Mechanism of feedback regulation of insulin receptor substrate-1 phosphorylation in primary adipocytes

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Serine and threonine phosphorylation of IRS-1 (insulin receptor substrate-1) has been reported to decrease its ability to be tyrosine-phosphorylated by the insulin receptor. Insulin itself may negatively regulate tyrosine phosphorylation of IRS-1 through a PI3K (phosphoinositide 3-kinase)-dependent feedback pathway. In the present study, we examined the regulation and role of IRS-1 serine phosphorylation in the modulation of IRS-1 tyrosine phosphorylation in physiologically relevant cells, namely freshly isolated primary adipocytes. We show that insulin-stimulated phosphorylation of Ser³¹² and Ser⁶¹⁶ in IRS-1 was relatively slow, with maximal phosphorylation achieved after 20 and 5 min respectively. The effect of insulin on phosphorylation of both these sites required the activation of PI3K and the MAPKs (mitogen-activated protein kinases) ERK1/2 (extracellular-signal-regulated kinase 1 and 2), but not the activation of mTOR (mammalian target of rapamycin)/p70S6 kinase, JNK (c-Jun N-terminal kin-

ase) or p38MAPK. Although inhibition of PI3K and ERK1/2 both substantially decreased insulin-stimulated phosphorylation of Ser³¹² and Ser⁶¹⁶, only wortmannin enhanced insulin-stimulated tyrosine phosphorylation of IRS-1. Furthermore, inhibition of mTOR/p70S6 kinase, JNK or p38MAPK had no effect on insulin-stimulated IRS-1 tyrosine phosphorylation. The differential effect of inhibition of ERK1/2 on insulin-stimulated IRS-1 phosphorylation of Ser³¹²/Ser⁶¹⁶ and tyrosine indicates that these events are independent of each other and that phosphorylation of Ser³¹²/Ser⁶¹⁶ is not responsible for the negative regulation of IRS-1 tyrosine phosphorylation mediated by PI3K in primary adipocytes.

Key words: insulin, insulin receptor substrate-1 (IRS-1), phosphoinositide 3-kinase (PI3K), serine phosphorylation, signalling, tyrosine phosphorylation.

INTRODUCTION

Insulin secretion and insulin action play an essential role in glucose homeostasis and impairment will result in the development of diabetes. The primary action of insulin is to decrease the glucose concentration in blood by stimulating glucose uptake into muscle and adipose tissues and by suppressing glucose production by the liver. Insulin binds to its receptor, leading to an increase in its intrinsic tyrosine kinase activity and subsequent phosphorylation of several cellular substrates such as Shc, APS [adapter with PH and SH3 (Src homology 3) domains], Cbl and IRS (insulin receptor substrate) proteins. Tyrosine phosphorylation of the IRS proteins results in the generation of binding sites for SH2 domain-containing proteins such as the p85-regulatory subunit of PI3K (phosphoinositide 3-kinase), Grb2 and SHP-2 (SH2 domain-containing phosphatase-2). The IRS proteins are crucial to the insulin-stimulated activation of PI3K, an essential component of the insulin signalling system leading to the stimulation of glucose uptake [1,2].

In addition to tyrosine phosphorylation sites, IRS-1 and IRS-2 contain many potential serine phosphorylation sites that may play regulatory roles during the insulin response. Various metabolites and proinflammatory cytokines that are produced in states of obesity, and associated with insulin resistance, stimulate serine phosphorylation of IRS-1, including non-esterified fatty acids and TNF α (tumour necrosis factor α) [3,4]. In addition, signalling pathways stimulated by insulin itself have been proposed to play a role in both the negative-feedback regulation of the insulin signal and cellular insulin resistance after hyperinsulinaemia [5–8].

Protein kinases that have been shown to phosphorylate IRS-1 include JNK (c-Jun N-terminal kinase) [9], mTOR (mammalian target of rapamycin) [10–12], PKC δ (protein kinase C δ) [13], PKC ζ [14], I κ B (inhibitory κ B) kinase [3], ERK (extracellular-signal-regulated kinase) [15], PKB [16] and AMPK (5'-AMP-activated kinase) [17]. So far, a number of specific serine phosphorylation sites on IRS-1 have been identified as targets involved in insulin resistance, including Ser³¹² [5,9,18–20] and Ser⁶¹⁶ [18,21], and the recently identified sites Ser³⁰⁷ [13,22] and Ser³²³ [13,23]. (The sequence numbers shown correspond to the human sequence of IRS-1.) Of these, Ser³¹² has been studied most intensively and several studies have shown that its phosphorylation attenuates tyrosine phosphorylation of IRS-1. Furthermore, TNF α and non-esterified fatty acids stimulate the phosphorylation of Ser³¹² on IRS-1 and mutation of this site prevents the inhibitory effect of TNF α on insulin-stimulated IRS-1 tyrosine phosphorylation [19]. It has been shown that JNK phosphorylates Ser³¹², resulting in the inhibition of the interaction between the PTB (phosphotyrosine-binding domain) domain of IRS-1 and the insulin receptor [5]. Moreover, insulin itself is capable of stimulating the phosphorylation of Ser³¹² through a PI3K-dependent feedback loop probably involving JNK [24].

