Insulin action in cultured human myoblasts: contribution of different signalling pathways to regulation of glycogen synthesis

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A key metabolic action of insulin is the stimulation of non-oxidative glucose utilization in skeletal muscle, by increasing both glucose uptake and glycogen synthesis. The molecular mechanism underlying this process has been investigated using a variety of experimental systems. We report here the use of cultured human myoblasts to study insulin control of glycogen synthesis in humans. In these cells insulin stimulates glycogen synthesis approx. 2.2-fold, associated with a similar activation of glycogen synthase (GS) which occurs within 5–10 min of the addition of insulin. Insulin also causes inactivation of glycogen synthase kinase-3 (GSK-3) and activation of protein kinase B, both processes being sufficiently rapid to account for the effects of insulin on GS. Activation by insulin of the protein kinases $p70^{s6K}$, $p90^{s6K}$ and extracellular signal-regulated kinase 2 (ERK2) is observed, but is significantly slower than the activation of GS. Selective inhibitors of the $p70^{s6K}$ pathway (rapamycin), the ERK2/$p90^{s6K}$ pathway (PD98059) and phosphatidylinositol 3-kinase (wortmannin) have been used to probe the contribution of these components to insulin signalling in human muscle. Wortmannin blocks activation of both glycogen synthesis and GS and inactivation of GSK-3. PD98059 is without effect on these events, while rapamycin is without effect on inactivation of GSK-3 but partially blocks activation of glycogen synthesis and GS. Taken together, these findings suggest that protein kinase B is responsible for the inactivation of GSK-3, but that an additional rapamycin-sensitive mechanism may contribute to the activation of GS and stimulation of glycogen synthesis.

INTRODUCTION

A key physiological action of insulin is the stimulation of glycogen synthesis, involving an increase in glucose uptake and activation of the enzyme glycogen synthase (GS), processes which are markedly diminished in non-insulin-dependent diabetes mellitus and first-degree relatives of this disease (reviewed in [1–3]). Work on insulin action in skeletal muscle in humans has focused principally on studies *in vivo*, often linked to subsequent analysis of biopsies, or the use of muscle strips *in vitro*. These approaches have several limitations, e.g. only a small amount of tissue can be obtained, the impracticability of repeating studies and the limited number of subjects willing to undergo these invasive procedures. Because of these limitations, most of the work on insulin action has been carried out in experimental animals, or using animal cell lines or purified enzymes.

Activation by insulin of GS results from net dephosphorylation of the enzyme, principally at sites 3a–3c [4], although dephosphorylation of additional sites may also play a role [5]. Phosphorylation at sites 3a–3c is catalysed by glycogen synthase kinase-3 (GSK-3) and dephosphorylation is catalysed by $PP1_G$, the glycogen-bound form of protein phosphatase 1 (reviewed in [6]). Following insulin administration, the activity of $PP1_G$ towards GS and phosphorylase kinase (but not glycogen phosphorylase) is stimulated as a result of phosphorylation at site 1 of the glycogen-binding subunit by $p90^{s6K}$ (also called MAPKAP kinase 1 or rsk-2) [7]. Activation by insulin of PP1 activity against glycogen phosphorylase has been reported in rat adipocytes [8] and more recently in 3T3-L1 adipocytes and rat L6 myotubes [9], but the molecular basis of this transient event has not been determined. Insulin has also been demonstrated to cause inactivation of GSK-3 in several cell types [10–13] and in rabbits *in vivo* [12]. As $p90^{s6K}$ can phosphorylate and inactivate GSK-3 *in vitro* [14], and as it lies downstream of the insulin-stimulated mitogen-activated protein kinase [MAP kinase; also known as extracellular signal-regulated kinase (ERK)] cascade [15], it provides an attractive mechanism whereby insulin could stimulate GS by both activating PP1 and inactivating GSK-3.

Recent evidence, however, has questioned the involvement of $p90^{s6K}$ and the MAP kinase pathway in signalling to glycogen synthesis (reviewed in [16]). Of particular significance is the finding that the compound PD98059, a selective inhibitor of MAP kinase kinase [17], has no effect on insulin stimulation of glycogen synthesis and GS in 3T3-L1 adipocytes and L6 myotubes [9], on glycogen synthesis in rat diaphragm [18] or on GSK-3 inactivation in L6 myotubes [19].

Two additional insulin-stimulated kinases, namely $p70^{s6K}$ and protein kinase B (PKB), have also been shown to phosphorylate and inactivate GSK-3 *in vitro* [14,19]. However, their relative contributions to the regulation of GSK-3 and glycogen synthesis in the intact cell remain controversial. Activation of $p70^{s6K}$ in cells is blocked selectively by the macrolide rapamycin [20]. This inhibitor has no observable effect on activation of GS in rat

Abbreviations used: GS, glycogen synthase; GSK-3, glycogen synthase kinase-3; PP1, protein phosphatase 1; $PP1_G$, glycogen-bound form of PP1; MAP kinase, mitogen-activated protein kinase; ERK, extracellular-signal-regulated kinase; PKB, protein kinase B; PKI, peptide inhibitor of cAMP-dependent protein kinase.

