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Heart 6-phosphofructo-2-kinase activation by insulin requires protein kinase B but not serum- and glucocorticoid-inducible protein kinase-3

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Running title: insulin activation of heart PFK-2 by PKB

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SYNOPSIS

Based on transfection experiments using a dominant-negative approach, our previous studies suggested that protein kinase B (PKB) was not involved in heart 6-phosphofructo-2-kinase (PFK-2) activation by insulin. Therefore, we first tested whether the serum- and glucocorticoid-inducible protein kinase-3 (SGK3) might be involved in this effect. Treatment of recombinant heart PFK-2 with [γ -³²P] labelled ATP and SGK3 *in vitro* led to PFK-2 activation and phosphorylation at Ser466 and Ser483. However, in HEK 293T cells co-transfected with SGK3 siRNA and heart PFK-2, insulin-induced heart PFK-2 activation was unaffected. The involvement of PKB in heart PFK-2 activation by insulin was re-evaluated using different models: (i) hearts from transgenic mice with a muscle/heart-specific mutation in the PDK1 substrate docking site injected with insulin; (ii) hearts from PKB β deficient mice injected with insulin; (iii) freshly isolated rat cardiomyocytes and perfused hearts treated with the selective Akti-1/2 PKB inhibitor prior to insulin, (iv) HEK 293T cells co-transfected with heart PFK-2 and PKB α/β siRNA or PKB α siRNA and incubated with insulin. Together, the results indicated that SGK3 is not required for insulin-induced PFK-2 activation and that this effect is likely mediated by PKB α .

Key words: Glycolysis, PFK-2, phosphorylation, SGK3, PKB, siRNA

Abbreviations used:

Akti-1/2, selective Akt isoform 1 and 2 (PKB α and PKB β) inhibitor; BH1(His)₆, polyhistidine-tagged recombinant bovine heart 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; HEK, human embryonic kidney; LDH, lactate dehydrogenase; PAS, Phospho Akt-Substrate; PDK1, phosphoinositide-dependent protein kinase-1; PFK-2, 6-phosphofructo-2-kinase; PKB, protein kinase B; p70^{S6K}, p70 ribosomal protein S6 kinase; SGK3, serum- and glucocorticoid-inducible protein kinase-3; siRNA, small interfering RNA; shRNA, small hairpin RNA

INTRODUCTION

Insulin stimulates heart glycolysis via the recruitment of GLUT4 transporters to the plasma membrane [1,2] and activation of 6-phosphofructo-2-kinase (PFK-2) [3]. The subsequent rise in fructose 2,6-bisphosphate (Fru-2,6-P₂) allosterically stimulates 6-phosphofructo-1-kinase and hence glycolysis. Insulin action involves phosphatidylinositol 3-kinase (PI 3-kinase) activation [4] which generates phosphatidylinositol-3,4,5-trisphosphate (PIP₃) in the plasma membrane. PIP₃ is a lipid second messenger, which recruits two serine/threonine protein kinases, namely phosphoinositide-dependent protein kinase-1 (PDK1) and protein kinase B (PKB), to the plasma membrane via their pleckstrin homology (PH) domains. Full activation of PKB requires phosphorylation of Thr308 by PDK1 [5,6] and phosphorylation at a second site, Ser473 in the hydrophobic motif, by PDK2. There is now strong evidence to suggest that the physiological PDK2 is the mammalian target of rapamycin (mTOR) rictor-mTOR (TORC2) protein complex [7-10].

PKB exists as three isoenzymes (PKB α , β and γ or Akt1, 2 and 3, respectively) and is considered to mediate most, if not all, short-term metabolic effects of insulin [11]. PKB α is expressed ubiquitously, PKB β is highly expressed in insulin responsive tissues such as adipose tissue, liver and skeletal muscle while PKB γ expression is predominant in brain. Deletion of PKB α in mice does not affect glucose metabolism [12-14], while knockout of PKB β causes a diabetes mellitus-type syndrome [15,16], implicating PKB β in the control of glucose transport by insulin. Small interfering RNA (siRNA)-directed gene silencing of Akt2 impaired insulin-stimulated glucose transport in 3T3-L1 adipocytes [17,18]. Also in these cells, pharmacological Akt inhibition with Akti-1/2, a selective inhibitor of PKB α and PKB β , ablated insulin-induced glucose uptake [19].

PKB α activates heart PFK-2 *in vitro* by phosphorylating Ser466 and Ser483 leading to a 2-fold increase in V_{\max} [20]. In isolated cardiomyocytes, the insulin-induced activation of PFK-2 was insensitive to rapamycin and PD98059, which inhibit p70 ribosomal S6 kinase (p70^{S6K}) and mitogen-activated protein kinase activation, respectively, but was blocked by the PI 3-kinase inhibitors wortmannin and LY294002 [21]. Also in hearts of muscle specific PDK1^{-/-} mice, insulin failed to activate PKB, p70^{S6K} and PFK-2 [22], consistent with the PI 3-kinase/PDK1 axis mediating these processes. Our previous studies indicated that PKB was not essential for insulin-induced heart PFK-2 activation based on co-transfection of a kinase-inactive dominant negative PKB construct in human embryonic kidney (HEK) 293 cells [23]. However, in a later study in HeLa cells co-transfected with active PKB and HA-tagged PFK-2 and stimulated with insulin-like growth factor-1, PFK-2 was reported to be phosphorylated [24]. We purified a wortmannin-sensitive and insulin-stimulated protein kinase (WISK) that phosphorylated and activated heart PFK-2 [25]. PKB isoforms were undetectable in the WISK preparation by immunoblotting and although PKC ζ was present, it is unlikely that this insulin-stimulated AGC kinase is required for heart PFK-2 activation [26]. The serum and glucocorticoid-regulated protein kinases (SGKs) are three serine-threonine AGC kinases homologous to PKB that are also potential candidates for insulin-induced heart PFK-2 activation, lying downstream of PDK1 and activated by T-loop phosphorylation [27]. One major structural difference between PKBs and SGKs is the absence of a PH domain, which at the amino-terminus of SGK3, but not of SGK1/2, is replaced by a Phox homology (PX) domain [28,29] that binds PI(3)P [30]. SGK3, but not SGK1 or SGK2, is expressed in heart [28] and has a molecular mass compatible with that of WISK [25,26].

In this paper, we reinvestigated heart PFK-2 activation by insulin. We first tested whether SGK3 is a PFK-2 kinase and whether its activation in heart by insulin was required for PFK-2 activation. Given the inherent drawbacks of the PKB dominant negative approach, we re-evaluated insulin-induced PFK-2 activation in four different systems: (i) hearts from transgenic mice with a muscle/heart-specific mutation in the PDK1 substrate docking site injected with insulin; (ii) hearts from PKB β deficient mice injected with insulin; (iii) freshly isolated rat cardiomyocytes and perfused hearts treated with Akti-1/2 prior to insulin, (iv) HEK 293T cells co-transfected with heart PFK-2 and PKB α/β siRNA or PKB α siRNA and incubated with insulin. The results indicate that SGK3 is not essential for heart PFK-2 activation by insulin and that this effect is likely mediated by PKB α .

