Multiple signalling pathways involved in the stimulation of fatty acid and glycogen synthesis by insulin in rat epididymal fat cells

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We have investigated the signalling pathways involved in the stimulation of glycogen and fatty acid synthesis by insulin in rat fat cells using wortmannin, an inhibitor of phosphatidylinositol 3-kinase, and rapamycin, which blocks activation of p70 ribosomal S6 protein kinase ($p70^{S6K}$). Insulin produced a decrease in the activity of glycogen synthase kinase-3 which is likely to be important in the observed stimulation of glycogen synthase. Both of these actions were found to be sensitive to inhibition by wortmannin. Activation of three processes is involved in the stimulation of fatty acid synthesis from glucose by insulin, namely glucose uptake, acetyl-CoA carboxylase and pyruvate dehydrogenase. Whereas wortmannin largely abolished the effects of insulin on glucose utilization and acetyl-CoA carboxylase activity, it was without effect on the stimulation of pyruvate dehydrogenase. Although epidermal growth factor stimulated mitogen-activated protein kinase to a greater extent than insulin, it was unable to mimic the effect of insulin on glycogen synthase, glycogen synthase kinase-3, glucose utilization, acetyl-CoA carboxylase or pyruvate dehydrogenase. Rapamycin also failed to have any appreciable effect on stimulation of these parameters by insulin, although it did block the effect of insulin on $p70^{S6K}$. We conclude that the activity of phosphatidylinositol 3-kinase is required for the effects of insulin on glycogen synthesis, glucose uptake and acetyl-CoA carboxylase, but is not involved in signalling to pyruvate dehydrogenase. Activation of mitogen-activated protein kinase or $p70^{S6K}$, however, does not appear to be sufficient to bring about the stimulation of fatty acid or glycogen synthesis. Altogether it seems likely that at least four distinct signalling pathways are involved in the effects of insulin on rat fat cells.

INTRODUCTION

Despite substantial recent advances in our understanding of intracellular signalling, the mechanisms by which insulin regulates metabolic processes are still unclear. Interaction of insulin with its receptor at the cell surface stimulates the intrinsic tyrosine kinase activity of the receptor and increases the tyrosine phosphorylation of a number of intracellular substrates such as insulin receptor substrate 1 (IRS-1) [1]. Increased phosphorylation of tyrosine residues on IRS-1 creates a number of recognition sites for proteins containing SH2 domains, including GRB-2 and the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase) (for a review see ref. [2]). The binding of GRB-2, through its association with the guanine nucleotide exchange factor, son-of-sevenless (mSOS), leads to increases in the level of active GTP-bound $p21^{ras}$. GTP–$p21^{ras}$ recruits the serine/threonine protein kinase Raf-1 to the plasma membrane, increasing the activity of this protein kinase by some, as yet undefined, mechanism, hence stimulating the mitogen-activated protein kinase (MAPK) cascade (see refs. [3] and [4] for reviews). Increased MAPK activity in response to insulin has been shown in many insulin-sensitive tissues and cell lines (see ref. [5]).

Association of the p85 subunit with IRS-1 increases the activity of the p110 catalytic subunit of PI 3-kinase [6,7]. At present it is not clear how the products of PI 3-kinase are involved in insulin action. The p110 subunit has been shown to exhibit serine protein kinase activity, although to date the only known substrates for this serine kinase activity are the p85 subunit [8] and IRS-1 [9–11]. The involvement of PI 3-kinase in insulin signalling pathways can be investigated using the fungal metabolite wortmannin, which is considered to be a specific inhibitor of the kinase at nanomolar concentrations [12–14]. PI 3-kinase may be involved in regulation of the MAPK cascade, as wortmannin has recently been shown to block almost completely the activation of MAPK by insulin in muscle L6 cells [15] and in Chinese hamster ovary cells stably overexpressing the human insulin receptor (CHO.T cells) [16]. Increases in the activity of PI 3-kinase have also been implicated in the activation of p70 ribosomal S6 protein kinase ($p70^{S6K}$) in response to insulin [17,18].

Although increases in the activity of MAPK may account for the mitogenic effects of insulin and may also be involved in the selective increase in the transcription of certain genes (see ref. [5]), the mechanism by which insulin brings about its major effects on metabolism, such as the stimulation of fatty acid and glycogen synthesis in adipose tissue, has yet to be elucidated. Insulin is thought to increase the rate of fatty acid synthesis from glucose in this tissue by acting on three main control points. First, it stimulates the rate of glucose transport into the cell, primarily via increased translocation of the GLUT4 glucose-transporter isoform to the plasma membrane, although increases in GLUT1 translocation may be responsible for some of the increase in glucose uptake (see ref. [19] for a review). At present there is no clear evidence for the involvement of increases in MAPK activity in the effect of insulin on GLUT4 translocation [20–22]. The effect of insulin on fatty acid synthesis also involves the activation of both pyruvate dehydrogenase (PDH) and acetyl-CoA carboxylase (ACC). Increases in PDH activity arise from increased dephosphorylation of the enzyme by a specific PDH

Abbreviations used: ACC, acetyl-CoA carboxylase; GSK-3, glycogen synthase kinase 3; IRS-1, insulin receptor substrate 1; MAPK, mitogen-activated protein kinase; PDH, pyruvate dehydrogenase; PI 3-kinase, phosphatidylinositol 3-kinase; PP-1G, protein phosphatase-1G; SH2, Src-homology-2; $p90^{rsk}$, p90 ribosomal S6 protein kinase; $p70^{S6K}$, p70 ribosomal S6 protein kinase; EGF, epidermal growth factor.