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adipocytes [21,22] and CHO cells [23], but blocks activation, at least partially, in 3T3-L1 adipocytes [24] and in isolated rat diaphragm [18]. The results from work in 3T3-L1 adipocytes and rat diaphragm are in apparent contrast with those from work in rat adipocytes [22] and rat L6 myotubes [12,19], where rapamycin has no effect on the inactivation of GSK-3 by insulin. However, in none of these studies have the effects of insulin and inhibitors on GS and GSK-3 been examined concurrently. In all cases studied to date, activation of GS, inactivation of GSK-3 and stimulation of glucose uptake by insulin is blocked by wortmannin, a fungal inhibitor of PtdIns 3-kinase [25], placing the relevant pathway(s) downstream of that enzyme.

In view of the apparently conflicting data from the different experimental models, it seems particularly relevant to study insulin signalling in human muscle. In order to achieve this, we and others have established primary human muscle cultures and have shown that these are insulin-responsive, providing an important model system in which to study the insulin signalling cascade in humans [13,26,27]. The major advantages of using cultured muscle cells are: a continual supply of human muscle for study, the conditions can be easily altered, and multiple time points from basal can be analysed. Furthermore, by using inhibitors and potentiators of insulin action or by modulation of key enzymes, the model allows for identification of sites within the insulin signal transduction pathways that may be defective in humans with insulin insensitivity and non-insulin-dependent diabetes mellitus.

EXPERIMENTAL

Materials

Antibodies against $p70^{s6K}$ kinase (C-18) and ERK2 (C-14) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The anti-$p90^{s6K}$ antibody was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Antibodies against a C-terminal peptide and the pleckstrin homology domain of PKB-α [19] were generously provided by Dr. Brian Hemmings (Friedrich Miescher-Institut, Basel, Switzerland). Antibodies against GSK-3 and isoforms were as described previously [12]. The specific 18-residue peptide inhibitor of cAMP-dependent protein kinase, termed PKI [28], and 'Crosstide' [19], a peptide substrate for PKB, were synthesized in the University of Newcastle Facility for Molecular Biology. The s6 kinase substrate peptide (RRRLSSLRA) was from Upstate Biotechnology, and the GSK-3 phosphopeptide substrate [11] was a gift from Professor Chris Proud (University of Kent, Canterbury, U.K.). Wortmannin and rapamycin were from Sigma (Poole, Dorset, U.K.), and PD98059 was from New England Biolabs. Actrapid insulin was from Novo Nordisk (Copenhagen, Denmark). Myelin basic protein was from Gibco BRL (Paisley, Scotland, U.K.).

Cell culture

Muscle biopsy samples (0.10–0.25 g) were obtained with prior consent from the gastrocnemius muscle of four healthy subjects with no family history of diabetes. Prior ethical committee approval was obtained. Tissue was rinsed in culture medium (Ham's nutrient culture mixture F10 with 20 % foetal calf serum, 1 % chick embryo extract, 10000 units/ml penicillin and 2 μg/ml streptomycin). All visible connective tissue and fat was removed using forceps, and the biopsy samples were chopped finely with scissors and transferred to a small sterile flask containing 0.05 unit/ml trypsin and 0.05 mM EDTA in PBS. After stirring gently at 37 °C for 15 min, the particulate debris was allowed to settle and the supernatant was removed and centrifuged at 550 ***g*** for 5 min. Pellets were collected and resuspended in conditioning medium. The cells were then grown to confluency in culture medium in 25 cm^2 culture flasks coated with 1 % gelatin, and were then transferred to 75 cm^2 flasks and ultimately to 6 cm^2 wells. Fusion into myotubes and immunocytochemical staining with anti-desmin and anti-(fibroblast surface protein) antibodies confirmed the predominant presence of myoblasts (results not shown). Experiments were performed on cells between the fifth and fifteenth passage in flasks or wells in which cells had reached greater than 90 % confluency. At 16 h prior to the studies, cells were transferred into serum-free medium. When required, insulin was added at a concentration of 1 μM. For inhibition studies, cells were preincubated with 100 nM wortmannin or 100 nM rapamycin for 15 min, or with 50 μM PD98059 for 1 h, prior to the addition of insulin. All assays were performed in duplicate on at least four separate occasions using cells from at least three different subjects.

Values given are means $\pm$ S.E.M., and statistical analysis was by Students *t*-test. A significance level of $P < 0.05$ was chosen. Results are expressed as units per mg of protein.

Preparation of myoblast extracts

Following incubation of cells under appropriate conditions, reactions were terminated by washing the cell monolayers rapidly five times with ice-cold PBS. Extraction buffer [100 mM Tris/HCl, 100 mM KCl, 2 mM EDTA, 25 mM KF, 0.1 % (v/v) Triton X-100, 1 mM benzamidine, 0.1 mM Na_3VO_4, 1 mg/ml glycogen, pH 7.3, containing 10 μg/ml pepstatin, 10 μg/ml antipain and 10 μg/ml leupeptin] was added (200 μl per 6 cm^2 plate), and cells were scraped and immediately frozen in liquid N_2. Prior to analysis, samples were thawed, dispersed by sonication for 60 s (Sonibath; Dawe) and then centrifuged at 13000 ***g*** for 5 min at 4 °C. The supernatants were retained and the protein concentration was determined by a dye-binding method [29].

Fractionation of myoblast extracts on Mono Q

Following centrifugation at 13000 ***g***, the supernatant was passed through a 0.2 μm-pore-size filter and the extract, containing approx. 1 mg of protein, was diluted in 5 ml of buffer A (50 mM sodium glycerophosphate, 1 mM EGTA, 1 mM benzamidine, 1 mM dithiothreitol, 0.1 mM Na_3VO_4, pH 7.4, containing 1 μg/ml each of pepstatin, antipain and leupeptin) and loaded on to an FPLC Mono Q column (Pharmacia) equilibrated in the same buffer. After washing extensively with buffer A, proteins were eluted with a linear salt gradient from 0 to 0.5 M NaCl in 20 ml of buffer A. Fractions of 0.5 ml were collected, and 10 μl of each fraction was assayed for PKB activity as described below or was subjected to immunoblotting using anti-PKB antibodies, as in [19].

