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Role of the PDK1–PKB–GSK3 pathway in regulating glycogen synthase and glucose uptake in the heart

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Abstract In order to investigate the importance of the PDK1–PKB–GSK3 signalling network in regulating glycogen synthase (GS) in the heart, we have employed tissue specific conditional knockout mice lacking PDK1 in muscle (mPDK1$^{-/-}$), as well as knockin mice in which the protein kinase B (PKB) phosphorylation site on glycogen synthase kinase-3α (GSK3α) (Ser21) and GSK3β (Ser9) is changed to Ala. We demonstrate that in hearts from mPDK1$^{-/-}$ or double GSK3α/GSK3β knockin mice, insulin failed to stimulate the activity of GS or induce its dephosphorylation at residues that are phosphorylated by GSK3. We also establish that in the heart, both GSK3 isoforms participate in the regulation of GS, with GSK3β playing a more prominent role. This contrasts with skeletal muscle where GSK3β is the major regulator of insulin-induced GS activity. Despite the inability of insulin to stimulate glycogen synthesis in hearts from the mPDK1$^{-/-}$ or double GSK3α/GSK3β knockin mice, these animals possessed normal levels of cardiac glycogen, demonstrating that total glycogen levels are regulated independently of insulin's ability to stimulate GS in the heart and that mechanisms such as allosteric activation of GS by glucose-6-phosphate and/or activation of GS by muscle contraction, could operate to maintain normal glycogen levels in these mice. We also demonstrate that in cardiomyocytes derived from the mPDK1$^{-/-}$ hearts, although the levels of glucose transporter type 4 (GLUT4) are increased 2-fold, insulin failed to stimulate glucose uptake, providing genetic evidence that PDK1 plays a crucial role in enabling insulin to promote glucose uptake in cardiac muscle.

Keywords: Insulin; Glycogen synthesis; Glycose uptake; 3-Phosphoinositide dependent protein kinase-1; Glycogen synthase kinase-3

1. Introduction

The phosphoinositide 3-kinase (PI 3-kinase), 3-phosphoinositide dependent protein kinase-1 (PDK1) and protein kinase B (PKB also known as Akt) signalling cascade is believed to play a key role in mediating the ability of insulin to simulate glucose uptake and glycogen synthesis in skeletal muscle [1]. Insulin stimulates glycogen synthase (GS), the rate-limiting enzyme in glycogen synthesis, by promoting its dephosphorylation at a cluster of C-terminal residues (Ser641, Ser645, Ser649 and Ser653). These residues are phosphorylated by glycogen synthase kinase-3α (GSK3α) and GSK3β [2]. Insulin is thought to induce dephosphorylation of GS by inhibiting GSK3α and GSK3β following the phosphorylation of these enzymes at an N-terminal Serine residue (Ser21 in GSK3α and Ser9 in GSK3β) by PKB [3]. Consistent with this model, inhibitors of GSK3 stimulate GS activity in skeletal muscle [4].

Recently, we generated homozygous knockin mice in which the PKB phosphorylation site on GSK3α (Ser21) and GSK3β (Ser9) was changed to Ala to prevent inactivation of this enzyme by insulin [5]. We found that in skeletal muscle of the single GSK3α$^{21A/21A}$ knockin mice, insulin induced normal dephosphorylation and activation of GS. However, in single GSK3β$^{9A/9A}$ or double GSK3α/β$^{21A/21A/9A/9A}$ knockin mice, insulin failed to induce dephosphorylation and activation of GS. These results indicate that insulin stimulates GS in skeletal muscle by specifically inactivating GSK3β. We also found that the protein levels of GSK3β in mouse skeletal muscle were 4-fold higher than GSK3α, which may account for the dominant role of GSK3β in the insulin-mediated regulation of GS [5].

Insulin stimulates glucose uptake in skeletal muscle as well as the heart, by inducing the translocation of the glucose transporter type 4 (GLUT4) glucose transporters, from intracellular vesicles, to the plasma membrane. PI 3-kinase is believed to regulate this process by stimulating the PKBβ isoform, since the ability of insulin to stimulate glucose uptake is impaired in mice lacking PKBβ [6], or in cells in which PKBβ has been depleted through RNAi-based approaches [7].

A common complication of type 2 diabetes is cardiovascular disease, making it important to investigate the signalling networks that control insulin-regulated processes in the heart. The purpose of this study was to define the role that the PDK1–PKB–GSK3 pathway plays in regulating GS activity and glucose transport specifically in the heart, utilizing previously described mice lacking PDK1 and hence the activity of all PKB isoforms [8], as well as GSK3 knockin mice in which GSK3 isoforms cannot be inactivated by insulin [5].

2. Materials and methods

2.1. Materials

Protein G–Sepharose, ^{14}C-UDP glucose, 2-deoxy-D-[1-^{3}H]-glucose, D-[^{14}C]-mannitol, ^{32}Pγ-ATP were purchased from Amersham Pharmacia Biotech, protease-inhibitor cocktail tablets and fatty acid-free bovine serum albumin (BSA) were from Roche, Tween-20, glycogen Type III from rabbit liver and non-radioactive glucose-6-phosphate

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Abbreviations: BSA, bovine serum albumin; G6P, glucose-6-phosphate; GLUT4, glucose transporter type 4; GSK3, glycogen synthase kinase-3; PDK1, 3-phosphoinositide dependent protein kinase-1; PKB, protein kinase B; PI 3-kinase, phosphatidylinositol 3-kinase

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(G6P) were from Sigma, human insulin from Novo-Nordisk obtained from Ninewells Pharmacy, Dundee, collagenase type II was from Worthington Biochemical Corporation. All peptides were synthesized by Dr. Graham Bloomberg at the University of Bristol (UK).