One of the first serine phosphorylation sites to be identified on IRS-1 was Ser⁶¹⁶, which becomes phosphorylated on activation of the MAPK (mitogen-activated protein kinase) family member ERK, and it negatively regulates IRS-1 tyrosine phosphorylation [15]. This site can also become phosphorylated after insulin stimulation [11,18] and may therefore participate in insulin-stimulated negative-feedback regulation of IRS-1.

Abbreviations used: ERK, extracellular-signal-regulated kinase; IRS, insulin receptor substrate; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; mTOR, mammalian target of rapamycin; NP40, Nonidet P40; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; PKC, protein kinase C; SH2/3, Src homology 2/3; SHP-2, SH2 domain-containing phosphatase-2; TNF α , tumour necrosis factor α .

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We previously reported that, in primary adipocytes, insulin-stimulated IRS-1 tyrosine phosphorylation was enhanced in the presence of the PI3K inhibitor wortmannin [25]. Since both Ser³¹² and Ser⁶¹⁶ phosphorylations have been reported to have a role in the negative regulation of IRS-1 in transformed cells in culture, we wished to investigate the role of these sites in more physiologically relevant cells, specifically primary rat adipocytes. In particular, we explored the role and regulation of Ser³¹² and Ser⁶¹⁶ phosphorylation in insulin-stimulated negative-feedback regulation of IRS-1 tyrosine phosphorylation.

EXPERIMENTAL

Materials

Male Wistar rats (160–210 g) were fed *ad libitum* with a stock diet (CRM; Bioshore, Manea, Cambridgeshire, U.K.). Wortmannin, rapamycin, SP600125 (JNK inhibitor II) and SB203580 were obtained from Calbiochem (Bad Soden, Germany) and the MEK (MAPK kinase) inhibitor UO126 was from Promega Biosciences (San Luis Obispo, CA, U.S.A.). The anti-IRS-1 (C-20), anti-P-c-Jun (KM-1) and anti-c-Jun (H-79) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The anti-phosphotyrosine 4G10 was obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.), whereas the phosphospecific antibodies against IRS-1 Ser³¹² and Ser⁶¹⁶ were obtained from Biosource International (Camarillo, CA, U.S.A.). The anti-P-ERK (Thr²⁰²/Tyr²⁰⁴) and the anti-ERK antibodies were from Cell Signaling Technology (Beverly, MA, U.S.A.).

Preparation and incubation of epididymal fat cells

Adipocytes were isolated from the epididymal fat pads of Wistar rats as described previously [26]. Cells were subsequently washed with Krebs–bicarbonate–Hepes buffer, pH 7.4 (130 mM NaCl, 4.7 mM KCl, 1.5 mM MgSO₄, 1.2 mM CaCl₂, 2.5 mM NaH₂PO₄, 15.5 mM NaHCO₃, 10 mM Hepes and 11 mM glucose) without BSA. Cells were incubated with 100 nM wortmannin, 50 nM rapamycin, 10 μ M UO126, 60 μ M SP600125 (JNK inhibitor II), 10 μ M SB203580 or DMSO (vehicle) for 30 min at 37°C before stimulation with 87 nM insulin for the indicated time. Alternatively, cells were incubated with different concentrations of SP600125 for 30 min at 37°C before stimulation with 87 nM insulin for 10 min. The reaction was terminated by extracting the cells (1:1 packed cells, v/v) in ice-cold NP40 (Nonidet P40) extraction buffer (50 mM Tris/HCl, pH 7.5, containing 1 mM EDTA, 120 mM NaCl, 50 mM NaF, 1 mM benzamidine, 1% NP40, 1 μ M microcystin, 7.2 mM 2-mercaptoethanol, 5 mM orthovanadate and 1 μ g/ml each of pepstatin, leupeptin and antipain). Cell extracts were centrifuged at 10000 g for 10 min at 4°C and the infranatant was taken for subsequent analysis.

