Glucocorticoid-induced insulin resistance of protein synthesis is independent of the rapamycin-sensitive pathways in rat skeletal muscle

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Abstract

This study was designed to evaluate the role of p70 S6 kinase ($p70^{S6K}$), p90 S6 kinase ($p90^{RSK}$) and mitogen-activated protein (MAP) kinase pathways in the insulin resistance of muscle protein synthesis observed during glucocorticoid treatment. Dexamethasone treatment decreased the effect of insulin on protein synthesis (-35·2%) in epitrochlearis muscle incubated *in vitro*. This resistance is associated with a total blockage of the stimulation of $p70^{S6K}$ by insulin without any significant decrease in the amount of the kinase. However, the effect of rapamycin (inhibitor of several intracellular pathways including $p70^{S6K}$ pathways) on muscle protein synthesis was not modified by dexamethasone in rat muscles. This suggested that 'rapamycin-sensitive pathways' associated with the insulin stimulation of protein synthesis were not altered by glucocorticoids and thus are not responsible for the insulin resistance observed. As incubation of muscles with a MAP kinase inhibitor (PD98059) did not modify the stimulation of protein synthesis by insulin and as glucocorticoids did not alter the effect of insulin on $p90^{RSK}$ activity, our results provide evidence that glucocorticoid-induced alterations in muscle protein synthesis regulation by insulin do not involve factors or kinases that are dependent on MAP kinase and/or $p90^{RSK}$.

Journal of Endocrinology (1999) **162,** 77–85

Introduction

Circulating levels of glucocorticoids are elevated in Cushing's syndrome, infections, stress and various traumatic conditions and clinical treatments (Dujovne & Azarnoff 1975, Vaughan *et al.* 1982, Legaspi *et al.* 1985, Bondy 1985, Tessitore *et al.* 1993). The association of glucocorticoid excess and muscle atrophy is now well established, and the mechanisms underlying muscle wasting consistently involve inhibition of skeletal muscle protein synthesis and/or increased proteolysis (Tomas *et al.* 1979, Rannels & Jefferson 1980, McGrath & Goldspink 1982, Odedra *et al.* 1983, Kayali *et al.* 1987). However, glucocorticoid-induced muscle atrophy may result from both the steroid effect *per se* and/or modification of anabolic hormone action on protein metabolism (i.e. insulin). Indeed, steroids are also known to be potent diabetogenic agents resulting from both hepatic and peripheral resistance to the action of insulin (Amatruda *et al.* 1985). It is well known that the actions of insulin on muscle glucose uptake (Weinstein *et al.* 1995), glycogen synthesis (Leighton *et al.*1987), protein synthesis (Southorn *et al.* 1990, Dardevet *et al.* 1998) and proteolysis (Louard *et al.* 1994, Dardevet *et al.* 1998) are altered after glucocorticoid treatment.

Cellular responses to insulin involve interaction of the hormone with its receptor and subsequent activation of intracellular signalling pathways. One of the earliest post-receptor events is phosphorylation of the insulin receptor substrate-1 (IRS-1) on tyrosine residues which in turn acts as a 'docking protein' to activate a number of different proteins, including phosphatidylinositol 3 kinase (PI3 kinase) and growth factor receptor-binding protein-2 (see Myers & White (1993) and Denton & Tavaré (1995) for reviews). The latter protein is thought to be linked to the activation of Ras, which subsequently activates the mitogen-activated protein (MAP) kinase pathways. PI3 kinase activation represents a general mechanism in muscle insulin signalling toward glucose metabolism (Okada *et al.* 1994, Cheatham *et al.* 1994, Yeh *et al.* 1995), protein synthesis (Dardevet *et al.* 1996, Thompson & Palmer 1998) or proteolysis (Dardevet *et al.* 1996, Thompson & Palmer 1998). The downstream events linked to PI3 kinase remain unclear but its activation is required for insulin stimulation of the p70 S6 kinase ($p70^{S6K}$) in skeletal muscle (Dardevet *et al.* 1996) and various cells (Cheatham *et al.* 1994, Cross *et al.*1994, Welsh *et al.* 1994). Interestingly, blunting the activation of $p70^{S6K}$ with rapamycin impaired the stimulatory effect of insulin on protein synthesis in rat skeletal muscle

0022–0795/99/0162–0077

(Dardevet *et al.* 1996) as well as in C2C12 myoblasts (Palmer *et al.* 1997) suggesting involvement of this kinase in the regulation of muscle protein synthesis by insulin.

The mechanism by which steroids alter insulin action on skeletal muscle protein synthesis remains unclear. Indeed, the first cellular events involved in the action of insulin, i.e. a change in insulin receptor number (Block & Buse 1989, Giorgino *et al.* 1993, Saad *et al.* 1993), insulin receptor autophosphorylation (Block & Buse 1989, Giorgino *et al.* 1993, Saad *et al.* 1993) and tyrosine phosphorylation of IRS-1 (Giorgino *et al.* 1993, Saad *et al.* 1993) are not modified by glucocorticoid treatment. Up to now, the only significant effect of glucocorticoids was a decrease in the association/activation of PI3 kinase with IRS-1 in response to insulin (Giorgino *et al.* 1993, Saad *et al.* 1993). However, it is not known if such an alteration modifies activation of the $p70^{S6K}$ pathways or MAP kinase pathways by insulin. In an attempt to understand the origin of the insulin resistance of muscle protein synthesis after glucocorticoid treatment, we examined in the present study the action of dexamethasone (DEX) on the activity of selected intracellular protein kinases such as $p70^{S6K}$ (rapamycin-sensitive pathway), $p90^{RSK}$ and MAP kinases (rapamycin-insensitive pathway) in rat epitrochlearis muscle *in vitro*.

Materials and Methods

Animals

Young (4–5-week-old) male Sprague–Dawley rats were purchased from Iffa-Credo (L'Arbresle, France) and housed under controlled environmental conditions (temperature, 22 °C; 12 h dark period starting at 1800 h). They were given free access to commercial laboratory chow and water before the experiments were performed. Rats were randomly divided into a control and a DEX-treated group. DEX (a synthetic glucocorticoid analogue that does not bind to plasma binding proteins) was given daily (at 0900 h) via the drinking water. DEX concentration was adjusted every day on the basis of drinking water intake the day before. Animals received 2·19 ± 0·1 mg/kg per day for 4 days. As DEX was reported to decrease food intake, the control group was pair-fed to the DEX-treated group. Food intake was maintained at similar levels in the two groups during the DEX treatment as shown in Table 1. Thus differences between pair-fed and treated groups do not originate from different food intakes. DEX induced a progressive weight loss of 20% over the treatment period and the pair-fed animals stopped growing (Table 1). Animals were anaesthetized with sodium pentobarbital (6·0 mg/100 g body weight) after an overnight fast. Epitrochlearis muscles were dissected intact for incubation (see below). Separate animal protocols were performed to examine the effects of the inhibitors tested and to assess kinase activities (16 animals per groups).

