RESEARCH COMMUNICATION

Insulin stimulation of glycogen synthesis and glycogen synthase activity is blocked by wortmannin and rapamycin in 3T3-L1 adipocytes: evidence for the involvement of phosphoinositide 3-kinase and p70 ribosomal protein-S6 kinase

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We have investigated the involvement of phosphoinositide (PI) 3-kinase and p70 ribosomal protein-S6 kinase ($p70^{s6k}$) in mediating insulin stimulation of glycogen synthesis in 3T3-L1 adipocytes using specific inhibitors. Wortmannin inhibited PI 3-kinase activity ($IC_{50} \approx 10$ nM), inhibition being complete at 100 nm. Wortmannin (100 nM) completely blocked the ability of insulin to activate glycogen synthase in 3T3-L1 adipocytes and the ability of insulin to stimulate glucose incorporation into glycogen in 3T3-L1 fibroblasts. Rapamycin, which blocks insulin-stimulated activation of $p70^{s6k}$, decreased insulin activation of glycogen synthase in a dose-dependent manner ($IC_{50} \sim 0.8$ ng/ml), with a maximum approx. 75% inhibition of insulin's stimulatory effect. Rapamycin inhibited insulin-stimulated glucose incorporation into glycogen to a similar extent and with similar dose-dependency, while having no effect on insulin-stimulated glucose transport. We conclude that PI 3-kinase and $p70^{s6k}$ are involved in the signalling pathways by which insulin stimulates glycogen synthase in 3T3-L1 adipocytes.

INTRODUCTION

Insulin elicits a wide variety of metabolic and growth-related effects in target tissues, including inhibition of lipolysis and stimulation of glucose transport, glycogen synthesis, lipogenesis, gene transcription and DNA synthesis. These effects are exerted at different subcellular locations (plasma membrane, cytosol, mitochondrion, nucleus) and in different time frames (seconds to hours), and variously reflect control of enzyme activity by reversible phosphorylation, translocation of membrane vesicles or modulation of transcriptional events [1]. This diversity of effects has encouraged the belief that the insulin receptor may initiate multiple signalling pathways regulating different processes.

A potential source of this diversity of signalling is insulin-receptor substrate-1 (IRS-1), which has been identified as a major intracellular substrate of the insulin receptor in a wide range of tissues [2]. Insulin stimulates multi-site tyrosine phosphorylation of IRS-1, and phospho-IRS-1 then acts as a docking site for a number of different proteins with potentially important signalling functions [3,4]. These proteins include phosphoinositide (PI) 3-kinase [5,6], the tyrosine-specific phosphatase SHPTP2/Syp [7] and the adaptor proteins Nck [8] and Grb2 [9]. The association with Grb2/Sos (son of sevenless GDP exchange factor) is thought to be linked to activation of Ras and subsequently the mitogen-activated protein (MAP) kinase cascade [2,9,10]. Importantly, studies in rabbit muscle have linked the MAP kinase cascade to activation of glycogen synthase via protein phosphatase-1G (PP1G), which dephosphorylates and activates glycogen synthase [11]. These studies have been the focus of much attention, as they provided the first example of a continuous signalling pathway from the insulin receptor to a metabolic end response. However, recent data have suggested that activation of MAP kinase is not sufficient to activate glycogen synthase in 3T3-L1 adipocytes [12], suggesting that there is more that one signalling mechanism controlling the activity of glycogen synthase.

There is growing evidence that PI 3-kinase plays a central role in insulin signalling to metabolic pathways. The role of PI 3-kinase in insulin-stimulated glucose transport has been most conclusively established by using two structurally unrelated PI 3-kinase inhibitors, wortmannin [13,14] and LY294002 [15], as well as by studies over-expressing dominant negative p85 subunits of PI 3-kinase [16]. Wortmannin and LY294002 have also been found to block insulin's activation of p70 ribosomal protein-S6 kinase ($p70^{s6k}$), indicating that this serine/threonine kinase lies downstream of PI 3-kinase [15,17]. The observation that $p70^{s6k}$ could inactivate glycogen synthase kinase-3 (GSK-3) [18], at least *in vitro*, led us to investigate a possible role for PI 3-kinase and $p70^{s6k}$ in insulin stimulation of glycogen synthesis. In the present study we provide evidence for the first time the PI 3-kinase and $p70^{s6k}$ are involved in the pathways which insulin utilizes to activate glycogen synthase and stimulate glycogen synthesis.