Immunoprecipitation

Proteins were immunoprecipitated by rotating 250 μ l of total cell extract with the anti-IRS-1 antibody and 20 μ l of Protein A–Sepharose (50%, w/v) at 4°C. The Protein A–Sepharose beads were isolated by centrifugation and washed three times with NP40 extraction buffer. Subsequently, Laemmli sample buffer was added and proteins were separated by SDS/PAGE for immunoblotting.

Immunoblotting

Laemmli sample buffer was added to the cell extracts and proteins were separated by SDS/PAGE (7.5% gel) and transferred on to PVDF membranes. The membranes were blocked in 10% (w/v)

BSA dissolved in TBS-T (Tris-buffered saline with 0.1% Tween 20; 20 mM Tris/HCl, 137 mM NaCl and 0.1% Tween 20) and subsequently incubated with primary and secondary antibodies, which were diluted in TBS-T containing 5% BSA. Blots were washed for at least 2 h in TBS-T for the anti-phosphotyrosine antibody 4G10 and washed at least five times for 5 min, after each antibody incubation, for all other antibodies. Membranes were developed using an ECL[®] (enhanced chemiluminescence) detection system (AP Biotech, Amersham Biosciences, Little Chalfont, Bucks., U.K.). Primary antibodies were used at a concentration of 1 μ g/ml. Horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) was diluted 1:10000.

Quantification and statistics

Quantitative assessment of phosphorylated protein bands detected by Western blotting was performed by densitometry of films and subsequent analysis using the program ImageQuant (Molecular Dynamics, Little Chalfont, Bucks., U.K.). Results are expressed as a percentage of the insulin response in the absence of inhibitors. Statistical significance was determined using two-tailed Student's *t* test for paired data and *P* < 0.05 was considered to be statistically significant.

RESULTS

Time course of insulin-stimulated phosphorylation of IRS-1 on Ser³¹² and Ser⁶¹⁶ and activation of ERK, p70S6 kinase and JNK in primary adipocytes

IRS-1 contains many potential serine and threonine phosphorylation sites that may be involved in the regulation of the insulin response. In particular, phosphorylation of IRS-1 on Ser³¹² and Ser⁶¹⁶ has been shown to regulate negatively insulin signalling [5,9,21]. To investigate whether insulin stimulates serine phosphorylation of these sites in primary adipocytes, we used phosphospecific antibodies that recognize IRS-1 when phosphorylated on Ser³¹² and Ser⁶¹⁶. Figures 1(A) and 1(B) demonstrate that insulin stimulates the phosphorylation of both Ser³¹² and Ser⁶¹⁶ in these physiologically relevant cells. Phosphorylation of Ser³¹² on IRS-1 was relatively slow, with maximal phosphorylation achieved 20–30 min after stimulation, whereas insulin-stimulated phosphorylation of Ser⁶¹⁶ was slightly faster and maximal at 5–10 min.

mTOR and members of the MAPK family such as JNK and ERK have been implicated in the serine phosphorylation of IRS-1 in other cell types [11,12,15,24]. To delineate the potential role of these kinases in the phosphorylation of Ser³¹² and Ser⁶¹⁶, their temporal activation by insulin was compared with the phosphorylation of Ser³¹² and Ser⁶¹⁶. The phosphorylation states of p70S6 kinase (Thr³⁸⁹), c-Jun (Ser⁶³) and ERK (Thr²⁰²/Tyr²⁰⁴) are widely considered to be corollaries of the activity of mTOR, JNK and ERK respectively and so were determined by immunoblotting using phosphospecific antibodies. Insulin stimulated a relatively slow phosphorylation of p70S6 kinase in primary adipocytes, which was maximal after 10 min (Figure 1C). The effect of insulin on phosphorylation of the JNK substrate c-Jun was also relatively slow and continued to increase up to 60 min after stimulation (Figure 1D). In contrast, insulin-stimulated ERK phosphorylation was rapid but transient, becoming maximal as early as 2 min after stimulation and returning to basal level within 30 min (Figure 1E). Wortmannin inhibited the insulin-stimulated phosphorylation of all three of these phosphoproteins, demonstrating a key role for PI3K in the regulation of these kinases in primary adipocytes.