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phosphatase [23], whereas changes in ACC activity are associated with increased phosphorylation by a specific insulin-stimulated ACC kinase [24,25].

Insulin increases the rate of glycogen synthesis in muscle, liver and adipose tissue primarily via dephosphorylation of a number of sites on the C-terminus of glycogen synthase (for recent reviews see refs. [3] and [26]). The phosphorylation of these C-terminal serine residues is controlled by the relative activities of glycogen synthase kinase-3 (GSK-3) and the glycogen-associated form of protein phosphatase-1 (PP-1G) [27–30]. GSK-3 activity is decreased in response to insulin in a number of cell types [15,31,32], and a potential explanation for this phenomenon is suggested by the ability of both p90 ribosomal S6 protein kinase ($p90^{rsk}$) and $p70^{S6K}$ to phosphorylate and inactivate GSK-3 *in vitro* [33,34]. The activity of $p90^{rsk}$ is increased by insulin on phosphorylation by MAPK [35,36]. The $p70^{S6K}$ is phosphorylated and activated by insulin via a signalling pathway distinct from the MAPK cascade [17]. A number of recent studies using cultured cells and rapamycin, an immunosuppressant that blocks activation of $p70^{S6K}$ without affecting activation of MAPK [37], suggest that stimulation of $p70^{S6K}$ may not be involved in the regulation of GSK-3 activity by insulin *in vivo* [15,16]. Activation of muscle PP-1G in response to insulin has been proposed to be a consequence of phosphorylation of the subunit that binds the phosphatase to glycogen particles by $p90^{rsk}$ [38,39].

The aim of the present study was to characterize further the signalling pathways involved in the stimulation of fatty acid and glycogen synthesis by insulin. Because established cell lines are dividing and are not necessarily fully differentiated, insulin signalling pathways in such cells may be different from those present in the cells of insulin target tissues *in vivo*. In the present study we have used fresh rat epididymal fat pads and isolated fat cells. The inhibitors wortmannin and rapamycin, along with epidermal growth factor (EGF), a potent activator of MAPK in fat cells [40,22], have been used to extend the work of others mainly in cultured cells [41,42] and to explore the relative importance of PI 3-kinase, MAPK and $p70^{S6K}$ in a wide range of effects of insulin in rat fat cells.

EXPERIMENTAL

Materials

Male Wistar rats (180–200 g) were fed *ad libitum* up to the time of killing on a stock laboratory diet (CRM; Bioshore, Lavender Hill, Manea, Cambs., U.K.). [γ-^{32}P]ATP and the ECL Western-blotting detection kit were obtained from Amersham International (Amersham, Bucks., U.K.). Collagenase was purchased from Worthington Diagnostic Systems (Freehold, NJ, U.S.A.) and pepstatin, leupeptin and antipain were from Cambridge Research Biochemicals (Harston, Cambridge, U.K.). The synthetic peptides used to assay MAPK, GSK-3 and $p70^{S6K}$ activities were synthesized by Dr. G. Bloomberg of this department. EGF was obtained from Collaborative Biomedical Products (Bedford, MA, U.S.A.). Rapamycin was purchased from Affiniti Research Products Limited (Nottingham, U.K.). Microcystin and dithiothreitol were obtained from Calbiochem (Nottingham, U.K.) and GSH was from Boehringer-Mannheim (Lewes, E. Sussex, U.K.). The anti-(C-terminal $p70^{S6K}$) antibody used for immunoblotting was purchased from UBI (Lake Placid, NY, U.S.A.), and the anti-$p70^{S6K}$ antibody used for immunoprecipitation was raised by E.J.F. in rabbits immunized with a synthetic peptide based on residues 502–525 from the human $p70^{S6K}$ cDNA [43]. The anti-GSK-3 serum was a gift from Dr. J. Vandenheeden (Katholieke Universiteit, Leuven, Belgium). All other chemicals and biochemicals, including wortmannin, were from Sigma Chemical Co. or BDH (both of Poole, Dorset, U.K.). Wortmannin and rapamycin (5 mM and 100 mM stock solutions respectively in DMSO) were stored in aliquots at −20 °C.

