Contraction inhibits insulin-stimulated insulin receptor substrate-1/2-associated phosphoinositide 3-kinase activity, but not protein kinase B activation or glucose uptake, in rat muscle

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The initial stages of insulin-stimulated glucose uptake are thought to involve tyrosine phosphorylation of insulin receptor substrates (IRSs), which recruit and activate phosphoinositide 3-kinase (PI 3-kinase), leading to the activation of protein kinase B (PKB) and other downstream effectors. In contrast, contraction stimulates glucose uptake via a PI 3-kinase-independent mechanism. The combined effects of insulin and contraction on glucose uptake are additive. However, it has been reported that contraction causes a decrease in insulin-stimulated IRS-1-associated PI 3-kinase activity. To investigate this paradox, we have examined the effects of contraction on insulin-stimulated glucose uptake and proximal insulin-signalling events in isolated rat epitrochlearis muscle. Stimulation by insulin or contraction produced a 3-fold increase in glucose uptake, with the effects of simultaneous treatment by insulin and contraction being additive. Wortmannin completely blocked the additive effect of insulin in

contracting skeletal muscle, indicating that this is a PI 3-kinase-dependent effect. Insulin-stimulated recruitment of PI 3-kinase to IRS-1 was unaffected by contraction; however, insulin produced no discernible increase in PI 3-kinase activity in IRS-1 or IRS-2 immunocomplexes in contracting skeletal muscle. Consistent with this, contraction inhibited insulin-stimulated p70^{S6K} activation. In contrast, insulin-stimulated activation of PKB was unaffected by contraction. Thus, in contracting skeletal muscle, insulin stimulates glucose uptake and activates PKB, but not p70^{S6K}, by a PI 3-kinase-dependent mechanism that is independent of changes in IRS-1- and IRS-2-associated PI 3-kinase activity.

Key words: exercise, insulin action, metabolism, signal transduction, transport.

INTRODUCTION

Insulin and contraction are the two most potent stimulators of skeletal muscle glucose uptake. Skeletal muscle represents the major site for insulin-stimulated glucose disposal in the post-prandial period, whereas glucose is utilized as a source of energy during exercise. Although both insulin and contraction increase the translocation of GLUT4 to the plasma membrane, the precise mechanisms involved are not yet fully understood. Activation of the insulin receptor tyrosine kinase results in tyrosine phosphorylation of insulin receptor substrates (IRSs), which serve as docking proteins for proteins containing Src homology 2 (SH2) domains, of which the most extensively characterized are the class Ia phosphoinositide 3-kinases (PI 3-kinases) (for review, see [1]). The p85 regulatory subunit of PI 3-kinase binds to phosphotyrosine motifs of IRS-1, and IRS-2, resulting in activation of the p110 catalytic subunit. Activation of PI 3-kinase appears necessary for insulin-stimulated glucose uptake, since inhibition of PI 3-kinase, using chemical inhibitors or dominant-negative p85, blocks insulin-stimulated GLUT4 translocation and glucose uptake [1]. Activated PI 3-kinase generates phosphoinositide intermediates (PtdInsP₃), which serve to recruit and facilitate the activation of downstream effectors. One such effector is the serine/threonine kinase protein kinase B (PKB; Akt), which has been implicated in insulin signalling to

glucose transport in several studies using constitutively active forms of PKB or inhibitor studies employing dominant-negative PKB constructs, PKB substrate peptides or anti-PKB antibody ([2,3], and references therein).

In contrast with insulin, contraction stimulates glucose uptake in a PI 3-kinase-independent manner [4,5]. While the signalling mechanisms involved in contraction-stimulated glucose uptake remain poorly understood, it has been suggested that the 5'-AMP-activated protein kinase is involved [6,7]. This difference in the proximal steps of the signalling pathways for insulin and contraction has been proposed as a possible explanation for their additive effects. Such additive effects have been reported for glucose uptake [6–8] as well as GLUT4 translocation [9,10].