Together, these results show that insulin-stimulated phosphorylation of IRS-1 on Ser³¹² correlates most closely with the

Figure 1 Time course of insulin-stimulated phosphorylation of Ser³¹² and Ser⁶¹⁶ on IRS-1, p70S6 kinase, c-Jun and ERK

Primary adipocytes were stimulated with insulin (87 nM) for the indicated time and extracted. IRS-1 immunoprecipitates (**A**, **B**) and total lysate (**C**–**E**) were subjected to SDS/PAGE followed by immunoblotting with anti-IRS-1 P^{Ser312} (phospho-Ser³¹²) (**A**), anti-IRS-1 P^{Ser616} (**B**), anti-p70S6 kinase P^{Thr389} (**C**), anti-c-Jun P^{Ser63} (**D**) and anti-ERK P^{Thr202}/P^{Tyr204} (**E**) antibodies. The bar graphs represent quantification of the phosphorylation of IRS-1 (ratio of phosphorylated/total) expressed as a percentage of the signal obtained with insulin alone at 60 min (means $\pm$ S.E.M., $n = 3$).

activation of p70S6 kinase, whereas phosphorylation of IRS-1 on Ser⁶¹⁶ correlates most closely with ERK activation.

Insulin-stimulated IRS-1 Ser³¹² and Ser⁶¹⁶ phosphorylation requires the activation of PI3K and ERK, but is independent of mTOR

To delineate more carefully the signalling mechanism by which insulin stimulates Ser³¹² and Ser⁶¹⁶ phosphorylation in rat adipocytes, we used the inhibitors wortmannin, rapamycin and UO126 to block the activation of PI3K, mTOR/p70S6 kinase and ERK respectively. Interestingly, the insulin-stimulated increase in both Ser³¹² and Ser⁶¹⁶ phosphorylations was blocked in the presence of wortmannin (Figures 2A and 2B), which also prevented insulin-stimulated p70S6 kinase and ERK phosphorylation (Figures 2C and 2D). mTOR has recently been found to be responsible for

Figure 2 Insulin stimulates the phosphorylation of Ser³¹² and Ser⁶¹⁶ on IRS-1 through a PI3K- and ERK-dependent pathway in primary adipocytes

Primary adipocytes were pretreated with vehicle (DMSO), wortmannin (100 nM), rapamycin (50 nM) or UO126 (10 μ M) for 30 min at 37°C and subsequently stimulated with or without insulin (87 nM) for 10 min. Cells were extracted and IRS-1 immunoprecipitates (**A**, **B**) or total lysates (**C**, **D**) were subjected to SDS/PAGE followed by immunoblotting with the indicated antibodies (anti-IRS-1 P^{Ser312}, anti-IRS-1 P^{Ser616}, anti-p70S6 kinase P^{Thr389} and anti-ERK P^{Thr202}/P^{Tyr204}). The membranes were stripped and reprobed with appropriate antibodies to confirm loading. The bar graphs (**A**, **B**) represent quantification of the serine phosphorylation of IRS-1 (ratio of phosphorylated/total) expressed as a percentage of the signal obtained with insulin alone (means $\pm$ S.E.M., $n = 4$). * $P < 0.05$ is considered to be significantly different with respect to the insulin response, as determined by the two-tailed paired Student's t test.

most of the PI3K-dependent Ser³¹² phosphorylation in 3T3-L1 adipocytes and H4IIE rat hepatoma cells [11,12,18]. In contrast with these reports, however, we found no significant inhibitory effect of the mTOR inhibitor rapamycin on Ser³¹² (or Ser⁶¹⁶) phosphorylation in primary adipocytes (Figures 2A and 2B; 84 ± 17 and 107 ± 12 % of control respectively) under conditions where the inhibitor completely blocked insulin-stimulated p70S6 kinase phosphorylation (Figure 2C). On the other hand, the MEK inhibitor UO126 partially decreased Ser³¹² phosphorylation

Figure 3 Effects of the JNK inhibitor SP600125 on insulin-stimulated phosphorylation of c-Jun and phosphorylation of Ser³¹² and Ser⁶¹⁶ on IRS-1

Primary adipocytes were pretreated with different concentrations of SP600125 as indicated (A) or alternatively with vehicle (DMSO), wortmannin (100 nM), SP600125 (60 μ M) or SB203580 (10 μ M) (B, C) for 30 min at 37 °C and subsequently stimulated with or without insulin (87 nM) for 10 min. Cells were extracted and total lysate (A) or IRS-1 immunoprecipitates (B, C) were subjected to SDS/PAGE followed by immunoblotting with the indicated antibodies (anti-c-Jun P-Ser⁶³, anti-IRS-1 P-Ser³¹² and anti-IRS-1 P-Ser⁶¹⁶). The membranes were stripped and reprobed with appropriate antibodies to confirm loading. The bar graphs (B, C) represent quantification of the serine phosphorylation of IRS-1 (ratio of phosphorylated/total) expressed as a percentage of the signal obtained with insulin alone (means $\pm$ S.E.M., $n = 3$). * $P < 0.05$ is considered to be significantly different with respect to the insulin response, as determined by the two-tailed paired Student's t test.

