akt phosphorylation. This difference could be due to the different antibodies employed in the two assays.

The effect of insulin on glycogen synthesis is thought to involve Akt-mediated inactivation of GSK-3, one the kinases that phosphorylate and inactivate glycogen synthase (*10*). We therefore measured the level of GSK-3 inactivation in WT and −/− cells following insulin and IGF-1 stimulation. In WT cells, insulin caused a 30% decrease in the GSK-3 activity. The effect of IGF-1 in WT hepatocytes was decreased by 40% compared to that of insulin. Both ligands reached the maximal effect on GSK-3 activity after 20 min; however, the onset of insulin's effect was more rapid, being apparent after stimulation for 1 min (Figure 5A). In −/− cells, insulin failed to affect GSK-3 activity, whereas the effect of IGF-1 was decreased by 50% compared to the effect of insulin in WT cells (Figure 5B). Thus, the level of inhibition of GSK-3 activity by IGF-1 was reduced by ~40% in WT cells and by ~50% in −/− cells compared to the level of insulin inhibition of the enzyme in WT cells. Interestingly, the effect of IGF-1 receptors on GSK-3 activity was similar in both WT and −/− cells.

## DISCUSSION

Insulin and IGF-1 receptors belong to the same subfamily of receptor tyrosine kinases. However, they are thought to mediate different effects on target tissues, as evidenced from the phenotypes of the relevant knock-out mice (*3*, *4*, *17*). These functional differences are intriguing, since both receptors utilize the same set of intracellular substrates and activate similar signaling pathways. Several theories have been advanced to explain the difference between insulin and IGF-1 receptor signaling (*1*, *7*). Some have proposed that the different tissue distribution of receptors would influence the response (*18*); some have argued for a role of hybrid receptors (*19*), and some have favored the explanation that the different receptors generate qualitatively different signals, for example, in the subcellular distribution (*20*) or duration of the stimulus (*21*). A complicating factor in the interpretation of these studies is that they are either conducted in nonphysiologic target cells by way of overexpression of receptors or carried out in target cells by inhibition of receptor function with dominant negative constructs. Both approaches have technical pitfalls, which may alter the fine balance required for a physiologic response. Some studies have addressed this problem via transfection of chimeric receptors into target cells. Interestingly, these studies indicate that chimeric TrkC−insulin receptors are more potent than chimeric TrkC−IGF-1 receptors in stimulating glycogen synthesis (*22*). Using the same cell lines described in this report, we have suggested that the specificity of signaling may be explained by the preferential use of different substrates by insulin and IGF-1 receptors, the former being preferentially coupled to IRS-2 and the latter to IRS-1 (8). This conclusion is also borne out by the results of targeted ablation of IRS-1 and IRS-2 in mice (*23*−*25*).

The mechanism by which insulin stimulates glycogen synthesis in different tissues has been intensively investigated. Recent work has suggested that insulin activates the PI 3-kinase-dependent kinase Akt, which in turn is able to phosphorylate and inactivate GSK-3, thereby leading to a reduced level of phosphorylation of sites 4, 3a, 3b, and 3c on glycogen synthase and to increased glycogen synthase activity (*9*, *10*). It appears, however, that this mechanism is not the only one by which insulin regulates glycogen

