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Heart 6-phosphofructo-2-kinase activation by insulin requires PKB (protein kinase B), but not SGK3 (serum- and glucocorticoid-induced protein kinase 3)

Véronique MOUTON*[1], Louise TOUSSAINT*†[1], Didier VERTOMMEN*, Marie-Agnès GUEUNING*, Liliane MAISIN*, Xavier HAVAUX‡, Cossette SANCHEZ-CANEDO‡, Luc BERTRAND‡, Franck DEQUIEDT†, Brian A. HEMMINGS§, Louis HUE* and Mark H. RIDER*[2]

*Université catholique de Louvain and de Duve Institute, 75 Avenue Hippocrate, B-1200 Brussels, Belgium, †Cellular and Molecular Biology Unit, FUSAGx, 13 Avenue Marechal Juin, 5030 Gembloux, Belgium, ‡Université catholique de Louvain Division of Cardiology, 55 Avenue Hippocrate, CARD5550, B-1200 Brussels, Belgium, and §Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, 4058 Basel, Switzerland

On the basis of transfection experiments using a dominant-negative approach, our previous studies suggested that PKB (protein kinase B) was not involved in heart PFK-2 (6-phosphofructo-2-kinase) activation by insulin. Therefore we first tested whether SGK3 (serum- and glucocorticoid-induced protein kinase 3) might be involved in this effect. Treatment of recombinant heart PFK-2 with [γ-^{32}P]ATP and SGK3 *in vitro* led to PFK-2 activation and phosphorylation at Ser^{466} and Ser^{483}. However, in HEK-293T cells [HEK (human embryonic kidney)-293 cells expressing the large T-antigen of SV40 (simian virus 40)] co-transfected with SGK3 siRNA (small interfering RNA) 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 (phosphoinositide-dependent protein kinase 1)-substrate-docking site injected with insulin; (ii) hearts from PKBβ-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 treatment; and (iv) HEK-293T cells co-transfected with heart PFK-2, and PKBα/β siRNA or PKBα siRNA, 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 PKBα.

Key words: glycolysis, protein kinase B (PKB), 6-phosphofructo-2-kinase (PFK-2), serum- and glucocorticoid-induced protein kinase 3 (SGK3), small interfering RNA (siRNA).

INTRODUCTION

Insulin stimulates heart glycolysis via the recruitment of GLUT4 (glucose transporter 4) transporters to the plasma membrane [1,2] and activation of PFK-2 (6-phosphofructo-2-kinase) [3]. The subsequent rise in Fru-2,6-P_2 (fructose 2,6-bisphosphate) allosterically stimulates PFK-1 (6-phosphofructo-1-kinase) and hence glycolysis. Insulin action involves PI3K (phosphoinositide 3-kinase) activation [4], which generates PIP_3 (phosphatidylinositol-3,4,5-trisphosphate) in the plasma membrane. PIP_3 is a lipid second messenger, which recruits two serine/threonine protein kinases, namely PDK1 (phosphoinositide-dependent protein kinase 1) and PKB (protein kinase B), to the plasma membrane via their PH (pleckstrin homology) domains. Full activation of PKB requires phosphorylation of Thr^{308} by PDK1 [5,6] and phosphorylation at a second site, Ser^{473} in the hydrophobic motif, by PDK2. There is now strong evidence to suggest that the physiological PDK2 is the mTOR (mammalian target of rapamycin)–Rictor (rapamycin-insensitive companion of mTOR) complex TORC2 (mTOR complex 2) [7–10].