Interestingly, insulin-stimulated IRS-1-associated PI 3-kinase activity was moderately decreased following a single bout of exercise in both rat and human skeletal muscle [11,12], although insulin-stimulated glucose uptake was increased [12]. Given the proposed central role of IRS-1-associated PI 3-kinase activity in mediating insulin's metabolic effects, we thought that this was somewhat paradoxical. Therefore, in the present study, we have investigated the interplay between insulin and contraction on the activation of the insulin signalling pathway, particularly IRS-1- and IRS-2-associated PI 3-kinase activity, in rat epitrochlearis muscle *ex vivo*.

Abbreviations used: IRS, insulin receptor substrate; PI 3-kinase, phosphoinositide 3-kinase; PKB, protein kinase B; SH2, Src homology 2.

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EXPERIMENTAL

Materials

Reagents were from Sigma (Poole, Dorset, U.K.) unless otherwise stated. Anti-IRS-1 antibody has been described previously [13]. The anti-phosphotyrosine antibody (4G10) and anti-Akt1, anti-p70^{S6K} and anti-IRS-2 antibodies were from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Anti-phospho-Akt antibodies (Akt phosphorylated on Thr³⁰⁸ or Ser⁴⁷³) and anti-phospho-p70^{S6K} antibodies (p70^{S6K} phosphorylated on Thr³⁸⁹) were from New England Biolabs (Hitchin, Herts., U.K.). Monoclonal anti-p85 antibody was generously donated by Dr Peter Shepherd (UCL, London, U.K.).

Animals

Male Wistar rats (Bk1:Wist) were obtained from B & K Universal AS (Nittedal, Norway) and housed in laboratory animal facilities for at least 1 week before the experiment. A 12 h light/dark cycle was set (light from 06:00 to 18:00 h), with a room temperature of 21 °C and humidity of $\geq 55\%$. The rats had free access to standard rat chow (B & K Universal Ltd, Grimston, U.K.) and water. The experiments were performed during the light part of the cycle (between 10:00 and 14:00 h), and the rats weighed between 120 and 150 g on the day of the experiment. The experiments were conducted in conformity with the laws and regulations controlling experiments/procedures of live animals in Norway, and procedures were approved by the local laboratory animal science specialist.

Muscle preparation and incubation

The rats were anaesthetized with an intraperitoneal injection of 10 mg of pentobarbital (50 mg/ml), and the epitrochlearis was dissected out and suspended on a contraction apparatus between two platinum electrodes at its approximate resting length. The apparatus was then placed in a test tube and gas containing 95% O₂ and 5% CO₂ was passed continuously through the incubation buffer, as described previously [8]. The muscles were preincubated for 30 min in 3 ml of Krebs/Henseleit buffer containing 5.5 mM glucose, 2 mM sodium pyruvate, 5 mM Hepes and 0.1% BSA (Fraction V), pH 7.4. All incubations were performed at 30 °C. Insulin (Monotard; Novo Nordisk, Copenhagen, Denmark) was used at 10 m-units/ml. Where indicated, wortmannin (1 μ M) was added for the final 10 min of the preincubation period and throughout the incubation period.

Muscle contraction

After preincubation, some of the muscles were stimulated to contract isometrically. The muscles were stimulated with impulse trains of 200 ms (100 Hz; square wave pulses of 0.2 ms duration and 10 V amplitude), delivered at a rate of one train per 2 s for 30 min. The pulses were generated by equipment built at The National Institute of Occupational Health, Oslo, Norway.

Glucose uptake

Glucose uptake was determined as described previously [14]. In brief, 0.25 μ Ci/ml 2-[1,2-³H(n)]deoxy-D-glucose (25.5 Ci/mmol; DuPont NEN) and 0.1 μ Ci/ml [1-¹⁴C]-D-mannitol (51.5 mCi/mmol; DuPont NEN) were added to the buffer (containing 5.5 mM glucose), and glucose uptake was calculated from the intracellular accumulation of 2-[³H]deoxy-D-glucose. After incubations, the muscles were removed from the contraction apparatus, blotted on filter paper and frozen in liquid nitrogen. Muscles were freeze-dried, weighed and dissolved in 600 μ l of

1 M KOH for 20 min at 70 °C. Of this, 400 μ l was neutralized with 100 μ l of 4 M HCl and added to 3 ml of scintillation solution (Ultima Gold XR; Packard), mixed and counted for radioactivity.

