GRK2 Negatively Regulates Glycogen Synthesis in Mouse Liver FL83B Cells

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G-protein-coupled receptor (GPCR) kinases (GRKs) are serine/threonine kinases that desensitize agonist-occupied classical GPCRs. Although the insulin receptor (IR) is a tyrosine kinase receptor, the IR also couples to G-proteins and utilizes G-protein signaling components. The present study was designed to test the hypothesis that GRK2 negatively regulates IR signaling. FL83B cells, derived from mouse liver, were treated with insulin and membrane translocation of GRK2 was determined using immunofluorescence and Western blotting. Insulin caused an increase in the translocation of GRK-2 from cytosol to the plasma membrane. To determine the role of GRK2 in IR signaling, GRK2 was selectively down-regulated (∼by 90%) in FL83B cells using a small interfering RNA technique. Basal as well as insulin-induced glycogen synthesis (measured by D-[U-14C]glucose incorporation) was increased in GRK2-deficient cells compared with control cells. Similarly, GRK2 deficiency increased the basal and insulin-stimulated phosphorylation of Tyr612 in insulin receptor subunit 1 was significantly increased while phosphorylation of Ser307 was decreased in GRK2-deficient cells compared with control cells. Chronic insulin treatment (24 h) in control cells caused an increase in GRK2 (56%) and a decrease in IR (50%) expression associated with the absence of an increase in glycogen synthesis, suggesting impairment of IR function. However, chronic insulin treatment (24 h) did not decrease IR expression or impair IR effects on glycogen synthesis in GRK2-deficient cells. We conclude that (i) GRK2 negatively regulates basal and insulin-stimulated glycogen synthesis via a post-IR signaling mechanism, and (ii) GRK2 may contribute to reduced IR expression and function during chronic insulin exposure.

The primary biological effect of insulin is maintenance of glucose homeostasis. The effects of insulin are mediated by binding to the insulin receptor, a member of the tyrosine kinase receptor family (1). The insulin receptor is a heterotetrameric receptor, consisting of two extracellular α and two transmembrane β subunits (2). Insulin binding to the α-subunit of insulin receptor causes autophosphorylation of the β-subunit at specific tyrosine residues (3). Autophosphorylation of the β-subunit increases the tyrosine kinase activity of the receptor toward other protein substrates, resulting in the tyrosine phosphorylation of a family of insulin receptor substrate proteins including IRS1,2 IRS2, and Shc. The IRS proteins serve as docking proteins for other intracellular proteins containing Src homology 2 domains such as phosphatidylinositol 3-kinase (PI 3-kinase) and Grb2. The binding of these proteins to IRS proteins transmits the signal downstream, leading to the metabolic and mitogenic effects of insulin (4). For example, the interaction of phosphorylated IRS1/2 with PI 3-kinase leads to activation of protein kinase B/Akt, producing many effects such as GLUT4 translocation, protein synthesis, and inactivation of glycogen synthase kinase 3 (GSK3). The GSK3, a serine/threonine kinase, phosphorylates glycogen synthase and inactivates it. As glycogen synthase is a rate-limiting enzyme in glycogen synthesis, inactivation of GSK3 by insulin removes the inhibitory effect of GSK3 on glycogen synthase and leads to increased glycogen synthesis (5–7).

In addition to signaling via its tyrosine kinase activity, the insulin receptor also couples to the G-proteins Gα and Gq (8, 9). This IR/G-protein coupling is required for some actions of insulin. For example, insulin resistance develops in adipocytes when Gq is down-regulated (10). Moreover, in 3T3L1 adipocytes IR signaling via Gαq/11 is required for insulin-induced GLUT4 translocation (11). The signaling of receptors via G-proteins is regulated by G protein-coupled receptor kinases (GRKs), a family of serine/threonine kinases that participate in G-protein-coupled receptor (GPCR) desensitization. GRK2 can produce GPCR desensitization either by phosphorylation-dependent or phosphorylation-independent mechanisms. Phosphorylation of GPCRs by GRKs results in the uncoupling of GPCRs from heterotrimeric G-proteins and receptor internalization (12). Recently, GRK2 also has been shown to decrease GPCR signaling by sequestering Gαq/11 proteins via their RGS domain (13, 14). This phosphorylation-independent mechanism was recently shown to account for the negative regulation of insulin-stimulated glucose transport by GRK2 in 3T3L1 adipocytes (15).