Immunoprecipitation and assay of kinases

Samples (30 μl) of cell extract supernatants containing approx. 5 μg of protein were incubated with antibodies to $p70^{s6K}$ (0.2 μg), $p90^{s6K}$ (0.5 μg) or ERK2 (0.4 μg) in a total volume of 40 μl for 2 h at 0 °C. Protein A (2 mg) immobilized on Sepharose 4B-Cl prepared at 100 mg/ml in extraction buffer was added, and the incubation was continued at 4 °C for 1 h with occasional shaking.

The immobilized immune complexes were recovered by centrifugation at 13000 ***g***, and washed twice with extraction buffer and then once with buffer A. The pellet was resuspended to 20 μl in buffer A, and the kinases were assayed in a total volume of 40 μl

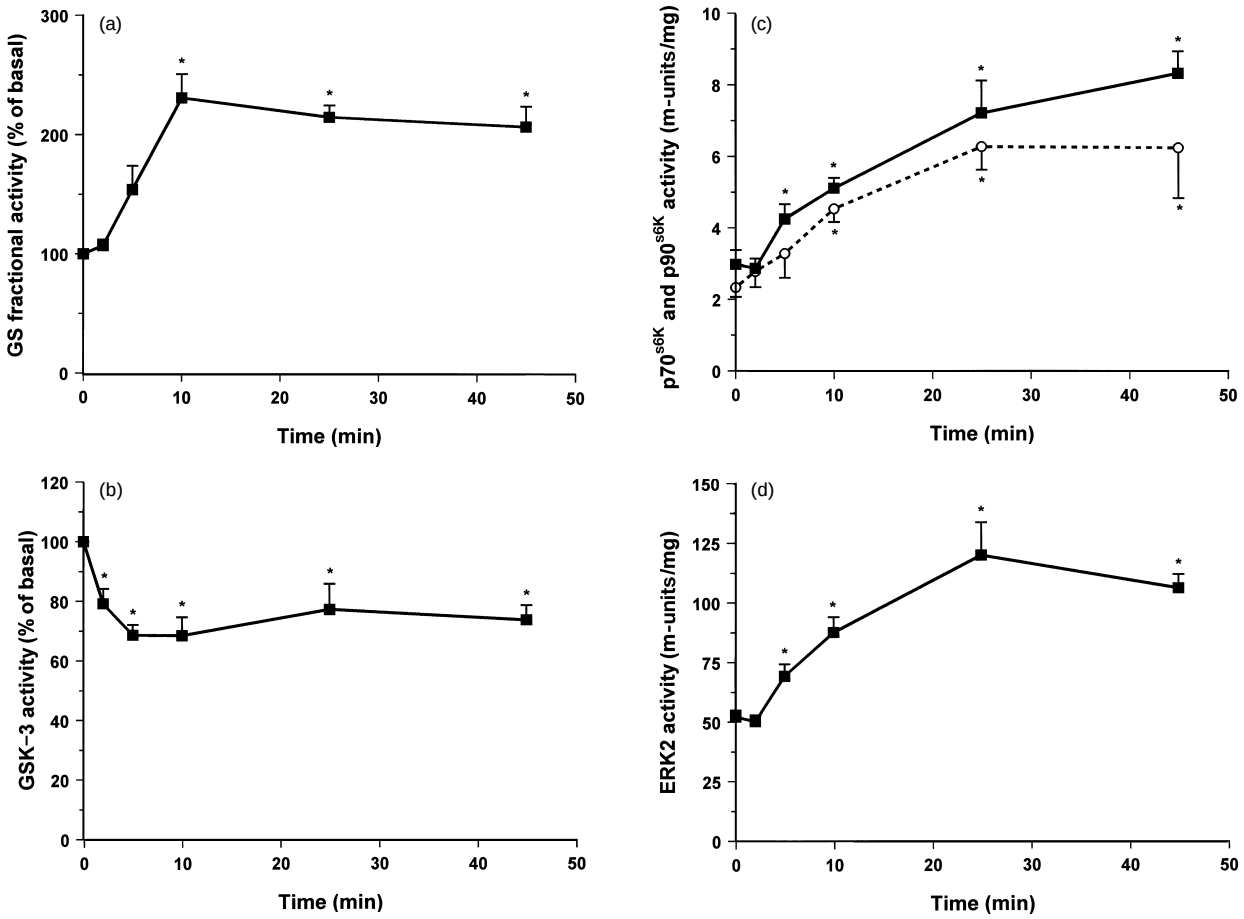

Figure 1 Time courses of insulin action on target enzymes in human myoblasts

Cells were incubated with insulin (1 μM) for the indicated times, and extracts were prepared and assayed as described in the Experimental section. Activities: (**a**) GS (fractional), (**b**) GSK-3, (**c**) $p70^{s6K}$ (■) and $p90^{s6K}$ (○), (**d**) ERK2. For GS and GSK-3, the activity is presented as a percentage of the basal value. All values are expressed as means ± S.E.M.; $n = 5$ for (**a**), $n = 7$ for (**b**) and $n = 4$ for (**c**) and (**d**). In each case cells from at least three different subjects were used. At zero time the fractional activity of GS was 0.163 ± 0.019 and the activity of GSK-3 was 3.09 ± 0.64 m-units/mg. Statistical significance ($P < 0.05$) compared with the basal value is indicated by *.