MATERIALS AND METHODS

Wortmannin, insulin, phosphatidylinositol and newborn-calf serum were purchased from Sigma (Poole, Dorset, U.K.). Rapamycin was from Calbiochem (Nottingham, U.K.). Deoxy[^{3}H]-glucose was obtained from New England Nuclear, and [γ-^{32}P]ATP, [^{14}C]glucose and [^{3}H]UDP-glucose were from Amersham. 3T3-L1 cells were obtained from American Type Culture Collection. Fetal-calf serum and Dulbecco's modified Eagle's medium (DMEM) were obtained from ICN–Flow. Wortmannin

Abbreviations used: PI 3-kinase, phosphoinositide 3-kinase; IRS-1, insulin-receptor substrate-1; DMEM, Dulbecco's modified Eagle's medium; MAP kinase, mitogen-activated protein kinase/extracellular-regulated kinase (erk); Mapkap-1, MAP-kinase-activated protein kinase-1; GSK-3, glycogen synthase kinase-3; $p70^{s6k}$, p70 ribosomal protein-S6 kinase; PP1-G, protein phosphatase-1G.

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(1 mM in dimethyl sulphoxide) was stored in batches at −20 °C for up to 1 month. Polyclonal antibodies to the p85 subunit of PI 3-kinase and phosphotyrosine were obtained from Transduction Laboratories and ICN–Flow respectively. Polyclonal antibody to IRS-1 was raised in rabbits to a glutathione-s-transferase fusion protein corresponding to the C-terminal 336 amino acid residues 899–1235 of the rat IRS-1 sequence (gift from M. Coghlan).

3T3-L1 fibroblasts were grown to confluence in DMEM containing 25 mM glucose and 10% newborn-calf serum, and maintained at confluence for 3–4 days. Differentiation was induced with DMEM containing 10% fetal-calf serum, 10 mM Hepes, 5 μg/ml insulin, 0.5 mM isobutylmethylxanthine and 0.25 μM dexamethasone for 2 days, after which dexamethasone and isobutylmethylxanthine were removed. Cells were cultured for a further 2 days in DMEM/10% fetal-calf serum/insulin and then subsequently in DMEM/10% fetal-calf serum. Cells were routinely used 7–10 days after differentiation was induced, and only plates in which > 95% of cells showed adipocyte morphology were used.

PI 3-kinase activity was assayed by t.l.c. determination of ^{32}P incorporation into phosphatidylinositol as previously described [19].

Glycogen synthesis in intact cells was assayed by determining the amount of [^{14}C]glucose incorporated into glycogen as previously described [20]. Glycogen synthase activity was determined as previously described [21]. Briefly, supernatants were prepared from adipocytes that has been scraped from the plate and disrupted by six passages through a 23-gauge needle. The ability of these to stimulate the incorporation of [^{3}H]UDP-glucose into glycogen was determined in the presence and absence of glucose 6-phosphate, and the activity ratios (activity − glucose 6-phosphate/activity + glucose 6-phosphate) were calculated. Glucose uptake was determined by using the 2-deoxy[^{3}H]glucose uptake method as described [22]. Wortmannin and rapamycin were added 5 min before insulin. Appropriate vehicle controls were used in every experiment.

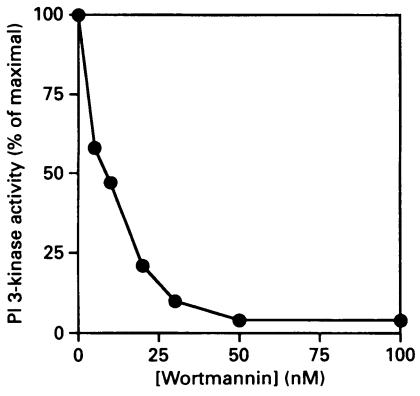

Figure 1 Wortmannin inhibits PI 3-kinase activity

Dishes (100 mm) of 3T3-L1 adipocytes were stimulated with 5 nM insulin for 10 min, and phosphotyrosine containing-proteins were precipitated from cell lysates with PY20 anti-phosphotyrosine antibody. Immunoprecipitates were assayed for PI 3-kinase activity (as described in the Materials and methods section) in the presence of the indicated concentrations of wortmannin. Results show a representative experiment; similar results were obtained in three separate experiments.