EXPERIMENTAL

Materials

Radiochemicals and other reagents were from sources described previously [26]. Akti-1/2 Akt/PKB inhibitor VIII was from Calbiochem. Active PKB α [31], wild-type SGK3 plasmid (pEBG2t-GST-SGK3) [28], anti-total SGK3 [28] and anti-total PKB β and PKB γ [32] antibodies were prepared as described and kindly donated by Prof. Dario Alessi (University of Dundee). A polyclonal anti-phosphoThr320 SGK3 antibody was raised against the phosphorylated peptide AISDTTTT(P)FCG (residues 313–323 of human SGK3 containing the activation loop phospho-Thr320) plus an N-terminal Cys residue for coupling to keyhole limpet haemocyanin with 3-maleimidobenzoic acid N-hydroxysuccinimide ester and immunization in sheep. The phospho- and non-phospho (C-AISDTTTTFCG) peptides (10 mg of each) were coupled to 2 ml of Thiol Sepharose suspension (from 1.4 g of dried gel swollen in 5 ml of water) in coupling buffer (0.1 M Tris-Cl pH 7.5, 1 mM EDTA, 0.3 M NaCl) for 2h with rotation at room temperature. The resins were washed 3x with coupling buffer (with centrifugation at 3000 rpm x 20 s to collect the gel), twice with 0.1 M sodium citrate pH 4.5 and resuspended in 0.1 M sodium citrate pH 4.5, 1 mM 2-mercaptoethanol for incubation for 90 min at room temperature. The resins were extensively washed with phosphate-buffered saline (PBS) containing 0.1% (w/v) Tween 20. Sera (16 ml aliquots) were first incubated with gel containing the immobilized nonphosphopeptide (overnight at 4°C) before applying the supernatant to a column containing the immobilized phosphopeptide. After extensive washing with PBS-Tween, anti-phospho antibodies were eluted with 1 ml additions of 100 mM glycine and collected in tubes containing 40 μ l of 1 M Tris base. The eluted protein fractions were combined, concentrated by ultrafiltration and dialyzed against PBS for storage in aliquots at -20°C. Anti-total PFK-2 antibody was raised against full-length purified recombinant bovine heart PFK-2 (see below) in rabbits. Anti-phospho Akt-substrate (anti-PAS) antibody and anti-phosphoSer473 PKB antibodies were from Cell Signalling. Anti-PH domain PKB and anti-PKB α antibodies were from Upstate. Anti-total p70^{SGK} antibody was from Santa Cruz. The shRNA plasmid vectors coding for mammalian Akt1/PKB α siRNA [pKD-AKT1/PKB α -v2], mammalian negative control siRNA [pKD-NegCon-v1], mammalian SGK3 siRNA [pKD-SGK3-v1] and Akt siRNA double stranded RNAs targeting both PKB α and PKB β isoforms and the corresponding control siRNAs were from Cell Signalling. For p70^{SGK}, SGK3 and PKB assay, the substrate peptides were KKRNRRLSVA (derived from ribosomal protein S6), PVRMRRNSFT (peptide containing Ser466 of heart PFK-2) and RPRAATF [33], respectively and were kindly synthesized by V. Stroobant (Ludwig Institute for Cancer Research, Brussels, Belgium). Purified recombinant His-tagged BH1 bovine heart PFK-2 isoform [23] and recombinant activated GST-SGK3 [28] from HEK293 cells incubated with insulin were produced as indicated.

Culture and transfection of HEK 293T cells

HEK 293T cells were cultured in 10 cm diameter dishes in Dulbecco's minimal essential medium (DMEM) containing 10% (w/v) fetal calf serum. They were transfected on the following day by the JetPEI procedure according to the manufacturer's instructions (PolyPlus Transfection, France). Cells were transfected with wild-type bovine heart PFK-2 [BH1(His)₆, 0.5 μ g], Akt1/PKB α shRNA plasmid vector (5 μ g), negative control shRNA plasmid vector (5 μ g), SGK3 shRNA plasmid vector (5 μ g), empty vector (PCMV5, 5 μ g), Akt siRNA (50 nM) and control siRNA (50 nM). Transfection was performed at 37 °C in Dulbecco's minimal essential medium containing 10% (w/v) fetal calf serum. After 32h (for protein overexpression), 48h (for PKB α/β silencing with Akt siRNA), 56h (for SGK3 silencing with shRNA vector) or 96h (for PKB α silencing with shRNA vector), the cells were deprived of serum in DMEM for at least 3h and then incubated with or without insulin (0.1 μ M) for 10min. Cells were lysed in 600 μ l of ice-cold Buffer A (50mM Hepes, pH 7.5, 50 mM KF, 1 mM potassium phosphate, 1 mM EDTA, 1 mM EGTA, 1 μ M microcystin, 1 mM Na₃VO₄, 0.1% (v/v) 2-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine hydrochloride, and 1 μ g/ml leupeptin) and stored at -80 °C.

Rat heart perfusion and incubation of isolated cardiomyocytes

Hearts from fed male Wistar rats were perfused by the Langendorff method in Krebs-Henseleit bicarbonate buffer containing 5 mM glucose and 1 μ M Akti-1/2 or DMSO vehicle for 15 min prior to perfusion with or without 0.1 μ M insulin for 10 min [25]. Hearts were freeze-clamped and homogenized in 4 vol of buffer containing 0.2 M sucrose, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 5 mM sodium pyrophosphate, 5 mM sodium β -glycerophosphate, 50 mM HEPES pH 7.5, 15 mM 2-mercaptoethanol, 2 mM benzamidine hydrochloride, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin and 0.2 mM phenylmethanesulphonyl fluoride using an Ultra-Turrax homogenizer (3 x 15 s on ice). Homogenates were centrifuged at 24000g_{max} for 30 min. Isolated cardiomyocytes were prepared [21] and preincubated with 10 μ M Akti-1/2 inhibitor or DMSO vehicle for 15 min prior to treatment with or without insulin (0.1 μ M) for 5 min. Cells were either stopped in hot alkali for Fru-2,6-P₂ measurement [21] or extracted in buffer (1 ml for 60 mg wet weight of cells) containing 20 mM HEPES pH 7.5, 100 mM KCl, 1 mM EDTA, 20 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethanesulphonyl fluoride, 0.1 mM Fru-6-P/0.3 mM Glc-6-P using an Ultra-Turrax homogenizer (3 x 20 s on ice). Following centrifugation as described above, the supernatants were removed and stored frozen at -80°C for subsequent enzyme assay and immunoblotting.

Insulin administration to control and transgenic mice

Wild-type, PKB β knock out, PDK1^{fl/L155E}MckCre^{-/-} and PDK1^{fl/L155E}MckCre^{+/-} mice were starved overnight and anaesthetized (Rompun 20 mg/Kg + Imalgene 100 mg/Kg intraperitoneal) prior to the intravenous injection of insulin (1 mU/Kg) via the vena cava. At the indicated times, hearts were freeze-clamped between tongs pre-cooled in liquid N₂ and homogenized in 4 vol of buffer containing 20 mM HEPES pH 7.6, 30 mM KCl, 20 mM NaF, 1 mM EDTA, 1 mM dithiothreitol, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, 0.1 mM phenylmethanesulphonyl fluoride and 0.1 mM Fru-6-P/0.3 mM Glc-6-P. Extracts were prepared as described above for Fru-2,6-P₂ measurement, PFK-2, PKB and p70^{S6K} assay, and immunoblotting (see below).

Measurement of Fru-2,6-P₂ content and enzyme assay

Fru-2,6-P₂ content and lactate dehydrogenase (LDH) activity were measured as described in [3]. PFK-2 activity was measured [3,26] in 5-20% polyethylene glycol (PEG) 8000 fractions of extracts from cardiomyocytes or HEK 293T cells over-expressing heart PFK-2. PKB and p70^{S6K} [34] were assayed following immunoprecipitation of 200 μ g of extract protein with 0.5 μ g of anti-PH domain PKB antibody or 1 μ g of anti-p70^{S6K} antibody pre-bound to 20 μ l of a 1:1 slurry of Protein G-Sepharose in a final volume of 50 μ l of phosphorylation buffer [20] supplemented with 2.5 μ M cAMP-dependent protein kinase inhibitor peptide, 40 μ M PKB substrate peptide or 200 μ M p70^{S6K} substrate peptide and 0.1 mM [γ -³²P] MgATP (1000 cpm/pmol). After 20 min at 30°, 15 μ l aliquots were taken and spotted onto Whatman P81 phosphocellulose papers and plunged into ice-cold 75 mM phosphoric acid. After extensive washing, ³²P incorporation was measured by liquid scintillation counting. One unit of enzyme activity corresponds to the formation of 1 μ mol (PFK-2, LDH) or 1 nmol (protein kinase) of product per min under the assay conditions.