Incubation of epididymal fat pads and preparation of isolated adipocytes

Epididymal fat pads were preincubated at 37 °C for 15 min in gassed Krebs–Henseleit buffer [44] containing 10 mM Hepes and 5.6 mM glucose before incubation in fresh buffer containing further additions as described in the Figure legends. After incubation, pads were extracted by homogenization in the appropriate ice-cold extraction buffer (2 ml/g of tissue) using a polytron homogenizer PT10. Pad extracts were centrifuged (10000 *g*; 4 °C; 10 min) and the infranatant removed for enzyme activity measurements. Isolated adipocytes were prepared from epididymal fat pads as described previously [45]. Cells (routinely 150–200 mg cell dry weight/ml) were preincubated in gassed Krebs–Henseleit buffer containing 10 mM Hepes, 2 mM glucose and 1 % BSA for 15 min and then incubated in the presence of further additions as described in the Figure legends. Adipocytes were then extracted by vortexing in the appropriate ice-cold extraction buffer in a glass tube [46] and centrifuged as described above for epididymal fat-pad extracts. All incubations of fat-pad and adipocyte extracts contained 0.1 % (v/v) DMSO as this was the vehicle used to prepare stock solutions of both wortmannin and rapamycin. Control studies showed that this concentration of DMSO had no appreciable effect on any of the parameters measured. Preliminary investigations showed that the full effect of wortmannin on the stimulation of glucose utilization by insulin was obtained if the adipocytes were incubated with the inhibitor for 30 min before the addition of insulin. The dose–response curve for wortmannin on glucose utilization was similar to that reported previously [14], with an IC_{50} of approx. 30 nM. Under the conditions used here, wortmannin does not alter adipocyte ATP levels (T. A. Diggle, unpublished work).

Assay of MAPK, GSK-3 and $p70^{S6K}$

MAPK activity in isolated adipocytes was measured in anti-(p42/p44 MAPK) immunoprecipitates as described by Young et al. [47], except that a synthetic peptide substrate based on the sequence around Thr-669 of the EGF receptor (KRELVEPLTP-SGEAPNQALLR) was used at 0.2 mM instead of the more commonly used myelin basic protein. This peptide has been shown to be a more specific substrate for MAPK than myelin basic protein [48]. Cells (150–200 mg dry weight) were extracted in 1 ml of kinase extraction buffer (50 mM β-glycerophosphate, 1.5 mM EDTA, 1 mM benzamidine, 0.5 mM Na_3VO_4, 1 mM dithiothreitol, 1 μM microcystin, 0.1 mM PMSF and 1 μg/ml pepstatin, leupeptin and antipain, pH 7.4). MAPK was immunoprecipitated from 500 μl of cell extract by incubation with 10 mg of Protein A–Sepharose and 5 μl of anti-(p42/p44 MAPK) serum [47] for 2 h at 4 °C. Immunoprecipitates were washed three times in extraction buffer before resuspension to a final volume of 80 μl.

The activity of GSK-3 in isolated adipocyte extracts was determined as described previously [16] using the synthetic peptide substrate YRRAAVPPSPSLSRHSSPHQS(P)EDEEE (60 μM) and the negative control peptide YRRAAVPPSPSLS-RHSSPHQAEDEEE in which the 'priming' serine phosphate residue is replaced with alanine. The same peptides were also used to measure GSK-3 activity in anti-GSK-3 immunoprecipitates as described by Van Lint et al. [49].

The activity of $p70^{S6K}$ in adipocytes was determined in anti-$p70^{S6K}$ immunoprecipitates as described above for MAPK, except

that the S6 peptide KEAKEKRQEQIAKKRRLSSLRASTSKS-ESSQK was used as the peptide substrate.

Western blotting for p70^{S6K}, MAPK and GSK-3 was carried out after separation of proteins by SDS/PAGE (10% gel). The relevant antisera were used at a 1:1000 dilution and immunoreactant proteins were visualized using a chemiluminescence detection system as described previously [50].

Measurement of glucose utilization and enzyme activities

The utilization of glucose by the epididymal fat pads was assessed by measuring disappearance of glucose from the incubation medium [51]. For the measurement of ACC activity, fat pads were extracted in ACC extraction buffer (0.25 M sucrose, 10 mM Tris, 20 mM Mops, 2 mM EGTA, 10 mM GSH, 3% defatted BSA, 2 mM benzamidine and 1 μg/ml each pepstatin, leupeptin and antipain, pH 7.4) and ACC activity was measured both before and after incubation with its allosteric activator, citrate (20 mM), as described previously [25]. For the measurement of PDH activity, fat pads previously frozen in liquid N_2, were extracted in PDH extraction buffer (100 mM KH_2PO_4, 2 mM EDTA, 1 mM dithiothreitol and 50 μl/ml rat serum, pH 7.3). The activity of PDH was measured as described by Rutter et al. [52] before and after incubation with PDH phosphatase. Glycogen synthase activity was measured in isolated adipocyte extracts by the method of Thomas et al. [53]. Briefly, cells were extracted with 300 ml/150–200 mg dry weight glycogen synthase extraction buffer (0.25 M sucrose, 50 mM Mops, 5 mM EDTA, 25 mM NaF, 5 mM dithiothreitol and 50 μl/ml rat serum, pH 7.0) and the activity ratio was determined by following the incorporation of UDP-[U-^{14}C]glucose into glycogen in the presence or absence of the allosteric activator, glucose 6-phosphate (15 mM).