2.2. Antibodies

The following antibodies were raised in sheep and affinity purified on the appropriate antigen: total PDK1 (residues 540–559 of mouse PDK1, RKIQEVWRQQYQSNPDAAVQ), total PKB (isolated PH domain of PKBα), total GSK3α (residues 314–327 of mouse GSK3α, LLGQPIFPGDSGVD used for immunoprecipitation), total GSK3β (full length human protein used for immunoprecipitation) total GS antibody was a mouse monoclonal antibody and was purchased from Chemicon (#MAB3106). The phospho-Ser641 of GS (Ser641-P, residues 635–650 of mouse GS, RYPRPVpSVPPSPSLSR), phospho-Ser645 of GS (Ser645-P, residues 635–650 of mouse GS, RYPRPVSVPPpSPSLSR), phospho-Thr308 PKB (Thr308-P, residues 301–313 of human PKBα, KDGATMKpTFCGTP) and phospho-Ser473 PKB (Ser473-P, residues 467–477 of human PKBα, KHFPQFpSYSAS). The total PKBα antibody used to immunoprecipitate PKBα was a mouse monoclonal antibody raised against residues 1–149 of human PKB and was purchased from Upstate Inc. (#05–591). The following antibodies were purchased from the indicated companies: phospho-Ser21/Ser9 GSK3α/GSK3β (Ser21-P/Ser9-P Cell Signaling Technology#9336), the Pan-GSK3 isoform antibody (Biosource #44–610), and the GLUT4 antibody (abcam #ab654).

2.3. Glycogen determination

The amount of glycogen was measured as described previously [9]. Triplicate samples per heart were analysed and data presented as μmol of glucosyl units per mg of heart tissue.

2.4. Genotype analysis and breeding of mice

All animal studies and breeding performed in this study were approved by the University of Dundee ethical committee and performed under a UK Home Office project license. PDK1$^{fl\Delta neo/fl\Delta neo}$ mice were generated and genotyped as previously described [10] and backcrossed for 6 generations to the C57BL/6j strain. These were crossed to transgenic mice expressing Cre recombinase under muscle creatine kinase promoter (MckCre) [11], which had been backcrossed for 8 generations to the C57BL/6j strain. The GSK3 knockin mice were bred and genotyped as described previously [5].

2.5. Buffers

Lysis Buffer: 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% (w/v) Triton X-100, 0.1% (v/v) β-mercaptoethanol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 μM microcystin-LR and one tablet of 'complete' proteinase inhibitor per 50 ml of buffer.

2.6. Preparation of tissue extracts, immunoblotting and protein kinase assays

Following an overnight fast, a bolus of insulin (150 mU/g) or saline solution was injected intraperitoneally to anaesthetized mice. At the indicated times, the heart was rapidly extracted, freeze clamped in liquid nitrogen, stored at −80 °C and homogenized to a powder in liquid nitrogen. A 10-fold mass excess of ice-cold Lysis Buffer, was added to the powdered tissue, briefly vortexed and then centrifuged at 4 °C for 10 min at 13000 × *g* to remove insoluble material. The supernatant was snap frozen in aliquots in liquid nitrogen and stored at −80 °C. The activation state of PKB, was assessed following its immunoprecipitation from cell extracts by assaying its activity using the Crosstide peptide (GRPRTSSFAEG) as described previously [12]. The activity of GSK3α and GSK3β was measured following the immunoprecipitation of each isoform with specific antibodies and assayed using the Phospho-GS2 (YRRAAVPPSPSLSRHSSPHQpSEDEEE, 20 μM) substrate as described before [5].

2.7. Immunoblotting

Unless indicated otherwise, 20 μg of protein lysate in SDS Sample Buffer was subjected to SDS/polyacrylamide gel electrophoresis and transferred to nitrocellulose. For all blots, the nitrocellulose membranes were incubated at 4 °C for 16 h using the indicated antibodies (1 μg/ml for the sheep antibodies or 1000-fold dilution for commercial antibodies) in the presence of 10 μg/ml of the de-phosphopeptide antigen used to raise the antibody for sheep phospho-specific antibodies. The blots were incubated in 50 mM Tris/HCl, pH 7.5, 0.15 M NaCl, 0.2% (by vol) Tween containing 5% (by mass) skimmed milk. Detection of total or phosphorylated protein was performed using horse radish peroxidase conjugated secondary antibodies and the enhanced chemiluminescence reagent. Quantitative immunoblot analysis was carried out employing a LI-COR Odyssey infrared detection system following the manufactures guidelines (see http://www.licor.com/).

2.8. Cardiomyocyte isolation and glucose transport activity

Cardiomyocytes from mice were prepared by collagenase digestion as described previously [13] except that 20 mM inosine was included in the final cell suspension [14]. Glucose transport in isolated cardiomyocytes was measured as described previously [14].

2.9. Glycogen synthase assay

Following an overnight fast, a bolus of insulin (150 mU/g) or saline solution was injected intraperitoneally to anaesthetized mice. At the indicated times, the heart was rapidly extracted and freeze clamped in liquid nitrogen, stored at −80 °C and homogenized to a powder in liquid nitrogen and the GS activity was measured as described previously [15]. The GS activity ratio is defined as activity measured in the absence of G6P divided by activity measured in the presence of G6P.

3. Results

3.1. Analysis of GSK3, GS and glycogen content in mPDK1$^{-/-}$ hearts

We have previously described the generation and phenotype of muscle-specific PDK1 knockout mice (termed mPDK1$^{-/-}$) that completely lack PDK1 expression in cardiac muscle [8]. As mPDK1$^{-/-}$ mice become ill, develop heart failure and die between 6 and 11 weeks of age [8], we ensured that we utilized mice of between 4 and 5 weeks of age that displayed no signs of ill health. In mPDK1$^{-/-}$ mice, PDK1 expression is completely ablated in heart muscle [8] (inset in Fig. 1B), but low levels of PDK1 is still expressed in skeletal muscle resulting in PDK1 dependent pathways being triggered by insulin in the skeletal muscle of these mice (A.M., unpublished data).

