The Activation of Glycogen Synthase by Insulin Switches from Kinase Inhibition to Phosphatase Activation during Adipogenesis in 3T3-L1 Cells*

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The effects of insulin and platelet-derived growth factor (PDGF) on glycogen synthase activation were compared in 3T3-L1 fibroblasts and adipocytes. In the fibroblasts, PDGF elicited a stronger phosphorylation of mitogen-activated protein kinase (MAPK) and AKT than did insulin. Both agents caused a comparable stimulation of receptor autophosphorylation, MAPK, and phosphatidylinositol 3-kinase (PI3-K) activation in the adipocytes. However, adipogenesis resulted in the uncoupling of PI3-K activation by PDGF from subsequent AKT phosphorylation. The relative contributions of glycogen synthase kinase-3 (GSK-3) inactivation and protein phosphatase-1 (PP1) activation in the regulation of glycogen synthase in both cell types were evaluated. Insulin and PDGF caused a small increase in glycogen synthase activity in the fibroblasts. Additionally, both agents caused a similar inhibition of GSK-3, while having no effect on PP1 activity. Following differentiation, insulin treatment resulted in a 5-fold stimulation of glycogen synthase, whereas PDGF was without effect. Both agents caused a comparable inhibition of GSK-3 activity in the adipocytes, whereas only insulin activated PP1. Finally, wortmannin completely blocked the stimulation of PP1 by insulin in 3T3-L1 adipocytes, indicating that PI3-K inhibition can impinge on PP1 activation. Cumulatively these results suggest that the weak activation of glycogen synthase in 3T3-L1 fibroblasts is mediated by GSK-3 inactivation, whereas in the more metabolically active adipocytes, the insulin-specific activation of glycogen synthase is mediated by PP1 activation.

Insulin is the most potent anabolic hormone known, stimulating both glucose and lipid utilization by acutely modulating rate-controlling enzymes of metabolism (1). One such enzyme, glycogen synthase, is regulated both allosterically, by binding of glucose 6-phosphate (G6P),1 and covalently, by the phosphorylation of multiple serine residues (2, 3). The phosphorylation of four key sites on glycogen synthase inhibits enzymatic activity (reviewed in Ref. 2). Insulin treatment results in the dephosphorylation of all 4 residues, leading to an increase in the G6P-independent glycogen synthase activity. The dephosphorylation of glycogen synthase by insulin is believed to be largely mediated by activation of protein phosphatase-1 (PP1). Glycogen synthase is an excellent in vitro substrate for PP1, and insulin stimulates PP1 activity against glycogen synthase in skeletal muscle (4).

Although the precise mechanisms by which insulin activates glycogen synthase remain unknown, numerous studies have suggested that the activation of PI3-K by insulin may be a critical step. The generation of phosphatidylinositol triphosphate can lead to the translocation and phosphorylation of AKT kinase (reviewed in Refs. 5 and 6). Activation of AKT may trigger the stimulation of glucose transport (7–10), although the exact target of AKT in the control of GluT4 translocation is unclear. The only in vivo substrate identified for AKT thus far is GSK-3 (11), a kinase that phosphorylates a variety of substrates, including glycogen synthase. AKT-mediated phosphorylation of GSK-3 results in its inactivation in vitro (11). Moreover, insulin has been shown to rapidly inactivate GSK-3 in several cell types (12–18). The PI3-K inhibitors wortmannin and LY294002, which block the inactivation of GSK-3, also completely inhibit the activation of glycogen synthase by insulin in a variety of cell models (14, 15, 19, 20). Additionally, overexpression of GSK-3β in 293 cells caused a reduction in basal glycogen synthase activity (21). Thus, the PI3-K/AKT/GSK-3 pathway has been proposed to partially mediate the insulin-dependent activation of glycogen synthase (5).

Despite these findings, however, several lines of evidence argue against a primary role for GSK-3 inactivation in the regulation of glycogen synthase by insulin. Overexpression of constitutively activated PI3-K in 3T3-L1 adipocytes increased basal glucose uptake 5-fold but had no effect on basal or insulin-stimulated glycogen synthase activity (8). Additionally, GSK-3 can phosphorylate only two of the four regulatory sites on glycogen synthase (reviewed in Ref. 2), and at least one other unidentified kinase can phosphorylate the same residues (22). Finally, the treatment of primary rat adipocytes with isoproterenol caused a decrease in GSK-3 activity similar to that observed with insulin but did not activate glycogen synthase (16), indicating that GSK-3 can be markedly inactivated without any change in glycogen synthase activity.