Immunoblotting

For HEK 293T cells, extracts (30 μ g of protein for PKB and SGK3 detection; 5 μ g of protein for PFK-2 detection) were subjected to SDS-PAGE in 10% (w/v) acrylamide. Proteins were electro-eluted onto polyvinylidene fluoride membranes, which were probed with primary antibodies. Immunoreactive bands were detected by enhanced chemiluminescence after incubation with a secondary peroxidase-coupled antibody. For immunodetection of SGK3 in hearts from control/PDK-1 knock-in mice or perfused rat hearts, extracts (100-130 μ g) were loaded on 10% (w/v) acrylamide or 7.5% (w/v) acrylamide pre-cast gels, respectively, for SDS-PAGE, electro-elution and blocking of polyvinylidene fluoride membranes with 5% (w/v) bovine serum albumin in Tris-buffered saline containing 0.1% (w/v) Tween 20. Membranes were probed with anti-phosphoThr320 SGK3 (1/100 dilution for a first

purification of antibody on extracts from control/PDK-1 knock-in mice and 1/1000 dilution of a second purification of antibody on extracts from perfused hearts) or anti-total SGK3 (1/500 or 1/800 dilution, respectively). Immunoreactive bands were detected by enhanced chemiluminescence (Pierce) after incubation with a secondary peroxidase-coupled anti-sheep antibody used at 1/10000 and 1/80000 dilution, respectively.

***In vitro* phosphorylation of recombinant bovine heart PFK-2**

Recombinant heart PFK-2 (2 μ g) was incubated with activated GST-SGK3 (12 mUnits) purified from insulin-stimulated transfected HEK 293T cells (see above) and 0.1 mM [γ - 32 P] ATP (1000 cpm/pmol) in a final volume of 100 μ l of phosphorylation buffer [20]. At various times, aliquots (15 μ l) were taken for SDS-PAGE in 10 % (w/v) acrylamide mini-gels. The stoichiometry of 32 P incorporation was calculated as described [26]. To measure effects of phosphorylation on PFK-2 activity, recombinant heart enzyme (1 μ g) was incubated with PKB (12 mUnits) or SGK3 (10 mUnits) in a final volume of 20 μ l of phosphorylation buffer [20] and 1 mM MgATP at 30°C, prior to dilution for PFK-2 assay [26].

Phosphorylation site identification by mass spectrometry

Recombinant heart PFK-2 (5 μ g) was incubated as described above with SGK3 (50 mUnits) and 0.1 mM [γ - 32 P] MgATP (specific radioactivity 2000 cpm/pmol) for 60 min at 30°C. After SDS-PAGE, bands corresponding to phosphorylated PFK-2 were cut from the gel and digested with trypsin [35]. Peptides were separated by reverse-phase narrowbore HPLC at a flow rate of 200 μ l/min and radioactive peaks were detected by Cerenkov counting and analysed by nano-electrospray ionisation tandem mass spectrometry (nano-ESI-MS/MS) in a LCQ Deca XP Plus ion-trap mass spectrometer (ThermoScientific, San Jose, CA).

Other methods

Protein concentration was estimated [36] with γ -globulin as standard. PFK-2 activities were expressed as specific activities or per unit of LDH to correct for differences in homogenization and recovery or as arbitrary units in transfected HEK 293T cells after correcting specific activities for PFK-2 content as measured by immunoblotting polyethylene glycol fractions with the anti-bovine heart PFK-2 antibody and quantification of blots by scanning densitometry. The results are expressed as the mean \pm S.E.M. for the indicated number of individual experiments. The statistical significance of the results was assessed by the Student's two-sided unpaired or paired *t* test as indicated. Band intensities of immunoblots were quantified by scanning films and processing the image intensities with the program Image J (1.33 for Mac OS X).

RESULTS

Role of SGK3 in heart PFK-2 activation by insulin

In HEK 293T cells transfected with a plasmid encoding full-length SGK3 fused to GST (GST-SGK3), insulin (0.1 μ M) led to a 2-fold activation of SGK3 after 5 min and to a parallel increase in the phosphorylation state of the activation loop Thr320 residue of SGK3 (Fig. 1S, supplemental data). Recombinant GST-SGK3 purified from insulin-treated HEK 293T cells phosphorylated bovine heart PFK-2 in a time-dependent manner (Fig. 1A). The stoichiometry of phosphorylation tended towards 0.9 mol of phosphate incorporated/mol of subunit protein after 40 min of incubation, which is close to the stoichiometry (0.8 mol/mol) obtained with PKB [25]. Phosphorylation of heart PFK-2 by SGK3 or PKB increased the V_{\max} of PFK-2 2-fold (Fig. 1B). For phosphorylation site identification by mass spectrometry, heart PFK-2 was maximally phosphorylated by activated GST-SGK3, digested with trypsin and the radioactive peptides were separated by HPLC (Fig. 1C). The first peak contained a peptide ($_{465}$ -NSFTPLSSSNTIR- $_{477}$) in which Ser466 was phosphorylated, as determined by tandem mass spectrometry, whereas the second peak contained a peptide ($_{480}$ -NYSVGSRPLQPLSPLR- $_{496}$) in

which Ser483 was phosphorylated. These phosphorylation sites are identical to the *in vitro* sites for PKB [25,26] and to those identified in intact HEK 293 cells transfected with heart PFK-2 and incubated with insulin [23].

Because cultured cardiomyocytes are difficult to transfect efficiently, we investigated the role of SGK3 in insulin-induced heart PFK-2 activation in transfected HEK 293 cells. These cells do not express insulin receptors, but do have IGF1 receptors which bind insulin, and contain all the elements of the insulin signalling pathway. Although HEK 293 cells have successfully been used by others [37] and ourselves [23] to study insulin signalling downstream of PDK1, these cells do not express a functional metabolic end-point for insulin and the stoichiometry and expression of insulin signalling components might not be similar to those of primary cells that are physiological targets for insulin. HEK 293T cells were co-transfected with heart PFK-2 and with a plasmid coding for SGK3 siRNA prior to insulin treatment. Immunoblotting with anti-total SGK3 antibody revealed that SGK3 levels were decreased by at least 80% in cells transfected by SGK3 siRNA (Fig. 2A). In non-transfected cells, immunoblotting with anti-phosphoThr320 SGK3 antibody revealed a 25-fold increase in band intensity in response to insulin treatment (not shown). Also, insulin activated endogenous immunoprecipitated PKB (Fig. 2B) and p70^{S6K} (Fig. 2C) both in cells transfected with heart PFK-2 and in cells co-transfected with heart PFK-2 and siRNA SGK3. Thus, there was no effect of SGK3 siRNA transfection on insulin-induced PKB and p70^{S6K} activation. Moreover, co-transfection of SGK3 siRNA failed to prevent insulin-induced activation of transfected PFK-2 (Fig. 2D). These experiments suggest that SGK3 is not required for insulin-induced heart PFK-2 activation.

Effect of insulin on PFK-2 activation in hearts of transgenic mice bearing a muscle-specific mutation in the PDK1 substrate docking site