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2 The abbreviations used are: IRS, insulin receptor substrate; PI 3-kinase, phosphatidylinositol 3-kinase; GSK3, glycogen synthase kinase 3; GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinases; IR, insulin receptor; siRNA, small interfering RNA; PBS, phosphate-buffered saline; ANOVA, analysis of variance.
The present study was designed to determine whether GRK2 modulates insulin receptor signaling and insulin-stimulated glycogen synthesis in mouse liver FL83B cells. Our data show that insulin induces the translocation of GRK2 from cytosol to the plasma membrane. Depleting cells of GRK2 caused an increase in basal as well as insulin-stimulated phosphorylation of GSK3α, leading to increased glycogen synthesis. GRK2 depletion also increased the basal and insulin-induced tyrosine phosphorylation of IRS1 at residue 612, whereas the phosphorylation at serine residue 307 was decreased in GRK2-deficient cells. Finally, we found that chronic insulin treatment (24 h) decreased IR expression and IR-stimulated glycogen synthesis. These changes in IR expression and IR-stimulated glycogen synthesis by chronic insulin treatment were not observed in GRK2-depleted cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—The FL83B cell line was obtained from American Tissue Culture Collection (Manassas, VA). Anti-GRK2 antibody, anti-insulin receptor β-subunit antibody, and anti-GRK3 antibody were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-Cy3 antibody was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Anti-GSK3α, anti-p-S21 GS3α, anti-IRS1, anti-p-S307 IRS1, and anti-p-Y612 IRS1 antibodies were purchased from BIOSOURCE (Camarillo, CA). Phosphorylated tyrosine 612 is the pYM anti-pY612 IRS1 antibodies were purchased from BIOSOURCE (Camarillo, CA). Phosphorylated tyrosine 612 is the pYM consensus motif that binds to PI 3-kinase. GRK2 siRNA was purchased from Dharmacon Inc. (Chicago, IL). Oligo-consensus motif that binds to PI 3-kinase. GRK2 siRNA was purchased from Pierce.

**Plasma Membrane Preparation**—The cells were treated without/with insulin (100 nm for 5 or 10 min), collected in ice-cold buffer containing 10 mm potassium acetate and 18 mm HEPES, protease inhibitor mixture, pH 7.2, and processed for plasma membrane preparation (16). Briefly, the samples were incubated on ice for 10 min and then passed 10 times through a 22-gauge needle. The buffer concentration of the resulting cell lysate was adjusted to 25 mm potassium acetate and 125 mm HEPES. The samples were centrifuged at 3,000 × g for 5 min at 4 °C. The postnuclear supernatant was collected and centrifuged at 66,000 × g for 30 min at 4 °C. The resulting pellet was suspended in 25 mm potassium acetate, 125 mm HEPES, pH 7.2, and used as plasma membrane fraction. The purity of the plasma membrane fraction was checked using Rab-5 antibody.

**Immunoblotting**—The cells were treated without/with insulin (100 nm) acutely (5 or 10 min) or chronically (24 h) at 37 °C. After treatment, a cell lysate was collected in lysis buffer (mmol/liter: HEPES 50, NaCl 150, Na2VO4 4, Na3H2P2O7 20, NaF 10, EDTA 10, Triton X-100 1%, glycerol 10%). Equal amounts of protein were subjected to SDS-PAGE and electroblotted to polyvinylidene fluoride membranes (blot). The blots were blocked with 4% fat-free milk and then incubated with primary antibody (anti-insulin receptor β-subunit, anti-phosphotyrosine, anti-GRK2, anti-GRK3, anti-GSK3α, anti-pS307 IRS1, anti-Y612 IRS1, or anti-IRS1 antibody). The blots were then washed, treated with anti-rabbit or anti-mouse IgG coupled to horseradish peroxidase, and developed with enhanced chemiluminescence reagents. The bands were visualized on x-ray films and the intensity of each band was measured by densitometry using a Kodak Image analyzer. The specificity of the antibodies for GRK2 and insulin receptor β-subunit was confirmed.
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by using the appropriate blocking peptides that resulted in the disappearance of the appropriate protein band (data not shown).