Preparation of muscle extracts for signalling studies

After incubations, muscles were frozen in liquid nitrogen and stored at -80 °C. Subsequent steps were performed at 4 °C. Samples were homogenized in ice-cold buffer (1% Triton X-100, 50 mM Hepes, 150 mM NaCl, 10 mM Na₄P₂O₇, 10 mM EDTA, 1 μ g/ml each of antipain, pepstatin and leupeptin, 2.5 mM benzamidine, 0.5 mM PMSF and 1 μ M microcystin) using a glass homogenizer. Homogenates were rotated for 1 h and insoluble matter was removed by centrifugation at 13000 g for 10 min. Protein concentration was determined using the Bradford dye-binding procedure (Bio-Rad).

Western blot analyses

Immunoblotting was performed as described previously [15]. In brief, total muscle protein lysates (≈ 50 μ g) or p70^{S6K} or IRS-1 immunoprecipitates (from ≈ 350 or 1000 μ g respectively) were resolved by SDS/PAGE, transferred to Immobilon-P PVDF membranes (Millipore Corp., Bedford, MA, U.S.A.) and membranes probed with the appropriate primary and horseradish peroxidase-conjugated or ¹²⁵I-labelled secondary antibodies. Antibody binding was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.) or imaging using a Fujix Bas 2000 PhosphorImager (Fuji Photo Film Co., Tokyo, Japan).

Immunoprecipitation and assay of PI 3-kinase activity

Samples were immunoprecipitated for 2 h unless otherwise stated. Immunoprecipitations were performed using ≈ 350 μ g of muscle protein, and PI 3-kinase assays were carried out essentially as described previously [13].

RESULTS AND DISCUSSION

Wortmannin inhibits the additive effects of insulin and contraction on glucose uptake

The effects of insulin and/or contraction on glucose uptake in isolated rat epitrochlearis muscle are shown in Figure 1. Treatment with insulin alone stimulated glucose uptake approx. 3-fold, as did treatment with contraction alone. Combined stimulation with insulin and contraction had an additive, 5-fold, effect. In agreement with other studies, the PI 3-kinase inhibitor wortmannin was without effect on contraction-stimulated glucose uptake, whereas it completely inhibited insulin's ability to stimulate glucose uptake [4,5]. Wortmannin also completely inhibited the additive effect of insulin on contraction-stimulated glucose uptake, indicating that this additive effect was PI 3-kinase dependent.

Insulin-stimulated IRS-1-associated PI 3-kinase activity is completely inhibited by contraction

Numerous studies have suggested that IRS-1-associated PI 3-kinase activity is central to many of insulin's effects; however, insulin-stimulated IRS-1-associated PI 3-kinase activity has been reported to be reduced in both rat and human skeletal muscle following a single bout of exercise [11,12]. Having shown that simultaneous stimulation by contraction and insulin had additive effects on glucose uptake in isolated rat epitrochlearis muscle, we

Figure 1 Wortmannin inhibits the additive effects of insulin and contraction on glucose uptake in isolated rat epitrochlearis muscle

Epitrochlearis muscles were incubated for 30 min with tracer 2-[^3H]deoxyglucose, and glucose uptake was measured in the absence (filled bars) or presence (hatched bars) of wortmannin in resting or contracting (Con) muscle in the absence or presence of insulin (Ins), as indicated. Values are means $\pm$ S.E.M. ($n = 5-7$ muscles for each). *Value for Con + Ins is significantly higher than that for Con alone or Ins alone ($P < 0.05$; Mann-Whitney test). dw, dry weight.

then examined IRS-1-associated PI 3-kinase activity in immunoprecipitates of IRS-1 (Figure 2A). Stimulation with insulin alone caused a 5-fold increase in IRS-1-associated PI 3-kinase activity, whereas treatment by contraction alone had no significant effect.