In order to further elucidate which kinase downstream of PDK1 could be involved in insulin-induced PFK-2 activation, we took advantage of a conditional knock-in mouse model in which muscle and heart PDK1 harbored a mutation of the key residue Leu155 to Glu in the PIF-pocket responsible for binding its downstream targets [38]. Following phosphorylation of the hydrophobic motif Ser/Thr residue of SGK1, p70^{S6K} and p90 ribosomal S6 kinase (p90^{RSK}) by PDK2, the PIF-pocket of PDK1 binds to the phosphorylated residue in the hydrophobic motif which then allows PDK1 to activate the downstream kinase by phosphorylating its activation loop Thr residue. Mutation of Leu155 to Glu prevents SGK1, p70^{S6K} and p90^{RSK} activation by insulin, but not PKB, which is activated by an alternative mechanism. The PKB PH domain binds phosphatidylinositol-(3,4,5)-P₃ thereby inducing a conformational change allowing PDK1 to phosphorylate its activation loop Thr308 residue. Mice were obtained in which the wild-type PDK1 allele had been flanked with LoxP sites (PDK1^{fl/fl}) and bred with PDK1^{L155E/+} knock-in mice to generate PDK1^{fl/L155E} animals, which were in turn crossed with transgenic mice expressing the Cre recombinase under the muscle creatine kinase (Mck) promoter (which induces expression of the Cre recombinase specifically in skeletal muscle and heart) [38]. In the resulting PDK1^{fl/L155E}MckCre^{+/-} mice, PDK1 expression was ablated and expression of PDK1 only occurred from the L155E knock-in allele not flanked with LoxP sites. Intravenous injection of insulin to control PDK1^{fl/L155E}MckCre^{-/-} mice activated PKB more than 5-fold after 5 min (Fig. 3A) and activated p70^{S6K} almost 13-fold after 20 min (Fig. 3B). In hearts from mice bearing the PIF-pocket mutation (PDK1^{fl/L155E}MckCre^{+/-}), PKB was activated 4-fold by insulin after 5 min (Fig. 3A), whereas p70^{S6K} activation by insulin was completely ablated (Fig. 3B). In heart extracts from PDK1^{fl/L155E}MckCre^{-/-} and PDK1^{fl/L155E}MckCre^{+/-} mice, the insulin-induced increase in GSK3 β Ser9 phosphorylation was comparable (not shown), indicating that the PDK1 PIF-pocket mutation did not affect the phosphorylation of PKB targets. Indeed, the time courses of PFK-2 (Fig. 3C) activation and the subsequent rise in Fru-2,6-P₂ (Fig. 3D) were similar in hearts from PDK1^{fl/L155E}MckCre^{-/-} and PDK1^{fl/L155E}MckCre^{+/-} mice. By contrast, the increase in SGK3 activation loop Thr320 phosphorylation in control PDK1^{fl/L155E}MckCre^{-/-} mice seen 10 min after insulin injection was completely abrogated in PDK1^{fl/L155E}MckCre^{+/-} mice harbouring the PIF-pocket mutation (Fig. 3E). Together the findings suggest that kinases downstream of PDK1 other than PKB and containing a hydrophobic phosphorylation site motif, such as SGK3, are not necessary for insulin-induced PFK-2 activation. The results also indicate that as for SGK1, insulin-induced SGK3 activation by PDK1 is

PIF-pocket dependent and further argue against the participation of SGK3 in heart PFK-2 activation by insulin.

Effect of insulin on PFK-2 activation in hearts of PKB β deficient mice

The studies on insulin-induced PFK-2 activation in hearts from mice bearing the PIF-pocket mutation strongly suggest that PKB, and not SGK3, is mediating this effect. Therefore, we examined PFK-2 activation by insulin in hearts from PKB β deficient mice. These mice are viable but insulin resistant, whereas mice deficient in PKB α have growth defects, dying not long after birth and so cannot be studied for insulin-induced heart PFK-2 activation. Following intravenous injection of insulin to anaesthetized wild-type and PKB β ^{-/-} mice, the hearts were freeze-clamped and extracted for PKB activity measurement after immunoprecipitation with anti PH-domain PKB antibody which recognizes PKB α and PKB β . Insulin induced a 3-fold activation of PKB in hearts from wild-type mice but surprisingly, in hearts from PKB β deficient mice, a 7-fold activation of PKB by insulin was seen (Fig. 4A). This increase in PKB activity in hearts from the knockout mice suggests that the deletion of PKB β results in over-compensation by PKB α . The absence of PKB β in heart extracts from PKB β ^{-/-} mice was apparent after immunoblotting with the isozyme-specific antibody (Fig. 4B). However, due to the fact that the available anti-PKB α antibody was not specific and cross-reacts with PKB β , we could not test for over-compensation by PKB α either by immunoblotting or by immunoprecipitation/PKB assay. In parallel with the rise in insulin-induced PKB activity in hearts from PKB β ^{-/-} mice, the increase in PFK-2 activity (Fig. 4C) and Fru-2,6-P₂ content (Fig. 4D) was greater in hearts from PKB β deficient mice. This correlation supports the implication of PKB in insulin-induced heart activation and indicates that this effect is not mediated by PKB β .

Effect of insulin on PFK-2 activation in rat cardiomyocytes treated with Akti-1/2 inhibitor

Cultured cardiomyocytes are difficult to transfect for PKB isoform expression knock-down by RNA interference. Therefore, freshly isolated rat cardiomyocytes were pre-incubated with and without the highly selective Akti-1/2 inhibitor, then with and without insulin. We first verified that any stress induced by treatment with the inhibitor had no effect on the activation of the AMP-activated protein kinase, which like PKB also phosphorylates and activates heart PFK-2 [39]. Acetyl-CoA carboxylase-2 phosphorylation, measured by immunoblotting, was used as a surrogate marker of AMP-activated protein kinase activation and was found to be unaffected by preincubation with the Akti-1/2 inhibitor (not shown). Insulin treatment activated PKB about 4-fold after immunoprecipitation from cardiomyocyte extracts, the effect being drastically reduced by preincubation with the Akti-1/2 inhibitor (Fig. 5A). Likewise, immunoblotting revealed that the Akti-1/2 inhibitor blocked insulin-induced PKB Ser473 phosphorylation (Fig. 5B). Moreover, the insulin-induced rise in Fru-2,6-P₂ (Fig. 5C) and PFK-2 activation (Fig. 5D) were abrogated in cardiomyocytes preincubated with Akti-1/2, suggesting that PKB is involved in heart PFK-2 activation.

Intact rat hearts were perfused with Akti-1/2 to assess the effect of the compound on the activation state of PKB and SGK3 in response to insulin. Like in cardiomyocytes (Fig. 5A), insulin induced a robust (6-fold) activation of PKB in perfused hearts, which was markedly decreased by pre-treatment with Akti-1/2 (Fig. 6A). As observed in control PDK1^{fl/L155E}MckCre^{-/-} mice injected with insulin (Fig. 3E), perfusion of rat hearts with insulin led to an increase in SGK3 Thr320 activation loop phosphorylation (Fig. 6B). However, the insulin-induced increase in SGK3 Thr320 phosphorylation was unaffected by pre-treatment with Akti-1/2 (Fig. 6B).

Effect of insulin on heart PFK-2 activation in HEK 293T cells co-transfected with PKB siRNAs

HEK 293T cells were co-transfected with a vector coding for polyhistidine-tagged recombinant heart PFK-2 [23] and either control siRNA or PKB α/β siRNA to knock down the expression of both PKB isoforms. Immunoblotting with anti-total PH-domain PKB antibody indicated that PKB α/β siRNA treatment decreased the PKB content of the cells by more than 80% without affecting PKC ζ expression as a loading control (Fig. 7A). In order to evaluate the efficiency of PKB knock-down, the

phosphorylation state of Thr389 of p70^{S6K}, an indirect downstream target, was measured by immunoblotting. In cells treated with control siRNA, insulin increased p70^{S6K} Thr389 phosphorylation by about 50%, which was completely abrogated by transfection of PKB α / β siRNA (Fig. 7B). Moreover, in PKB α / β siRNA-treated HEK 293T cells, heart PFK-2 activation by insulin was likewise abolished (Fig. 7C). The results suggest that PKB α and/or PKB β is involved in insulin-induced heart PFK-2 activation.

In HEK 293T cells with co-transfected with His-tagged heart PFK-2 and PKB α siRNA, a PKB α knock-down of at least 50% was achieved after 96 h of transfection (Fig. S2A), however the anti-PKB α antibody used for immunoblotting was not specific and cross-reacts to some extent with PKB β . Total PKB expression was unchanged (Fig. S2A) and there was no effect of PKB α knock-down on PKB Ser473 phosphorylation (Fig. S2B). Also, by immunoblotting with the specific antibodies, we were unable to detect any change in expression of PKB β or PKB γ following PKB α knock-down (not shown). The 96h time frame of transfection needed to achieve a reasonable knock-down in PKB α expression resulted in a large overexpression of co-transfected PFK-2, such that no or little effect of insulin treatment on PFK-2 activity was evident. Therefore, we resorted to the use of the anti-PAS antibody to monitor PFK-2 phosphorylation in response to insulin. PKB α siRNA transfection abrogated insulin-induced PFK-2 phosphorylation (Fig. S2C), thus implicating this PKB isoenzyme in heart PFK-2 activation by insulin.