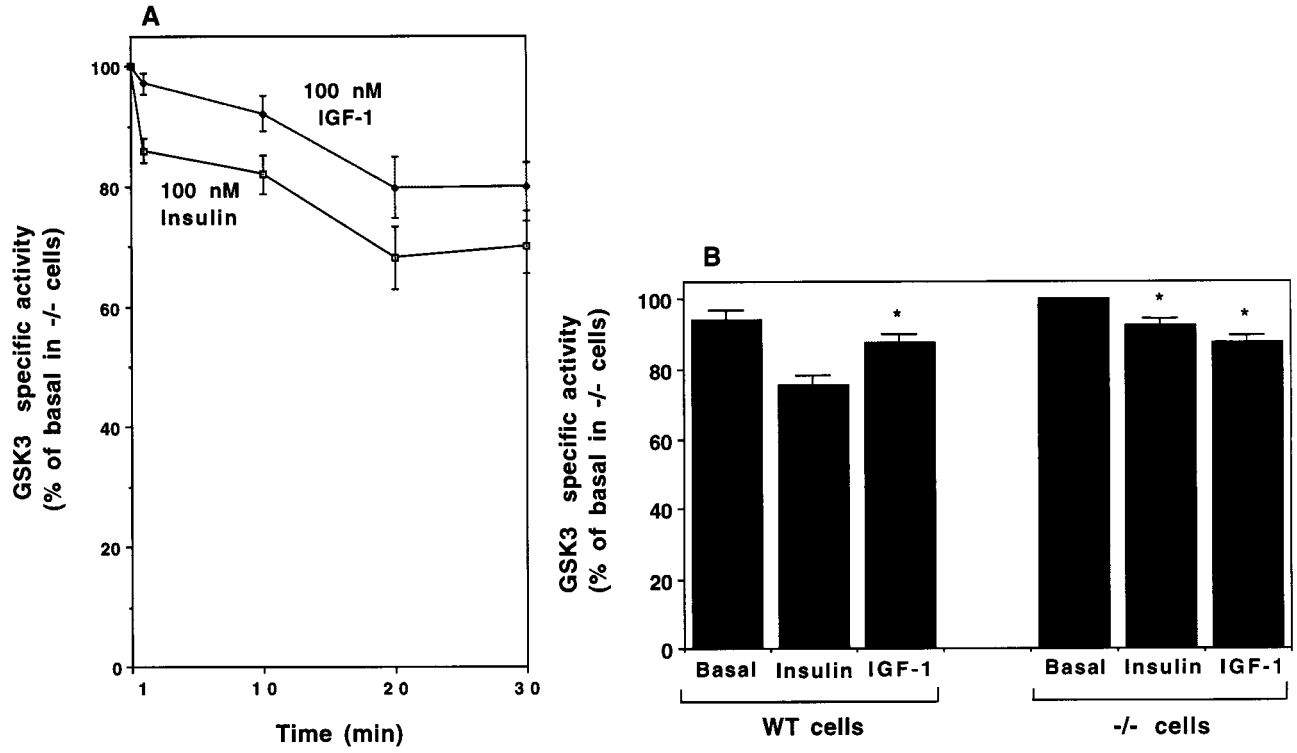


FIGURE 5: (A) Effect of insulin and IGF-1 on GSK-3 activity in WT hepatocytes. Cells were incubated in serum-free medium overnight, and insulin (100 nM) or IGF-1 (100 nM) was added for the indicated time periods. Thereafter, GSK-3 was precipitated using a mixture of a polyclonal antibody to GSK-3α and a monoclonal antibody to GSK-3β, and its kinase activity was measured using a phosphopeptide derived from the consensus glycogen synthase phosphorylation site. Data are plotted as the percentage decrease from basal (non-insulin-stimulated) values. (B) Effect of insulin and IGF-1 on GSK-3 activity in WT and −/− cells. Confluent monolayers were incubated overnight in serum-free medium, and then treated with insulin (100 nM) or IGF-1 (100 nM) for 30 min. GSK-3 was isolated by immunoprecipitation, and GSK-3 activity was measured as indicated for panel A. An asterisk indicates a statistically significant difference ($P < 0.05$) as determined by ANOVA between the effect of insulin and that of IGF-1 in WT cells, and between the effect of insulin in WT cells and that of insulin or IGF-1 in −/− cells.

synthesis. On the basis of studies with the inhibitor rapamycin, it appears that p70 S6 kinase is also involved in this process in 3T3-L1 adipocytes (*26*) and in muscle (*27*), but not in hepatocytes (*11*). Furthermore, it has been proposed that insulin activates one or more glycogen synthase phosphatases, even though the exact sequence of mediators in this process remains controversial. Interestingly, this mechanism appears to play a pivotal role in the stimulation of glycogen synthesis by insulin in 3T3-L1 cells, as they undergo differentiation from fibroblasts into adipocytes (*28*).

In primary cultures of rat hepatocytes, both insulin and EGF stimulate Akt activity and GSK-3 phosphorylation. Yet only insulin is able to stimulate glycogen synthesis (*11*). This study indicates that GSK-3 phosphorylation is not sufficient for insulin action in this cell type. A novel finding of our studies is that insulin and IGF-1 receptors differ in their ability to inactivate GSK-3, even though they are both able to activate Akt to the same extent.

The difference between insulin and IGF-1 in inactivating GSK-3 is not simply a reflection of a different receptor number in the two cell types, since IGF-1 is less potent than insulin also in WT cells, where presumably some of its effect is mediated through insulin receptors. Moreover, the effect of insulin on Akt activity in WT cells is similar to that of IGF-1 in −/− cells. Thus, the difference in GSK-3 inactivation cannot be entirely explained by a difference in the extent of Akt activation. These findings, along with the inhibitor experiments, suggest that Akt is necessary, but not sufficient to inactivate GSK-3 in response to insulin. We conclude that there is a direct correlation between GSK-3 activity and the extent of glycogen synthesis, but not between the extents of Akt activation and GSK-3 inhibition. We propose that additional pathways play a role in the stimulation of glycogen synthesis by insulin in liver cells.

The identity of these pathways can be surmised on the basis of the experiments carried out in the presence of rapamycin. The effect of insulin on glycogen synthesis in WT cells is exquisitely sensitive to rapamycin, compared to the effect of IGF-1 in −/− cells. It is possible that insulin activates a rapamycin-sensitive kinase that phosphorylates GSK-3. An alternative possibility is that the rapamycin-sensitive pathway activates a glycogen synthase phosphatase, but this possibility would not account for the larger inhibitory effect of insulin on GSK-3 compared to that on IGF-1. Our data differ from those of Peak et al. (*11*), who have reported that glycogen synthesis in response to insulin in primary hepatocytes is not inhibited by rapamycin. There might be subtle differences between primary hepatocytes and SV40-transformed hepatocytes that account for this discrepancy. A possible explanation is that the level of expression of IGF-1 receptors in primary hepatocytes is increased, and is able to confer rapamycin independence on glycogen synthesis.

In these experiments, insulin reproducibly caused a ~2-fold increase in the level of glycogen synthesis. While this may appear to be a modest effect, it should be remembered

that, in mice, hepatic glycogen is about 15-fold more abundant than muscle glycogen (D. Accili, unpublished data). Therefore, even a small variation in the level of hepatic glycogen synthesis has the potential of affecting whole body glucose homeostasis in a profound manner. In view of the increasing use of genetically engineered mice as a tool in analyzing glucose metabolism, and in view of recent observations pointing to the liver as a primary determinant in this process (*29*−*31*), we believe that our data have broad relevance in investigations of insulin action and insulin resistance in mice.

In summary, our interpretation of these findings is that both insulin and IGF-1 receptors activate PI 3-kinase-dependent pathways that lead to glycogen synthesis. However, while the effect of IGF-1 can be explained entirely by its ability to activate Akt in a PI 3-kinase-dependent manner, the effect of insulin requires the PI 3-kinase-dependent activation of Akt and a rapamycin-sensitive pathway. Whether the rapamycin-dependent pathway involves activation of p70 S6 kinase and whether GSK-3 is a direct target of p70 S6 kinase remain open questions. Further progress will require the identification of the rapamycin-sensitive components of the insulin receptor pathway.

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