containing 50 mM sodium glycerophosphate, pH 7.4, 50 μM [γ-^{32}P]ATP (approx. 4000 c.p.m./pmol), 10 mM $MgCl_2$, 0.5 mM benzamidine, 0.5 mM dithiothreitol, 0.5 mM EGTA, 0.05 mM Na_3VO_4, 2.5 μM PKI, and including either 50 μM s6 kinase substrate peptide for the assay of $p70^{s6K}$ and $p90^{s6K}$, or 0.25 mg/ml myelin basic protein for the assay of ERK2. After a 30 min incubation at 30 °C, the radiolabelled peptide product was recovered by centrifugation, and 25 μl of the supernatant was spotted on to Whatman P81 phosphocellulose paper squares. After washing in 175 mM phosphoric acid with four changes, the papers were dried and phosphate incorporation was determined by liquid scintillation counting.

GSK-3 immunoprecipitations were carried out using a mixture of anti-GSK-3α and anti-GSK-3β antibodies pre-absorbed to Pansorbin. Cell extracts were prepared in extraction buffer as described above but with the addition of 100 nM okadaic acid. Aliquots containing approx. 10 μg of protein were diluted to 150 μl by addition of buffer A. Following conjugation with antibody/Pansorbin for 2 h at 4 °C, samples were centrifuged at 13000 ***g*** to recover the immune complex and the supernatant was removed. The pellet was washed once with extraction buffer and twice with buffer A. The pellet was then resuspended to 10 μl in buffer A and GSK-3 was assayed in a total volume of 20 μl containing 25 mM sodium glycerophosphate, pH 7.4, 100 mM NaCl, 25 μM GSK-3 phosphopeptide, 50 μM [γ-^{32}P]ATP, 10 mM $MgCl_2$, 0.5 mM benzamidine, 0.5 mM dithiothreitol, 0.5 mM EGTA, 0.05 mM Na_3VO_4 and 2.5 mM PKI. After a 30 min incubation at 30 °C, the radiolabelled peptide product was recovered and quantified as above.

PKB was immunoprecipitated in an identical manner to that for GSK-3, except that anti-(PKB pleckstrin homology domain) antibodies (1 μl) were used instead of anti-GSK-3 antibodies. PKB activity was assayed against 100 μM Crosstide [19] under the conditions described above. Immunoprecipitation conditions were optimized for each kinase, as determined by either immunoblotting of the supernatants or failure to detect activity in the supernatant or in a subsequent immunoprecipitate.

Assay of GS

Following the indicated treatments, cell extracts were prepared in extraction buffer and GS activity was assayed as incorporation of [^{14}C]glucose from UDP-[U-^{14}C]glucose into glycogen. Assays were performed in the presence of low (0.1 mM) and high (10 mM) concentrations of glucose 6-phosphate to give active

and total activities of GS, and the results are expressed as fractional activity [30].

Glycogen synthesis

Glycogen synthesis was determined as [^{14}C]glucose incorporation into glycogen. Cells were incubated for 2 h in culture medium containing [U-^{14}C]glucose (5 mM glucose; 1.25 μCi/ml) with or without insulin. The experiment was terminated by removing the medium and rapidly washing the cells five times in ice-cold PBS. Cells were lysed by the addition of 20 % (w/v) KOH, which was neutralized after 1 h by the addition of 1 M HCl. The wells were aspirated and the contents boiled for 5 min. After addition of 1 mg/ml glycogen, precipitation was carried out with ethanol at 0 °C for 2 h. The samples were centrifuged at 1100 ***g*** for 10 min, pellets were redissolved in water and radioactivity was determined by liquid scintillation counting.

RESULTS

In cultured human myoblasts, insulin stimulated glycogen synthesis from extracellular glucose by approx. 2.2-fold from a basal value of 184.4 ± 66.0 pmol/min per mg of protein (mean ± S.E.M., $n = 4$) when measured over a 2 h period (results not shown). This was associated with activation of GS by a similar magnitude; this increase was observable after 5 min and remained even after 45 min (Figure 1a). Expression of the insulin-regulated glucose transporter GLUT4 is minimal in human myoblasts in culture [31], and glucose uptake was essentially insensitive to insulin (results not shown).

We have shown previously [13] that the activity of GSK-3 in human myoblasts is inhibited by insulin within 10 min. The time course of this effect is shown in Figure 1(b), in which it can be seen that the effect was rapid, being significant after 2 min, maximal after 5 min and maintained for up to 45 min. As discussed above, at least three protein kinases, namely p70^{s6K}, p90^{s6K} and PKB [14,19], have been implicated in the phosphorylation and inactivation of GSK-3. The effect of insulin on the activities of p70^{s6K} and p90^{s6K} is shown in Figure 1(c). Activation of both p70^{s6K} and p90^{s6K} was relatively slow, reaching near maximum only after 25 min. p90^{s6K} lies downstream of one or more MAP kinases. Immunoblot analysis using anti-pan-ERK antibody (Transduction Labs) confirmed the presence of at least four MAP kinase isoforms in human myoblasts, of which the predominant form was ERK2. Using in-gel assays with immobilized myelin basic protein as substrate, ERK2 was found to be the only form that was stimulated significantly by insulin (results not shown). Subsequently ERK2 activity was measured in immunoprecipitates obtained using anti-ERK2 antibodies. The effect of insulin on this activity is shown in Figure 1(d), in which it can be seen that the stimulatory effect of insulin was again relatively slow-acting, reaching a maximum after 25 min.