Table 1 Body weight and food intake of pair-fed and DEX-treated rats

Day	Pair-fed		DEX-treated	
	Weight (g)	**Food intake** (g)	**Weight** (g)	**Food intake** (g)
0	130·8 ± 1·42	15·0	130·4 ± 1·51	14·7 ± 0·17
1	137·4 ± 1·37	9·0	128·4 ± 1·79	8·8 ± 0·13
2	137·0 ± 1·14	9·0	120·0 ± 1·58	8·5 ± 0·34
3	137·4 ± 1·09	9·0	114·6 ± 1·58	8·8 ± 0·12
4	132·5 ± 0·97	4·0	104·3 ± 1·57	4·0

Effect of rapamycin and PD98059 on insulin-stimulated protein synthesis

The effect of the inhibitors was assessed by preincubating epitrochlearis muscle for 30 min in Krebs/Henseleit buffer (NaCl 120 mM, KCl 4·8 mM, $NaHCO_3$ 25 mM, $CaCl_2$ 2·5 mM, KH_2PO_4 1·2 mM and $MgSO_4$ 1·2 mM; pH 7·4) supplemented with 5 mM Hepes, 5 mM glucose, 0·17 mM leucine, 0·20 mM valine, 0·10 mM isoleucine and 0·1% BSA (99% fatty acid free) and saturated with 95% O_2/5% CO_2 gas mixture in the presence or absence of 200 nM rapamycin or 20 μM PD98059. Muscles were then transferred to fresh medium of the same composition supplemented with 0·5 mM L-[^{14}C]phenylalanine (0·15 μCi/ml) with or without 30 nM insulin and incubated for an additional 90 min period. At the end of the incubation, muscles were blotted and homogenized in 10% trichloroacetic acid (TCA). Samples were centrifuged at 10 000 ***g*** for 10 min at 4 °C and TCA-insoluble material was washed three times with 10% TCA. The resultant pellet was solubilized in 1 M NaOH at 37 °C for determination of protein and radioactivity incorporated into muscle protein. Tissue protein mass was determined using the bicinchoninic acid procedure (BCA; Pierce Chemical Co., Rockford, IL, USA), and protein-bound radioactivity was measured using scintillation counting. Protein synthesis was calculated by dividing the protein-bound radioactivity by the specific radioactivity of the phenylalanine in the incubation medium. It was expressed as nmol phenylalanine incorporated/per mg protein per 90 min.

Assessment of $p70^{S6K}$ and $p90^{RSK}$ activities

Epitrochlearis muscles were preincubated for 30 min in Krebs/Henseleit buffer supplemented with 5 mM Hepes, 5 mM glucose, 0·17 mM leucine, 0·20 mM valine, 0·10 mM isoleucine and 0·1% BSA (99% fatty acid free) and saturated with 95% O_2/5% CO_2 gas mixture. Insulin (30 nM) was then added to the medium and muscles were incubated for 20 min in the presence of the hormone. Muscles were homogenized in ice-cold extraction buffer (50 mM Tris/acetate, 50 mM NaF, 2·5 mM EDTA, 1 mM EGTA, 5 mM sodium pyrophosphate, 5 mM

β-glycerophosphate, 1 mM Na_3VO_4 , 2 mM dithiothreitol, 1 mM benzamidine, 4 μg/ml leupeptin, 0·5% Triton X-100, pH 7·2) and centrifuged at 10 000 ***g*** for 10 min at 4 °C.

The activity of $p70^{S6K}$ was assessed by an immune complex kinase assay. Normalized amounts of muscle protein (100 μg) were incubated for 4 h at 4 °C with 3 μl $p70^{S6K}$ antibodies preabsorbed to Protein A/agarose beads. The immune complexes were washed three times with S6 kinase assay buffer (25 mM Mops, 15 mM $MgCl_2$, 1 mM dithiothreitol, 0·1% BSA; pH 7·2). The beads were then resuspended in 50 μl S6 kinase assay buffer containing 0·1 mM S6 peptide RRRLSSLRA, 2 μM peptide inhibitor of cAMP-dependent protein kinase and 100 mM ATP (specific activity 3000 d.p.m./pmol). After a 60 min incubation at 30 °C, the phosphorylation reaction was stopped with 10 μl 50 mM unlabelled ATP. The reaction mixture was spotted on to Whatman P-81 phosphocellulose filter paper squares, which, after three washes in 75 mM phosphoric acid, were counted in a β-scintillation counter. Pilot experiments showed that ^{32}P incorporation into S6 substrate was linear for at least 60 min and proportional to the amount of protein used (100–200 μg).

The activity of $p90^{RSK}$ was also assessed by an immune complex kinase assay as described for the $p70^{S6K}$. Normalized amounts of muscle protein (50 μg) were immunoprecipitated and then allowed to phosphorylate. After a 20 min incubation at 30 °C, the phosphorylation reaction was stopped, and radioactivity incorporated into the substrate was counted. Pilot experiments showed that ^{32}P incorporation into S6 substrate was linear for at least 40 min and proportional to the amount of protein used (50–100 μg).

Western blotting

Aliquots of tissue supernatant (20 μg protein) were analysed by SDS/PAGE (7·5% polyacrylamide gel) under reducing conditions. Proteins were then transferred on to nylon membranes (Millipore Corp., Bedford, MA, USA) and subsequently blocked in TBS (20 mM Tris, 500 mM NaCl, pH 7·5) supplemented with 5% non-fat powdered milk. Membranes were then exposed to a polyclonal antibody that specifically recognizes $p70^{S6K}$ (SC-230; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or $p90^{RSK}$ (SC-231; Santa Cruz Biotechnology) for 16 h at 4 °C. After three washes in TBS with 0·05% Tween 20 supplemented with 1% milk, membranes were incubated for 45 min in the same buffer with a goat anti-rabbit antibody conjugated with horseradish peroxidase and developed using the enhanced chemoluminescence (ECL) detection system (Amersham) according to the manufacturer's directions.

Statistical analysis

Data are expressed as the means ± S.E.M. Statistical evaluation of the data was performed using Student's *t*-test. Differences among means were considered significant when $P<0·05$.