Surprisingly, when muscles were stimulated simultaneously by insulin and contraction, there was no increase in IRS-1-associated PI 3-kinase activity. This indicates that contraction completely inhibits insulin's ability to stimulate PI 3-kinase activity in IRS-1 complexes. We reasoned that the inhibitory effect of contraction may occur at a time point preceding the stimulation of glucose transport. To address this, we measured PI 3-kinase activity in IRS-1 immunoprecipitates after treatment for various times (Figure 2B). Insulin stimulation caused a rapid increase in IRS-1-associated PI 3-kinase activity, which was 5-fold at 2 min and peaked at approx. 7-fold at 15 min. Contraction inhibited insulin's effects at all time points.

Contraction causes only modest decreases in the phosphorylation of the insulin receptor and IRS-1, and in the recruitment of PI 3-kinase

To investigate the molecular mechanisms underlying the ability of contraction to inhibit insulin-stimulated IRS-1-associated PI 3-kinase activity, we determined the effects of insulin and/or contraction on insulin receptor and IRS-1 phosphorylation, and on recruitment of PI 3-kinase to IRS-1, by immunoblot analysis of total cell lysates or IRS-1 immunoprecipitates (Figure 3). Treatment with insulin alone caused a 3-4-fold increase in receptor autophosphorylation, whereas contraction alone was without effect. Combined treatment with insulin and contraction resulted in a slight but significant decrease (35%; $P < 0.03$) in receptor phosphorylation compared with insulin alone. Treatment with insulin alone resulted in a 2-fold increase in tyrosine

Figure 2 Effects of contraction on insulin-stimulated IRS-1-, IRS-2- and phosphotyrosine-associated PI 3-kinase activity

(A) Contraction inhibits insulin-stimulated IRS-1-associated PI 3-kinase activity. Muscles were rested or contracted (Con) in the absence or presence of insulin (Ins) for 30 min, and PI 3-kinase activity was measured in IRS-1 immunoprecipitates; $n = 8-10$ muscles for each; * $P < 0.0005$ for Ins compared with Basal, Con or Con + Ins (no significant differences between others). (B) Time course of the effects of inhibition by contraction of insulin-stimulated IRS-1-associated PI 3-kinase activity. IRS-1-associated PI 3-kinase activity was determined in muscles without treatment ($\square$) or following contraction ($\circ$), insulin ($\bullet$) or contraction + insulin ($\blacksquare$), for the times indicated; $n = 3-10$ muscles for each. (C) Contraction inhibits insulin-stimulated IRS-2-associated PI 3-kinase activity. Muscles were treated as in (A) for 2 min, and PI 3-kinase activity was determined in IRS-2 immunoprecipitates; $n = 6$ muscles for each; * $P < 0.002$ for insulin (Ins) compared with Basal, contraction (Con) or Con + Ins (no significant differences between others). (D) Contraction inhibits insulin-stimulated phosphotyrosine (pY)-associated PI 3-kinase activity. Muscles were treated as in (A), except that PI 3-kinase activity was measured in phosphotyrosine immunoprecipitates; $n = 8-12$ muscles for each; * $P < 0.001$ for insulin (Ins) compared with Basal, contraction (Con) or Con + Ins; † $P < 0.01$ for Con compared with Basal or Con + Ins (no significant difference between Basal and Con + Ins). All values are means $\pm$ S.E.M.

Figure 3 Effects of contraction and/or insulin on insulin receptor auto-phosphorylation, IRS-1 phosphorylation and p85 recruitment

Muscles were rested or contracted (Con) in the absence or presence of insulin (Ins) for 30 min. Insulin receptor phosphorylation was determined by SDS/PAGE and immunoblotting of total cell lysates. IRS-1 phosphorylation and p85 recruitment were determined in IRS-1 immunoprecipitates subjected to SDS/PAGE and immunoblotting. The blots shown are representative of at least four separate experiments. pY, phosphotyrosine; IR, insulin receptor.

phosphorylation of IRS-1, and this was mirrored by a comparable increase in the amount of p85 α co-precipitated with IRS-1 ($P < 0.03$ for each). Contraction alone had no significant effect on IRS-1 tyrosine phosphorylation or p85 α recruitment, and nor did it prevent insulin-stimulated tyrosine phosphorylation of IRS-1 or recruitment of p85 α (contraction reduced insulin-stimulated tyrosine phosphorylation of IRS-1 by 13% and p85 α recruitment by 16%). Treatment with wortmannin was without effect on the above (results not shown).