DISCUSSION

In a classic review of reversible phosphorylation of enzymes, Krebs and Beavo proposed a set of criteria for establishing that changes in enzyme activity as a result of phosphorylation/dephosphorylation by a protein kinase would be physiologically relevant [40]. The third and fourth criteria, which are the most difficult to satisfy, state that the enzyme should be shown to be phosphorylated/dephosphorylated *in vivo* or in an intact cell system with accompanying functional changes and that there should be a correlation between cellular levels of protein kinase effectors and the extent of phosphorylation of the enzyme, respectively. When Krebs and Beavo proposed their criteria for establishing physiologically relevant phosphorylation/dephosphorylation of enzymes, this was mainly based on the study of protein phosphorylation in response to changes in cyclic AMP. To verify that a particular protein kinase is the physiologically relevant kinase for a target enzyme in stimulated cells, changes in cellular kinase activity should be correlated with a change in activity of the target enzyme. Nowadays this can be done either by incubating cells with a selective kinase inhibitor, by transfecting cells with vectors overexpressing constitutively active and/or dominant-negative forms of the kinase, by knock-down of the kinase by transfecting small interfering RNAs, or by using mouse knockout or transgenic models.

Here we showed that in HEK 293T cells, insulin activated SGK3 and that treatment of heart PFK-2 with SGK3 *in vitro* led to PFK-2 activation and phosphorylation at Ser466 and Ser483, the same sites phosphorylated by PKB *in vitro*. Because cultured cardiomyocytes cannot be easily transfected, we investigated the role of SGK3 in insulin-induced heart PFK-2 activation in transfected HEK 293T cells. Co-transfection of HEK 293T cells with SGK3 siRNA indicated that SGK3 was not required for insulin-induced heart PFK-2 activation, a conclusion supported by studies on heart PFK-2 activation by insulin in the PDK1 PIF pocket mutation knock-in mice and use of the Akti-1/2 selective PKB inhibitor (see below). Our previous conclusions that PKB was not involved heart PFK-2 activation by insulin were based on experiments using a dominant-negative PKB construct in co-transfection experiments in HEK 293 cells [23], which led us to embark on the purification of WISK [25]. However, the dominant negative approach has inherent drawbacks such as incomplete competition with endogenous PKB, lack of sequestration of PKB in the right intracellular compartment or the fact that the dominant-negative PKB might not titrate out the relevant PKB isoenzyme to completely block insulin-induced PFK-2 activation. Clearly, in order to verify the last of the Krebs and Beavo criteria, as many different approaches as possible for correlating intracellular kinase activity with the biological response should be tested before definitive conclusions can be drawn. Therefore, we re-evaluated the role of PKB by studying PFK-2 activation by insulin in

different models, namely hearts from transgenic mice, cardiomyocytes and perfused hearts treated with Akti-1/2 PKB inhibitor and HEK 293T cells after PKB knock-down by siRNA. The fact that insulin-induced heart PFK-2 activation was still observed in conditional knock-in mice bearing a mutation in the PDK1 substrate binding motif (Fig. 3C,D) argues against the involvement of kinases other than PKB, containing a hydrophobic phosphorylation site motif but without a PH domain, for insulin-induced PFK-2 activation. Indeed, the increase in SGK3 activation loop Thr320 phosphorylation induced by insulin injection in control mice was abolished in the PDK1 PIF pocket knock-in mice (Fig. 3E). In freshly isolated rat cardiomyocytes incubated with the Akti-1/2 inhibitor, PFK-2 activation by insulin was almost ablated (Fig. 5) and in hearts perfused with Akti-1/2, insulin-induced PKB activation markedly decreased, whereas the increase in SGK3 Thr320 phosphorylation was unaffected (Fig. 6). Thus, while the PDK1 PIF pocket knock-in mouse model can differentiate between insulin effects on PKB and SGK3 *in vivo* (Fig. 3), the use of Akt1/2 in perfused hearts can distinguish between PKB activation and SGK3 Thr320 phosphorylation *ex-vivo* (Fig. 6). Experiments in HEK 293T cells after PKB α / β siRNA knock-down clearly implicated either PKB α or PKB β in heart PFK-2 activation by insulin (Fig. 7). Unexpectedly, in hearts from PKB β deficient mice, insulin-induced PFK-2 activation was not abolished and was even greater than in wild-type mice, presumably due to over-compensation by PKB α in hearts of the knock-out animals (Fig. 4). However, one can conclude that PKB β does not mediate heart PFK-2 activation, which we propose would rather involve PKB α , as supported by the fact that siRNA knock-down of this PKB isoenzyme in HEK 293T cells ablated heart PFK-2 phosphorylation (Fig. S2).

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FIGURE LEGENDS

Fig. 1 Phosphorylation and activation of heart PFK-2 by SGK3 *in vitro*. *A.* Recombinant heart PFK-2 was incubated with activated SGK3 as described under Experimental procedures. At the indicated times, ^{32}P -incorporation (means \pm S.E.M. of three separate experiments) was measured by phosphoimaging after SDS-PAGE. *B.* PFK-2 activity was measured under V_{\max} conditions (means \pm S.E.M. of three separate experiments) after phosphorylation of heart PFK-2 with SGK3 or PKB as described under Experimental procedures. *- indicates a significant effect of protein kinase treatment compared with the control ($p < 0.05$, paired *t*-test). *C.* Recombinant heart PFK-2 was incubated with SGK3 and 0.1 mM $[\gamma\text{-}^{32}\text{P}]$ MgATP for SDS-PAGE and in-gel trypsin digestion. Peptides were separated by reverse-phase narrowbore HPLC and eluting fractions were collected and counted by cerenkov radiation. The major radiolabelled peaks were analysed by tandem mass spectrometry to identify phosphorylation sites.

Fig. 2 Effect of SGK3 knock-down by siRNA on insulin-induced PFK-2 activation in transfected HEK293T cells. *A.* HEK 293T cells were co-transfected with vectors (see Experimental procedures) as indicated. Cell extracts were immunoblotted with anti-total SGK3 and anti-total PKC ζ (loading control) antibodies. Following scanning densitometry, band intensities obtained with the anti-total SGK3 antibody were calculated relative to band intensities obtained for the loading control (lower panel). Following transfection, the cells were incubated with (black bars) or without (open bars) 0.1 μM insulin for 10 min. *B.* PKB and *(C)* p70 $^{\text{S6K}}$ activities were measured in immunoprecipitates of cell lysates. *D.* PFK-2 activities were measured in cell extracts under V_{\max} conditions and expressed as arbitrary units after correction for PFK-2 expression by immunoblotting as described under Experimental Procedures. The results are the means \pm S.E.M. of three separate experiments. *- indicates a significant effect of insulin compared with the control ($p < 0.05$, paired *t*-test) and in *(A)*, a significant effect of transfection of SGK3 siRNA ($p < 0.05$, paired *t*-test).

Fig. 3 Effect of insulin on the activities of PKB, p70 $^{\text{S6K}}$ and PFK-2, effect on Fru-2,6-P $_2$ content and SGK3 Thr320 phosphorylation in hearts of transgenic mice bearing a muscle-specific mutation in the PDK1 substrate docking site. PDK1 $^{\text{fl/L155E}}$ MckCre $^{-/-}$ (control, open symbols) mice and PDK1 $^{\text{fl/L155E}}$ MckCre $^{+/+}$ (knock-in, filled symbols) mice bearing the PIF-pocket mutation were starved for 16h prior to intravenous injection with insulin. At the indicated times, the hearts were rapidly excised and freeze-clamped. *A.* PKB and *(B)* p70 $^{\text{S6K}}$ were immunoprecipitated from extracts and assayed by phosphorylation of synthetic peptides. *- indicates a significant effect of insulin on p70 $^{\text{S6K}}$ activity in heart extracts from control mice compared with extracts from knock-in mice ($p < 0.05$, unpaired *t*-test). *C.* Extracts were also prepared for PFK-2 assay and Fru-2,6-P $_2$ measurement *(D)*. The results are the means \pm S.E.M. of measurements on at least four different animals. *E.* Heart extracts from control PDK1 $^{\text{fl/L155E}}$ MckCre $^{-/-}$ (white bars) and PDK1 $^{\text{fl/L155E}}$ MckCre $^{+/+}$ knock-in mice (black bars) after 0 and 10 min of treatment with insulin were blotted with anti-phosphoThr320 and anti-total SGK3 (loading control) antibodies (a representative blot is shown in the lower panel). Following scanning densitometry, band intensities obtained with the anti-phospho Thr320 SGK3 antibody were expressed relative to band intensities of the loading controls (upper panel). *- indicates a significant effect of insulin on SGK3 Thr320 phosphorylation compared to the 0 min values ($p < 0.05$, unpaired *t*-test). The results are the means \pm S.E.M. of measurements on three different animals.