Results

Effect of DEX on insulin-stimulated muscle protein synthesis

Glucocorticoid treatment induced skeletal muscle protein wasting in both epitrochlearis muscle (22·9 ± 1·2 vs 16·7 ± 0·6 mg in pair-fed and treated rats respectively) and gastrocnemius muscle (774 ± 3 vs 542 ± 2 mg in pair-fed and treated rats respectively). In pair-fed animals, muscle protein synthesis was significantly stimulated ($P<0·05$) by insulin (0·248 ± 0·0057 vs 0·489 ± 0·026 nmol phenylalanine/mg protein per 90 min without and with insulin respectively). Food restriction did not significantly modify the effect of insulin on epitrochlearis protein synthesis when compared with a control *ad libitum* group (0·295 ± 0·023 vs 0·485 ± 0·048 nmol phenylalanine/mg protein per 90 min without and with insulin respectively). The basal rate of protein synthesis was significantly decreased in epitrochlearis muscle from rats treated with DEX (0·171 ± 0·0056 nmol phenylalanine/mg protein per 90 min; $P<0·05$ vs untreated rats) (Fig. 1). Insulin was still able to stimulate protein synthesis (0·327 ± 0·013 nmol phenylalanine/mg protein per 90 min; $P<0·05$ vs basal) but to a lesser extent than in pair-fed controls (Fig. 1). Indeed, the incremental increase in protein synthesis above basal due to the effect of insulin was significantly decreased by 35·2% ($P<0·05$) when animals were treated with DEX (0·241 ± 0·010 vs 0·156 ± 0·005 nmol phenylalanine/mg protein per 90 min for pair-fed and DEX-treated rats respectively).

Effect of PD98059 on insulin-stimulated protein synthesis

Addition of PD98059 (an inhibitor of MAP kinase pathway) to the incubation medium did not modify the rate of basal muscle protein synthesis (0·248 ± 0·006 vs 0·243 ± 0·010 005 nmol phenylalanine/mg protein per 90 min without or with the inhibitor respectively) nor the stimulatory effect of insulin in both pair-fed and Dex-treated rats (Fig. 1). This result shows that the MAP kinase pathway is not involved in the acute regulation of muscle protein synthesis by insulin and thus could not be responsible for the defective hormone action under steroid treatment. To check the efficiency of the inhibitor, we assessed, in a side experiment, insulin-stimulated glucose transport. PD98059, at the concentration used above, completely inhibited the effect of the hormone on muscle deoxyglucose (1·859 ± 0·179 and 3·121 ± 0·297 nmol deoxyglucose/mg muscle per 15 min without and with insulin in the absence of PD98059; 1·661 ± 0·107 and 2·108 ± 0·273 nmol deoxyglucose/mg muscle per 15 min without and with insulin in the presence of PD98059).

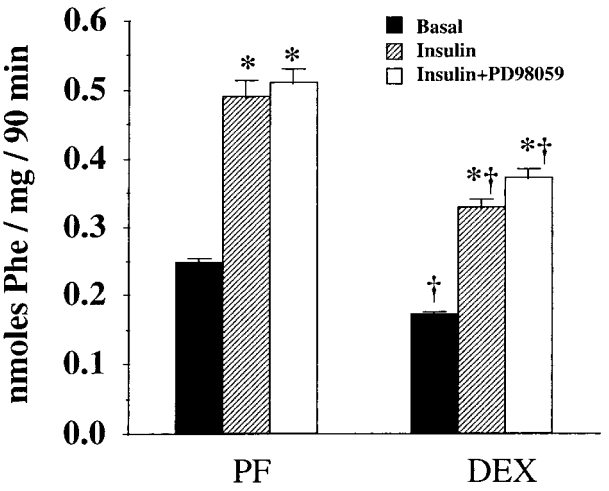

Figure 1 Effect of PD98059 on muscle protein synthesis. Epitrochlearis muscles from pair-fed (PF) and DEX-treated (DEX) rats were preincubated for 1 h in Krebs/Henseleit buffer supplemented with 5 mM Hepes, 5 mM glucose, 0·17 mM leucine, 0·2 mM valine, 0·1 mM isoleucine and 0·1% BSA and saturated with 95% O_2/5% CO_2 gas mixture in the presence or absence of 20 μM PD98059. Muscles were then transfered to fresh medium of the same composition supplemented with 0·5 mM L-[^{14}C]phenylalanine (0·15 μCi/ml) with or without 30 nM insulin and incubated for an additional 90 min period. Muscles were blotted and homogenized in 10% TCA. The resultant pellet was solubilized in 1 M NaOH at 37 °C for determination of muscle protein content and radioactivity incorporated into muscle proteins. Protein synthesis was expressed as nmol phenylalanine incorporated/mg protein per 90 min. Data are means ± S.E. for six to eight individual muscles. *Significantly different from basal value (P<0·05); †significantly different from the pair-fed value (P<0·05).

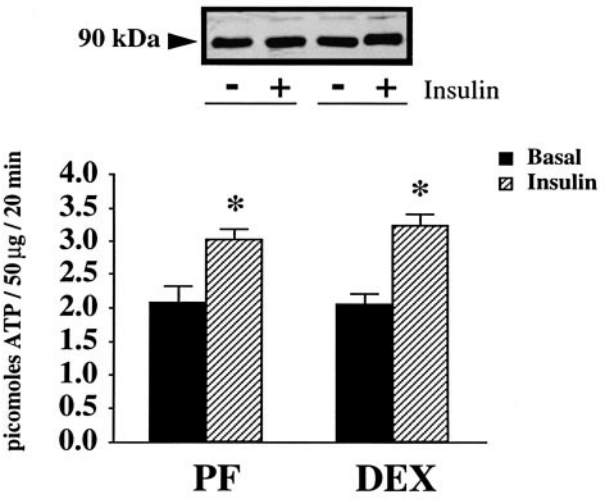

Figure 2 Activity of p90^{RSK} in rat skeletal muscle. Epitrochlearis muscles were preincubated for 30 min in Krebs/Henseleit buffer. Muscles were then incubated for 20 min in the presence or not of 30 nM insulin. Muscles were homogenized in ice-cold extraction buffer and centrifuged at 10 000 ***g*** for 10 min at 4 °C. The activity of p90^{RSK} was assessed by an immune complex kinase assay. After a 20 min incubation at 30 °C, the reaction mixture was spotted on to Whatman P-81 phosphocellulose filter paper squares and counted. Aliquots of tissue proteins were analysed by SDS/PAGE (7·5% gel) under reducing conditions, transfered to a nylon membrane and exposed to a polyclonal antibody that specifically recognizes p90^{RSK}. Data are means ± S.E. for eight to ten individual muscles. *Significantly different from basal value (P<0·05).