The modest changes described above are insufficient to explain the complete inhibition by contraction of insulin-stimulated IRS-1-associated PI 3-kinase activity. The finding that insulin-stimulated IRS-1 phosphorylation and recruitment of p85 α were unaffected by contraction is of particular interest, as this suggests that, in contracting skeletal muscle, the recruitment of PI 3-kinase to IRS-1 is insufficient to mediate activation of its lipid kinase activity. Several potential mechanisms may be invoked whereby contraction inhibits the insulin-stimulated activation, but not recruitment, of IRS-1-associated PI 3-kinase. Contraction may result in the covalent modification of PI 3-kinase, resulting in decreased lipid kinase activity. For example, contraction may promote phosphorylation of the p85 α regulatory subunit on Ser⁶⁰⁸, which has been shown to decrease the lipid kinase activity of the associated p110 catalytic subunit [16]. Alternatively, contraction may stimulate the recruitment of additional 'inhibitory' factors to the IRS-1·PI 3-kinase complex. Support for the involvement of an inhibitory factor comes from our observations that the inhibitory effects of contraction on insulin-stimulated IRS-1-associated PI 3-kinase activity were decreased when immunoprecipitation was prolonged. When immunoprecipitation was carried out overnight, rather than for 2 h, contraction inhibited insulin-stimulated IRS-1-associated PI 3-kinase activity by only 20–40% (J. P. Whitehead and M. A. Soos, unpublished work). Similar inhibition was reported by others when immunoprecipitations were performed overnight [11,12]. Prolonged immunoprecipitation may result in dissociation of an inhibitory factor and a subsequent increase in IRS-1-associated PI 3-kinase activity. One potential candidate is the 14-13-3 protein [17,18], which is reported to inhibit PI 3-kinase activity without altering PI 3-kinase association *per se* [19]. However, contraction did not affect the association of 14-13-3 with IRS-1 in immunoprecipitates of IRS-1 immunoblotted for 14-13-3 protein (results not shown).

There is overwhelming evidence for a central role for both IRS-1 and PI 3-kinase in mediating many of insulin's effects

[1,20]. Consistent with this, Yamauchi et al. [21] found skeletal muscle from IRS-1^{-/-} mice to be insulin-resistant, exhibiting impaired insulin-stimulated PI 3-kinase activation and glucose uptake. However, in a more recent study insulin-stimulated glucose uptake was found to be comparable in muscle from wild-type and IRS-1^{-/-} mice [22]. Furthermore, in the muscle-specific insulin-receptor knockout mice (MIRKO), the effects of exercise and insulin were found to be additive in the absence of significant insulin-stimulated IRS-1-associated PI 3-kinase activity [23]. Thus the absolute requirement for insulin-stimulated IRS-1-associated PI 3-kinase activity has not been demonstrated unequivocally. Indeed, several reports utilizing various cell culture models have questioned the central role of IRS-1-associated PI 3-kinase in insulin-stimulated glucose uptake. Utilizing microinjection of competitive inhibitory reagents into 3T3-L1 adipocytes, Olefsky and colleagues [24] found that disruption of the insulin receptor/IRS-1 interaction had no effect upon GLUT4 translocation. Furthermore, using adenovirus-mediated overexpression of IRS-1-interacting domains, IRS-1-associated PI 3-kinase activity was reduced by 90% without affecting insulin-induced activation of PKB or glucose transport [25]. Also, pretreatment of 3T3-L1 adipocytes with platelet-derived growth factor resulted in a significant decrease in insulin-stimulated IRS-1-associated PI 3-kinase activity, without affecting glucose transport [26]. These studies suggest that increased IRS-1-associated PI 3-kinase activity is not required for insulin-stimulated GLUT4 translocation and glucose uptake. However, while there is strong evidence to suggest that PI 3-kinase activation alone is insufficient to stimulate glucose uptake [27], the possibility cannot be ruled out that a small increase in IRS-1-associated PI 3-kinase activity, coupled with the appropriate additional signals, may be adequate for the efficient activation of PKB, GLUT4 translocation and glucose uptake.