To determine whether lack of cardiac PDK1 expression impaired activation of enzymes of the glycogen synthesis pathway, mPDK1$^{-/-}$ and control mPDK1$^{+/+}$ mice were injected with insulin for varying times (0, 10, 20 and 40 min) and heart extracts generated. For each time point and genotype, 3 mice were employed. PKBα activity was measured using a quantitative immunoprecipitation kinase assay. Consistent with our previous findings [8], insulin-stimulated PKB activity over 20-fold in control mPDK1$^{+/+}$ mice within 10 min, but no significant activation was observed up to 40 min in mPDK1$^{-/-}$ hearts (Fig. 1B). We next studied the phosphorylation of the inhibitory PKB phosphorylation sites on GSK3α (Ser21) and GSK3β (Ser9) employing phospho-specific antibodies. As expected, insulin-induced phosphorylation of both GSK3 isoforms within 10 min in the control mPDK1$^{+/+}$ hearts, but no detectable increase above the basal levels of phosphorylation of GSK3 isoforms was seen at any time point in the mPDK1$^{-/-}$ hearts injected with insulin. Employing phospho-specific antibodies recognizing the Ser641 and Ser645 GSK3 phosphorylation sites on GS, we demonstrated that in the control mPDK1$^{+/+}$ hearts, insulin induced a marked dephosphorylation of these residues within 10 min. In the mPDK1$^{-/-}$

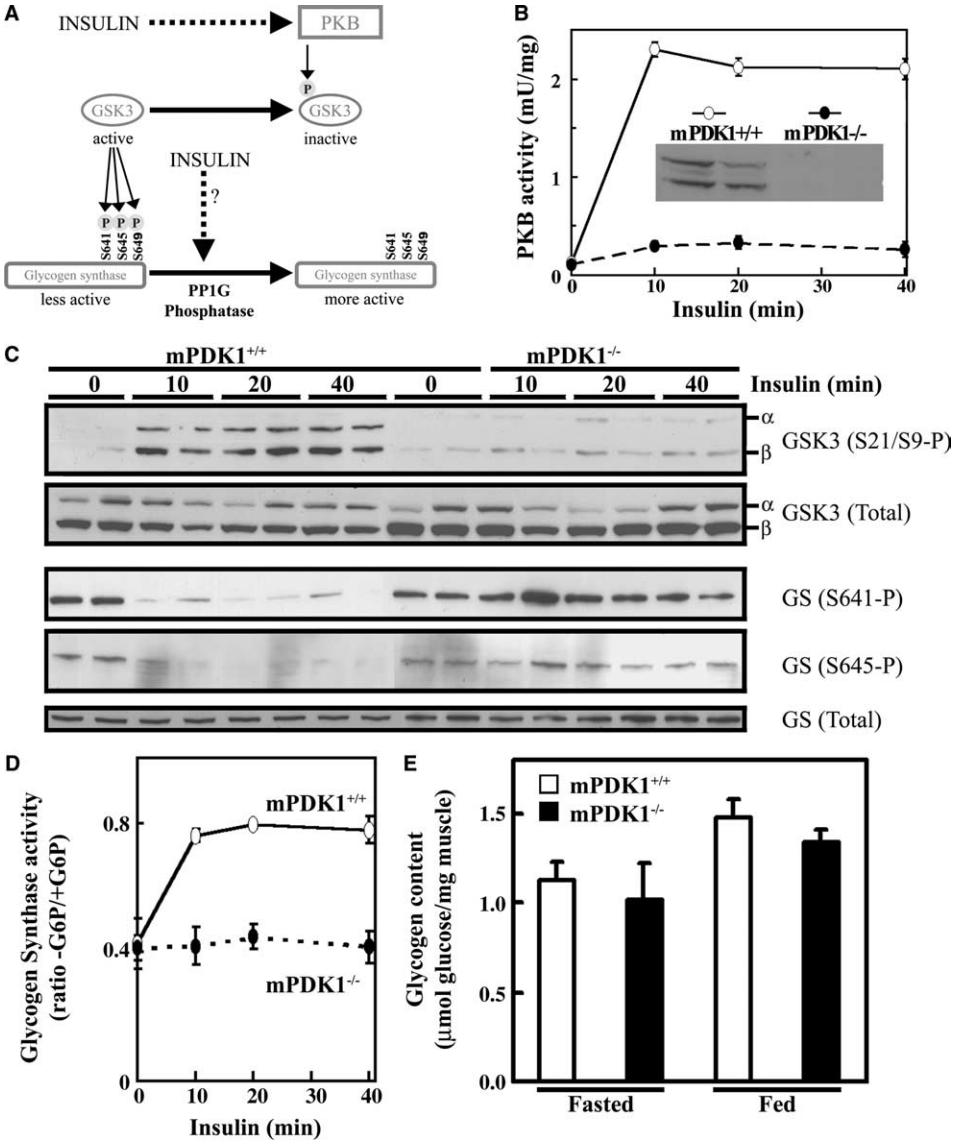

Fig. 1. Effect of insulin on PKB, GSK3 and GS in mPDK1$^{-/-}$ heart. (A) Diagram summarizing the mechanism by which insulin is thought to regulate GS. (B–D) The mPDK1$^{+/+}$ and mPDK1$^{-/-}$ mice were fasted overnight, anaesthetized and then injected intraperitoneally with insulin (150 mU/g) or saline solution (for the 0 min time point). At the indicated time (10 min for saline control), the heart was rapidly extracted and snap frozen in liquid nitrogen. (B) PKBα activity was measured following its immunoprecipitation. The results shown at each time point represent the means ± S.E.M. for three mice each assayed in triplicate. In the inset, 40 μg of heart extract from the indicated mice were immunoblotted with an anti-PDK1 antibody. (C) Heart extracts from the indicated mice were immunoblotted with the indicated antibodies. For each time point heart samples from 2 separate mice are shown. (D) GS activity was measured in the absence and presence of G6P. The data are presented as the means ± S.E.M. for heart isolated from 3 mice each assayed ±G6P in triplicate. (E) mPDK1$^{+/+}$ and mPDK1$^{-/-}$ mice were fasted overnight for 16 h (Fasted) or fed ad libitum (Fed). The mice were sacrificed and the heart was rapidly extracted and glycogen content determined. The glycogen content is expressed as μmol of glucosyl units per mg of muscle. The data are presented as the means ± S.E.M. for cardiac muscle isolated from 3 mice in each group each assayed in triplicate.