(48 $\pm$ 12 % of control) and completely blocked Ser⁶¹⁶ phosphorylation (Figure 2B). The latter is consistent with previous reports that have identified Ser⁶¹⁶ as an ERK-regulated phosphorylation site [15,21]. As expected, UO126 blocked insulin-stimulated ERK phosphorylation under these conditions (Figure 2D).

Taken together, these findings indicate a role for PI3K and its downstream effector ERK in insulin-stimulated Ser³¹² and Ser⁶¹⁶ phosphorylation.

Insulin-stimulated IRS-1 Ser³¹² and Ser⁶¹⁶ phosphorylation is independent of the MAPK family member JNK

A recent study demonstrated an important role for JNK in PI3K-dependent negative regulation of IRS-1 [24]. Since wortmannin inhibits insulin-stimulated JNK activation in primary adipocytes (Figure 1D), we next investigated whether a similar feedback inhibition is present in primary adipocytes, by using the recently described cell-permeant JNK inhibitor SP600125 [27]. The optimal inhibitor concentration required for these experiments was determined by incubating primary adipocytes with various concentrations of SP600125 followed by stimulation with insulin and subsequent assessment of phosphorylation of c-Jun. Figure 3(A) shows that the insulin-stimulated increase in c-Jun phosphorylation was substantially prevented by pretreatment with 60 μ M SP600125. This concentration of the inhibitor, however, had no

Figure 4 Effects of okadaic acid on insulin-stimulated Ser³¹² and Ser⁶¹⁶ phosphorylation of IRS-1

Primary adipocytes were pretreated with vehicle (DMSO) or okadaic acid for 5 min at 37 °C and subsequently stimulated with or without insulin (87 nM) for 10 min. Cells were extracted and IRS-1 immunoprecipitates were subjected to SDS/PAGE followed by immunoblotting with the anti-IRS-1 P-Ser³¹² (A) and anti-IRS-1 P-Ser⁶¹⁶ (B) antibodies. The membranes were stripped and reprobed with the anti-IRS-1 antibody to confirm loading. The bar graphs represent quantification of the serine phosphorylation of IRS-1 (ratio of phosphorylated/total) expressed as a percentage of the signal obtained with insulin after incubation with okadaic acid (means $\pm$ S.E.M., $n = 3$).

significant effect on insulin-stimulated phosphorylation of IRS-1 on Ser³¹² and Ser⁶¹⁶ (87 $\pm$ 12 and 83 $\pm$ 8 % of control respectively; Figures 3B and 3C).

The effect of okadaic acid on insulin-stimulated Ser³¹² and Ser⁶¹⁶ phosphorylation

The potential for the serine phosphorylation to have an effect on tyrosine phosphorylation of IRS-1 will depend largely on the degree to which phosphorylation occurs. To investigate the maximal possible serine phosphorylation of IRS-1, we incubated adipocytes in the presence of the Ser/Thr phosphatase inhibitor okadaic acid to prevent dephosphorylation. Figure 4(A) shows that okadaic acid had no significant effect on insulin-stimulated phosphorylation of Ser³¹², indicating that near-maximal phosphorylation has been achieved. Okadaic acid alone induced weak Ser³¹² phosphorylation, suggesting a relatively slow basal turnover in the phosphorylation of this site (Figure 4A). In contrast, okadaic acid increased the phosphorylation of Ser⁶¹⁶ to a similar extent as insulin and increased insulin-stimulated Ser⁶¹⁶ phosphorylation by 1.7-fold (Figure 4B). Together, these results indicate that insulin stimulates strong Ser³¹² and Ser⁶¹⁶ phosphorylation of IRS-1, which therefore has the potential to change its ability to be tyrosine-phosphorylated.