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

PKBα activates heart PFK-2 *in vitro* by phosphorylating Ser^{466} and Ser^{483} leading to a 2-fold increase in V_{max} [20]. In isolated

Abbreviations used: AGC-type kinase, protein kinase A/protein kinase G/protein kinase C-family kinase; BH, bovine heart; DMEM, Dulbecco's minimal essential medium; Fru-2,6-P_2, fructose 2,6-bisphosphate; GST, glutathione transferase; HEK, human embryonic kidney; IGF1, insulin-like growth factor 1; LDH, lactate dehydrogenase; Mck, muscle creatine kinase; mTOR, mammalian target of rapamycin; p70^{S6K}, p70 ribosomal S6 kinase; p90^{RSK}, p90 ribosomal S6 kinase; PAS, phospho-Akt substrate; PBST, PBS containing 0.1% Tween 20; PDK, phosphoinositide-dependent protein kinase; PEG, poly(ethylene glycol); PFK-2, 6-phosphofruco-2-kinase; PH, pleckstrin homology; PI3K, phosphoinositide 3-kinase; PIP_3, phosphatidylinositol-3,4,5-trisphosphate; PKB, protein kinase B; PKC, protein kinase C; PX, Phox homology; SGK, serum- and glucocorticoid-induced protein kinase; shRNA, small hairpin RNA; siRNA, small interfering RNA; WISK, wortmannin-sensitive and insulin-stimulated protein kinase.

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed (email mark.rider@uclouvain.be).

cardiomyocytes, the insulin-induced activation of PFK-2 was insensitive to rapamycin and PD98059, which inhibit $p70^{S6K}$ (p70 ribosomal S6 kinase) and MAPK (mitogen-activated protein kinase) activation respectively, but was blocked by the PI3K inhibitors wortmannin and LY294002 [21]. In addition in hearts of muscle-specific PDK1-knockout mice, insulin failed to activate PKB, $p70^{S6K}$ and PFK-2 [22], consistent with the PI3K/PDK1 axis mediating these processes.

Our previous studies indicated that PKB was not essential for insulin-induced heart PFK-2 activation on the basis of co-transfection of a kinase-inactive dominant-negative PKB construct in HEK (human embryonic kidney)-293 cells [23]. However, in a later study in HeLa cells co-transfected with active PKB and HA (haemagglutinin)-tagged PFK-2, and stimulated with IGF1 (insulin-like growth factor 1), PFK-2 was reported to be phosphorylated [24]. We purified WISK (wortmannin-sensitive and insulin-stimulated protein kinase), which phosphorylated and activated heart PFK-2 [25]. PKB isoforms were undetectable in the WISK preparation by immunoblotting, and although PKC (protein kinase C) ζ was present, it is unlikely that this insulin-stimulated AGC-type kinase (protein kinase A/protein kinase G/protein kinase C-family kinases) is required for heart PFK-2 activation [26]. The SGKs (serum- and glucocorticoid-induced protein kinases) are three serine/threonine AGC-type kinases homologous with PKB, which 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 N-terminus of SGK3, but not in SGK1/2, is replaced by a PX (Phox homology) domain [28,29] that binds PI(3)P (phosphatidylinositol 3-phosphate) [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 the present study we re-investigated 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 PKBβ-deficient mice injected with insulin; (iii) freshly isolated rat cardiomyocytes and perfused hearts treated with Akti-1/2 prior to insulin treatment; and (iv) HEK-293T [HEK-293 cells expressing the large T-antigen of SV40 (simian virus 40)] cells co-transfected with heart PFK-2, and PKBα/β siRNA or PKBα 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 to be mediated by PKBα.