Glycogen Synthesis Assay—The accumulation of glycogen in intact cells was determined using a previously described method (17). Briefly, plated cells (80—90% confluent) were incubated overnight in glucose-free media with serum. Then, the cells were treated without (basal) or with insulin (100 nm) for 10 min followed by incubation with D-[U-14C]glucose (2 μCi/ml) for 3 h at 37 °C. After the incubation, the cells were washed with ice-cold PBS, solubilized in 30% KOH, and heated at 100 °C for 30 min. Aliquots were then taken for protein estimation. Acetone and Na2SO4 were added to the remainder of each sample, and the samples were kept on ice for 30 min to allow precipitation of glycogen. The samples were centrifuged at 8,000 × g for 10 min, and D-[U-14C]glucose incorporation into glycogen was determined by scintillation counting. Glycogen synthesis is expressed as nanomoles of glucose incorporated/mg of protein/3 h. Protein in plasma membranes and cell lysates was measured using BCA protein assay kit, which is based on the biuret reaction detected with bicinchoninic acid (18).

Statistical Analysis—Student’s t test/one way ANOVA followed by Newman-Keuls multiple comparison test was used to compare variation between groups. Statistical analysis was done using GraphPad Prism, version 4.01 (Graph Pad Software, San Diego, CA). A value of p < 0.05 was considered statistically significant.

RESULTS

Effect of Insulin Treatment on GRK2 Localization—To determine whether insulin induces GRK2 translocation from cytosol to the plasma membrane, we utilized immunofluorescence and Western blot analysis. The FL83B cells were treated with insulin for 5 or 10 min. GRK2 was labeled with anti-GRK2 antibody and Cy3-conjugated secondary antibody and the cells were observed under the microscope. Insulin treatment increased the translocation of GRK2 from cytosol to the plasma membrane (Fig. 1A).

GRK2 localization also was determined in the isolated plasma membrane fraction by Western blotting. Insulin treatment (100 nm) caused an increase in GRK2 (~80 kDa) localization in the plasma membrane by 29 and 38% at 5 and 10 min, respectively (Fig. 1B). The purity of the plasma membrane fraction was tested by assessing the content of Rab-5, a cytosolic protein. Rab-5 antibody labeled a band in the cytosolic fraction, whereas no band was detected in the plasma membrane fraction (data not shown) suggesting that the plasma membrane fractions are devoid of cytosolic proteins.

Effect of GRK2 siRNA on GRK2 Expression—To down-regulate GRK2, we utilized the GRK2 siRNA technique. Treatment of FL83B cells with GRK2 siRNA (2 μM) for 48 h decreased the levels of GRK2 in the cell by more than 90% (Fig. 2A). This concentration of siRNA was used in all subsequent experiments. GRK2 siRNA did not affect the expression of GRK3 (Fig. 2A), a member of GRK2 subfamily, suggesting that our GRK2 siRNA is specific for GRK2. Similar treatment with scrambled sequence used as a control, had no effect on the GRK2 expression (Fig. 2B).

Effect of Insulin on Glycogen Synthesis in Control and GRK2-deficient Cells—Insulin treatment increased the incorporation of glucose into glycogen by 30 nmol/mg of protein over basal in control cells, whereas the increase was 52 nmol/mg of protein over basal in GRK2-deficient cells (Fig. 3). The insulin-stimulated increase in glycogen synthesis in scrambled sequence-treated cells (28 nmol/mg of protein over basal) was similar to that in control cells. However, the basal value for glycogen syn-
thsis in GRK2-deficient cells (36.5 ± 4.5 nmol/mg of protein) was higher than the basal value in the control (21.6 ± 3.5) and scrambled sequence-treated cells (23.3 ± 6.5). It should be noted that insulin receptor expression was not higher in GRK2-deficient cells (82.5%) as compared with the control cells (Fig. 7C).