ATP citrate-lyase phosphorylation

Adipocytes were incubated in low-phosphate (0.4 mM) Krebs–Henseleit buffer containing 10 mM Hepes, 5.6 mM glucose, 1% BSA and [^{32}P]P_i (500 c.p.m./pmol) for 2 h. Cells were then preincubated for 30 min with wortmannin or rapamycin as indicated before addition of insulin. After a further 10 min, cells were extracted in 1 ml/200–250 mg dry weight ACC extraction buffer. Extracts were then spun at 10000 *g* for 10 min at 4 °C to remove the fat and then solubilized in SDS/PAGE sample buffer. Phosphoproteins were separated on an SDS/7.5% polyacrylamide gel [54], and subsequently visualized by overnight radioautography using preflashed Amersham Hyperfilm.

RESULTS AND DISCUSSION

Effects of wortmannin and rapamycin on MAPK activity in adipocytes

Initial experiments were performed to determine the time course of MAPK activation seen in adipocytes on treatment with insulin or EGF. Both insulin and EGF rapidly activated MAPK in isolated rat adipocytes (Figure 1), with maximal stimulation of kinase activity seen after 2–5 min. EGF increased MAPK activity approximately 4-fold after 5 min, whereas the maximum effect seen with insulin was more modest (2.5-fold). These increases are similar to those reported by Sevetson and co-workers [40] and by Lin and Lawrence [22]. The time course for activation of MAPK in freshly isolated adipocytes was broadly similar to that reported for the effects of insulin and EGF on MAPK activity in 3T3-L1 adipocytes [20]. The identity of the kinases measured was confirmed by Mono Q chromatogaphy and immunoblotting (results not shown).

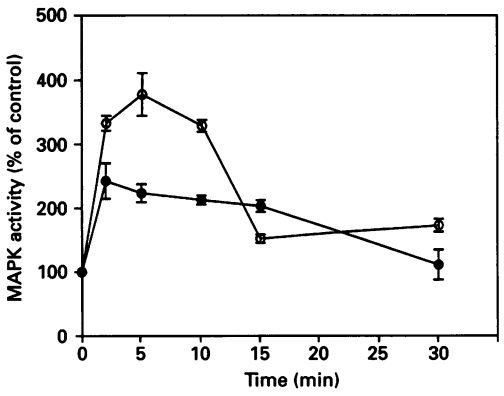

Figure 1 Time course for MAPK activation in response to insulin and EGF

Freshly isolated adipocytes were incubated with 83 nM insulin (●) or 100 nM EGF (○) for the times indicated. MAPK was assayed as described in the Experimental section. Results are expressed as a percentage of the control value before addition of hormones (1.07 pmol of ^{32}P incorporated into substrate peptide/min per g dry cell weight) and are means ± range for observations on two separate cell preparations.

Preincubation of adipocytes for 30 min with 100 nM wortmannin, although having no significant effect on basal MAPK activity, greatly decreased the stimulation seen in response to insulin, although it was unable to block completely the effect of EGF (Figure 2). Wortmannin also inhibits the stimulation of MAPK by insulin in CHO.T cells [16]. The effect of wortmannin on the MAPK cascade may, however, be cell- and/or stimulus-specific as it has been reported to have no effect on the stimulation of MAPK by EGF or platelet-derived growth factor in Swiss 3T3 cells or by nerve growth factor in PC12 cells [15]. In agreement with studies in 3T3-L1 adipocytes [55], rapamycin was without effect on the stimulation of MAPK activity in freshly isolated fat cells. As expected, under these conditions rapamycin (20 nM) inhibited the phosphorylation of p70^{S6K} seen in response to insulin. Phosphorylation was detected in a gel-shift assay by the appearance of a more slowly migrating form which was not evident in the presence of rapamycin (Figure 2, insert). The activity of p70^{S6K} was measured in immunoprecipitates of extracts prepared from cells incubated as described in the legend for Figure 2. The activity in control cells was 4.27 ± 0.47 pmol of ^{32}P incorporated into the substrate peptide/min per g dry cell weight (mean ± range for two separate cell experiments) and was increased 8.6 ± 1.0-fold by insulin. Rapamycin (20 nM) had no significant effect on the activity in control cells but greatly diminished the effects of insulin to 1.1 ± 0.2-fold. Treatment of cells with wortmannin also had no effect on the basal activity of p70^{S6K}, but it decreased the effect of insulin to 0.7 ± 0.1-fold.