Effect of DEX on muscle insulin-stimulated p90^{RSK} and p70^{S6K} activities

Insulin stimulated the activity of p90^{RSK} in epitrochlearis muscle from pair-fed rats (3·01 ± 0·170 vs 2·07 ± 0·256 pmol ATP/50 μg per 20 min with and without insulin respectively; P<0·05)(Fig. 2). Treatment with DEX modified neither the basal activity of p90^{RSK} nor its stimulation by insulin (2·04 ± 0·165 vs 3·23 ± 0·181 pmol ATP/50 μg per 20 min with and without insulin respectively; P<0·05)(Fig. 2). Quantification of total p90^{RSK} by Western blot revealed that the amount of kinase was not altered by the DEX treatment in epitrochlearis muscle (Fig. 2).

Stimulation of p70^{S6K} by insulin is characterized by an increase in its apparent molecular mass secondary to multiple phosphorylation sites of the kinase. Therefore we used this property (assessed by Western blot) as an indicator of hormone action on the kinase. DEX treatment did not modify the amount of p70^{S6K} protein recovered from epitrochlearis muscle (Fig. 3). The presence of insulin in the incubation medium greatly increased the phosphorylation of p70^{S6K} in pair-fed animals whereas no modification of the electrophoretic motility was recorded in DEX-treated epitrochlearis muscle (Fig. 3). This observation suggests that the stimulation of p70^{S6K} activity by insulin was impaired when rats were treated with steroids. The measurement of p70^{S6K} activity confirmed this observation as insulin greatly increased p70^{S6K} activity in pair-fed animals (3·44 ± 0·55 vs 0·71 ± 0·18 pmol ATP/100 μg per h with and without insulin respectively; P<0·05) whereas it had no significant stimulatory effect in DEX-treated rats (0·78 ± 0·12 vs 0·56 ± 0·12 pmol ATP/100 μg per h with and without insulin respectively) (Fig. 3). It is important to note that DEX did not modify the basal activity of p70^{S6K} in epitrochlearis muscle. This result suggests that the rapamycin pathway to which p70^{S6K} belongs may be in part responsible for the defect in insulin action on muscle protein synthesis in DEX-treated rats. In contrast with protein synthesis, food restriction greatly modifies both basal and insulin-stimulated p70^{S6K} in epitrochlearis muscle when compared with a control *ad libitum* group (1·296 ± 0·054 vs 7·010 ± 0·820 pmol ATP/100 μg per h without and with insulin respectively). Thus, in order to assess the effect of DEX alone on the intracellular pathways involved in the stimulation of muscle protein synthesis by insulin, it is essential to compare the DEX group with a pair-fed group.

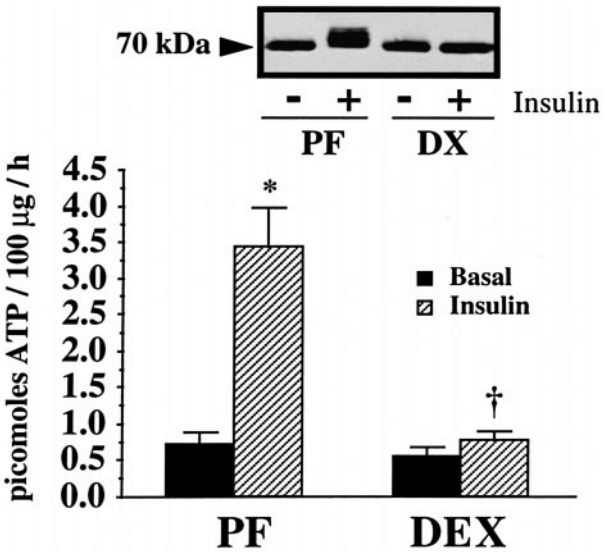

Figure 3 Activity of $p70^{S6K}$ in rat skeletal muscle. Epitrochlearis muscles were preincubated as described in Fig. 2. The activity of $p70^{S6K}$ was assessed by an immune complex kinase assay. After a 60 min incubation at 30 °C, the phosphorylation reaction was stopped with unlabelled ATP. The reaction mixture was spotted on to Whatman P-81 phosphocellulose filter paper squares and counted in a β-scintillation counter. Aliquots of tissue supernatant (20 µg protein) were analysed by SDS/PAGE (7·5% polyacrylamide gel) under reducing conditions. Proteins were then transfered to a nylon membrane and exposed to a polyclonal antibody that specifically recognizes $p70^{S6K}$. Data are means ± S.E. for eight to ten individual muscles. *Significantly different from basal value ($P<0·05$); †significantly different from the pair-fed value ($P<0·05$).

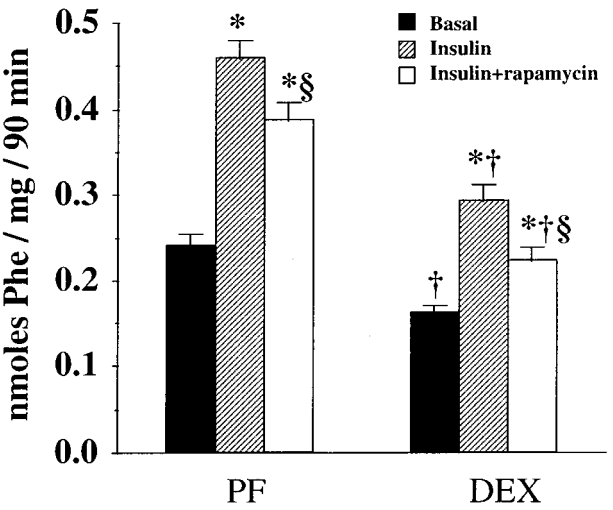

Figure 4 Effect of rapamycin on muscle protein synthesis. Epitrochlearis muscles from pair-fed (PF) and DEX-treated (DEX) rats were preincubated for 30 min in Krebs/Henseleit buffer supplemented with 5 mM Hepes, 5 mM glucose, 0·17 mM leucine, 0·2 mM valine, 0·1 mM isoleucine and 0·1% BSA and saturated with 95% O_2/5% CO_2 gas mixture in the presence or absence of 200 nM rapamycin. Muscles were then transfered to fresh medium of the same composition supplemented with 0·5 mM L-[^{14}C]phenylalanine (0·15 µCi/ml) with or without 30 nM insulin and incubated for an additional 90 min period. Muscles were blotted and homogenized in 10% TCA. The resultant pellet was solubilized in 1 M NaOH at 37 °C for determination of muscle protein content and radioactivity incorporated into muscle proteins. Protein synthesis was expressed as nmol phenylalanine incorporated/mg protein per 90 min. Data are means ± S.E. for eight to twelve individual muscles. *Significantly different from basal value ($P<0·05$); †significantly different from the pair-fed value ($P<0·05$); §significantly different from the corresponding value without rapamycin ($P<0·05$).