**Effect of Insulin on Ser Phosphorylation of GSK3α in Control and GRK2-deficient Cells**—As shown in Fig. 4, Ser(P)21-GSK3α antibody labeled a single band (~51 kDa). After the bands were recorded, the blot was stripped and reprobed with anti-GSK-3 antibody. Density of Ser(P)21-GSK-3 was normalized with total GSK-3. Data revealed that the basal phosphorylation of Ser21 at GSK-3 was increased in GRK2 deficient compared with control cells. Insulin (100 nM) treatment (5 and 10 min) caused an increase in the Ser21 phosphorylation of GSK3 over respective basals. However, the insulin-stimulated Ser 21 phosphorylation of GSK-3 was greater in GRK2-deficient cells (Fig. 4). Phosphorylation activates GSK3 activity leading to an increase in glycogen synthesis. Thus, the greater phosphorylation (basal and insulin-induced) of GSK3α is consistent with the enhanced glycogen synthesis in GRK2-deficient cells. The insulin-stimulated phosphorylation of GSK3β in GRK2-deficient hepatocytes was similar to that in control cells (data not shown).

**Effect of Insulin on Tyr Phosphorylation of IR in Control and GRK2-deficient Cells**—To determine whether the increase in insulin-induced glycogen synthesis in GRK2-deficient cells is
due to changes in the Tyr phosphorylation of the IR, we performed immuno precipitation and immunoblotting experiments (Fig. 5). In the immunoprecipitation samples, phospho-Tyr antibody labeled a single protein band (96 kDa). After the bands were recorded, the same blot was stripped and re-probed with IR antibody, which labeled a protein with the same size (96 kDa). Density of the phospho-Tyr protein bands was normalized with total IR band density. Insulin treatment increased Tyr phosphorylation of IR to a similar extent in both control and GRK2-deficient cells. The basal Tyr phosphorylation was not detectable in either cell groups. This suggests that GRK2 does not affect the Tyr phosphorylation of IR in hepatocytes.

Effect of Insulin on Tyr and Ser Phosphorylation of IRS1 in Control and GRK2-deficient Cells—To determine the Tyr phosphorylation of IRS1, we used phospho-Tyr$^{612}$-IRS1 specific antibody. The phosphorylation of IRS-1 on this residue increases its association with PI 3-kinase and hence increases insulin signaling (19). The phospho-Tyr$^{612}$-IRS1 band density was normalized with the total IRS1 protein band density. Insulin treatment increased Tyr phosphorylation of IRS1 to a similar extent in both control and GRK2-deficient cells. The basal Tyr phosphorylation was not detectable in either cell groups. This suggests that GRK2 does not affect the Tyr phosphorylation of IR in hepatocytes.

We determined the Ser phosphorylation of IRS1 by using a site-specific antibody that labels phospho-Ser$^{307}$ of IRS1. The phosphorylation of IRS1 at this site decreases its association with IR and hence decreases the insulin signaling (20). In control cells, the IRS1 is Ser$^{307}$ phosphorylated at the basal level, which was modestly reduced (8%) by insulin treatment. Basal Ser$^{307}$ phosphorylation in GRK2-deficient cells was significantly reduced (81%) compared with that in control basal. Insulin treatment did not affect Ser$^{307}$ phosphorylation further in GRK2-deficient cells (Fig. 6B).
Effect of Chronic Insulin Treatment on GRK2 and Insulin Receptor Expression—As shown in Fig. 7, A and B, insulin (100 nM) treatment for 24 h increased GRK2 protein expression by 56%; insulin treatment had no effect on the expression of GRK-3 (83 kDa), which is a member of the same subfamily. This suggests a selective effect of chronic insulin treatment on GRK-2 expression. On the other hand, chronic insulin treatment caused a 50% reduction in insulin receptor expression in control cells. However, in GRK-2-deficient cells, chronic treatment with insulin increased glycogen synthesis. This suggests a role of GRK2 in the desensitization of insulin action on glycogen synthesis.

Effect of Chronic Insulin Treatment on Glycogen Synthesis in Control and GRK2-deficient Cells—To determine whether chronic insulin treatment decreases glycogen synthesis and whether this decrease is restored in GRK2-deficient cells, we performed glycogen synthesis in control and GRK2-deficient cells. In the control group, the insulin treatment for 10 min, as expected, significantly increased the glycogen synthesis, whereas the chronic insulin treatment (24 h) failed to increase glycogen synthesis (Fig. 8). This suggests desensitization of the insulin action after a prolonged exposure of cells, as reported earlier (21). However, in GRK-2-deficient cells, chronic treatment with insulin increased glycogen synthesis. This suggests a role of GRK2 in the desensitization of insulin action on glycogen synthesis.