Activation of PKB by insulin has been demonstrated previously in only one system, namely rat L6 myotubes [19]. Following incubation of human myoblasts with insulin for 10 min, cellular extracts were prepared and fractionated by ion-exchange chromatography on Mono Q and fractions were assayed for PKB activity using the synthetic Crosstide peptide as substrate [19]. Three broad peaks of activity were detected, eluting between 220 and 330 mM salt (Figure 2, upper panel). Little or no activity was detected in samples prepared from cells incubated in the absence of insulin. Immunoblot analysis using antibodies against the C-terminus of PKB [19] showed the presence of a single immunoreactive polypeptide of 59 kDa, which was detectable only in those fractions with detectable PKB activity. Having established the presence of PKB in human myoblasts and the responsiveness of the enzyme to insulin, the time course of the effect was then examined (Figure 2, lower panel). A dramatic activation of PKB by insulin was observed; this effect was very rapid, with half-maximal activation within 2 min and near-maximal activation after 5 min.

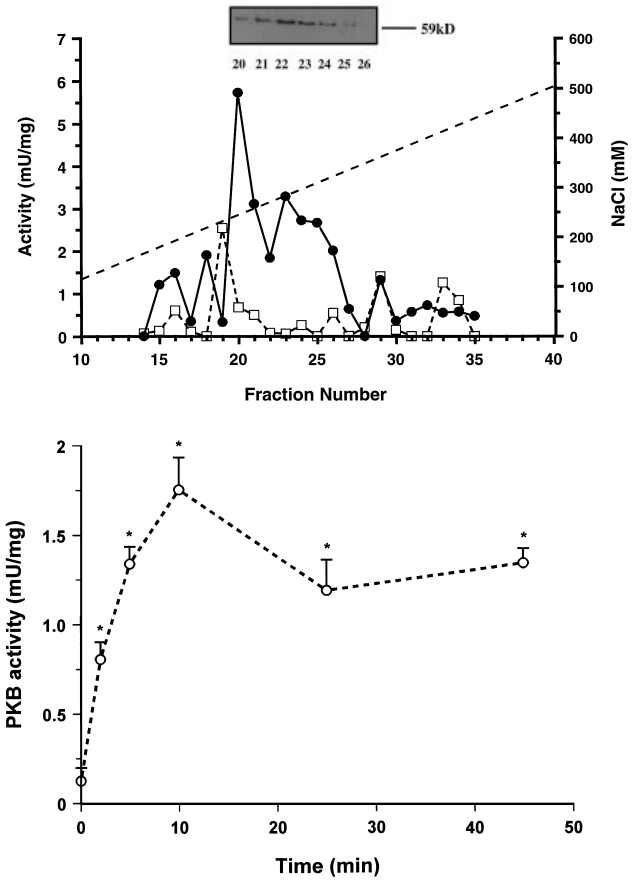

Figure 2 Activation by insulin of PKB in human myoblasts

Upper panel: extracts containing 1 mg of protein were prepared from human myoblasts incubated for 10 min either in the absence (□) or in the presence (●) of insulin (1 μM). The extracts were filtered and applied to a Mono Q (HR5/5) column, and activity was eluted with a linear NaCl gradient (broken line). Kinase activity was determined as described in the Experimental section. Inset: immunoblot detection of PKB using antisera to PKB and enhanced chemiluminescence detection. Numbers below lanes refer to fraction numbers; kD = kDa. Lower panel: cells were incubated with insulin (1 μM) for the indicated times and extracts were prepared and assayed as described in the Experimental section. Values are expressed as means ± S.E.M., $n = 4$, using cells from three different subjects. Statistical significance compared with the basal value is indicated by *.

Overall analysis of the time courses of the effects of insulin on the range of putative signalling components is consistent with GSK-3 and PKB being crucial, in that the changes in their respective activities were at least as fast as that of their target proteins. To investigate this further, the effects of specific inhibitors of selected components on the activity of the overall processes were investigated. In order to allow several enzymes to be assayed in the same cell extract, the cells were incubated in the presence of insulin for 10 min. Figures 1 and 2 show that a significant effect of insulin on the activity of each enzyme was observable at this time.

Three inhibitors were employed: rapamycin, which blocks activation of p70^{s6K} [20], PD98059, which blocks activation of MAP kinase and hence p90^{s6K} [17], and wortmannin, a potent