Insulin-stimulated IRS-2- and phosphotyrosine-associated PI 3-kinase activity is also inhibited by contraction

Recently the alternative insulin receptor substrate, IRS-2, has been implicated in insulin signalling to metabolic events, including glucose uptake [28]. As activation of IRS-2 is reported to be more transient than that of IRS-1 [29,30], we determined IRS-2-associated PI 3-kinase activity following treatment for 2 min (Figure 2C). Stimulation with insulin alone produced a 3-fold increase in IRS-2-associated PI 3-kinase activity. Treatment by contraction alone had little effect in the absence of insulin; however, contraction completely inhibited insulin's ability to stimulate IRS-2-associated PI 3-kinase activity. This suggests that the inhibition by contraction, which was observed for both IRS-1 and IRS-2 complexes, is mediated by a common mechanism. Our data rule out a requirement for increased IRS-2-associated PI 3-kinase activity in either insulin- or contraction-stimulated glucose uptake in skeletal muscle. In accordance with this, recent evidence from transgenic mice has indicated that IRS-2 does not play a major role in insulin signalling in skeletal muscle, but is more involved in mediating insulin's effects in the liver [31,32].

As IRS-1 and IRS-2 represent only two of the putative tyrosine-phosphorylated mediators of insulin's ability to stimulate class Ia PI 3-kinase, we determined PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates. Results appeared consistent with those with IRS-1 and IRS-2 immunoprecipitates, with contraction inhibiting insulin's ability to increase PI 3-kinase activity associated with tyrosine-phosphorylated proteins (Figure 2D; compare Basal with Con+Ins). However, contraction also caused a significant decrease in PI 3-kinase activity in the absence

Figure 4 Insulin-stimulated PKB phosphorylation is unaffected by contraction

(A) Effects of contraction and wortmannin on insulin-stimulated PKB phosphorylation. Muscles were rested or contracted (Con) in the absence or presence of insulin (Ins) and wortmannin for 30 min as indicated. PKB Thr³⁰⁸ phosphorylation (top panel), Ser⁴⁷³ phosphorylation (middle panel) and band shift (bottom panel) were determined by SDS/PAGE and immunoblot analysis of total cell lysates. Immunoblots shown are representative of at least four separate experiments. (B), (C) Time course of Ser⁴⁷³ phosphorylation. Samples were treated as in (A) for the indicated times, and Ser⁴⁷³ phosphorylation was determined. Representative blots are shown in (B) and quantified in (C): □, no treatment (basal); ○, following contraction; ●, + insulin; ■, contraction + insulin. Values are means $\pm$ S.E.M. ($n = 4$ muscles for each).

of insulin (Figure 2D; compare Basal with Con), which was not observed with IRS-1 and IRS-2 complexes. Consequently, insulin mediated a significant increase in PI 3-kinase activity in phosphotyrosine complexes in contracting muscle (Figure 2D; compare Con with Con + Ins). Thus, although there is no quantitative difference between phosphotyrosine-associated PI 3-kinase activity in untreated muscle and muscle stimulated simultaneously with contraction and insulin, the above data suggest that these may be qualitatively different. Such a qualitative difference would occur independently of changes in IRS-1- and IRS-2-associated PI 3-kinase activity, as contraction had no significant effect on these parameters in the absence of insulin.