DISCUSSION

GPCR kinase-2 (GRK2) is predominantly a cytosolic protein. Upon activation, GRK2 translocates to the plasma membrane and phosphorylates GPCRs at serine/threonine residues, leading to receptor desensitization. GRK2 also directly binds to the activated Goq/11 proteins and decreases signaling through the G-protein (14). Although, GRK2 has been known to regulate members of the GPCR superfamily (12), the results of the present study suggest that GRK2 may have an inhibitory role in regulating glycogen synthesis by affecting insulin receptor signaling in liver cells. Recently, a similar study showed that GRK2 negatively regulates insulin-stimulated glucose transport in adipocytes (15).

The insulin receptor is a member of the tyrosine kinase receptor superfamily. However, the insulin receptor also activates G protein-linked signaling pathways (8–10). In the present study increased GRK2 translocation to the plasma membrane, in response to insulin, suggests a link between the insulin receptor and GRK2. At least two mechanisms could contribute to this translocation. First, GRK2 translocation may be promoted by a Gβγ subunit released as a result of the interaction of the activated insulin receptor with G-proteins. Alternatively, activation of protein kinase C by the insulin receptor could promote GRK2 translocation (22, 23).

To determine the functional significance of GRK2 translocation in insulin receptor signaling/function, FL83B cells were made GRK2-deficient and then insulin receptor signaling and function were studied. Examining the metabolic effects of insulin, we found that GRK2 deficiency caused an increase in basal glycogen synthesis compared with basal in control cells. Insulin...
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In light of the potentiation of insulin-stimulated glycogen production produced by GRK2 depletion, we were surprised to find that GRK2 depletion had no effect on insulin-stimulated insulin receptor tyrosine phosphorylation. Classically, GRK2 activation leads to phosphorylation of GPCRs on serine/threonine residues and subsequent receptor desensitization (24). Increased Ser phosphorylation of the insulin receptor causes a simultaneous decrease in Tyr phosphorylation of insulin receptor and thus a decrease in receptor activity (25, 26). Therefore, we anticipated that the GRK2 depletion would reduce serine phosphorylation of the insulin receptor, enhance Tyr phosphorylation of the insulin receptor, and enhance insulin receptor signaling. However, insulin-stimulated Tyr phosphorylation was similar in control and GRK2-deficient cells. This suggests that GRK2 may not regulate insulin receptor signaling by affecting Tyr phosphorylation of the insulin receptor. Rather, the changes in basal and insulin-induced glycogen synthesis produced by GRK2 depletion may result from effects on post-insulin receptor signaling components such as IRS1. Evidence suggesting that the function of IRS1 might be modulated by GRK2 was obtained in GRK2-deficient cells.

We observed that the phosphorylation of IRS1 at Tyr\(^{612}\) was increased, whereas phosphorylation at Ser\(^{307}\) was decreased by GRK2 depletion. The increase in Tyr\(^{612}\) phosphorylation of IRS1 was observed under basal conditions and insulin-stimulated Tyr\(^{612}\) phosphorylation of IRS1 also was increased. IRS1 phosphorylation at this site promotes the binding of the p85 subunit of PI 3-kinase, leading to insulin-mediated activation of downstream effectors (18). In contrast to Tyr\(^{612}\), phosphorylation of Ser\(^{307}\) of IRS-1 was reduced by GRK2 depletion. This phosphorylation of IRS1 at Ser\(^{307}\) reduces the influence of insulin on effectors downstream of IRS1 (27, 28). For example, Ser\(^{307}\) phosphorylation of IRS1 decreases IRS1 association with the insulin receptor, decreases Tyr phosphorylation on IRS1 and thus negatively regulates insulin signaling (19). Taken together, the results of the present study support the hypothesis that GRK2 affects phosphorylation of Ser\(^{307}\) on IRS1, reducing insulin receptor-IRS1 interaction and reducing insulin receptor phosphorylation of Tyr\(^{612}\) on IRS1. In this manner GRK2 could negatively regulate insulin receptor signaling, both in the basal condition and in the presence of added insulin.