Table 1 Wortmannin blocks insulin activation of glycogen synthase in 3T3-L1 adipocytes

3T3-L1 adipocytes in 50 mm dishes were serum starved for 3 h in gluocose-free DMEM, then incubated with dimethyl sulphoxide or 100 nM wortmannin for 2 min before incubation in the absence or presence of 100 nM insulin for 30 min. Supernatants from disrupted cells were assayed for incorporation of UDP-glucose into glycogen in both the absence (−G6P) and presence (+G6P) of glucose 6-phosphate. Glycogen synthase activity was calculated as described in the Materials and methods section. Experiments were done in duplicate and results show the mean percentage activity (−G6P/+G6P) of 4 independent determinations (±S.D.).

	Glycogen synthase activity (%)	
	−Wortmannin	+Wortmannin
Basal	3.8±0.5	3.4±0.3
Insulin (100 nM)	8.4±1.2	3.4±0.4

Table 2 Wortmannin blocks insulin-stimulated glucose incorporation into glycogen in 3T3-L1 fibroblasts

For this experiment, 80–90%-confluent 3T3-L1 fibroblasts in 50 mm plates were incubated in serum-free DMEM (1000 mg/ml glucose) for 3 h. Wortmannin (100 nM) or dimethyl sulphoxide was added immediately before stimulation with 10 nM insulin. The amount of [^{14}C]glucose incorporated into glycogen was determined as described in the Materials and methods section and is expressed as % relative to basal. Results are mean of triplicate determinations (±S.D.), and similar results were obtained in three separate experiments.

	[^{14}C]Glucose incorporation (%)	
	−Wortmannin	+Wortmannin
Basal	100±9	82±6
Insulin (100 nM)	224±9	72±8

RESULTS AND DISCUSSION

We used the selective PI 3-kinase inhibitor wortmannin [23] to assess the involvement of PI 3-kinase in insulin stimulation of glycogen synthase in 3T3-L1 adipocytes. It has previously been demonstrated that at concentrations up to 100 nM wortmannin does not directly inhibit any other enzyme activity so far investigated, including the insulin-receptor tyrosine kinase or phosphatidylinositol 4-kinase [13], myosin light-chain kinase, protein kinase A, protein kinase C or cyclic-GMP-dependent protein kinase [24], the components of the MAP kinase cascade [25,26] or p70^{s6k} [25]. We found that wortmannin inhibited PI 3-kinase activity in anti-phosphotyrosine (Figure 1), anti-IRS-1 and anti-p85 (results not shown) immunoprecipitates from insulin-treated 3T3-L1 adipocytes, with an IC_{50} of approx. 10 nM. Wortmannin also inhibited insulin-stimulated glucose transport in 3T3-L1 adipocytes with a similar dose-dependency (results not shown). The potency of these inhibitory effects is similar to those reported recently for wortmannin in rat adipocytes [13] and 3T3-L1 adipocytes [14].

Glycogen synthase activity was stimulated 2–3-fold by insulin, and this stimulation was completely abolished in the presence of 100 nM wortmannin (Table 1). Further, the dose-dependency of wortmannin's inhibitory effects closely paralleled its inhibition of PI 3-kinase and glucose transport in 3T3-L1 adipocytes (results not shown). We sought to confirm that this alteration in glycogen synthase activity was manifested in whole cells as an inhibition of insulin-stimulated glycogen synthesis. Because wortmannin in-

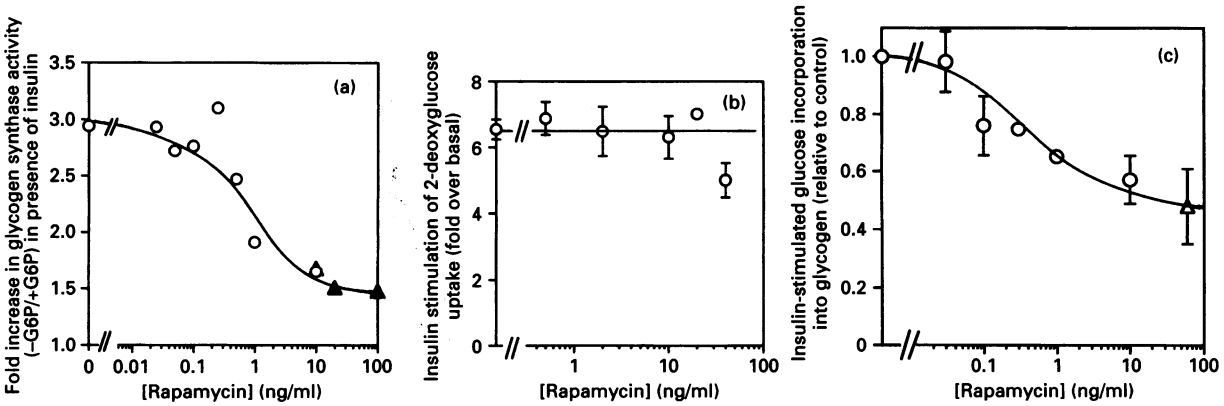

Figure 2 Rapamycin decreases insulin stimulation of glycogen synthase and glucose incorporation into glycogen, but not glucose transport, in 3T3-L1 adipocytes