Effect of rapamycin on insulin-stimulated protein synthesis

The effect of rapamycin was assessed in an additional experiment in which the decrease in basal muscle protein synthesis and the defective action of insulin on muscle protein synthesis (-39·9%) was similar to the previous study (Fig. 4). Addition of rapamycin to the incubation medium did not modify basal protein synthesis in epitrochlearis muscle from pair-fed animals (0·241 ± 0·013 vs 0·233 ± 0·019 nmol phenylalanine/mg protein per 90 min without and with inhibitor respectively). Insulin increased protein synthesis but in the presence of rapamycin its effect was significantly reduced, by 0·071 ± 0·004 nmol phenylalanine/mg protein per 90 min (0·459 ± 0·020 vs 0·388 ± 0·015 nmol phenylalanine/mg protein per 90 min respectively) (Fig. 4). Thus insulin partially stimulated muscle protein synthesis through a rapamycin-dependent pathway.

As observed with pair-fed animals, the addition of rapamycin did not modify basal protein but partially inhibited the insulin-stimulated protein synthesis in DEX-treated rats. This inhibition was similar to that recorded in pair-fed animals: 0·069 ± 0·002 nmol phenylalanine/mg protein per 90 min (from 0·293 ± 0·019 to 0·224 ± 0·015 nmol phenylalanine/mg protein per 90 min with and without rapamycin respectively) (Fig. 4). This result shows that the insulin effect that is dependent on the rapamycin-sensitive pathway is not significantly altered in response to DEX and thus not responsible for the resistance observed.

Discussion

Glucocorticoids have direct effects on muscle protein turnover by either inhibiting protein synthesis (Tomas *et al.* 1979, Rannels & Jefferson 1980, McGrath & Goldspink 1982, Odedra *et al.* 1983, Kayali *et al.* 1987) or increasing proteolysis (Tomas *et al.* 1979, McGrath & Goldspink 1982, Kayali *et al.* 1987). However, *in vivo*, they also act through their interaction with insulin. Accordingly, our study in rat epitrochlearis muscle showed that DEX significantly decreased basal muscle protein synthesis but also strongly depressed its stimulation by insulin. The effect of insulin on muscle protein metabolism *in vivo* is still an open question. Most of the experiments

demonstrated that infusion of exogenous insulin does not increase muscle protein synthesis except in young fasted growing rats (Garlick & Grant 1988, Mosoni *et al.* 1993). Thus it has been postulated that physiological concentrations of insulin already maximally stimulate protein synthesis *in vivo*. This was confirmed by the experiment of Sinaud *et al.* (1999) which demonstrated, in standard postprandial rats, that only a specific decrease in insulinaemia produced by diazoxide was able to show the role of insulin in the stimulation of muscle protein synthesis *in vivo*. The *in vitro* stimulation of protein synthesis by insulin in epitrochlearis muscle occurred in the range zero to physiological insulin concentration and thus confirms the previous hypothesis (Dardevet *et al.* 1994, Vary *et al.* 1998). In our experiment we used a dose of insulin that stimulated protein synthesis maximally (as *in vivo*) and we postulate that we were reproducing insulin stimulation that occurs *in vivo*. As physiological insulin levels already maximally stimulate muscle protein synthesis *in vivo*, the difference in insulin-stimulated protein synthesis that we observed *in vitro* should also be found *in vivo*. The results of both Odedra *et al.* (1983) and Southorn *et al.* (1990) agree with this. They demonstrated that the responsiveness of muscle protein synthesis to insulin was altered when rats were treated with steroids and that insulin infusion *in vivo* did not counteract the decrease in muscle protein synthesis.

Cellular responses to insulin involve interaction of the hormone with its receptor and subsequent activation of intracellular signalling pathways. It is now established that phosphorylation/dephosphorylation reactions play a major role in the control of protein synthesis by insulin. The involvement of PI3 kinase as a mediator of insulin action on muscle protein synthesis has been demonstrated in studies using a specific inhibitor of the kinase (Dardevet *et al.* 1996, Thompson & Palmer 1998). However, PI3 kinase represents a general mechanism in insulin signalling; for example, several studies have shown that this kinase is also important in the regulation of glucose uptake (Okada *et al.* 1994, Cheatham *et al.* 1994, Yeh *et al.* 1995, Dardevet *et al.* 1996). The downstream events linked to PI3 kinase remain poorly defined but in several cell lines (Cheatham *et al.* 1994, Cross *et al.* 1994, Welsh *et al.* 1994, Chung *et al.* 1994) as well as in rat skeletal muscle (Dardevet *et al.* 1996), PI3 kinase activation was found to be required for insulin stimulation of $p70^{S6K}$. Interestingly, using rapamycin, we previously showed that $p70^{S6K}$ (a rapamycin-sensitive kinase) may be partially involved in the signalling pathway linking the insulin receptor to the protein synthesis machinery in rat epitrochlearis muscle, as in the presence of the inhibitor, insulin stimulation of protein synthesis was decreased by 25% (Dardevet *et al.* 1996). In the present study, DEX treatment totally abolished the stimulation of $p70^{S6K}$ by insulin in rat epitrochlearis muscle without decreasing the enzyme content. Therefore such a modification could explain the insulin resistance of the muscle protein synthesis in DEX-treated rats. In order to verify whether such an alteration was indeed responsible for the glucocorticoid-induced insulin resistance of protein synthesis, we compared the effect of rapamycin on insulin-stimulated protein synthesis between pair-fed and DEX-treated rats. Surprisingly, the addition of rapamycin decreased the effect of insulin to the same extent in both groups of rats suggesting that the rapamycin-sensitive pathway was not involved in the insulin resistance observed in spite of total blockage of the stimulation of $p70^{S6K}$ by insulin. However, DEX-induced modification of the insulin-stimulated $p70^{S6K}$ activity may explain the alteration in other metabolic effects of insulin in skeletal muscle. Indeed DEX treatment decreased the action of insulin on glucose transport (Weinstein *et al.* 1995) or glycogen synthesis (Leighton *et al.* 1987) in which the involvement of a rapamycin-sensitive mechanism (including elements of the $p70^{S6K}$ signalling pathway) has been demonstrated (Azpiazu *et al.* 1996). Furthermore, insulin activates nuclear $p70^{S6K}$ (Kim & Kahn 1997) which has been shown to regulate the GLUT-1 and hexokinase II gene expression (Osawa *et al.* 1996, Taha *et al.* 1995). Thus, by inhibiting the $p70^{S6K}$ activation by insulin, DEX may indirectly modify glucose metabolism but not protein synthesis.