EXPERIMENTAL

Materials

Radiochemicals and other reagents were from sources described previously [26]. The Akti-1/2 PKB inhibitor VIII was from Calbiochem. Active PKBα [31], the wild-type SGK3-expressing plasmid (pEBG2t-GST-SGK3) [28], the anti-total SGK3 [28] and anti-total PKBβ and PKBγ [32] antibodies were a gift from Professor Dario Alessi (Department of Biochemistry, University of Dundee, Dundee, U.K.). A polyclonal anti-phospho-Thr^{320} SGK3 antibody was raised against the phosphorylated peptide C-AISDTTTTpFCG (residues 313–323 of human SGK3 containing the activation loop phosphorylated Thr^{320} plus an N-terminal cysteine residue for coupling to keyhole limpet haemocyanin with 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester) by immunization in sheep. The phosphorylated and non-phosphorylated 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/HCl, pH 7.5, 1 mM EDTA and 0.3 M NaCl) for 2 h with rotation at room temperature (25 °C). The resins were washed three times with coupling buffer (with centrifugation at 1500 ***g*** for 20 s to collect the gel), twice with 0.1 M sodium citrate, pH 4.5, and were then resuspended in 0.1 M sodium citrate, pH 4.5, containing 1 mM 2-mercaptoethanol, followed by incubation for 90 min at room temperature. The resins were extensively washed with PBST (PBS containing 0.1 % Tween 20). Aliquots of serum (16 ml) were then incubated with gel containing the immobilized non-phosphorylated peptide (overnight at 4 °C) before applying the supernatant to a column containing the immobilized phosphopeptide. After extensive washing with PBST, anti-phospho antibodies were eluted with additions of 1 ml of 100 mM glycine and collected in tubes containing 40 μl of 1 M Tris base. The eluted protein fractions were combined, concentrated by ultrafiltration and dialysed against PBS for storage in aliquots at − 20 °C. The anti-total PFK-2 antibody was raised against full-length purified recombinant BH (bovine heart) PFK-2 (see below) in rabbits; anti-PAS (phospho-Akt substrate) and anti-phospho-Ser^{473} PKB antibodies were from Cell Signaling Technology; anti-PH domain PKB and anti-PKBα antibodies were from Upstate Biotechnology; and anti-total $p70^{S6K}$ antibody was from Santa Cruz Biotechnology. The shRNA (small hairpin RNA) plasmid vectors encoding mammalian PKBα siRNA [pKD-AKT1/PKBα-v2], mammalian negative control siRNA [pKD-NegCon-v1], mammalian SGK3 siRNA [pKD-SGK3-v1] and Akt siRNA double-stranded RNAs, which targets both PKBα and PKBβ isoforms, and the corresponding control siRNAs were from Cell Signaling Technology. For the $p70^{S6K}$, SGK3 and PKB assays, the substrate peptides were KKRNRTL<u>S</u>VA (derived from ribosomal protein S6), PVRMRRN<u>S</u>FT (a peptide containing Ser466 of heart PFK-2) and RPRAA<u>T</u>F [33] respectively (the phosphorylated residue is underlined) and were kindly synthesized by V. Stroobant (Ludwig Institute for Cancer Research, Brussels, Belgium). The purified recombinant His_6-tagged BH1 PFK-2 isoform [23] and recombinant activated GST (glutathione transferase)–SGK3 [28] from HEK-293 cells incubated with insulin were produced as described previously.

Culture and transfection of HEK-293T cells

HEK-293T cells were cultured in 10-cm-diameter dishes in DMEM (Dulbecco's minimal essential medium) containing 10 % (w/v) fetal bovine serum. They were transfected on the following day by the JetPEI procedure according to the manufacturer's instructions (PolyPlus Transfection). Cells were transfected with wild-type BH PFK-2 (0.5 μg of BH1–His_6), 5 μg of PKBα shRNA plasmid vector, 5 μg of negative control shRNA plasmid vector, 5 μg of SGK3 shRNA plasmid vector, empty vector (5 μg of PCMV5), 50 nM Akt siRNA and/or 50 nM control siRNA as indicated. Transfection was performed at 37 °C in DMEM containing 10 % (w/v) fetal bovine serum. After 32 h (for protein overexpression), 48 h (for PKB α/β silencing with Akt siRNA), 56 h (for SGK3 silencing with shRNA vector) or 96 h (for PKBα silencing with shRNA vector), the cells were deprived of serum in DMEM for at least 3 h and then incubated with or without 0.1 μM insulin for 10 min. Cells were lysed in 600 μl of ice-cold Buffer A (50 mM Hepes, pH 7.5, 50 mM potassium fluoride, 1 mM potassium phosphate, 1 mM EDTA,

1 mM EGTA, 1 μM microcystin, 1 mM Na_3VO_4, 0.1 % (v/v) 2-mercaptoethanol, 1 mM PMSF, 1 mM benzamidine hydrochloride and 1 μg/ml leupeptin) and stored at -80 °C.