We have utilized the 3T3-L1 cell line model to evaluate the relative roles of kinase inhibition and phosphatase activation in the regulation of glycogen synthase activity. In the present work, we show that in preadipocytes GSK-3 inactivation most likely mediates the activation of glycogen synthase. In the more metabolically active 3T3-L1 adipocytes, however, the insulin-specific stimulation of glycogen synthase appears to be primarily mediated by PP1 activation rather than GSK-3 inactivation.

1 The abbreviations used are: G6P, glucose 6-phosphate; PP1, type 1 protein phosphatase; GSK-3, glycogen synthase kinase-3; PDGF, platelet-derived growth factor; PI3-K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase.
EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents and glycogen phosphorylase b were obtained from Life Technologies, Inc. Phosphorylase kinase, GSK-3α, p81 phosphocellulose squares, and monoclonal anti-phosphotyrosine antibody were from Upstate Biotechnology. Recombinant PDGF-BB was supplied by Intergen, and insulin was from Sigma. The GSK-3 peptide substrate RRAEELDSRASIPQL and the negative control peptide RRAEELDSRASIPQ (23) were synthesized by Bioyntec, Inc. (Lewesville, TX). UDP-[U-14C]glucose (286 mCi/mmol) was from ICN, whereas [γ-32P]ATP (3000 Ci/mmole) and ECL reagent were purchased from Amersham Pharmacia Biotech. Polyclonal anti-AKT and anti-phospho-AKT antibodies were obtained from New England Bio-labs, and polyclonal anti-phospho-MAPK was supplied by Promega. Horseradish peroxidase-conjugated IgG was from Bio-Rad.

Cell Culture and Extract Preparation—3T3-L1 fibroblasts were maintained and differentiated as described previously (24). Fibroblasts were used at 80–90% confluence, whereas adipocytes were used 7–11 days after completion of the differentiation protocol, when >95% of the cells expressed the adipocyte phenotype. Cells were serum-starved for 3 h in Dulbecco’s modified Eagle’s medium (5 mM glucose) containing 0.5% fetal bovine serum and 25 mM Hepes (pH 7.4). Following treatments, cells were rapidly washed three times with ice-cold phosphate-buffered saline. For immunoblotting experiments, cells were lysed in HNTG buffer (24) with 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 0.5 mM sodium orthovanadate added just before use. The cells were scraped off the plates and centrifuged at 15,000 × g for 3 h in Dulbecco’s modified Eagle’s medium (5 mM glucose) containing 0.5% fetal bovine serum and 25 mM Hepes (pH 7.4). Following treatments, cells were rapidly washed three times with ice-cold phosphate-buffered saline. For immunoblotting experiments, cells were lysed in HNTG buffer (24) with 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 0.5 mM sodium orthovanadate added just before use. The cells were scraped off the plates and centrifuged at 15,000 × g for 10 min. 4 × sample buffer was added to the supernatants, which were then boiled for 2 min.

Enzymatic Assays—For glycogen synthase assays, cells were harvested in 50 mM Hepes (pH 7.8), 10 mM EDTA, 100 mM NaF, and 2 mg/ml glycogen plus protease inhibitors, and glycogen-enriched pellets were prepared as described previously (25). Glycogen synthase activity was measured at 37 °C as described (24). 40–50 μg of the fibroblast or adipocyte samples were assayed for 30 min, whereas 10–15 μg of the adipocyte samples were assayed for 15 min. For PK1 assays, cells were collected in PP1 buffer, and PP1 assays were rapidly performed as described (26). 1–2 μg of cell extract from the fibroblasts or adipocytes was assayed at 37 °C for 7 and 2 min, respectively.

For GSK-3 assays, cells were collected in GSK-3 buffer (50 mM Hepes (pH 7.4), 1 mM EDTA, 10 mM β-glycerophosphate, 5 mM NaPi, 100 mM KCl, 0.5% Triton X-100) with protease inhibitors, 1 mM dithiothreitol and 0.5 mM sodium orthovanadate, added just before use. Samples were centrifuged at 15,000 × g for 10 min, and 2–4 and 7–10 μg of fibroblast and adipocyte lysates were assayed, respectively. GSK-3 assays were performed at 30 °C in 25-μl final volumes of 50 mM Hepes (pH 7.4), 10 mM MgCl2, 1 mM dithiothreitol, and 0.5 mg/ml peptide. Reactions were initiated by the addition of 100 μM [γ-32P]ATP (1000 cpm/pmol). After 15 min, the reactions were terminated by the addition of 5 μl of 200 mM EDTA plus 5 mM ATP and placing the tubes on ice. Samples were spotted on p81 phosphocellulose squares, washed in 100 mM phosphoric acid, and air dried overnight, and 32P incorporation was measured by liquid scintillation counting. 32P incorporation into the negative control peptide was subtracted from values obtained using the GSK-3 substrate peptide (23).