hearts derived from non-insulin saline injected mice, the level of phosphorylation of Ser641 and Ser645 sites was similar to that observed in the mPDK1$^{+/+}$ mice, however, insulin failed to induce any significant dephosphorylation of these residues in the mPDK1$^{-/-}$ hearts (Fig. 1C). Immunoblot analysis indicated that the levels of GS were identical in mPDK1$^{-/-}$ and mPDK1$^{+/+}$ hearts, although GSK3 isoforms were expressed at slightly higher levels in mPDK1$^{-/-}$ hearts used for these studies (Fig. 1C). We are not of the opinion that this difference is significant, as in other mPDK1$^{-/-}$ hearts we have not observed changes in the expression of GSK3 isoforms (A.M., data not shown [8]).

We next measured GS activity in heart extracts in the presence or absence of G6P, an allosteric activator of GS. In the control mPDK1$^{+/+}$ hearts, insulin induced a 2-fold increase in the GS activity ratio within 10 min, which was sustained for up to 40 min (Fig. 1D). In the mPDK1$^{-/-}$ hearts, the basal −/+ G6P GS activity ratio was identical to that observed in the wild type mPDK1$^{+/+}$ mice. Strikingly, however, insulin failed to stimulate GS activity in mPDK1$^{-/-}$ heart (Fig. 1D). We also

measured total glycogen content in mPDK1$^{-/-}$ and mPDK1$^{+/+}$ hearts, and found that the levels were not significantly different in both genotypes, fasted or fed ad libitum (Fig. 1E).

3.2. Analysis of GS and glycogen content in hearts of GSK3 knockin mice

Single homozygous GSK3α$^{21A/21A}$ and GSK3β$^{9A/9A}$ as well as double GSK3α/β$^{21A/21A/9A/9A}$ knockin mice were bred as described previously [5]. Mice were fasted overnight and injected with insulin, and PKB activity was assessed by analysis of its phosphorylation at its activating Thr308 and Ser473 residues. In the heart of the single GSK3α$^{21A/21A}$, GSK3β$^{9A/9A}$ and double GSK3α/β$^{21A/21A/9A/9A}$ knockin mice, insulin induced robust phosphorylation of PKB at Thr308 and Ser473 residues, similar to that seen in wild type control mice (Fig. 2A). We next assayed the insulin-induced phosphorylation of GSK3α at Ser21 and GSK3β at Ser9 employing phospho-specific antibodies. Insulin-stimulated phosphorylation of both GSK3 isoforms in wild type control mice (Fig. 2A). In the GSK3α$^{21A/21A}$ knockin mice, no phosphorylation of GSK3α at Ser21 was seen, while phosphorylation of GSK3β at Ser9 was normal. In the GSK3β$^{9A/9A}$ knockin mice, no phosphorylation of GSK3β at Ser9 was observed, while phosphorylation of GSK3α at Ser21 was unaffected. As expected, in the double knockin GSK3α/β$^{21A/21A/9A/9A}$ mice, phosphorylation of neither isoform of GSK3 was detected. The levels of GSK3α and GSK3β isoforms in the hearts of knockin mice were similar to those found in the wild type mice (Fig. 2A).

We next studied the level and activity of GS in cardiac muscle extracts. GS protein levels were normal in the muscles of knockin mice (Fig. 2B). In wild type mice, similar to that observed with the mPDK1$^{+/+}$ mice (Fig. 1D), insulin activated GS ~2-fold, as judged by an increase in the −/+ G6P GS activity ratio of 0.42–0.76, which was sustained for up to 40 min (Fig. 2C). This activation was accompanied by dephosphorylation of Ser641 and Ser645 (Fig. 2A). In the single GSK3α$^{21A/21A}$ knockin mice, the basal level of GS activity was normal, and insulin induced a ~2-fold activation after 10 min that was sustained for 20 min, but reduced after 40 min (Fig. 2C). Activation of GS in the single GSK3α$^{21A/21A}$ knockin mice was accompanied by the dephosphorylation of Ser641 and Ser645 (Fig. 2A). In the single GSK3β$^{9A/9A}$ knockin mice, the basal −/+ G6P GS activity ratio was 0.33 lower than the 0.42 observed in wild type or GSK3α$^{21A/21A}$ knockin mice. In the single GSK3β$^{9A/9A}$ knockin mice, insulin still stimulated GS, but to an activity ratio of only 0.6, less than that observed in the wild type or single GSK3α$^{21A/21A}$ animals. Importantly, however, in the double GSK3α/β$^{21A/21A/9A/9A}$ knockin mice, the basal activity of GS (0.34) was comparable to that