Inhibition of PI3K, but not mTOR/p70S6 kinase, ERK, JNK or p38MAPK, enhances insulin-stimulated tyrosine phosphorylation of IRS-1

We have previously reported that preincubation of freshly isolated adipocytes with wortmannin enhances the ability of insulin to

Figure 5 Rapamycin, UO126, SP600125 and SB203580 have no effect on the insulin-stimulated tyrosine phosphorylation of IRS-1

Primary adipocytes were pretreated with vehicle (DMSO), wortmannin (100 nM), rapamycin (50 nM), UO126 (10 μ M), SP600125 (60 μ M) or SB203580 (10 μ M) for 30 min at 37°C and subsequently stimulated with or without insulin (87 nM) for 10 min. Cells were extracted and total lysates were subjected to SDS/PAGE followed by immunoblotting for tyrosine-phosphorylated proteins with the anti-PTyr antibody 4G10. The membranes were stripped and reprobed with the anti-IRS-1 antibody to confirm loading.

stimulate tyrosine phosphorylation of IRS-1, while it simultaneously inhibits insulin-stimulated IRS-3 tyrosine phosphorylation (see Figures 5, 6A and 6B). The mechanism underlying this complex reciprocal feedback regulation is not known. To explore whether ERK might mediate the effect of PI3K, we investigated whether UO126 mimicked the effect of wortmannin on insulin-stimulated IRS-1 and IRS-3 tyrosine phosphorylation.

UO126 treatment of adipocytes not only inhibited insulin-stimulated ERK phosphorylation, but decreased it significantly below basal levels (Figure 2D). Despite this, insulin-stimulated IRS-1 tyrosine phosphorylation was not significantly affected by UO126 (Figure 5A) at any time point examined (Figures 6C and 6D). In addition, UO126 had no effect on insulin-stimulated IRS-3 tyrosine phosphorylation (Figures 5A and 6C).

Since p70S6 kinase and c-Jun phosphorylation were inhibited by wortmannin (Figures 1C and 1D), we further explored whether rapamycin and SP600125 could mimic the effects of wortmannin on IRS-1 and IRS-3 tyrosine phosphorylation. As shown in Figures 5(A) and 5(B), this was not the case. Furthermore, the p38MAPK inhibitor SB203580 was also ineffective (Figure 5B). This excludes mTOR/p70S6 kinase, ERK, JNK and p38MAPK as

Figure 6 Effects of wortmannin and UO126 on the time course of insulin-stimulated tyrosine phosphorylation

Primary adipocytes were pretreated with vehicle (DMSO), 100 nM wortmannin or 10 μ M UO126 for 30 min at 37°C and subsequently stimulated with insulin (87 nM) for the indicated times. Cells were extracted and total lysates were subjected to SDS/PAGE followed by immunoblotting for tyrosine-phosphorylated proteins with the anti-PTyr antibody 4G10. The membranes were stripped and reprobed with appropriate antibodies to confirm loading. Results are representative of three experiments. The bar graphs (B, D) represent quantification of the tyrosine phosphorylation of IRS-1 (ratio of phosphorylated/total) expressed as a percentage of the signal obtained with insulin alone at 2 min (means $\pm$ S.E.M., $n = 3$).

candidates for the wortmannin-sensitive pathway that modulates IRS-1 and IRS-3 tyrosine phosphorylation.

DISCUSSION

PI3K plays a central role in the negative regulation of IRS-1 tyrosine phosphorylation and subsequent insulin signalling in primary adipocytes [25].

Numerous studies have demonstrated that hyperphosphorylation of IRS-1 on serine and threonine residues inhibits insulin-stimulated IRS-1 tyrosine phosphorylation and subsequent downstream signalling cascades [5,7,8,28]. Insulin itself can stimulate Ser/Thr phosphorylation of IRS-1, which has been proposed to modulate insulin signalling negatively [5–8,28]. Specific phosphorylation sites in IRS-1 that have been identified in the negative regulation of insulin signalling include Ser³¹² and Ser⁶¹⁶.

In the present study, we investigated the role and regulation of insulin-stimulated Ser³¹² and Ser⁶¹⁶ phosphorylation of IRS-1 in primary rat adipocytes. We demonstrate that both of these sites are phosphorylated on insulin stimulation and that their phosphorylation is relatively slow, with maximal Ser³¹² and Ser⁶¹⁶ phosphorylation reached after 20 and 5 min respectively. A similar time course of phosphorylation has been reported in 3T3-L1 adipocytes, with Ser⁶¹⁶ being more rapidly phosphorylated compared with Ser³¹² [11].

The effect of insulin on these phosphorylation events was inhibited by wortmannin (Figure 2), demonstrating the requirement for PI3K activation. In further investigating the identity of the insulin-stimulated protein kinases that may be responsible for phosphorylation of Ser³¹² and Ser⁶¹⁶, we found that ERK activation in response to insulin occurred rapidly and preceded the phosphorylation of Ser⁶¹⁶. Furthermore, the inhibition of ERK phosphorylation and activation using the MEK inhibitor, U1026, completely prevented insulin-stimulated phosphorylation of Ser⁶¹⁶ (Figure 2B). This is consistent with the fact that IRS-1 Ser⁶¹⁶ has been previously identified as an ERK-regulated phosphorylation site [15,21]. This suggests, therefore, that ERK is responsible for insulin-stimulated Ser⁶¹⁶ phosphorylation in these cells, although it remains to be unequivocally established whether ERK directly phosphorylates this site.