Other Procedures—Immunoblotting was performed and [32P]phosphorylation was prepared as described previously (24). Protein measurements were done by the method of Bradford.

RESULTS AND DISCUSSION

Insulin and PDGF Differentially Regulate AKT during Adipogenesis of 3T3-L1 Cells—To evaluate the relative roles of signaling pathways in the regulation of glycogen synthase, we compared the actions of insulin and PDGF in subconfluent 3T3-L1 fibroblasts and fully differentiated adipocytes. Replicate plates of cells were treated for various times with either 100 nM insulin or 10 ng/ml PDGF, lysates were then prepared, and tyrosine phosphorylation was analyzed by immunoblotting. As shown in Fig. 1A, insulin receptor autophosphorylation was readily detected in cell lysates from the fibroblasts but dramatically increased following adipogenesis. In contrast, PDGF treatment of the fibroblasts caused a strong increase in the tyrosine phosphorylation of its receptor, which was markedly decreased in the adipocytes (Fig. 1A).

Both MAPK and AKT are phosphorylated and activated in response to insulin and PDGF in a variety of cell lines and tissues (5, 24, 27, 28). To study the phosphorylation of these enzymes, the lysates from Fig. 1A were transferred to nitrocellulose, and probed with either anti-phosphotyrosine (A), anti-phospho-MAPK (B) or anti-phospho-AKT (C) antibodies. Receptors for PDGF (PDGF) and insulin (IR) are denoted by arrows in A. Autoradiographs of immunoblots (IB) shown are representative of two independent experiments.

Fig. 1. Insulin and PDGF signaling in 3T3-L1 fibroblasts and adipocytes. 3T3-L1 fibroblasts and fully differentiated adipocytes were serum-starved for 3 h and then stimulated with either 100 nM insulin or 10 ng/ml PDGF for the indicated times. Cells were collected in HNTG buffer, and 40 μg of protein from each sample were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with either anti-phosphotyrosine (A), anti-phospho-MAPK (B) or anti-phospho-AKT (C) antibodies. Receptors for PDGF (PDGF) and insulin (IR) are denoted by arrows in A. Autoradiographs of immunoblots (IB) shown are representative of two independent experiments.
insulin treatment dramatically increased AKT phosphorylation, whereas PDGF had a lesser effect (Fig. 1C, Ref. 15). This decrease in the stimulation of AKT phosphorylation by PDGF was unexpected, since PDGF and insulin cause an equivalent activation of PI3-K in these cells (28–30), which is thought to lie upstream of AKT activation. However, two reports have demonstrated that PDGF and insulin activate different intracellular pools of PI3-K in 3T3-L1 adipocytes (29, 30), which may explain the differences in AKT activation by insulin and PDGF.

**GSK-3 Activity Is Diminished during Adipogenesis of 3T3-L1 Cells**—We evaluated the contribution of GSK-3 inactivation to the regulation of glycogen synthase in 3T3-L1 cells. Replicate plates of subconfluent fibroblasts and fully differentiated adipocytes were lysed, and the two enzyme activities were measured in parallel. As previously reported, 3T3-L1 differentiation resulted in a 5-fold increase in total glycogen synthase activity (Fig. 2A, GS), with a corresponding rise in synthase protein levels (26). In contrast, total GSK-3 activity measured in cell lysates using a specific peptide substrate was decreased over 75% following differentiation (Fig. 2A, GSK-3). The down-regulation of GSK-3 enzymatic activity is mirrored by a dramatic decrease in both GSK-3α and β protein levels during differentiation (31) (data not shown). These initial results suggested that in 3T3-L1 adipocytes, GSK-3 most likely is not a primary regulator of glycogen synthase activity.

The inhibition of GSK-3 activity by PDGF and insulin was examined in the two cell types. In the fibroblasts, PDGF treatment produced a 40% reduction in GSK-3 activity, whereas insulin caused a 25% decrease (Fig. 2B, Fibroblasts). In the adipocytes, insulin reduced GSK-3 activity by 50%, whereas PDGF caused a 30% inhibition (Fig. 2B, Adipocytes). Although insulin caused a larger percentage inactivation of GSK-3 in the adipocytes than in the fibroblasts (50% versus 25%, respectively), the absolute decrease in GSK-3 specific activity was reduced substantially following differentiation (Fig. 2B).