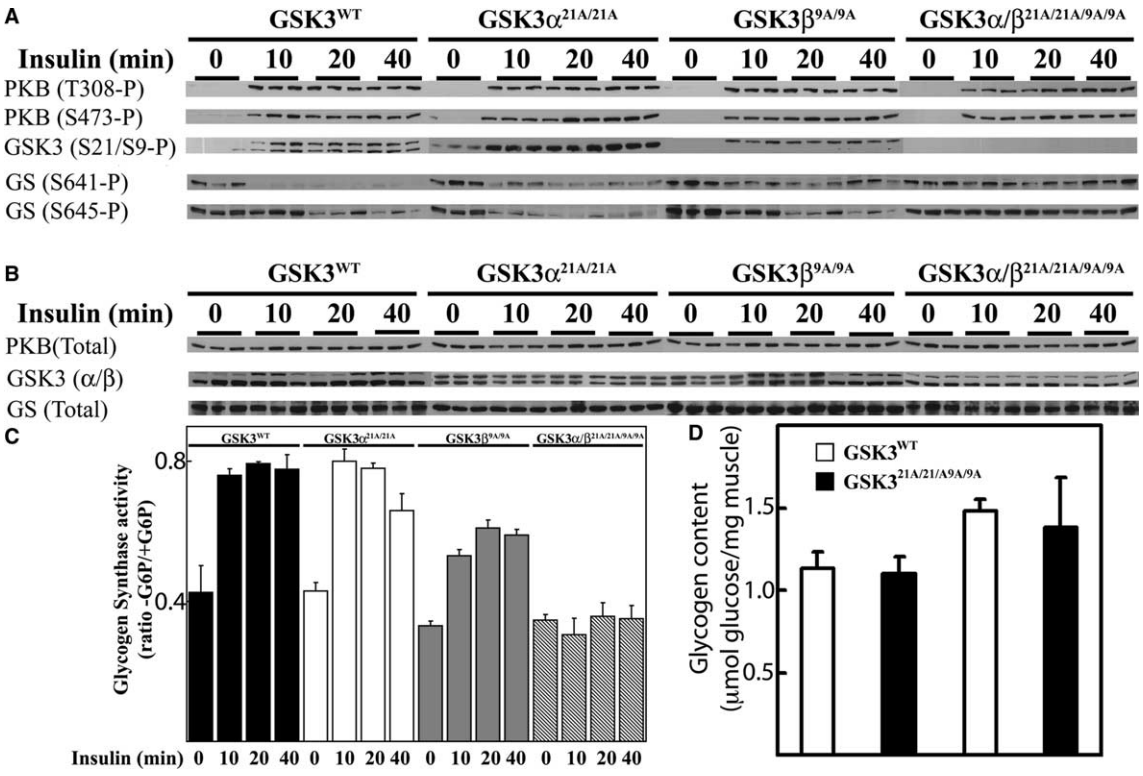

Fig. 2. PKB and GSK3 activity in GSK3 knockin mice. The indicated wild type (WT) and GSK3 knockin mice were fasted overnight, anaesthetized and injected intraperitoneally with insulin (150 mU/g) or saline solution (for the 0 min time point), to anaesthetized mice. At the indicated time (10 min for saline control), the hearts were rapidly extracted and snap frozen in liquid nitrogen. (A, B) Heart extracts were immunoblotted with the indicated antibodies. For each time point cardiac muscle samples from 3 separate mice are shown. (C) GS activity was measured in the absence and presence of G6P. The data are presented as the means ± S.E.M. for heart isolated from 3 mice each assayed ±G6P in triplicate. For the experiments in A, B and C, data were obtained with the three sets of knockin mice were each compared to wild type littermate control mice. Since these data from the sets of wild type controls did not differ significantly from each other, only the results obtained for the wild type GSK3α$^{21A/21A}$ littermate controls are shown. (D) The wild type and double knockin GSK3α/β$^{21A/21A/9A/9A}$ were fasted overnight for 16 h (Fasted) or fed ad libitum (Fed). The mice were sacrificed and the heart was rapidly extracted and glycogen content determined. The glycogen content is expressed as μmol of glucosyl units per mg of heart. The data are presented as the means ± S.E.M. for cardiac muscle isolated from 3 mice in each group each assayed in triplicate.

observed in the single GSK3β$^{9A/9A}$ knockin animals, but, insulin failed to induce a significant activation or dephosphorylation of the Ser641 and Ser645 residues of GS (Fig. 2A). We also quantified glycogen levels in double GSK3α/β$^{21A/21A/9A/9A}$ knockin hearts, and found that glycogen content was similar to that found in wild type hearts from fasted or fed mice (Fig. 2D).

3.3. Relative expression and activity of GSK3α and GSK3β in cardiac muscle

In the skeletal muscle of single GSK3β$^{9A/9A}$ knockin mice [5], in contrast to what is observed in the heart (Fig. 2), insulin failed to stimulate GS. To verify whether the ability of insulin to stimulate GS in cardiac muscle of single GSK3β$^{9A/9A}$ knockin mice compared to skeletal muscle, could be caused by differences in the level of expression of GSK3α and GSK3β isoforms in muscle and heart, we compared the relative levels of GSK3α and GSK3β in these tissues. Using an antibody recognizing GSK3α and GSK3β isoforms, we found that both GSK3 isoforms were expressed at ~2-fold higher levels in heart compared to skeletal muscle (Fig. 3A). The ratios of GSK3α and GSK3β were not markedly different between heart and skeletal muscle (Fig. 3A). The specific activity of GSK3α and GSK3β as judged by quantitative immunoprecipitation kinase assay, was 2–3-fold higher in heart compared to skeletal muscle (Fig. 3B).

3.4. Analysis of GLUT4 levels and glucose uptake in mPDK1$^{-/-}$ cardiomyocytes

Insulin stimulates glucose uptake in muscle by inducing the recruitment of the GLUT4 glucose transporter to the plasma membrane. We first compared the levels of expression of GLUT4 in mPDK1$^{-/-}$ and mPDK1$^{+/+}$ hearts in total tissue extracts using quantitative immunoblot LI-COR analysis. The GLUT4 levels were found to be ~2-fold higher in total extracts of those derived from mPDK1$^{-/-}$ hearts compared to wild type mPDK1$^{+/+}$ hearts (Fig. 4A). We also generated plasma membrane fractions from hearts, and observed that the GLUT4 levels were also ~2-fold higher in membranes derived from mPDK1$^{-/-}$ hearts, compared to those from control hearts (A.M. data not shown). We next measured glucose uptake in isolated cardiomyocytes that were incubated for 30 min in the presence or absence of insulin, followed by 10 min in