Although rapamycin causes a deactivation of $p70^{S6K}$, the direct target of this inhibitor is in fact the protein known as mTOR (mammalian target of rapamycin). The role of mTOR as the rapamycin target responsible for inhibition of $p70^{S6K}$ was demonstrated by Brown *et al.* (1995) who showed that mutant TORs, which lack the ability to bind rapamycin, prevent the inhibition of $p70^{S6K}$ by the drug. However, recent studies (Hara *et al.* 1997, Von Manteuffel *et al.* 1997) demonstrated that rapamycin-resistant mutants of $p70^{S6K}$ are not able to prevent the rapamycin-dependent dephosphorylation of eukariotic initiation factor 4E binding protein 1 (eIF4E-BP1), a target of insulin in the regulation of protein synthesis. Thus, even if mTOR is an upstream regulator of the $p70^{S6K}$, other signalling pathways arise directly from mTOR and act independently of the $p70^{S6K}$. Our experiment is consistent with this observation, as we showed in DEX-treated rats that rapamycin was still able to inhibit the stimulation of muscle protein synthesis by insulin in the absence of stimulation of $p70^{S6K}$. As the lack of stimulation of $p70^{S6K}$ by insulin in DEX-treated animals did not modify the rapamycin-associated decrease in protein synthesis, the involvement of $p70^{S6K}$ in the regulation of muscle protein synthesis by insulin was questionable. Furthermore, as food restriction altered the stimulation of $p70^{S6K}$ by insulin without having any effect on muscle protein synthesis, this observation supports our hypothesis that this kinase may not be directly involved in the regulation of muscle protein synthesis by insulin.

Insulin activates multiple cellular protein kinases (Myers & White 1993). These include MAP kinases which are

part of a signal-transduction pathway that is distinct from the rapamycin-sensitive pathway. Indeed, it has been shown that rapamycin does not block the activation of MAP kinase by insulin or the downstream kinase $p90^{RSK}$ (Chung *et al.* 1992, Price *et al.* 1992, Fingar *et al.* 1993). Furthermore, because PI3 kinase activation was required for insulin stimulation of muscle protein synthesis (Dardevet *et al.* 1996, Palmer *et al.* 1997), and because wortmanin (inhibitor of PI3 kinase) has been shown to block the activation of MAP kinase by insulin in L6 myocytes (Cross *et al.* 1994) and CHO cells (Welsh *et al.* 1994), the role of this pathway in the DEX-induced insulin resistance of muscle protein synthesis was assessed in our model. Addition of the MAP kinase (MEK) inhibitor did not modify the stimulatory effect of insulin on muscle protein synthesis, suggesting that the MAP kinase pathway and its downstream elements were not involved in the regulation of the translation machinery. Our observations are in agreement with the study of Azpiazu *et al.* (1996) who concluded that the MAP kinase pathway was not necessary for insulin action on muscle protein synthesis in rat diaphragm. In consequence, the resistance of muscle protein synthesis to insulin in DEX-treated rats could not originate from a defect in this pathway. Furthermore, we showed that the stimulation by insulin of a downstream kinase of the MAP kinase pathway ($p90^{RSK}$) was not alterated by DEX treatment. However, the role of $p90^{RSK}$ in the regulation of muscle protein synthesis has been proposed because it activates the glycogen synthase kinase 3 which is known to activate eIF2B (eukaryotic initiation factor 2B) *in vitro* (Welsh & Proud 1993). The latter is a target of insulin in its regulation of the formation of the 43S preinitiation complex (Kimball *et al.* 1994). However, DEX-induced insulin resistance of muscle protein synthesis was also independent of $p90^{RSK}$ as treated animals did not show any alteration in the stimulation of this kinase by insulin. Further studies are nevertheless necessary to confirm whether or not $p90^{RSK}$ represents an insulin-stimulated kinase leading to stimulation of muscle protein synthesis.

Insulin modulates protein synthesis by enhancing initiation of translation of mRNA (Kimball *et al.* 1994). The rate-limiting step for translation is the initiation phase which involves recognition of capped mRNA and binding to the 40S ribosome (Altmann & Trachsel 1993, Rhoads 1993, Sonenberg 1994). These processes require the participation of the initiation factor eIF4F, a complex composed of eIF4E (the cap binding protein), eIF4G and eIF4A (RNA helicase). eIF4E is the least abundant of the initiation factors and thus plays a critical regulatory role in protein synthesis. Modulation of its concentration by gene transfection leads to parallel modification of protein synthesis (Rhoads 1993, Sonenberg 1994). eIF4E activity is modulated by a protein known as 4E-BP-1 (also termed PHAS-I) which specifically binds to eIF4E and decreases translation (Lin *et al.* 1994, Pause *et al.* 1994). Phosphorylation of PHAS-I in response to insulin resulted from a dissociation of the PHAS-I–eIF4E complex and thus increased free eIF4E for the formation of the complex eIF4F (Lin *et al.* 1994, 1995, Graves *et al.* 1995). To our knowledge, the effect of steroids on the insulin regulation of the complex PHAS-I–eIF4E has not been studied but we hypothesize that such a mechanism may occur in the generation of the insulin resistance of muscle protein synthesis in our model. Indeed, such a mechanism has been recently demonstrated in diabetes type I, another well-characterized insulin-resistant state. Interestingly, even if PHAS-I represents a substrate of MAP kinase *in vitro* (Haystead *et al.* 1994), several studies have shown that insulin-stimulated phosphorylation of the PHAS-I–eIF4E was independent of the MAP kinase pathway in 3T3-L1 adipocytes (Lin *et al.* 1995) or rat diaphragm (Azpiazu *et al.* 1996) and was only partially blocked by rapamycin (Azpiazu *et al.* 1996). Further work will be required to demonstrate the contribution of such a mechanism in the generation of the DEX-induced insulin resistance of protein synthesis in rat epitrochlearis muscle.

In conclusion, our experiments show that glucocorticoid-induced insulin resistance of muscle protein synthesis is independent of the rapamycin-sensitive pathway despite a total inhibition of $p70^{S6K}$ activation by insulin. In addition, our results on the $p90^{RSK}$ and MAP kinases show that they also were not responsible for the insulin resistance observed. Investigations are needed to determined which intracellular kinases or factors are involved. From previous (Dardevet *et al.* 1996) and the present results, it was found that that these kinases are dependent on PI3 kinase but independent of rapamycin and MAP kinase. Protein kinase B (PKB) may be a good candidate because its activation by insulin was blocked by LY294002 (inhibitor of the PI3 kinase) and unaffected by rapamycin and PD98059 (inhibitor of MAP kinase pathways; see Cohen *et al.* (1997) for a review). Furthermore, this kinase has been linked to the glycogen synthase kinase 3 in skeletal muscle (Cohen *et al.* 1997). Whether or not PKB is responsible for the insulin resistance of protein synthesis in DEX-treated rats remains to be determined.