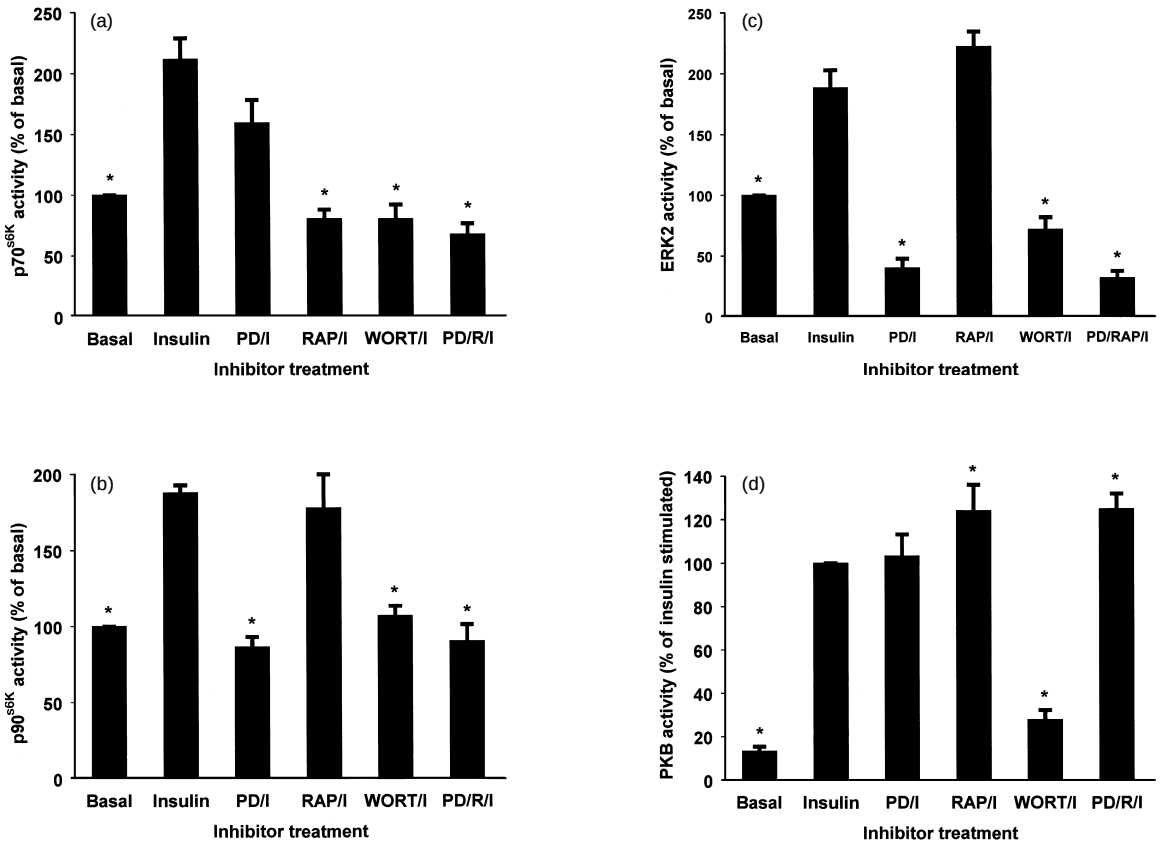

Figure 3 Effects of inhibitors on regulation by insulin of $p70^{s6K}$, $p90^{s6K}$, ERK2 and PKB

Cells were incubated in the absence (Basal) or in the presence of insulin for 10 min with no inhibitors present (Insulin), or following preincubation with 50 μM PD98059 (PD/I), 100 nM rapamycin (RAP/I), 100 nM wortmannin (WORT/I) or 50 μM PD98059 plus 100 nM rapamycin (PD/R/I). Extracts were prepared and assayed for (**a**) $p70^{s6K}$, (**b**) $p90^{s6K}$ (**c**) ERK2 and (**d**) PKB activity. Values are expressed as percentage stimulation of the basal value, except for PKB which is expressed as a percentage of the value in the presence of insulin. All results are expressed as means $\pm$ S.E.M. for five (**a**–**c**) or six (**d**) cell preparations from at least three subjects. In the absence of insulin (basal), the activities were: (**a**) 2.91 $\pm$ 1.18, (**b**) 4.61 $\pm$ 1.38, (**c**) 58.43 $\pm$ 11.82 and (**d**) 0.29 $\pm$ 0.07 m-units/mg. Statistical significance ($P < 0.05$) compared with the value in presence of insulin is indicated by *.

inhibitor of PtdIns 3-kinase [25]. First it was considered necessary to confirm that the inhibitors act in human myoblasts in their predicted manner. It is shown in Figure 3(a) that activation of $p70^{s6K}$ was blocked completely by rapamycin and by wortmannin. Surprisingly, PD98059 appeared to have a partial inhibitory effect on activation of $p70^{s6K}$, although this did not reach statistical significance ($P = 0.051$). Conversely, activation by insulin of both $p90^{s6K}$ and ERK2 was completely prevented by the presence of PD98059 or wortmannin, both of which inhibited ERK2 to below basal levels, suggesting that this kinase may be partially active even in the absence of insulin. There were no significant inhibitory effects of rapamycin on insulin activation of $p90^{s6K}$ and ERK2 (Figures 3b and 3c respectively). Activation of PKB by insulin was unaffected by PD98059 or rapamycin; indeed, the effects of insulin were slightly elevated in the presence of rapamycin, either alone or in combination with PD98059 (Figure 3d). The action of insulin on PKB was essentially blocked by wortmannin, again locating this enzyme downstream of PtdIns 3-kinase.

The effects of the inhibitors on the actions of insulin on glycogen synthesis, GS and GSK-3 were then examined. Stimulation of glycogen synthesis was unaffected by PD98059 but blocked completely by wortmannin. Rapamycin partially blocked the stimulation by insulin, when added either alone or in combination with PD98059 (Figure 4, top panel). A similar profile was obtained for the effects of the inhibitors on insulin stimulation of GS activity. Again PD98059 was without effect, wortmannin blocked the action of insulin, and rapamycin attenuated the insulin response, but not as markedly as the effect on glycogen synthesis (Figure 4, middle panel). The effects of the inhibitors on the inhibition of GSK-3 by insulin are shown in Figure 4 (bottom panel). Neither PD98059 nor rapamycin blocked the effect of insulin when added either alone or in combination. Wortmannin prevented completely the inactivation of GSK-3 by insulin; indeed it caused a small increase in the basal activity.