Insulin-stimulated PKB phosphorylation is unaffected by contraction

The ability of insulin to stimulate PKB activity in insulin-responsive tissues is well documented. More recently, contraction has been reported to have little [33] or no [34–37] effect on PKB activity. However, given that insulin's stimulation of PKB is dependent on PI 3-kinase, and that contraction inhibits insulin-stimulated PI 3-kinase activity in IRS-1 and IRS-2 complexes and reduces the activity in phosphotyrosine complexes, we examined the effects of insulin and/or contraction on PKB activation, as determined by PKB phosphorylation (on Thr³⁰⁸ or Ser⁴⁷³) or band-shift in immunoblot analyses (Figure 4). Treatment with insulin alone caused a dramatic increase in the phosphorylation of both Thr³⁰⁸ and Ser⁴⁷³ (20–30-fold), with approx. 50 % of PKB shifting to a higher-molecular-mass form. Treatment by contraction alone was without effect on the phosphorylation of Thr³⁰⁸ and on PKB band-shift, although a small (3-fold) increase in PKB Ser⁴⁷³ phosphorylation was detected. Given that phosphorylation of PKB on Thr³⁰⁸ is a prerequisite for its activation, these data are consistent with contraction being unable to mediate significant activation of PKB and are concordant with several recent reports [34–37]. Contraction also had no significant effect on insulin's ability to stimulate PKB phosphorylation (on Thr³⁰⁸ or Ser⁴⁷³) or band-shift, as determined in muscles treated simultaneously with insulin and contraction. In contrast, wortmannin inhibited insulin's stimulation of PKB phosphorylation in muscles stimulated by insulin alone or by insulin and contraction. These findings indicate that PKB activation occurs via an insulin-stimulated PI 3-kinase-dependent pathway, which is independent of IRS-1 and IRS-2, and are consistent with PKB being involved in insulin-stimulated glucose uptake in both resting and contracting skeletal muscle.

Insulin-stimulated p70^{S6K} phosphorylation is inhibited by contraction

In order to determine whether preservation of signalling is exclusive to PKB and glucose uptake, we examined the effects of contraction on insulin's ability to stimulate p70^{S6K}, which also lies downstream of PI 3-kinase and is closely associated with the regulation of protein synthesis [38]. While the precise PI 3-kinase-dependent pathways regulating p70^{S6K} activation remain unresolved, several lines of evidence suggest that the PDK (phosphoinositide-dependent kinase 1)/PKB system and mammalian target of rapamycin (mTOR) are involved [39–41]. The activity of p70^{S6K} is regulated by phosphorylation at various sites; however, phosphorylation at Thr³⁸⁹ correlates most clearly with p70^{S6K} activity *in vivo* [42]. We therefore examined the effects of treatment with insulin and/or contraction for 30 min on p70^{S6K} activation, as determined by p70^{S6K}(Thr³⁸⁹) phospho-immunoblotting of p70^{S6K} immunoprecipitates (Figure 5). Stimulation with insulin alone induced an approx. 4-fold increase, and this was inhibited by wortmannin. Treatment by contraction alone was without effect. Simultaneous treatment with insulin and contraction reduced p70^{S6K} phosphorylation by 80 % when compared with stimulation with insulin alone ($P < 0.02$). Similar results were obtained when treatment was performed for 15 min (not shown). This indicates that the efficient activation of p70^{S6K} by insulin requires a robust IRS-1- and/or IRS-2-associated PI 3-kinase signal. In addition, the finding that p70^{S6K} activation is inhibited provides strong evidence that the inhibition of PI 3-kinase activity observed in immunoprecipitated complexes is representative of the situation in intact muscles, and not merely an artefact of the assay.

Figure 5 Insulin-stimulated p70^{S6K} phosphorylation is inhibited by contraction

Muscles were rested or contracted (Con) in the absence or presence of insulin (Ins) and wortmannin for 30 min as indicated. p70S6K Thr³⁶⁹ phosphorylation was determined by SDS/PAGE and immunoblot analysis of p70^{S6K} immunoprecipitates. Immunoblots shown are representative of at least four separate experiments. The lower panel represents quantified data collated from $n = 4$ –6 muscles for each treatment. Values are means $\pm$ S.E.M.; * $P < 0.02$ Ins compared with Basal, Con or Con + Ins (no significant differences between others).