References

Altmann M & Trachsel H 1993 Regulation of translation initiation and modulation of cellular physiology. *Trends in Biochemical Science* **18** 429–432.

Amatruda JM, Livingston JN & Lockwood DH 1985 Cellular mechanisms in selected states of insulin resistance: human obesity, glucocorticoid excess, and chronic renal failure. *Diabetes Metabolism Reviews* **1** 293–317.

Azpiazu I, Saltiel AR, Depaoliroach AA & Lawrence JC 1996 Regulation of both glycogen synthase and PHAS-I by insulin in rat skeletal muscle involves mitogenactivated protein kinase-independent and rapamycin-sensitive pathways. *Journal of Biological Chemistry* **271** 5033–5039.

Block NE & Buse MG 1989 Effects of hypercotisolemia and diabetes on skeletal muscle insulin receptor function *in vitro* and *in vivo*. *American Journal of Physiology* **256** E39–E48.

Bondy PK 1985 Disorders of the adrenal cortex. In *Williams' Textbook of Endocrinology*, edn 7, pp 816–890. Eds JD Wilson & DW Foster. Philadelphia: Saunders.

Brown EJ, Beal PA, Keith CT, Chen J, Shin TB & Schreiber SL 1995 Control of p70 S6 kinase by kinase activity of FRAP *in vivo*. *Nature* **377** 441–446.

Cheatham B, Vlahos CJ, Cheatham L, Wang L, Blenis J & Kahn CR 1994 Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. *Molecular and Cellular Biology* **14** 4902–4911.

Chung J, Kuo CJ, Crabtree GR & Blenis J 1992 Rapamycin-FKBP specifically blocks growth dependent activation of and signaling by the 70 kd S6 protein kinases. *Cell* **69** 1227–1236.

Chung JK, Grammer TC, Lemon KP, Kazlauskas A & Blenis J 1994 PDGF- and insulin-dependent p70(S6k) activation mediated by phosphatidylinositol-3-OH kinase. *Nature* **370** 71–75.

Cohen P, Alessi DR & Cross DAE 1997 PDK1, one of the missing links in insulin signal transduction? *FEBS Letters* **410** 3–10.

Cross DAE, Alessi DR, Vandenheede JR, McDowell HE, Hundal HS, Cohen P 1994 The inhibition of glycogen synthase kinase-3 by insulin or insulin-like growth factor 1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between ras and raf. *Biochemical Journal* **303** 21–26.

Dardevet D, Sornet C, Attaix D, Baracos VE & Grizard J 1994 Insulin-Like Growth Factor-1 and insulin resistance in skeletal muscles of adult and old rats. *Endocrinology* **134** 1475–1484.

Dardevet D, Sornet C, Vary T & Grizard J 1996 Phosphatidylinositol 3-kinase and p70 S6 kinase participate in the regulation of protein turnover in skeletal muscle by insulin-like growth factor I. *Endocrinology* **137** 4087–4094.

Dardevet D, Sornet C, Savary I, Debras E, Patureau Mirand P & Grizard J 1998 Glucocorticoid effects on insulin- and IGF-I-regulated muscle protein metabolism during aging. *Journal of Endocrinology* **156** 83–89.

Denton RM & Tavaré JM 1995 Does mitogen-activated-protein kinase have a role in insulin action? The cases for and against. *European Journal of Biochemistry* **227** 597–611.

Dujovne LA & Azarnoff DL 1975 Clinical complications of corticosteroid therapy: a review. In *Steroid Therapy*, pp 27–47. Ed DL Azarnoff. Philadelphia: Saunders.

Fingar DC, Hausdorff SF, Blenis J & Birnbaum MJ 1993 Dissociation of p70 ribosomal protein S6 kinase from insulin-stimulated glucose transport in 3T3-L1 adipocytes. *Journal of Biological Chemistry* **268** 3005–3008.

Garlick PJ & Grant I 1988 Amino acid infusion increases the sensitivity of muscle protein synthesis *in vivo* to insulin: effect of branch-chain amino acids. *Biochemical Journal* **254** 579–584.

Giorgino F, Almahfouz A, Goodyear LJ & Smith RJ 1993 Glucocorticoid regulation of insulin receptor and substrate IRS-1 tyrosine phosphorylation in rat skeletal muscle *in vivo*. *Journal of Clinical Investigation* **91** 2020–2030.

Graves LM, Bornfeldt KE, Argast GM, Krebs EG, Kong XM, Lin TA & Lawrence JC 1995 cAMP- and rapamycin-sensitive regulation of the association of eukaryotic initiation factor 4E and the translational regulator PHAS-I in aortic smooth muscle cells. *Proceedings of the National Academy of Sciences of the USA* **92** 7222–7226.

Hara K, Yonezawa K, Kozlowski MT, Sugimoto T, Andrabi K, Weng QP, Kasuga M, Nishimoto I & Avruch J 1997 Regulation of eIF-4E BP1 phosphorylation by mTOR. *Journal of Biological Chemistry* **272** 26457–26463.

Haystead TAJ, Haystead CMM, Hu CB, Lin TA & Lawrence JC Jr 1994 Phosphorylation of PHAS-I by mitogen-activated protein (MAP) kinase identification of a site phosphorylated by MAP kinase *in vitro* and in response to insulin in rat adipocytes. *Journal of Biological Chemistry* **269** 23185–23191.

Kayali AG, Young VR & Goodman MN 1987 Sensitivity of myofibrillar proteins to glucocorticoid-induced muscle proteolysis. *American Journal of Physiology* **252** E621–E626.

Kim SJ & Kahn CR 1997 Insulin stimulates p70 S6 kinase in the nucleus of cells. *Biochemical and Biophysical Research Communications* **234** 681–685.

Kimball SR, Vary TC & Jefferson LS 1994 Regulation of protein synthesis by insulin. *Annual Review of Physiology* **56** 321–348.

Legaspi A, Albert JD, Calvano SE, Brennan MF & Lowry SF 1985 Proteolysis of skeletal muscle in response to acute elevation of plasma cortisol in man. *Surgical Forum* **36** 16–18.

Leighton B, Challiss RAJ, Lozeman PJ & Newsholme EA 1987 Effects of dexamethasone treatment on insulin-stimulated rates of glycolysis and glycogen synthesis in isolated incubated skeletal muscles of the rat. *Biochemical Journal* **246** 551–554.