The signalling pathway responsible for insulin-stimulated Ser³¹² phosphorylation is less clear. Although the MEK inhibitor significantly inhibited this phosphorylation event, the inhibition was incomplete, suggesting that ERK-dependent as well as non-ERK-dependent pathways are involved (Figure 2A). The amino acid sequence surrounding Ser³¹² does not conform strictly to the consensus required for phosphorylation by ERK, so the ERK-dependent pathway may involve another kinase downstream of ERK. JNK has been reported to mediate feedback inhibition of IRS-1 tyrosine phosphorylation by promoting the phosphorylation of Ser³¹² [24]. We found that insulin-stimulated c-Jun phosphorylation in primary adipocytes was largely dependent on PI3K activity and the MEK/ERK pathway, since wortmannin and UO126 strongly decreased its phosphorylation (Figure 1D). This is in agreement with a recent study in 32D^{IR} cells where the PI3K/MEK/ERK pathway seems to be involved in insulin-stimulated JNK activation [24]. However, JNK is unlikely to be responsible for Ser³¹² phosphorylation in primary adipocytes, since the recently described JNK inhibitor SP600125 had no significant effect on the phosphorylation of this site despite preventing insulin-stimulated c-Jun phosphorylation. Furthermore, the insulin-dependent phosphorylation of c-Jun, and hence activation of JNK, was considerably slower than the phosphorylation of IRS-1 on Ser³¹² (and Ser⁶¹⁶; note that SP600125 also had no effect on IRS-1 Ser⁶¹⁶ phosphorylation).

It has recently been reported that mTOR is responsible for most of the PI3K-dependent insulin-stimulated Ser³¹² phosphorylation in 3T3-L1 adipocytes [11,29] and H4IIE rat hepatoma cells [18]. The phosphorylation of p70S6 kinase exhibited a temporal profile similar to that of insulin-stimulated IRS-1 Ser³¹² phosphorylation. At first sight, therefore, the results suggest that mTOR/p70S6 kinase could represent a candidate signalling pathway that may, at least in part, be responsible for mediating the insulin-stimulated

ERK-independent IRS-1 Ser³¹² phosphorylation. However, this possibility was ruled out because we found no significant inhibitory effect of the mTOR inhibitor rapamycin on IRS-1 Ser³¹² phosphorylation in primary adipocytes under conditions where phosphorylation of p70S6 kinase was completely blocked (Figure 2).

The kinases responsible for insulin-stimulated Ser³¹² phosphorylation of IRS-1 in primary adipocytes remain to be identified. An alternative candidate kinase to phosphorylate Ser³¹² is PKC ζ , which is phosphorylated and activated in a PI3K-dependent manner on insulin stimulation of adipocytes [30,31]. However, a recent study showed that Ser³¹² is not phosphorylated by PKC ζ *in vitro* [23].

Since Ser³¹² can potentially become phosphorylated by a number of different protein kinases, we cannot rule out the possibility that multiple kinases can simultaneously phosphorylate this site in response to insulin. Thus no inhibitor would be effective in decreasing Ser³¹² phosphorylation because of this functional redundancy. This would be consistent with the fact that insulin-stimulated phosphorylation of Ser³¹² in 3T3-L1 adipocytes requires the activation of both mTOR and JNK [11,24,29], whereas TNF α -stimulated phosphorylation of Ser³¹² requires dual phosphorylation by JNK and I κ B kinase [32].