Effects of wortmannin and rapamycin on the control of glycogen synthesis by insulin

Figure 3 shows that in isolated adipocytes insulin decreased the activity of GSK-3. The identity of the kinase measured using this peptide was confirmed as GSK-3 from its elution position on Mono S chromatography and immunoblotting (results not shown). The decrease in GSK-3 activity in response to insulin was also apparent in anti-GSK-3 immunoprecipitates (results not shown). Decreases in the activity of GSK-3 in rat adipose tissue on treatment with insulin were originally detected by Ramakrishna and Benjamin [32] as changes in the activity of an ATP citrate-lyase kinase termed the 'multifunctional protein

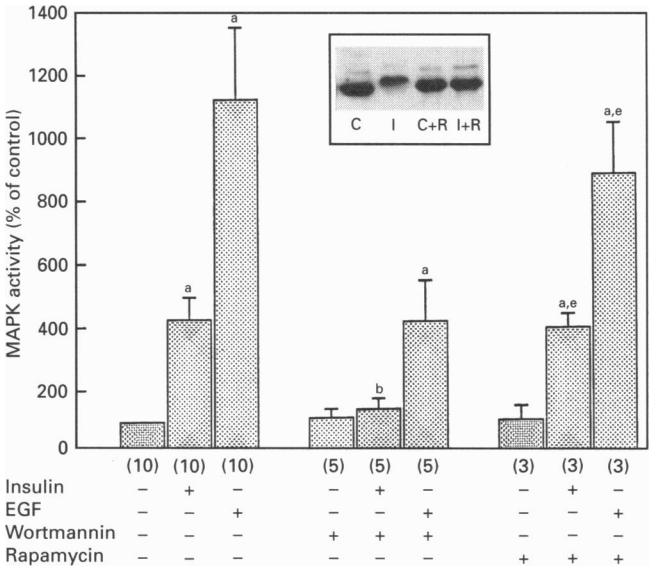

Figure 2 Effect of wortmannin and rapamycin on the stimulation of MAPK by insulin and EGF

Adipocytes were incubated with wortmannin (100 nM) or rapamycin (100 nM) for 30 min before the addition of 83 nM insulin or 100 nM EGF for 10 min. Results are expressed as a percentage of the control value (no additions) for MAPK activity (2.27 ± 0.65 pmol of ^{32}P incorporated into substrate peptide/min per g dry cell weight; $n = 10$) and are means $\pm$ S.E.M. for the number of separate cell preparations shown in parentheses. Significance as assessed by Student's t test is indicated by a single letter code as follows: a, $P < 0.05$ compared with control; b, $P < 0.05$ compared with insulin-stimulated result; c, $P < 0.05$ compared with EGF-stimulated result; d, $P < 0.05$ control + wortmannin compared with insulin or EGF + wortmannin; e, $P < 0.05$ control + rapamycin compared with insulin or EGF + rapamycin. The insert shows the effect of rapamycin on the phosphorylation of $p70^{S6K}$ by insulin. Cells were incubated with or without insulin and rapamycin as above. The phosphorylation state of $p70^{S6K}$ in the cell extracts was determined by Western blotting using an anti-$p70^{S6K}$ antibody as described in the Experimental section. The tracks shown are: C, control; I, insulin; C + R, control plus rapamycin (20 nM); I + R, insulin plus rapamycin. Similar results were obtained from four separate cell preparations.

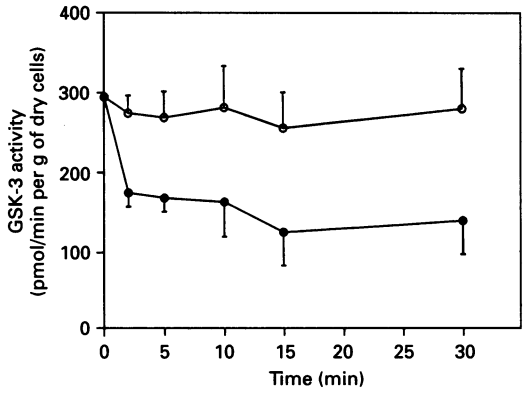

Figure 3 Time course of the effect of insulin and EGF on GSK-3 activity

GSK-3 activity was determined in cell extracts prepared from adipocytes incubated as described in the legend for Figure 1 with 83 nM insulin (●) or 100 nM EGF (◆). Results are expressed as means $\pm$ S.E.M. for observations on four separate cell preparations.

kinase'. More recently it has been shown that this kinase is the α-isoform of GSK-3 [56]. Insulin has also been shown to decrease GSK-3 activity in a number of other cell types [15,16]. EGF,