(a) Glycogen synthase. 3T3-L1 adipocytes in 50 mm dishes were serum-starved for 3 h in glucose-free DMEM. Ethanol (1 μl/ml) or rapamycin at the indicated concentration was added 5 min before exposure to 100 nM insulin or vehicle for 30 min. Supernatants from disrupted cells were assayed for incorporation of UDP-glucose into glycogen in both the absence and presence of glucose 6-phosphate (G6P). Glycogen synthase fractional activities were calculated as described in the Materials and methods section. Fold stimulation was calculated by comparing fractional activity in the presence of insulin with its relevant vehicle control. A representative experiment showing the dose-dependent inhibition of glycogen synthase is depicted (○). In this experiment the glycogen synthase activity ratio was 2.4% in the basal state, and in the presence of insulin it was 7% (2.9-fold stimulation). Similar results were obtained in three separate experiments. A further series of three experiments [of which a representative experiment is shown (▲)] confirmed that the inhibition was not further increased by higher doses of rapamycin. (b) 2-Deoxy[^{3}H]glucose transport. The effect of rapamycin on insulin-stimulated 2-deoxyglucose uptake was determined in 3T3-L1 adipocytes as described in the Materials and methods section. Results represent means ± S.D. of triplicate determinations. (c) Glucose incorporation into glycogen. 3T3-L1 adipocytes in 12-well dishes were incubated in serum-free DMEM (1000 mg/ml glucose) for 3 h. Ethanol (1 μl/ml) or the indicated concentration of rapamycin was added, followed 5 min later by 10 nM insulin. The amount of [^{14}C]glucose incorporated into glycogen was determined as described in the Materials and methods secretion. The effect of rapamycin on insulin stimulation of glucose incorporation into glycogen was assessed by comparison with maximal insulin stimulation in the absence of rapamycin. Values plotted are means ± S.D. of tripicate determinations. Similar results were obtained in three separate experiments.

hibited insulin-stimulated glucose transport, it was not possible to determine separately the effect of wortmannin on insulin-stimulated glycogen synthesis in adipocytes. However, in non-confluent 3T3-L1 fibroblasts 50 nM insulin did not significantly stimulate glucose transport (results not shown), whereas it stimulated incorporation of glucose into glycogen 2–3-fold (Table 2). This stimulation was totally abolished by 100 nM wortmannin (Table 2), indicating that wortmannin was inhibiting insulin's stimulation of glycogen synthesis independently of effects on glucose transport.

The fact that concentrations of wortmannin in the low-nanomolar range inhibit the insulin-stimulated activation of glycogen synthase provides the first evidence that PI 3-kinase is involved in the intracellular signalling pathways regulating glycogen synthesis. As we find that wortmannin also inhibits glycogen synthesis in 3T3-L1 pre-adipocytes, this novel pathway is probably not restricted to differentiated adipocytes. These findings may have important clinical implications, as insulin stimulation of PI 3-kinase activity is decreased in animal models of insulin resistance and diabetes [27,28].; If this is also the case in human diabetes, then it suggests a possible mechanism for the widely reported decrease in insulin's stimulation of glycogen synthase in diabetic subjects [29–34].