Fig. 4 Effect of insulin on PKB and PFK-2 activity and Fru-2,6-P $_2$ content in hearts of PKB β deficient mice. Wild-type (PKB $\beta^{+/+}$) and PKB β deficient (PKB $\beta^{-/-}$) mice were starved for 16h prior to

intravenous injection of insulin (black bars) or were untreated (open bars). After 10 min, the hearts were rapidly excised and freeze-clamped. *A*. Extracts were immunoprecipitated for PKB assay, immunoblotted with anti-PKB β antibody (*B*, representative blots from three wild-type and three PKB $\beta^{-/-}$ mice are shown) and assayed for (*C*) PFK-2 or (*D*) Fru-2,6-P₂ content. The results are the means \pm S.E.M. of measurements on at least four different animals. *- indicates a significant effect of insulin compared with the controls ($p < 0.05$, unpaired *t*-test). ** - indicates a significantly greater effect of insulin in PKB $\beta^{-/-}$ mice compared with wild-type mice ($p < 0.05$, unpaired *t*-test).

Fig. 5 Effect of insulin on PKB and PFK-2 activity and Fru-2,6-P₂ content in rat cardiomyocytes treated with Akti-1/2 PKB inhibitor. Freshly isolated rat cardiomyocytes were preincubated with 10 μ M Akti-1/2 inhibitor or DMSO vehicle for 15 min prior to treatment with (black bars) or without (open bars) insulin (0.1 μ M) for 5 min. *A*. Extracts were immunoprecipitated for PKB assay, (*B*) immunoblotted with anti-phosphoSer473 PKB and β -actin as a loading control (a representative blot is shown) and assayed for (*C*) Fru-2,6-P₂ content or (*D*) PFK-2 activity. The results are the means \pm S.E.M. of measurements on four different cell preparations. *- indicates a significant effect of insulin compared with the controls ($p < 0.05$, paired *t*-test). ** - indicates a significant decrease in the effect of insulin in cells treated with Akti-1/2 compared with control incubations ($p < 0.05$, paired *t*-test).

Fig. 6 Effect of insulin on PKB activity and SGK3 Thr320 phosphorylation in rat hearts perfused with Akti-1/2 PKB inhibitor. Rat hearts were perfused with 5 mM glucose and DMSO vehicle or 10 μ M Akti-1/2 inhibitor for 15 min prior to perfusion with (black bars) or without (open bars) insulin (0.1 μ M) for 10 min then freeze clamped and homogenized. *A*. Extracts were immunoprecipitated for PKB assay or (*B*) immunoblotted with anti-phosphoThr320 and anti-total SGK3 (loading control) antibodies (a representative blot is shown in the lower panel). Following scanning densitometry, band intensities obtained with the anti-phospho Thr320 SGK3 antibody were expressed relative to band intensities of the loading controls (upper panel). The results are the means \pm S.E.M. of four separate experiments and *- indicates a significant effect of insulin compared with the controls ($p < 0.05$, unpaired *t*-test). ** - indicates a significant decrease in the effect of insulin in hearts treated with Akti-1/2 compared with the controls ($p < 0.05$, unpaired *t*-test).

Fig. 7 Effect of PKB α/β knock-down by siRNA on insulin-induced PFK-2 activation in transfected HEK293T cells. HEK 293T cells were co-transfected with vectors (see Experimental procedures) as indicated. *A*. Extracts were immunoblotted with anti-total PH-domain PKB and anti-total PKC ζ (loading control) antibodies (a representative blot from two experiments is shown in the lower panel). Following scanning densitometry, band intensities obtained with the anti-total PKB antibody were calculated relative to band intensities obtained with the loading control (upper panel). The cells were serum-starved and incubated with (black bars) or without (open bars) 0.1 μ M insulin for 10 min. *B*. Extracts were immunoblotted with anti-phosphoThr389 p70^{S6K} and anti-loading control (PKC ζ) antibodies for quantification of p70^{S6K} phosphorylation relative to the loading control as described above (a representative blot from two experiments is shown). *C*. PFK-2 activities were measured in cell extracts under V_{max} conditions and expressed as arbitrary units after correction for PFK-2 expression by immunoblotting as described under Experimental Procedures. The results are the means \pm S.E.M. of three separate experiments. *- indicates a significant effect of insulin compared with the control ($p < 0.05$, paired *t*-test). ** - indicates a significant decrease in the effect of insulin in cells transfected with PKB siRNA compared with cells transfected with control siRNA ($p < 0.05$, paired *t*-test).