Lin TA, Kong XM, Haystead TAJ, Pause A, Belsham G, Sonenberg N & Lawrence JC Jr 1994 PHAS-1 as a link between mitogen-activated protein kinase and translation initiation. *Science* **266** 653–656.

Lin TA, Kong XM, Saltiel AR, Blackshear PJ & Lawrence JC 1995 Control of PHAS-I by insulin in 3T3-L1 adipocytes synthesis, degradation, and phosphorylation by a rapamycin-sensitive and mitogen-activated protein kinase-independent pathway. *Journal of Biological Chemistry* **270** 18531–18538.

Louard RJ, Bhushan R, Gelfand RA, Barrett EJ & Sherwin RS 1994 Glucocorticoids antagonize insulin's antiproteolytic action on skeletal muscle in humans. *Journal of Clinical Endocrinology and Metabolism* **79** 278–284.

McGrath JA & Goldspink DF 1982 Glucocorticoid action on protein synthesis and protein breakdown in isolated skeletal muscles. *Biochemical Journal* **206** 641–645.

Mosoni L, Patureau Mirand P, Houlier ML & Arnal M 1993 Age-related changes in protein synthesis measured *in vivo* in rat liver and gastrocnemius muscle. *Mechanisms of Ageing and Development* **68** 209–220.

Myers MG & White MF 1993 The new elements of insulin signaling-insulin receptor substrate-1 and proteins with SH2 domains. *Diabetes* **42** 643–650.

Odedra BR, Bates PC & Millward DJ 1983 Time course of the effect of catabolic doses of corticosterone on protein turnover in rat skeletal muscle and liver. *Biochemical Journal* **214** 617–627.

Okada T, Kawano Y, Sakakibara T, Hazeki O & Ui M 1994 Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes: studies with a selective inhibitor wortmannin. *Journal of Biological Chemistry* **269** 3568–3573.

Osawa H, Sutherland C, Robey RB, Printz RL & Granner DK 1996 Analysis of the signaling pathway involved in the regulation of hexokinase II gene transcription by insulin. *Journal of Biological Chemistry* **271** 16690–16694.

Palmer RM, Thompson MG, Knott RM, Campbell GP, Thom A & Morrison KS 1997 Insulin and insulin-like growth factor-I responsiveness and signalling mechanisms in C2C12 satellite cells: effect of differentiation and fusion. *Biochimica et Biophysica Acta* **1355** 167–176.

Pause A, Belsham GJ, Gingras AC, Donzé O, Lin TA, Lawrence JC & Sonenberg N 1994 Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5′-cap function. *Nature* **371** 762–767.

Price DJ, Russell Grove J, Calvo V, Avruch J & Bierer BE 1992 Rapamycin-induced inhibition of the 70-kilodalton S6 protein kinase. *Science* **257** 973–976.

Rannels SR & Jefferson LS 1980 Effects of glucocorticoids on muscle protein turnover in perfused rat hemicorpus. *American Journal of Physiology* **238** E564–E572.

Rhoads RE 1993 Regulation of eukaryotic protein synthesis by initiation factors. *Journal of Biological Chemistry* **268** 017–302.

Saad MJA, Folli F, Kahn JA & Kahn CR 1993 Modulation of insulin receptor, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in liver and muscle of dexamethasone-treated rats. *Journal of Clinical Investigation* **92** 2065–2072.

Sinaud S, Balage M, Bayle G, Dardevet D, Vary TC, Kimball SR, Jefferson LS & Grizard J 1999 Diazoxide-induced insulin deficiency greatly reduced muscle protein synthesis in rats. Involvement of eIF-4E. *American Journal of Physiology* **276** E50–E61.

Sonenberg N 1994 Regulation of translation and cell growth by eIF-4E. *Biochimie* **76** 839–846.

Southorn BG, Palmer RM & Garlick PJ 1990 Acute effects of corticosterone on tissue protein synthesis and insulin-sensitivity in rats *in vivo*. *Biochemical Journal* **272** 187–191.

Taha C, Mitsumoto Y, Liu Z, Skolnik EY & Klip A 1995 The insulin-dependent biosynthesis of GLUT1 and GLUT3 glucose transporters in L6 muscle cells is mediated by distinct pathways-roles of p21(ras) and p70 S6 kinase. *Journal of Biological Chemistry* **270** 24678–24681.

Tessitore L, Costelli P & Baccino FM 1993 Humoral mediation for cachexia in tumour-bearing rats. *British Journal of Cancer* **67** 15–23.

Thompson MG & Palmer RM 1998 Signalling pathways regulating protein turnover in skeletal muscle. *Cell Signal* **10** 1–11.

Tomas FM, Munro HN & Young VR 1979 Effect of glucocorticoid administration on the rate of muscle protein breakdown *in vivo* in rats, as measured by urinary excretion of Ntau-methylhistidine. *Biochemical Journal* **178** 139–146.

Vary TC, Dardevet D, Grizard J, Voisin L, Buffière C, Denis P, Breuillé D & Obled C 1998 Differential regulation of skeletal muscle protein turnover by insulin and IGF-I after bacteremia. *American Journal of Physiology* **275** E584–E593.

Vaughan GM, Becker RA, Allen JP, Goodwin CV, Pruitt BA & Mason AD 1982 Cortisol and corticotropin in burned patients. *Journal of Trauma* **22** 263–273.

Von Manteuffel SR, Dennis PB, Pullen N, Gingras AC, Sonenberg N & Thomas G 1997 The insulin-induced signalling pathway leading to S6 and initiation factor 4E binding protein 1 phosphorylation bifurcates at a rapamycin-sensitive point immediately upstream of p70(s6k). *Molecular and Cellular Biology* **17** 5426–5436.

Weinstein SP, Paquin T, Pritsker A & Haber RS 1995 Glucocorticoid-induced insulin resistance: dexamethasone inhibits the activation of glucose transport in rat skeletal muscle by both insulin and non-insulin-related stimuli. *Diabetes* **44** 441–445.

Welsh GI & Proud CG 1993 Glycogen synthetase kinase-3 is rapidly inactivated in response to insulin and phosphorylates eukaryotic initiation factor elF-2B. *Biochemical Journal* **294** 625–629.

Welsh GI, Foulstone EJ, Young SW, Tavare JM & Proud CG 1994 Wortmannin inhibits the effects of insulin and serum on the activities of glycogen synthase kinase-3 and mitogen-activated protein kinase. *Biochemical Journal* **303** 15–20.

Yeh JI, Gulve EA, Rameh L & Birnbaum MJ 1995 The effects of wortmannin on rat skeletal muscle dissociation of signaling pathways for insulin- and contraction-activated hexose transport. *Journal of Biological Chemistry* **270** 2107–2111.

Received 3 November 1998
Accepted 11 February 1999