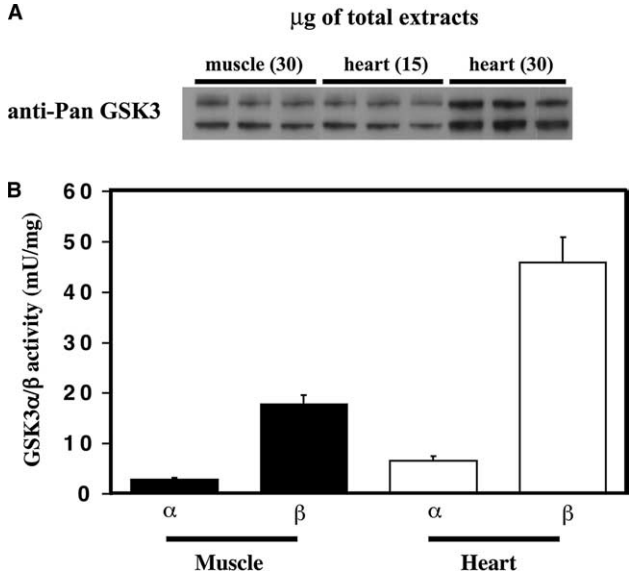

Fig. 3. Relative levels of GSK3α and GSK3β in heart and skeletal muscle. (A) The indicated amounts of mouse skeletal muscle and heart derived from 3 wild type mice were subjected to quantitative immunoblot analysis with the Pan-GSK3 isoform antibody. (B) GSK3α and GSK3β were immunoprecipitated from the same amount of skeletal and heart muscle employing isoform-specific antibodies and their activity was measured. The data are presented as the means ± S.E.M. for muscle isolated from 3 mice each assayed triplicate.

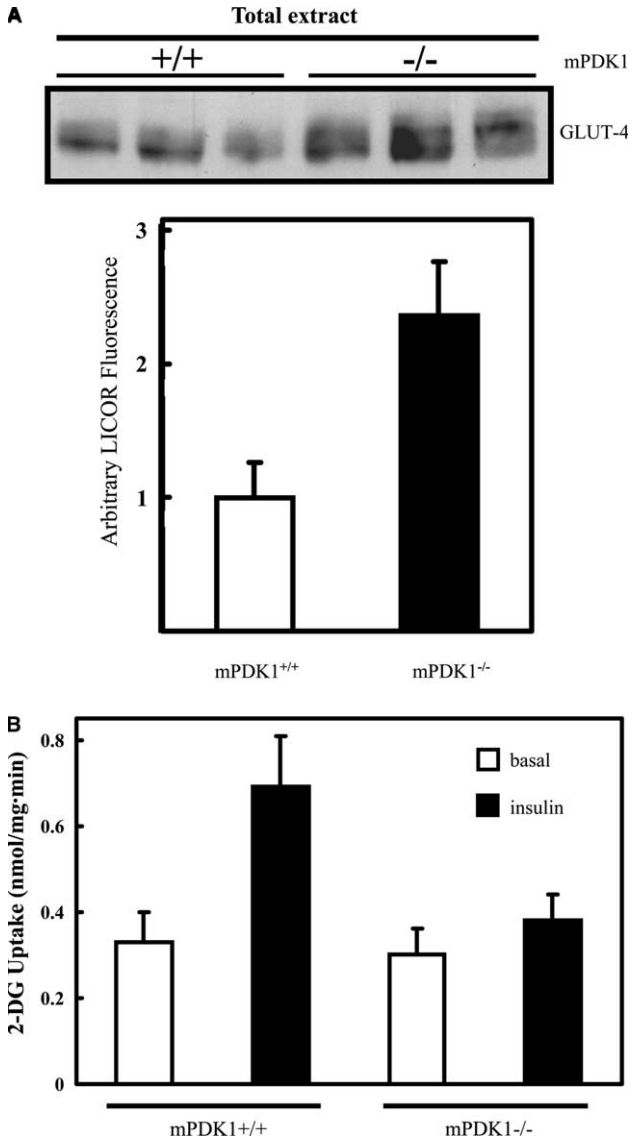

Fig. 4. GLUT4 levels and insulin stimulated glucose uptake in mPDK1$^{-/-}$ cardiomyocytes. (A) The indicated total extract derived from 3 separate mPDK1$^{-/-}$ and mPDK1$^{+/+}$ hearts was subjected to immunoblot analysis with anti-GLUT4 antibody. Quantification of the immunoblots was performed using the LI-COR system. The upper panel displays the primary immunoblot analysis, whilst in the lower panel the quantified GLUT4 amount (means ± S.E.M.) for heart isolated from 3 mice is displayed. (B) Cardiomyocytes were isolated from mPDK1$^{-/-}$ and mPDK1$^{+/+}$ hearts and glucose transport activities were determined without or with 30 nM insulin, as described in the experimental procedures. The data are presented as the means ± S.E.M. for cardiomyocytes isolated from 3 mice for each genotype.

[^{3}H]-labelled 2-deoxyglucose. In control mPDK1$^{+/+}$ cardiomyocytes, insulin stimulated glucose uptake 2-fold. In mPDK1$^{-/-}$ cardiomyocytes, the basal level of glucose transport was similar to control cells, but insulin failed to induce a significant increase in glucose uptake (Fig. 4B).

4. Discussion

Our findings provide strong evidence that inactivation of GSK3 is the major regulatory step in the mechanism by which insulin stimulates GS in cardiac muscle. It has also been suggested in skeletal muscle that insulin could stimulate GS by increasing the activity of a glycogen-associated form of protein phosphatase-1 protein by an unknown mechanism [16,17]. If this was the case in cardiac muscle, our findings suggest that the activity of this phosphatase would also need to be controlled by GSK3 isoforms. As Ser641 and Ser645 are not dephosphorylated in response to insulin in the double GSK3α/β$^{21A/21A/9A/9A}$ knockin mice, our findings indicate that GSK3 isoforms are the major enzymes phosphorylating Ser641 and Ser645 and regulating GS activity in response to insulin in cardiac muscle.

An interesting observation was that GS was not activated by insulin in skeletal muscle of single GSK3β$^{9A/9A}$ knockin mice [5], but was still partially activated in the cardiac muscle of these animals (Fig. 2C). Thus, the prominence of GSK3β in regulating GS activity is different in skeletal and cardiac muscle. These observations might be accounted for the 2–3-fold greater abundance of GSK3α in heart (Fig. 3), which might enhance the contribution that GSK3α plays in regulating GS activity.