Fig. 1

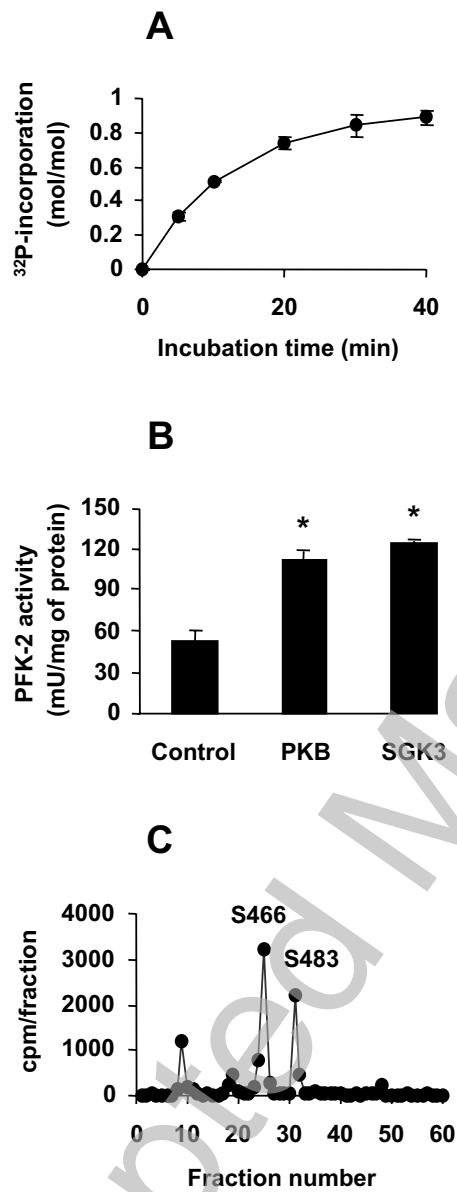


Fig. 2

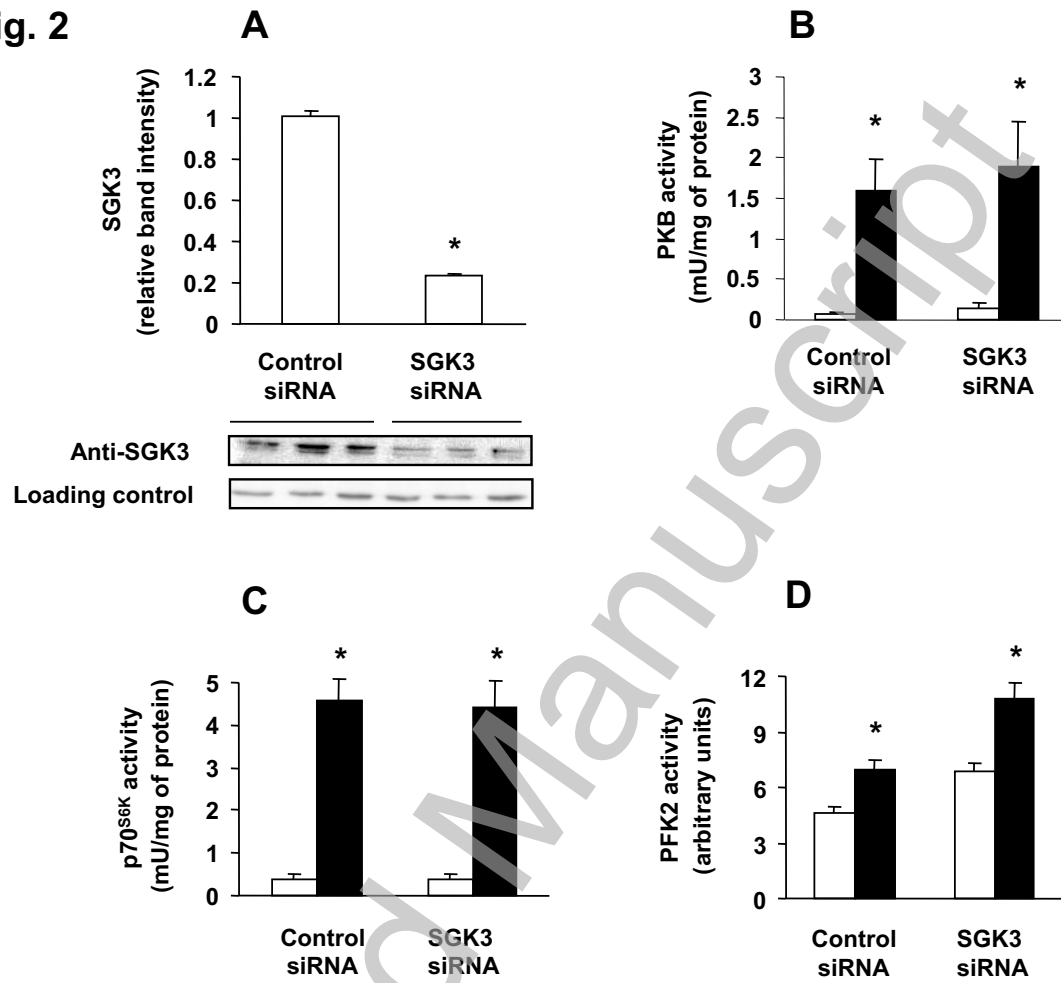
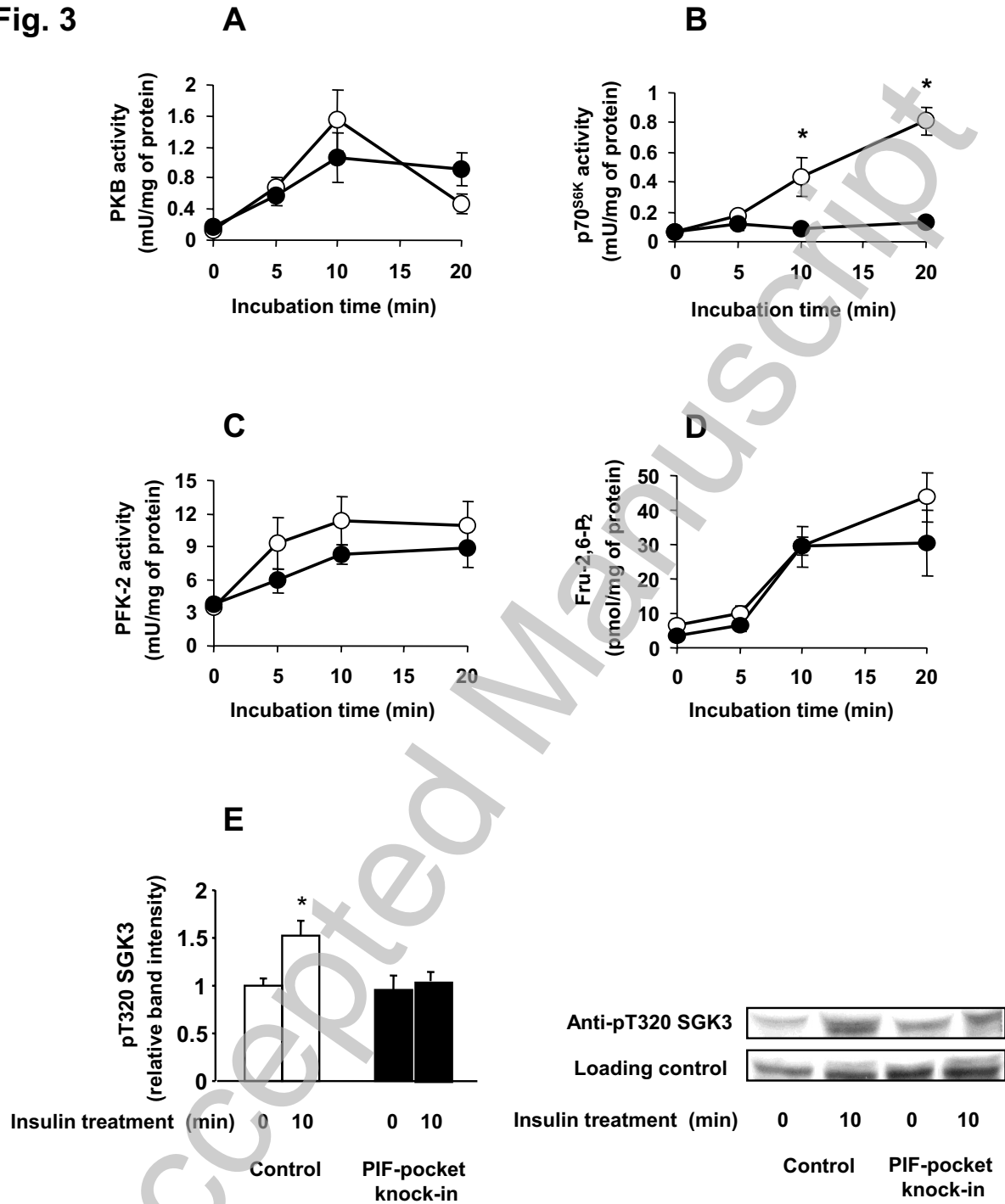
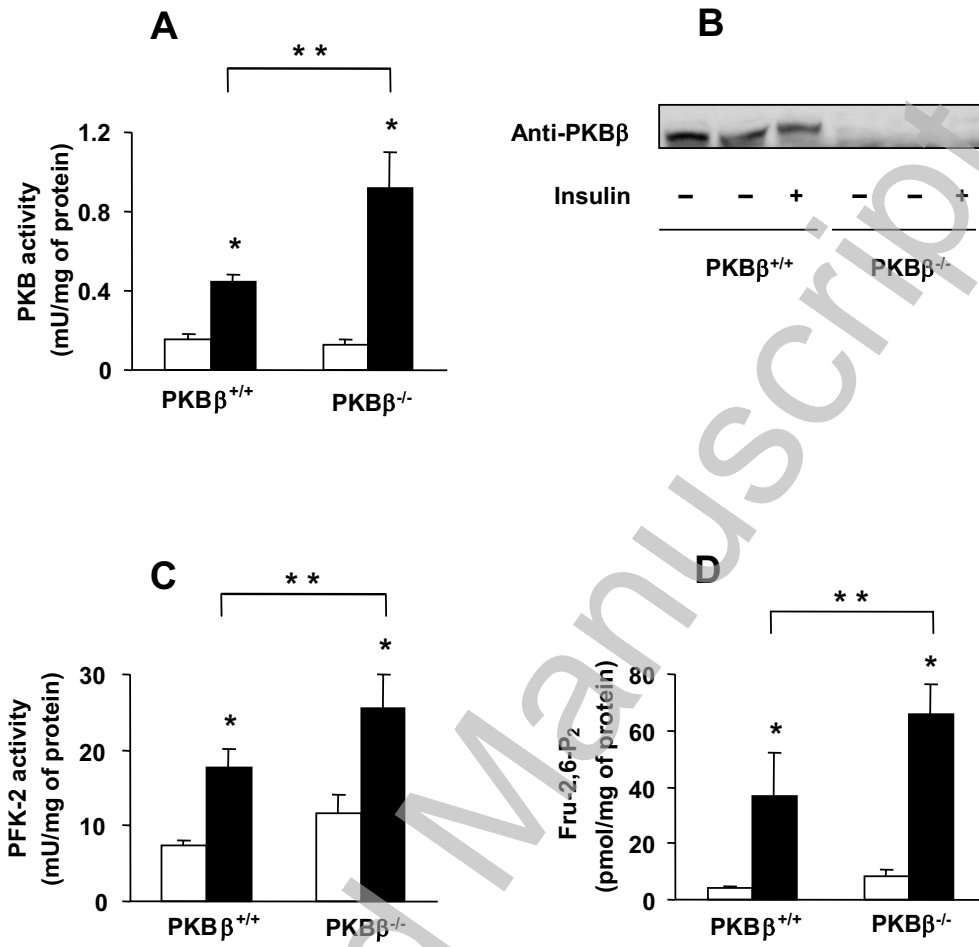