Our previous studies have demonstrated that wortmannin enhances the ability of insulin to stimulate IRS-1 tyrosine phosphorylation and inhibits insulin-stimulated IRS-3 phosphorylation (see also Figure 6A). The mechanism underlying this complex regulation is not known; however, it indicates an important role for PI3K in inhibiting tyrosine phosphorylation of IRS-1 and enhancing IRS-3 phosphorylation. One possibility is that wortmannin blocks an insulin-stimulated PI3K-dependent feedback pathway that inhibits IRS-1 tyrosine phosphorylation and enhances IRS-3 tyrosine phosphorylation. The insulin-stimulated negative-feedback regulation of IRS-1, however, will have to be very rapid, since the effect of wortmannin on IRS-1 tyrosine phosphorylation is already apparent at the earliest time point (2 min) after insulin stimulation. Insulin-stimulated phosphorylation of IRS-1 Ser³¹² and Ser⁶¹⁶ is relatively slow and phosphorylation of these sites is therefore unlikely to be involved in the effect of wortmannin on IRS-1 tyrosine phosphorylation in primary adipocytes. This is further supported by the finding that UO126 has no effect on insulin-stimulated tyrosine phosphorylation of IRS-1, whereas it mimics the inhibitory effect of wortmannin on insulin-stimulated IRS-1 Ser³¹² and Ser⁶¹⁶ phosphorylation. Phosphorylation of these sites may, however, be involved in feedback regulation during more prolonged insulin stimulation, which would be consistent with findings in Fao and CHO-T cells, where wortmannin prevented the decrease in insulin-stimulated tyrosine phosphorylation of IRS-1 after 60 min [16]. Whether a similar negative-feedback regulation is present in primary adipocytes is not clear, although we found no significant difference in tyrosine phosphorylation of IRS-1 after 60 min insulin stimulation in the presence of wortmannin or UO126 (Figure 6).

A more probable explanation for the effect of wortmannin is that a tonic, or increased, basal level of PI3K activity exists in these cells which represses IRS-1 tyrosine phosphorylation and enhances IRS-3 phosphorylation in response to a subsequent insulin challenge. This regulation may be mediated by serine/threonine phosphorylation of the IRS proteins and would be relieved by wortmannin treatment of the cells. A tonic, wortmannin-inhibitable, level of PI3K activity may itself lead to an increased basal level of ERK phosphorylation and activity that regulates IRS phosphorylation. Indeed, treatment of adipocytes with the MEK inhibitor UO126 not only inhibited insulin-stimulated ERK

phosphorylation, but decreased it significantly below basal levels (Figure 2D). Despite this, insulin-stimulated IRS-1 tyrosine phosphorylation was not significantly affected by UO126 (Figure 5A) at any time point examined (Figures 6C and 6D). In addition, UO126 was without effect on insulin-stimulated IRS-3 tyrosine phosphorylation. This indicates that the ability of wortmannin to modulate IRS-1 and IRS-3 tyrosine phosphorylation does not involve PI3K-mediated regulation of ERK. We further found that none among mTOR/p70S6 kinase, JNK and p38MAP kinase was responsible for mediating the effects of wortmannin, since rapamycin, SP600125 and SB203580 were all without effect.

The kinase downstream of PI3K and the potential serine/threonine phosphorylation sites that are responsible for the negative regulation of IRS-1 tyrosine phosphorylation in primary adipocytes therefore remain to be identified. One potential candidate is PKC ζ , which negatively regulates insulin-stimulated tyrosine phosphorylation of IRS-1 by phosphorylation of Ser²³ [23] and is activated in a PI3K-dependent manner on insulin stimulation of adipocytes [30,31]. Another potential candidate is the intrinsic serine kinase activity of PI3K itself, whose ability to phosphorylate IRS-1 may be blocked by wortmannin [33]. Furthermore, as the balance of enzyme activity between kinases and phosphatases determines the effect of insulin, wortmannin may exert its effect by inhibiting the activity or activation of phosphatases that negatively regulate insulin signalling, such as protein tyrosine phosphatase 1B and protein phosphatase 2A [34,35]. Alternatively, wortmannin may alter basal serine phosphorylation levels of IRS-1 by modulating the levels of plasma membrane-derived phosphoinositide lipids in the cell and thereby the localization of IRS-1, since its PH (pleckstrin homology) domain is known to be important in recruiting this substrate to the plasma membrane. These possibilities require further investigation in primary adipocytes.

In summary, we have shown that insulin induces a slow stimulation of Ser³¹² and Ser⁶¹⁶ phosphorylation on IRS-1 in a PI3K-dependent manner. In contrast with previous observations using transformed cell lines such as 3T3-L1 adipocytes and H4IIE rat hepatoma cells, mTOR and JNK are not involved in Ser³¹² phosphorylation, at least in response to insulin. Moreover, neither phosphorylation of Ser³¹² and Ser⁶¹⁶ nor the activation of mTOR, ERK or JNK is responsible for the enhanced insulin-stimulated tyrosine phosphorylation of IRS-1 brought about by wortmannin in primary adipocytes. Nor are these kinases responsible for the regulatory effects of PI3K on IRS-3 phosphorylation. These results differ fundamentally from observations in cultured transformed cell types and exemplify the need to focus on the use of more physiologically relevant cell types if we are to begin to understand how IRS-1 signalling is regulated in diabetes.

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