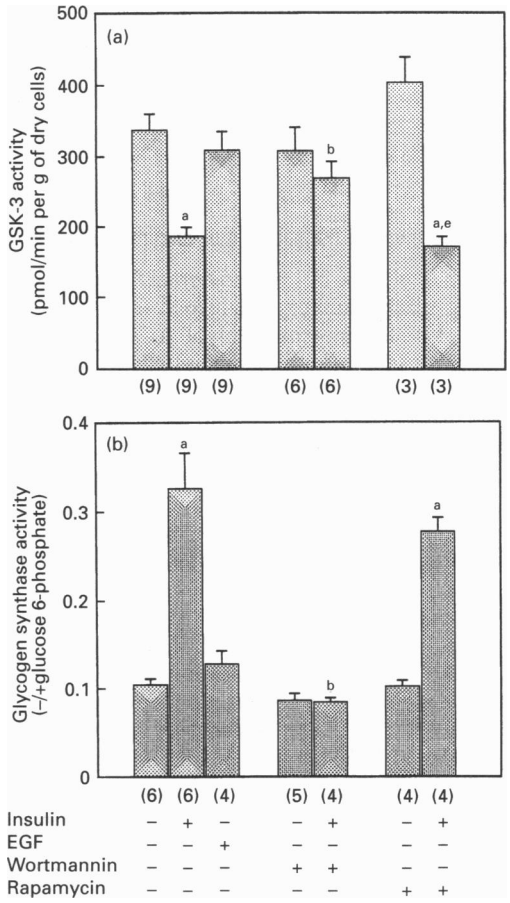

Figure 4 Effect of wortmannin and rapamycin on changes in GSK-3 and glycogen synthase activities in response to insulin and EGF

GSK-3 (**a**) and glycogen synthase (**b**) activities were determined in cell extracts prepared from adipocytes incubated as described in the legend for Figure 2. The activity of glycogen synthase is expressed as a ratio of its activity in the absence of glucose 6-phosphate to that in the presence of 15 mM glucose 6-phosphate. Extracts prepared from untreated control cells showed glycogen synthase activities of 34.02 ± 2.60 and 3.53 ± 0.37 nmol/min per g cell dry weight ($n = 6$) in the presence and absence of glucose 6-phosphate respectively. All results are expressed as means $\pm$ S.E.M. of the number of separate cell preparations shown in parentheses. Significance is indicated where appropriate as given in the legend for Figure 2.

however, had no detectable effect on GSK-3 activity in adipocytes, although it has been reported to decrease its activity in A431 and NIH/3T3 cells [57,58]. Figure 4(a) shows that the effect of insulin was abolished by preincubation with wortmannin. Rapamycin was unable to block the inhibition of GSK-3 seen in response to insulin (Figure 4a).

In agreement with the observed changes in GSK-3 activity, insulin increased the activity ratio of glycogen synthase approximately 3.4-fold over basal levels (Figure 4b). Although rapamycin had no effect on the stimulation of glycogen synthase by insulin, the increase in glycogen synthase activity was abolished by wortmannin. As expected from its lack of effect on GSK-3 activity, EGF did not stimulate glycogen synthase in adipocytes (see also ref. [22]).

Effects of wortmannin and rapamycin on the control of lipogenesis by insulin

Wortmannin has recently been shown to block the increase in 2-deoxyglucose uptake by isolated adipocytes in response to insulin

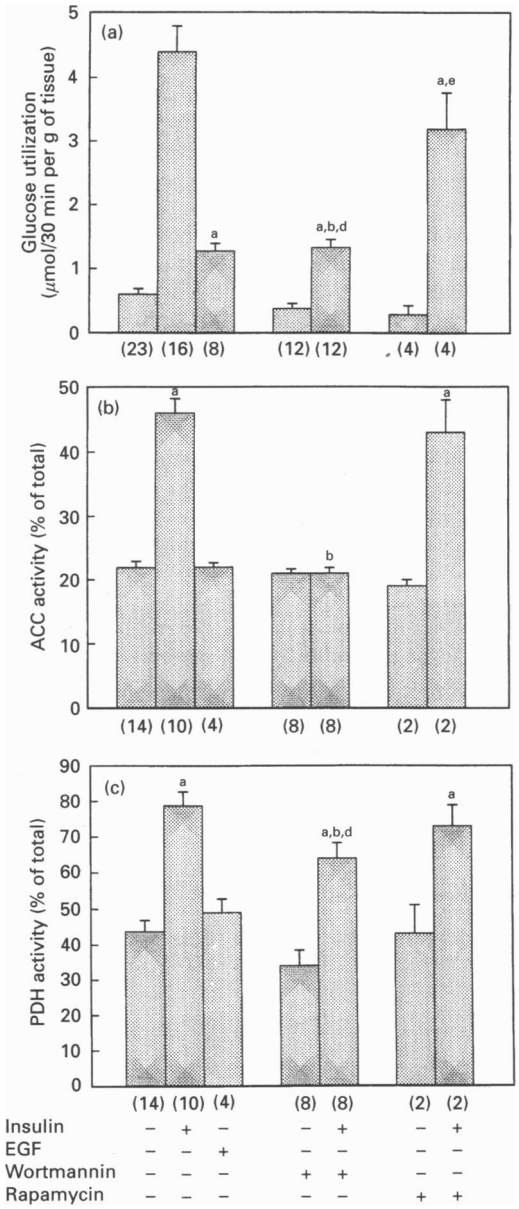

Figure 5 Effect of insulin, EGF, wortmannin and rapamycin on glucose utilization and on the activities of ACC and PDH