Direct control of glycogen synthase activity is exerted by co-ordinated phosphorylation and inactivation of GSK-3β, thus blocking continued phosphorylation of glycogen synthase, combined with phosphorylation and activation of PP-1G, which increases the rate of dephosphorylation of glycogen synthase [11,25,35–37]. As PI 3-kinase is essentially a lipid kinase, it seems unlikely to play a direct role in regulating these phosphorylation events. However, there is evidence that signalling events downstream of PI 3-kinase are involved in regulating enzymes directly controlling glycogen synthase activity, as wortmannin blocks insulin-stimulated inactivation of GSK-3β in L6 myoblasts [25] and CHO cells [37]. The hypothetical involvement of the MAP kinase cascade in this process is suggested by the fact that MAP-kinase-activated protein kinase-1β (Mapkap kinase-1β) has been shown to regulate GSK-3β and PP1-G activity [35], and it is well established that the MAP kinase cascade is involved in the control of Mapkap kinase 1β activity [35]. Further, wortmannin has been reported to block the activation of the MAP kinase pathway in L6 myoblasts [25] and CHO cells [37], therefore suggesting a PI 3-kinase-dependent pathway for stimulation of glycogen synthase. However, four lines of evidence suggest that such a MAP kinase/GSK-3β/PP1-G dependent pathway for glycogen synthase activation may not be present in 3T3-L1 adipocytes. These are: (a) activation of MAP kinase is not sufficient to activate glycogen synthase in 3T3-L1 adipocytes [12]; (b) the MAP kinase cascade can be activated in the face of total inhibition of PI 3-kinase, indicating that this enzyme may not be an absolute requirement for the activation of MAP kinase cascade in 3T3-L1 adipocytes [15]; (c) it has been reported that insulin does not stimulate protein phosphatase 1 activity in the particulate fraction of 3T3-L1 adipocytes [38]; and (d) GSK-3β levels fall dramatically during differentiation and are very low in 3T3-L1 adipocytes [39].

These observations suggested the involvement of other serine/threonine kinase cascades in insulin stimulation of glycogen synthesis. One candidate was p70^{s6k}, which is acutely activated by insulin and is an element of a serine/threonine kinase signalling system that acts independently of the MAP kinase cascade [40]. The observation that p70^{s6k} can inactivate GSK-3 *in vitro* suggested a possible role for this enzyme in regulating glycogen metabolism [18]. Further, it has also been observed that wortmannin in low-nanomolar concentrations blocks the activation of p70^{s6k} by insulin in a range of cell types, including 3T3-L1 adipocytes [15,17,25,37]. To investigate whether p70^{s6k} was a downstream element of the PI 3-kinase-dependent pathway

controlling glycogen synthase activity in these cells, the macrolide inhibitor rapamycin was used. Rapamycin has been shown to block specifically the activation of $p70^{s6k}$ by insulin without inhibiting insulin's effects on PI 3-kinase, MAP kinase, protein kinase C, Ca^{2+}-regulated kinases, raf kinase or $pp90^{rsk}$ [17,41,42]. Rapamycin was found to attenuate insulin's activation of glycogen synthase in a dose-dependent manner, with maximal doses of rapamycin inhibiting glycogen synthase activation by 75% without affecting the basal glycogen synthase activity (Figure 2a). As rapamycin has no effect on insulin-stimulated glucose transport in 3T3-L1 adipocytes (Figure 2b and [41], the effect of rapamycin on glycogen synthesis could be determined (Figure 2c). These results show that rapamycin also inhibits insulin-stimulated glycogen synthesis, again without affecting basal levels. If the underlying effect of increased glucose transport is taken into account, this decrease is of a similar magnitude to the effect on glycogen synthase. The inhibitory effect of rapamycin both on glycogen synthase activation and on stimulation of glycogen synthesis has an IC_{50} of ~ 0.8 ng/ml, which is very similar to the IC_{50} previously reported for rapamcyin's inhibition of $p70^{s6k}$ and mitogensis in 3T3 and D10 cells [42,43]. These data strongly suggest that the major pathway by which insulin activates glycogen synthesis in 3T3-L1 adipocytes is mediated through the activation of $p70^{s6k}$. Interestingly, rapamycin did not completely block insulin's ability to activate glycogen synthase, even at 100 ng/ml, a concentration which has previously been shown to block $p70^{s6k}$ activation completely in 3T3-L1 adipocytes [41,43]. One explanation for this could be that insulin may be controlling glycogen synthase activity by two separate PI 3-kinase-dependent pathways which have differential sensitivity to rapamycin. Although our data do not provide any direct evidence for this, it is apparent that in other cell types rapamycin-insensitive pathways exist for insulin-stimulated activation of glycogen synthase [44] and GSK-3β [25,37].

In summary, we provide evidence for the first time of the involvement of both PI 3-kinase and $p70^{s6k}$ in signalling pathways by which insulin acts to control glycogen synthesis. The data also highlight a post-receptor divergence in insulin's intracellular signalling pathways, since although insulin stimulation of both glucose transport and glycogen synthase requires PI 3-kinase, only glycogen synthase activation also requires $p70^{s6k}$.

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