Fig. 3



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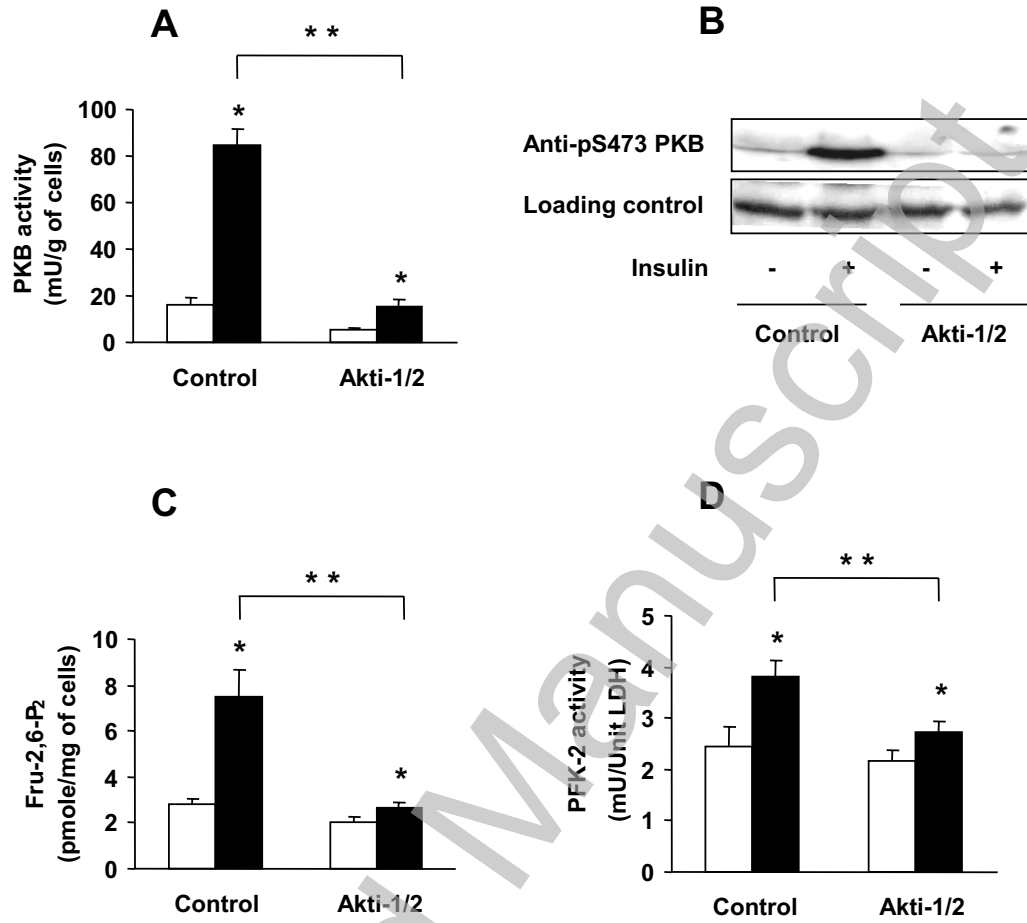
Fig. 4



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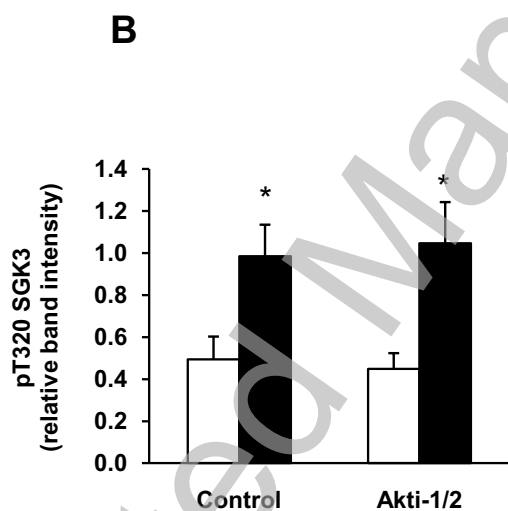
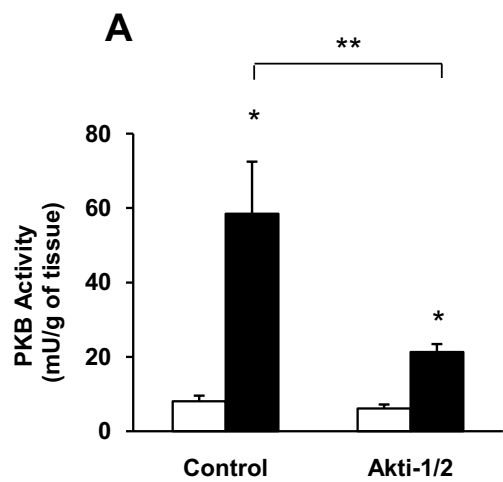
Fig. 5



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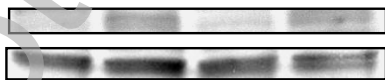
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Fig. 6



Anti-pT320 SGK3

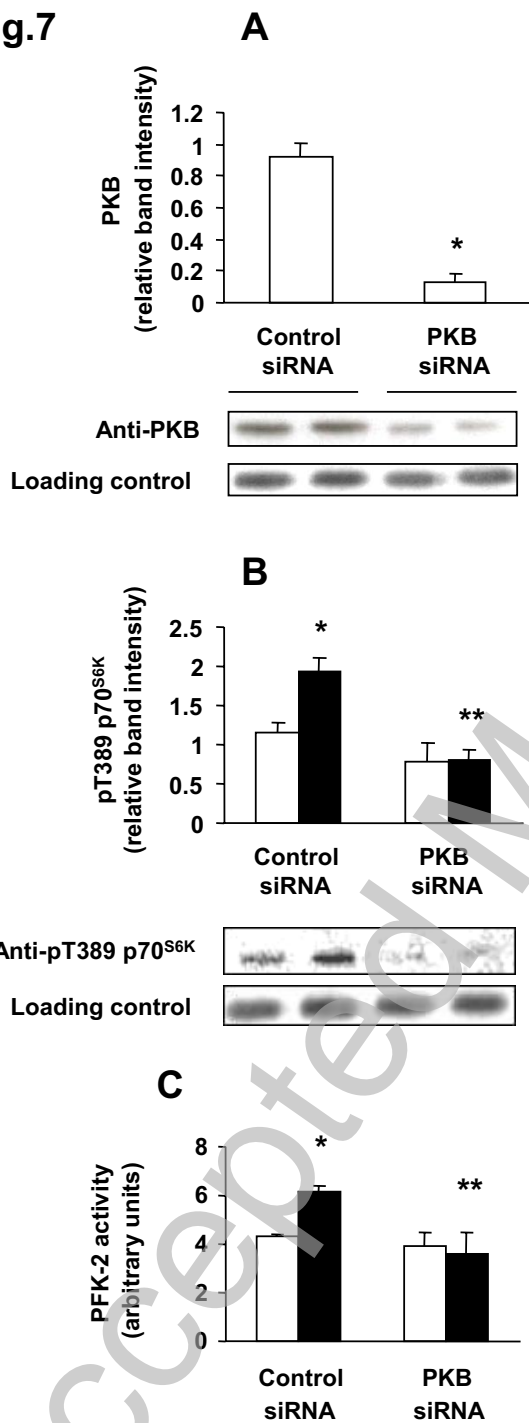
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Fig.7



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