Epididymal fat pads were preincubated for 30 min in the presence or absence of 200 nM wortmannin or 200 nM rapamycin before incubation for 30 min with 83 nM insulin or 100 nM EGF. After this time, samples of the incubation media were taken for analysis of glucose utilization (**a**), and the pads were extracted as described in the Experimental section. ACC results are expressed as a percentage of total activity in the presence of 20 mM citrate (**b**), and PDH results are expressed as a percentage of total activity in the presence of PDH phosphatase (**c**). Values shown are means ± S.E.M. for the number of separate observations shown in parentheses, and where appropriate significance is indicated by a single letter as described in the legend for Figure 2.

[14]. In this study we have taken the rate of glucose disappearance from the incubation medium as a measure of glucose transport into epididymal fat pads. Figure 5(a) shows that wortmannin reduced the stimulation of glucose utilization by insulin from 6.7-fold in the absence of inhibitor to 3.5-fold in its presence. In agreement with the work of Stagsted and co-workers [59] using isolated rat adipocytes, EGF produced a small but significant increase in the disappearance of glucose from the medium. In contrast, Okada et al. [14], and Lin and Lawrence [22] were unable to detect any effects of EGF on either the translocation of the GLUT4 isoform of the glucose transporter to the adipocyte plasma membrane or the uptake of 2-deoxyglucose into adipocytes.

Whereas wortmannin only partially blocked insulin-stimulated glucose utilization, it totally abolished the 1.7-fold increase in ACC activity seen in rat epididymal fat pads in response to insulin (Figure 5b). Similar effects were seen in isolated fat cells (results not shown). Under the conditions used here, we were unable to show any effect of EGF on ACC activity in epididymal fat pads (see Figure 5b) or in freshly isolated adipocytes (results not shown). It has been reported that EGF both increases the incorporation of radioactivity from [^{3}H]glucose into total lipid in adipocytes and apparently increases the activity of ACC to a similar extent to insulin [60]. The reason for the discrepancy between this work and that presented here is unclear.

In direct contrast with the effects of wortmannin on glucose utilization and ACC activity, the inhibitor had no effect on the 1.8-fold increase in PDH activity seen in fat pads treated with insulin (Figure 5c). Incubation with wortmannin decreased the level of PDH activity slightly in both control and insulin-stimulated cells. This may be the result of the decreases in glucose transport (see Figure 5a) which would be expected to diminish the intracellular concentration of pyruvate, resulting in decreased PDH kinase activity.

Similar studies to those reported in Figure 5 were carried out in which glucose in the incubation medium was replaced by fructose (5.6 mM). Wortmannin was again found to block completely the effect of insulin on ACC activity, whereas the effect on PDH activity was unaffected (results not shown). Previous studies have shown that, although insulin has little effect on fructose uptake into rat fat cells, the effects of insulin on ACC and PDH activities are similar to those in the presence of glucose [61,62].

Preincubation of fat pads with rapamycin had no significant effect on the stimulation of glucose utilization, ACC or PDH by insulin (Figures 5a–5c).

ATP citrate-lyase is another enzyme involved in fatty acid synthesis. Insulin causes large increases in its phosphorylation without apparently changing its activity [63,64]. The kinase involved has not been fully characterized [65,66]. Figure 6 shows that wortmannin, but not rapamycin, largely abolishes the increase in phosphorylation seen with insulin, whereas it has no effect on the basal level of ATP citrate-lyase phosphorylation in untreated cells. Figure 6 also shows that wortmannin, but not rapamycin, reversed the small increase in overall phosphorylation of ACC seen on treatment with insulin.

General conclusions

Signalling pathways involved in insulin action have been studied by others using the inhibitors wortmannin and rapamycin and comparing the effects of insulin and EGF (see the Introduction). In the present study, we have extended these approaches to the study of a wide range of insulin-sensitive processes involved in the stimulation of both glycogen and fatty acid synthesis in fresh rat epididymal fat cells or pads. A number of conclusions can be drawn.