DISCUSSION

The data presented here indicate that cultured myoblasts represent a valuable experimental system for the study of insulin signalling in human muscle. They show insulin-responsiveness in terms of several parameters, including glycogen synthesis, activation of GS and activation/inactivation of regulatory enzymes known to be influenced by insulin. Furthermore, the time course and magnitude of the responses of glycogen synthesis and GS are similar to those observed in other experimental systems, including *in vivo* in humans [32,33]. The myoblasts are, however, immature cells and differ in several respects from mature muscle. Pertinent to the findings here is that they do not express GLUT4 to any

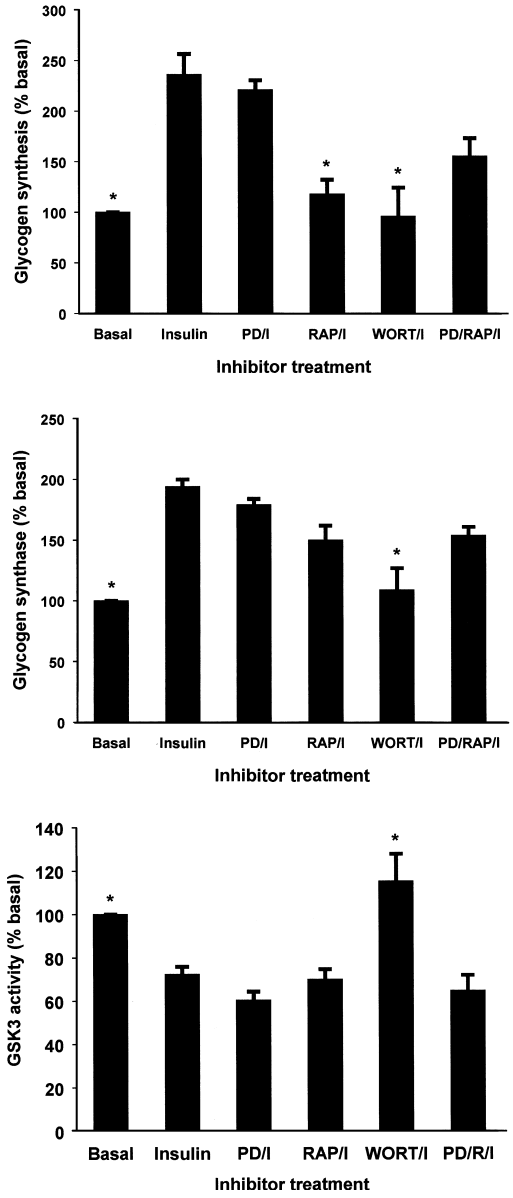

Figure 4 Effects of inhibitors on activation by insulin of glycogen synthesis, GS and GSK-3

Cells were incubated as described in the legend to Figure 3. The effects of inhibitors on glycogen synthesis (top panel) were determined after 2 h and those on GS (middle panel) and GSK-3 (bottom panel) after a 10 min incubation with insulin. Values are expressed as a percentage of the basal value, with means $\pm$ S.E.M. for four (glycogen synthesis and GS) or five (GSK-3) cell preparations from at least three subjects. In the absence of insulin (basal), glycogen synthesis was 36.9 ± 13.62 pmol/min per mg, the fractional activity of GS was 0.20 ± 0.02 and the activity of GSK-3 was 2.97 ± 1.03 m-units/mg. Statistical significance ($P < 0.05$) compared with the value in the presence of insulin alone is indicated by *.

extent and do not demonstrate insulin-responsive glucose uptake. Paradoxically, this provides an appropriate system in which to study effects of insulin on glycogen synthesis in the absence of effects of glucose uptake, and provides evidence that activation of GS results in increased glycogen synthesis independent of an increase in glucose uptake.

Insulin activation of GS is a rapid event in these cells, with maximal effects being observed within 10 min. This activation is preceded by inactivation of GSK-3 and activation of PKB, consistent with these enzymes being involved in mediating insulin signalling to GS. In contrast, activation of $p70^{s6K}$ and $p90^{s6K}$ is relatively slow, indicating that they do not play a significant role in the initial activation of GS, although it is possible that they contribute to the continued activation of GS in the myoblasts.

A key role for PKB in signalling to GSK-3 and GS is further supported by the inhibitor studies, in particular the ability of insulin to exert its effects on these enzymes in the absence of signalling through $p70^{s6K}$ and $p90^{s6K}$. The dramatic activation of PKB by insulin is observed only when okadaic acid is present in the extraction buffer, with much reduced activation being observed in its absence (results not shown). This is consistent with phosphorylation of Ser/Thr residues being associated with activation of PKB, as suggested previously [19].

As far as we are aware, the current study is the first to look at the effects of insulin and inhibitors on GS and GSK-3 in the same experimental system. The data obtained using PD98059 support the view that, in human muscle, the MAP kinase signalling cascade acting via ERK2 and $p90^{s6K}$ is neither necessary nor sufficient to inhibit GSK-3 and stimulate GS and glycogen synthesis in response to insulin. This is consistent with recent observations in other experimental systems [9,18,19]. A surprising observation is that, although rapamycin had no effect on inactivation of GSK-3 by insulin, it did attenuate the stimulation of glycogen synthesis. It also had an apparent inhibitory effect on activation of GS, although this did not reach statistical significance. Several plausible explanations exist for this, including the possibility that a kinase (or kinases) additional to GSK-3 act(s) in a regulatory capacity on GS and that this kinase is inactivated in response to insulin via a rapamycin-sensitive signalling cascade. This would reconcile some of the apparent discrepancies observed previously in different experimental systems. Clearly a role for PP1 activation is also possible but, in view of the lack of effect of PD98059 on GS activation, this would presumably not involve phosphorylation of the G subunit by $p90^{s6K}$ [7].