Conclusions

In the present study, simultaneous treatment with insulin and contraction produced additive effects on glucose uptake, even though contraction completely inhibited the ability of insulin to stimulate PI 3-kinase activity associated with IRS-1 and IRS-2, yet p85 α recruitment was unaffected. Consistent with the observed inhibition of PI 3-kinase activity, insulin's ability to stimulate p70^{S6K} was significantly inhibited by contraction. Conversely, insulin's ability to stimulate PKB was unaffected by contraction, yet remained wortmannin-sensitive in contracted, as well as rested, muscles. The latter findings are in keeping with those from studies of 3T3-L1 and primary rat adipocytes, where inhibition of insulin-stimulated IRS-1-associated PI 3-kinase activity by various treatments was without effect on PKB activation and glucose uptake, but markedly reduced p70^{S6K} activation [24–26]. This indicates that adipocytes and skeletal muscle exhibit the same requirement for a robust activation of PI 3-kinase to mediate the efficient activation of p70^{S6K}, whereas such PI 3-kinase activation does not appear necessary for the efficient activation of PKB and glucose uptake.

While contraction markedly reduced insulin-stimulated PI 3-kinase activity in phosphotyrosine immunoprecipitates to levels comparable with those seen in untreated muscles, this was significantly greater than that in contracted muscles without insulin. Thus it remains possible that a qualitative difference in PI 3-kinase activity between untreated muscles and those stimulated by contraction and insulin may be sufficient to generate insulin-stimulated PKB activation and glucose uptake. Such a qualitative difference may be due to the recruitment and activation of PI 3-kinase by alternative phosphotyrosine complexes at different subcellular locations. While a small fraction of

insulin-stimulated PI 3-kinase activity associates with the insulin receptor, previous studies have shown that contraction inhibits this receptor-associated PI 3-kinase activity [11]. Thus insulin-receptor-associated PI 3-kinase activity is unlikely to explain our observations. IRS-3 and IRS-4 recruit and stimulate PI 3-kinase activity in an insulin-dependent fashion, analogous to IRS-1 and IRS-2 [43,44]. In addition, both stimulate translocation of GLUT4 in adipocytes [45]. However, they are not thought to be expressed in skeletal muscle [43,46], and IRS-3^{-/-} and IRS-4^{-/-} mice do not exhibit major defects in glucose homeostasis [47,48]. More recently identified alternative insulin receptor substrates, such as APS (adaptor protein containing pleckstrin homology and SH2 domains), are expressed in skeletal muscle, although the precise role of such proteins remains to be elucidated [49]. Additionally, it remains possible that PI 3-kinase activity may be stimulated in complexes that are not immunoprecipitated by anti-phosphotyrosine antibodies. Phosphorylation of substrates on a restricted number of sites may be masked by subsequent recruitment and activation of molecules such as PI 3-kinase. Indeed, Brown et al. [50] recently described a tyrosine-phosphorylated protein that was co-precipitated with the class II PI 3-kinases, but was not precipitated by anti-phosphotyrosine antibodies. Finally, it remains possible that, during simultaneous stimulation by insulin and contraction, PI 3-kinase activity may be stimulated by an alternative mechanism to that utilized during stimulation with insulin alone.

From the data presented, increases in IRS-1- and IRS-2-associated PI 3-kinase activity do not appear necessary to promote efficient glucose uptake or PKB activation in contracting skeletal muscle. In contrast, IRS-1- and/or IRS-2-associated PI 3-kinase activity appears essential for effective activation of p70^{S6K}. Future work will involve identification of the molecules responsible for insulin-stimulated PI 3-kinase activity in contracting skeletal muscle in complexes distinct from IRS-1 and IRS-2, as well as further investigation into the mechanism underlying the ability of muscle contraction to inhibit insulin-stimulated IRS-1- and IRS-2-associated PI 3-kinase activity.

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