**Activation of Glycogen Synthase Is Mediated by PP1 in 3T3-L1 Adipocytes**—The effects of insulin and PDGF on glycogen synthase activity were measured during differentiation of 3T3-L1 cells. Both agents were equally potent in the fibroblasts, causing a 6-fold increase in glycogen synthase activity. In the 3T3-L1 adipocytes, however, basal glycogen synthase activity was significantly higher than even the stimulated activity observed in the fibroblasts. Although this most likely results from the increase in glycogen synthase expression following differentiation (26), the down-regulation of basal GSK-3 activity may also be partially responsible. More representative of the physiological state, a 15-min incubation of the adipocytes with 100 nM insulin caused a marked increase in glycogen synthase activity, whereas PDGF was without significant effect (Fig. 2C, Adipocytes). Since PDGF caused a 30% decrease in GSK-3 activity yet did not activate glycogen synthase, the 50% decrease in GSK-3 activity evoked by insulin probably does not account for a significant portion of the stimulation of glycogen synthase activity. These data indicate that other signaling pathways must be used by insulin in 3T3-L1 adipocytes to regulate glycogen synthase.

The contribution of PP1 activation to the dephosphorylation of glycogen synthase was next examined. In 3T3-L1 fibroblasts, neither PDGF nor insulin caused a detectable increase in PP1 activity (Fig. 3A, Fibroblasts). However, insulin treatment of the adipocytes caused a 3-fold increase in PP1 activity, measured at short assay time points, while PDGF had no effect on PP1 activity (Fig. 3A, Adipocytes). A role for PI3-K and AKT in the stimulation of PP1 activity in the adipocytes cannot be excluded. To examine this possibility, 3T3-L1 adipocytes were treated in the absence and presence of 100 nM wortmannin for 10 min, which completely blocks subsequent PI3-K activation (19). Cells were then stimulated with 100 nM insulin, and PP1 activity was measured in vitro. Wortmannin pretreatment totally inhibited the activation of PP1 by insulin (Fig. 3B), indicating a possible role for PI3-K activation in the regulation of PP1 activity. However, since overexpression of constitutively active PI3-K in 3T3-L1 adipocytes had no effect on either basal or insulin-stimulated glycogen synthase activity (8), the effects of wortmannin may reflect a nonspecific blockade of other signaling molecules.

Results presented here suggest that glycogen synthase is
activated by two different mechanisms in 3T3-L1 fibroblasts and adipocytes. In the fibroblasts, both insulin and PDGF cause a similar inactivation of GSK-3 and a comparable inactivation of glycogen synthase. While GSK-3 inactivation can cause a stimulation of glycogen synthase, the inactivation of other glycogen synthase kinases may also be involved. In the 3T3-L1 adipocytes, several lines of evidence argue against GSK-3 inactivation mediating glycogen synthase activation. 1) Following differentiation there is a dramatic 75% decrease in GSK-3 activity. In stark contrast, glycogen synthase activity and protein levels increase at least 5-fold (Fig. 2A, Ref. 26). 2) Insulin caused a significantly smaller absolute decrease in GSK-3 specific activity in the adipocytes than in the fibroblasts, yet insulin caused a far larger increase in glycogen synthase activity in the adipocytes (Fig. 2, B and C). 3) PDGF decreased GSK-3 activity 30% in the adipocytes yet had no significant effect on glycogen synthase activation. Therefore, the 50% inactivation of GSK-3 by insulin probably accounts for a small part of the increase in glycogen synthase activity (Fig. 2, B and C). Whereas GSK-3 inactivation is observed both with insulin and PDGF, only insulin can increase PPI activity, consistent with the specific activation of glycogen synthase in the 3T3-L1 adipocytes (Figs. 2B and 3). 5) The inhibition of glycogen synthase activation by the PI3-K inhibitor wortmannin had suggested a role for GSK-3 inactivation in this insulin effect (5, 14). However, these conclusions need to be re-examined since wortmannin completely blocked PP1 activation by insulin. These results suggest that in cell lines with a low basal metabolic activity, GSK-3 inactivation can cause a stimulation of glycogen synthase. However, in the more physiological 3T3-L1 adipocytes, PP1 activation rather than GSK-3 inactivation appears to be the major mechanism by which insulin specifically dephosphorylates and activates glycogen synthase.

**Addendum**—Complementary results have recently been reported by Ueki et al. (32).

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