(i) Activation of MAPK may not be involved in the insulin-sensitive processes linked to the stimulation of glycogen or fatty acid synthesis. This follows from the near complete lack of effect of EGF on glucose utilization, GSK-3, glycogen synthase, PDH

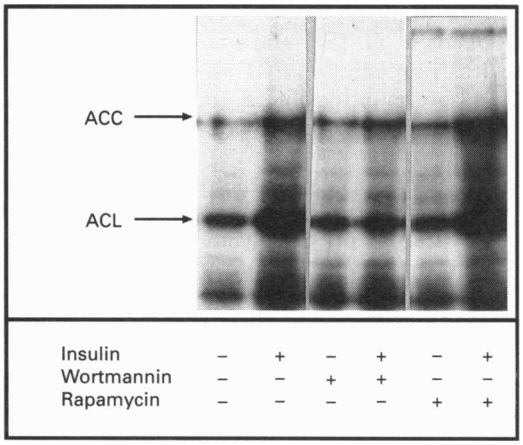

Figure 6 Effect of wortmannin and rapamycin on the phosphorylation of ATP citrate-lyase in response to insulin

Adipocytes were incubated with [^{32}P]P_i as described in the Experimental section before addition of 100 nM wortmannin or 100 nM rapamycin for 30 min. Cells were then incubated with 83 nM insulin for a further 10 min before extraction. Crude cell extracts (20 μl) were then subjected to SDS/PAGE (7.5% gel) and radiolabelled phosphoproteins were visualized by radioautography. The positions of ACC and ATP citrate-lyase (ACL) are indicated by the arrows.

and ACC despite the fact that EGF activates MAPK to a greater extent than insulin. Similar conclusions have been reached previously by Lin and Lawrence [22] concerning the possible involvement of MAPK in the activation of glucose utilization and glycogen synthase. Alternatively, it is possible that EGF causes some other unknown intracellular change or changes which counteract those in MAPK, but it seems unlikely that this could be the case for all of these separate processes. The lack of effect of EGF on GSK-3 is the most surprising as it has been concluded that activation of MAPK and hence $p90^{rsk}$ is responsible for changes in GSK-3 activity in A431, muscle L6, CHO.T and NIH/3T3 cells [3,16,57,58].

(ii) Activation of $p70^{S6K}$ is not involved in the insulin-sensitive processes linked to the stimulation of glycogen or fatty acid synthesis. This can be deduced from the lack of any appreciable effects of a high concentration of rapamycin (100 nM) despite its ability to block the effect of insulin on $p70^{S6K}$. Others have also shown that $p70^{S6K}$ is unlikely to play a role in the control of glycogen synthase by insulin in freshly isolated adipocytes [22] or in PC12 cells [67]. A recent report, however, has shown that rapamycin diminishes, but does not completely block, the effects of insulin on glycogen synthase in 3T3-L1 adipocytes [41]. Thus multiple pathways of varying importance in different cell types may be involved in the regulation of glycogen synthase by insulin. In the cultured 3T3-L1 cells the activity ratio of glycogen synthase is much less than in fresh adipocytes, and this may be an indication that different signalling pathways are operating.

(iii) Activation of PI 3-kinase appears to be necessary for many insulin-sensitive processes in fat cells but not the activation of PDH. This follows from the ability of wortmannin to block the effects of insulin on glucose utilization, the phosphorylation of ATP citrate-lyase and the activities of MAPK, GSK-3, glycogen synthase and ACC. Previous studies on rat adipocytes have shown that wortmannin inhibits the effects of insulin on 2-deoxyglucose transport [14], phosphodiesterase activity [68] and lipolysis [14,68]. The assumption is made that the effects of wortmannin at the low concentrations used in this and other studies are restricted to the inhibition of PI 3-kinase. Inhibition of PI 3-kinase in 3T3-L1 adipocytes by a different inhibitor, LY294002 [17], has also been shown to lead to inhibition of the effects of insulin on glucose transport and GLUT4 translocation. The links between PI 3-kinase and these insulin-sensitive processes are essentially unknown. Signalling to MAPK appears to be different from those to the other wortmannin-sensitive insulin effects (glucose utilization, GSK-3, glycogen synthase and ACC) as activation of MAPK does not appear to be important in these effects of insulin [see discussion of the effects of EGF in (i) above]. In complete contrast with all the other effects of insulin studied, the degree of activation of PDH was essentially unaltered in the presence of wortmannin. Previous studies from this laboratory have shown that insulin increases the activity of PDH by increasing the activity of PDH phosphatase rather than decreasing the activity of PDH kinase [23,69]. The increase in PDH phosphatase activity appears to be largely due to an increase in the sensitivity of this intramitochondrial enzyme to Mg^{2+} but it is not known how insulin causes this alteration [23,70].

Overall, at least four different signalling pathways are involved in the actions of insulin on fat cells. Two of these are the pathways that lead to the activation of MAPK and $p70^{S6K}$. However, neither of these appears to be important in the effects of insulin on glycogen and fatty acid synthesis in fat cells. A third signalling pathway (or group of pathways) leads to the activation of glucose transport, glycogen synthase and ACC; this pathway (or pathways) appears to involve PI 3-kinase (but not MAPK or $p70^{S6K}$). Finally, there is a separate signalling pathway leading to the activation of PDH which does not appear to involve PI 3-kinase, GSK-3, MAPK or $p70^{S6K}$.

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