Heart from mPDK1$^{-/-}$ and GSK3α/β$^{21A/21A/9A/9A}$ mice possessed normal levels of glycogen despite insulin being unable to stimulate activation of GS (Figs. 1E and 2D). We previously reported that the single and double GSK3 knockin mice displayed normal blood glucose and insulin levels and are not glucose intolerant [5]. Moreover, these animals also have normal levels of glycogen in their skeletal muscle [5], indicating that mechanisms independent from the PDK1–PKB–GSK3 pathway play an important role in maintaining normal glycogen levels in both skeletal and cardiac muscle. GS activity is not only activated by dephosphorylation, but also by G6P [18]. At saturating concentrations of G6P and UDP-glucose, the activities of the dephosphorylated and phosphorylated forms of GS are identical in vitro. Thus, the similar levels of glycogen observed in the wild type and GSK3 knockin mice might be explained, if activation of GS by G6P can compensate for the failure of insulin to induce the dephosphorylation of GS, in the skeletal muscle of knockin mice expressing GSK3 mutants that cannot be inactivated by insulin. Muscle contraction also stimulates glucose uptake independently from PI 3-kinase, through a pathway involving the AMP-activated protein kinase [19,20]. Moreover, muscle contraction also potently stimulates GS through a poorly understood mechanism, which might result from depletion of cellular glycogen and/or activation of a protein phosphatase [21]. It is therefore possible that the continuous contraction of cardiac muscle in the beating heart ensures that sufficient glucose is transported into cardiomyocytes, and that glycogen is synthesized without the need of insulin to stimulate these processes through the PDK1–PKB–GSK3 pathway. We have previously demonstrated that the muscle contraction stimulates GS activity normally in the skeletal muscle of double GSK3 knockin mice [5]. We are unable to rule out the possibility that degradation of glycogen is altered in the mPDK1$^{-/-}$ or GSK3 knockin mice, that could account for normal levels of glycogen. However, to our knowledge the PDK1–PKB–GSK3 signalling pathway has not been reported to control glycogen breakdown.

Long-chain fatty acids rather than glycogen are considered to be the major substrates for ATP production in adult heart [22,23]. If glycogen is not an important fuel source in the adult mouse heart, this might also account for our finding that the PDK1–PKB–GSK3 pathway does not play a major role in maintaining normal glycogen levels in the heart. However, glucose does become a significant source of energy when blood glucose and insulin concentrations are high, such as after a meal [22,23]. Moreover, when the workload of the heart is increased, such as after stimulation by epinephrine or increased exercise, glycogen provides a readily accessible source of additional energy [24,25]. In future work, it would be interesting to monitor glycogen levels under these conditions in the GSK3 knockin hearts. During embryo development glycogen is thought to play a major role in fuelling contraction, and consistent with this, mice lacking the muscle isoform of GS and hence glycogen, display abnormal cardiac development [26]. We have not examined glycogen levels of developing embryos in the GSK3 knockin mice, but these animals are born at about the expected Mendelian frequency and displayed no marked cardiac phenotypes [5].

The role of PDK1 in regulating insulin-stimulated glucose transport has previously been analysed in cultured differentiated 3T3L1 adipocytes [27,28] and immortalized brown adipocytes [29], but to our knowledge not in muscle. Two groups have overexpressed PDK1 in 3T3L1 adipocytes and reported that this did not stimulate glucose transport, leading them to conclude that PDK1 was not required for this process [27,28]. These findings were surprizing given the strong evidence outlined in the introduction that PKB activation mediates insulin stimulated glucose uptake. As PKB can only be activated by PDK1 in the presence of the phosphatidylinositol-3,4,5-trisphosphate second messenger, generated through activation of PI 3-kinase by insulin (reviewed in [30]), it is possible that overexpression of PDK1 in adipose-like cells is not sufficient to stimulate PKB in the absence of PI 3-kinase activation. Recently, Kasuga and co-workers [29] demonstrated that insulin failed to stimulate glucose uptake in PDK1 knockout immortalized brown adipocytes, showing that PDK1 does indeed play an important role in stimulating glucose uptake in fat cells. Our findings in isolated mPDK1$^{-/-}$ cardiomyocytes confirm the vital role that PDK1 plays in regulating glucose uptake in a muscle cell type for the first time, consistent with the notion that PKB plays an important role in regulating insulin-stimulated glucose transport.

We also noted that the levels of the GLUT4 glucose transporter were elevated ~2-fold in cardiac muscle lacking PDK1. Interestingly, mice lacking expression of the insulin receptor in the heart was also found to possess ~2-fold higher levels of GLUT4 protein expression in the heart [31]. These findings suggest that GLUT4 levels are negatively controlled by insulin working through a PDK1 regulated pathway, and might represent a mechanism by which a deficiency of the insulin-signalling network could result in a compensatory increase

in GLUT4 levels, to enhance glucose transport into cardiomyocytes. It should be noted, however, in cardiomyocytes derived from hearts lacking either the insulin receptor [31] or PDK1 (Fig. 4), the basal level of glucose uptake in the absence of insulin, is not enhanced, despite the increased levels of GLUT4. However, it is possible that in the contracting intact heart, higher levels of GLUT4 could help to compensate for the lack of insulin stimulated glucose uptake, which could also aide in maintaining normal glycogen levels.