In conclusion, human myoblasts are an appropriate model system in which to study insulin signalling in human tissue. Time-course and inhibitor studies with these cells support the role of PKB in regulating GSK-3 and glycogen synthesis. The possibility of culturing cells from insulin-resistant subjects also offers the potential for identifying molecular mechanisms contributing to such resistance.

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REFERENCES

1 Häring, H. U. and Mehnert, H. (1993) Diabetologia **36**, 176–182
2 Kahn, C. R. (1994) Diabetes **43**, 1066–1084
3 Cheatham, B. and Kahn, C. R. (1995) Endocr. Rev. **16**, 117–142
4 Parker, P. J., Caudwell, F. B. and Cohen, P. (1983) Eur. J. Biochem. **130**, 227–234
5 Lawrence, Jr., J. C. and Zhang, J. (1994) J. Biol. Chem. **269**, 11595–11600
6 Cohen, P. (1993) Biochem. Soc. Trans. **21**, 555–567
7 Dent, P., Lavionne, A., Nakielny, S., Caudwell, F. B., Watt, P. and Cohen, P. (1990) Nature (London) **348**, 302–308
8 Begum, N. (1995) J. Biol. Chem. **270**, 709–714
9 Lazar, D. F., Wiese, R. J., Brady, M. J., Mastick, C. C., Waters, S. B., Yamauchi, K., Pessin, J. E., Cuatrecasas, P. and Saltiel, A. R. (1995) J. Biol. Chem. **270**, 20801–20807
10 Welsh, G. I. and Proud, C. G. (1993) Biochem. J. **294**, 625–629

11 Welsh, G. I., Foulstone, E. J., Young, S. W., Tavaré, J. M. and Proud, C. G. (1994) Biochem. J. **303**, 15–20
12 Cross, D. A.E, Alessi, D. R., Vandenheede, J. R., McDowell, H. E., Hundal, H. S. and Cohen, P. (1994) Biochem. J. **303**, 21–26
13 Borthwick, A. C., Wells, A. M., Rochford, J. J., Hurel, S. J., Turnbull, D. M. and Yeaman, S. J. (1995) Biochem. Biophys. Res. Commun. **210**, 738–745
14 Sutherland, C. and Cohen, P. (1994) FEBS Lett. **338**, 37–42
15 Seger, R. and Krebs, E. G. (1995) FASEB J. **9**, 726–735
16 Denton, R. M. and Tavaré, J. M. (1995) Eur. J. Biochem. **227**, 597–611
17 Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J. and Saltiel, A. R. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 7686–7689
18 Azpiazu, I., Saltiel, A. R., DePaoli-Roach, A. A. and Lawrence, Jr., J. C. (1996) J. Biol. Chem. **271**, 5033–5039
19 Cross, D. A. E., Alessi, D. R., Cohen, P., Andjelkovich, M. and Hemmings, B. A. (1995) Nature (London) **378**, 785–789
20 Chung, J., Kuo, C. J., Crabtree, G. R. and Blenis, J. (1992) Cell **69**, 1227–1236
21 Lin, T. A. and Lawrence, Jr., J. C. (1994) J. Biol. Chem. **269**, 21255–21261
22 Moule, S. K., Edgell, N. J., Welsh, G. I., Diggle, T. A., Foulstone, E. J., Heesom, K. J., Proud, C. G. and Denton, R. M. (1995) Biochem. J. **311**, 595–601

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23 Sakaue, J., Hara, K., Noguchi, T., Matozaki, T., Kotani, K., Ogawa, W., Yonezawa, K., Waterfield, M. D. and Kasagu, M. (1995) J. Biol. Chem. **270**, 11304–11309
24 Shepherd, P. R., Navé, B. T. and Siddle, K. (1995) Biochem. J. **305**, 25–28
25 Acaro, A. and Wymann, M. P. (1993) Biochem. J. **296**, 297–301
26 Sarabia, V., Ramlal, T. and Klip, A. (1990) Biochem. Cell. Biol. **68**, 536–542
27 Henry, R. R., Abrams, L., Nikoulina, S. and Ciaraldi, T. P. (1995) Diabetes **44**, 936–946
28 Cheng, H.-C., Kemp, B. E., Pearson, R. B., Smith, A. J., Misconi, L., Van Patten, S. M. and Walsh, D. A. (1986) J. Biol. Chem. **261**, 989–992
29 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
30 Guinovart, J. J., Massague, J., Cuided, C. J., Salsaas, E. and Itarte, E. (1979) FEBS Lett. **106**, 284–288
31 Sarabia, V., Lam, L., Burdett, E., Leiter, L. A. and Klip, A. (1992) J. Clin. Invest. **90**, 1386–1395
32 Shulman, G. I., Rothman, D. L., Jue, T., Stein, P., DeFronzo, R. A. and Shulman, R. G. (1990) N. Engl. J. Med. **322**, 223–228
33 Barriocanal, L. A., Borthwick, A. C., Stewart, M., Wells, A., Hurel, S. J., Yeaman, S. J. and Taylor, R. (1995) Diabetic Med. **12**, 1110–1115