The influence of GRK2 on insulin receptor signaling through IRS Ser phosphorylation is potentially relevant to the pathophysiology of diabetes. Increased Ser phosphorylation of IRSs is observed in muscles of patients with type 2 diabetes mellitus (29). However, it is unknown whether GRK2 directly affects IRS1 phosphorylation or whether GRK2 phosphorylates another intermediary signaling molecule that then is responsible for Ser phosphorylation of IRS-1. GSK3\(\alpha\) via negative feedback regulation also phosphorylates IRS1 at serine residues and attenuates insulin receptor signaling (30). Therefore, the decrease in GSK3\(\alpha\) activity observed in GRK2-deficient cells could result in reduced Ser phosphorylation of IRS1 and contribute to the increase in IRS1 Tyr phosphorylation that we observed.

In addition to the kinase activity, GRK2 also suppresses GPCR signaling by binding to Go\(\text{q/11}\) protein and preventing
it from propagating the signal downstream. Insulin receptor is known to couple to Goαq/11 and Gαi proteins. The Goαq/11 subunit is required for insulin signaling in 3T3L1 adipocytes and down-regulation of Gαi leads to insulin resistance in mice (29). Recently a study demonstrated that GRK-2 in 3T3L1 adipocytes inhibits insulin-induced GLUT4 translocation by binding to Goαq/11 (15). This inhibition was independent of GRK2 kinase activity and did not affect Tyr phosphorylation of the insulin receptor, which supports our findings in that GRK2 deficiency did not affect basal or insulin-induced Tyr phosphorylation.

A final area of investigation in the present study was the influence of GRK2 on the response to chronic insulin receptor activation. Chronic insulin exposure down-regulates the insulin receptors and decreases insulin receptor signaling. Moreover, several studies have shown that chronic hyperinsulinemia is associated with reduced insulin receptor sensitivity in animal models and humans (21). In agreement with previous reports we observed that chronic insulin treatment decreased insulin receptor expression and was not associated with increased glycogen synthesis, suggesting insulin receptor desensitization. These responses were accompanied by increased expression of GRK2. In GRK2-deficient cells chronic insulin treatment had no effect on insulin receptor expression and was associated with increased glycogen synthesis. Because GRK2-deficient cells exhibited an increase basal glycogen synthesis, this could partially contribute to the increase in glycogen synthesis after insulin treatment in these cells. However, the absence of insulin receptor down-regulation in GRK2-deficient cells suggests that GRK2 may have a role in regulating insulin receptor expression and desensitization during prolonged insulin exposure. One potential explanation is that two different mechanisms regulate the response of the insulin receptor upon chronic insulin exposure. First, desensitization of the insulin receptor signal could be mediated in part by the post-insulin receptor mechanism involving IRS1 Ser phosphorylation as previously discussed. The second could be a regulation of receptor synthesis or degradation, leading to reduced receptor number with chronic insulin treatment. Our observation of enhanced GRK2 expression with chronic insulin treatment is important in that enhanced expression of GRK2 also is reported in disease conditions such as cardiac failure and myocardial ischemia. Moreover, tissue-specific inhibition of GRK2 in transgenic animals has led to enhanced contractility of heart muscles (31, 32). Enhanced GRK2 expression also is observed in tissues from obese Zucker rats, a model of insulin resistance and hyperinsulinemia (33). Over-expression of GRK2 due to chronic insulin receptor stimulation may contribute to disease states such as insulin resistance and diabetes. It would be interesting to examine the levels of GRK2 in experimental models of diabetes and to see if controlled inhibition of GRK2 could improve the disease state.

In summary, we demonstrate a relationship between the cellular levels of GRK2 and insulin-dependent and -independent glycogen synthesis in liver cells. Depletion of cellular GRK2 reduces IRS1 Ser phosphorylation and increases IRS1 Tyr phosphorylation, which in turn, appears to activate downstream insulin receptor effectors. Upon activation by insulin, this pathway is further enhanced with a net gain in insulin receptor function in terms of glycogen synthesis. Chronic insulin treatment causes elevation in GRK2 expression, which may have a role in the insulin receptor desensitization and down-regulation observed in cells after prolonged insulin exposure. Inasmuch as the present studies provide the first report indicating an inhibitory role of GRK2 in the regulation of glycogen synthesis in liver cells, more studies are needed to define the precise mechanism(s) of this phenomenon.

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