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References

[1] Saltiel, A.R. and Kahn, C.R. (2001) Insulin signalling and the regulation of glucose and lipid metabolism. Nature 414, 799–806.
[2] Cohen, P. (1999) The Croonian Lecture 1998. Identification of a protein kinase cascade of major importance in insulin signal transduction. Philos. Trans. R. Soc. Lond. B Biol. Sci. 354, 485–495.
[3] Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M. and Hemmings, B.A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378, 785–789.
[4] Cohen, P. and Goedert, M. (2004) GSK3 inhibitors: development and therapeutic potential. Nat. Rev. Drug. Discov. 3, 479–487.
[5] McManus, E.J., Sakamoto, K., Armit, L.J., Ronaldson, L., Shpiro, N., Marquez, R. and Alessi, D.R. (2005) Role that phosphorylation of GSK3 plays in insulin and Wnt signalling defined by knockin analysis. EMBO J. 24, 1571–1583.
[6] Cho, H. et al. (2001) Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). Science 292, 1728–1731.
[7] Zhou, Q.L. et al. (2004) Analysis of insulin signalling by RNAi-based gene silencing. Biochem. Soc. Trans. 32, 817–821.
[8] Mora, A. et al. (2003) Deficiency of PDK1 in cardiac muscle results in heart failure and increased sensitivity to hypoxia. EMBO J. 22, 4666–4676.
[9] Roe, J.H. and Dailey, R.E. (1966) Determination of glycogen with the anthrone reagent. Anal. Biochem. 15, 245–250.
[10] Lawlor, M.A. et al. (2002) Essential role of PDK1 in regulating cell size and development in mice. EMBO J. 21, 3728–3738.
[11] Bruning, J.C., Michael, M.D., Winnay, J.N., Hayashi, T., Horsch, D., Accili, D., Goodyear, L.J. and Kahn, C.R. (1998) A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. Mol. Cell 2, 559–569.
[12] Williams, M.R., Arthur, J.S., Balendran, A., van der Kaay, J., Poli, V., Cohen, P. and Alessi, D.R. (2000) The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells. Curr. Biol. 10, 439–448.
[13] Fischer, Y., Thomas, J., Holman, G.D., Rose, H. and Kammermeier, H. (1996) Contraction-independent effects of catecholamines on glucose transport in isolated rat cardiomyocytes. Am. J. Physiol. 270, C1204–C1210.
[14] Yang, J., Gillingham, A.K., Hodel, A., Koumanov, F., Woodward, B. and Holman, G.D. (2002) Insulin-stimulated cytosol alkalinization facilitates optimal activation of glucose transport in cardiomyocytes. Am. J. Physiol. Endocrinol. Metab. 283, E1299–E1307.
[15] Thomas, J.A., Schlender, K.K. and Larner, J. (1968) A rapid filter paper assay for UDPglucose-glycogen glucosyltransferase, including an improved biosynthesis of UDP-14C-glucose. Anal. Biochem. 25, 486–499.
[16] Delibegovic, M., Armstrong, C.G., Dobbie, L., Watt, P.W., Smith, A.J. and Cohen, P.T. (2003) Disruption of the striated muscle glycogen targeting subunit PPP1R3A of protein phosphatase 1 leads to increased weight gain, fat deposition, and development of insulin resistance. Diabetes 52, 596–604.
[17] Suzuki, Y. et al. (2001) Insulin control of glycogen metabolism in knockout mice lacking the muscle-specific protein phosphatase PP1G/RGL. Mol. Cell. Biol. 21, 2683–2694.
[18] Lawrence Jr., J.C. and Roach, P.J. (1997) New insights into the role and mechanism of glycogen synthase activation by insulin. Diabetes 46, 541–547.
[19] Winder, W.W. and Hardie, D.G. (1999) AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. Am. J. Physiol. 277, E1–E10.
[20] Hardie, D.G. (2004) The AMP-activated protein kinase pathway – new players upstream and downstream. J. Cell. Sci. 117, 5479–5487.
[21] Nielsen, J.N. and Richter, E.A. (2003) Regulation of glycogen synthase in skeletal muscle during exercise. Acta Physiol. Scand. 178, 309–319.
[22] Taegtmeyer, H. (1994) Energy metabolism of the heart: from basic concepts to clinical applications. Curr. Probl. Cardiol. 19, 59–113.
[23] Taegtmeyer, H. (2004) Glycogen in the heart – an expanded view. J. Mol. Cell. Cardiol. 37, 7–10.
[24] Goodwin, G.W., Ahmad, F. and Taegtmeyer, H. (1996) Preferential oxidation of glycogen in isolated working rat heart. J. Clin. Invest. 97, 1409–1416.
[25] Goodwin, G.W., Ahmad, F., Doenst, T. and Taegtmeyer, H. (1998) Energy provision from glycogen, glucose, and fatty acids on adrenergic stimulation of isolated working rat hearts. Am. J. Physiol. 274, H1239–H1247.
[26] Pederson, B.A., Chen, H., Schroeder, J.M., Shou, W., DePaoli-Roach, A.A. and Roach, P.J. (2004) Abnormal cardiac development in the absence of heart glycogen. Mol. Cell. Biol. 24, 7179–7187.
[27] Yamada, T. et al. (2002) Role of PDK1 in insulin-signaling pathway for glucose metabolism in 3T3- L1 adipocytes. Am. J. Physiol. Endocrinol. Metab. 282, E1385–E1394.
[28] Egawa, K. et al. (2002) Membrane localization of 3-phosphoinositide-dependent protein kinase-1 stimulates activities of Akt and atypical protein kinase C but does not stimulate glucose transport and glycogen synthesis in 3T3-L1 adipocytes. J. Biol. Chem. 277, 38863–38869.
[29] Sakaue, H., Nishizawa, A., Ogawa, W., Teshigawara, K., Mori, T., Takashima, Y., Noda, T. and Kasuga, M. (2003) Requirement for 3-phosphoinositide-dependent kinase-1 (PDK-1) in insulin-induced glucose uptake in immortalized brown adipocytes. J. Biol. Chem. 278, 38870–38874.
[30] Mora, A., Komander, D., Van Aalten, D.M. and Alessi, D.R. (2004) PDK1, the master regulator of AGC kinase signal transduction. Semin. Cell. Dev. Biol. 15, 161–170.
[31] Belke, D.D. et al. (2002) Insulin signaling coordinately regulates cardiac size, metabolism, and contractile protein isoform expression. J. Clin. Invest. 109, 629–639.