Rat heart perfusion and incubation of isolated cardiomyocytes

All animal experimentation was approved by the Ethical Committee of the Faculty of Medicine, Université catholique de Louvain. Hearts from fed male Wistar rats were perfused by the Langendorff method in Krebs–Henseleit bicarbonate buffer containing 5 mM glucose, and 1 μM Akti-1/2 or DMSO vehicle, for 15 min prior to perfusion with or without 0.1 μM insulin for 10 min [25]. Hearts were freeze-clamped and homogenized in 4 volumes of buffer containing 50 mM Hepes, pH 7.5, 0.2 M sucrose, 1 mM EDTA, 1 mM EGTA, 20 mM sodium fluoride, 5 mM sodium pyrophosphate, 5 mM sodium 2-glycerophosphate, 15 mM 2-mercaptoethanol, 2 mM benzamidine hydrochloride, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 0.2 mM PMSF using an Ultra-Turrax homogenizer (three times for 15 s on ice). Homogenates were centrifuged at 24000 ***g*** for 30 min. Isolated cardiomyocytes were prepared [21] and pre-incubated with 10 μM Akti-1/2 inhibitor or DMSO vehicle for 15 min prior to treatment with or without 0.1 μM insulin for 5 min. For Fru-2,6-P_2 measurement the cells were placed in hot alkali [21]. For enzyme assays the cells were extracted in buffer [1 ml for 60 mg of cells (wet weight)] containing 20 mM Hepes, pH 7.5, 100 mM KCl, 1 mM EDTA, 20 mM sodium floride, 1 mM dithiothreitol, 1 mM PMSF, and 0.1 mM fructose 6-phosphate/0.3 mM glucose 6-phosphate using an Ultra-Turrax homogenizer (three times for 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$\beta^{-/-}$, PDK1$^{fl/L155E}$MckCre$^{-/-}$ and PDK1$^{fl/L155E}$-MckCre$^{+/-}$ (where Mck is muscle creatine kinase) mice were starved overnight and anaesthetized (20 mg/kg of body weight Rompun and 100 mg/kg of body weight Imalgene intraperitoneally) prior to the intravenous injection of insulin (1 m-unit/kg of body weight) via the vena cava. At the indicated times, hearts were freeze-clamped between tongs pre-cooled in liquid nitrogen and homogenized in 4 volumes of buffer containing 20 mM Hepes, pH 7.6, 30 mM KCl, 20 mM sodium floride, 1 mM EDTA, 1 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 0.1 mM PMSF and 0.1 mM fructose 6-phosphate/0.3 mM glucose 6-phosphate. Extracts were prepared as described above for Fru-2,6-P_2 measurement, the PFK-2, PKB and p70^{S6K} assays, and immunoblotting (see below).

Measurement of Fru-2,6-P_2 content and enzyme assay

Fru-2,6-P_2 content and LDH (lactate dehydrogenase) activity were measured as described previously [3]. PFK-2 activity was measured in 5–20 % PEG [poly(ethylene glycol)] 8000 fractions of extracts from cardiomyocytes or HEK-293T cells overexpressing heart PFK-2 as described previously [3,26]. PKB and p70^{S6K} [34] were assayed following immunoprecipitation of 200 μg of extract protein with 0.5 μg of anti-PH domain PKB antibody or 1 μg of anti-p70^{S6K} antibody pre-bound to 20 μl of a 1:1 slurry of Protein G–Sepharose in a final volume of 50 μl of phosphorylation buffer [20] supplemented with 2.5 μM cAMP-dependent protein kinase inhibitor peptide, 40 μM PKB substrate peptide or 200 μM p70^{S6K} substrate peptide, and 0.1 mM [γ-^{32}P]ATP (1000 c.p.m./pmol). After 20 min at 30 °C, 15 μl aliquots were taken and spotted on to Whatman P81 phosphocellulose papers and plunged into ice-cold 75 mM phosphoric acid. After extensive washing, ^{32}P incorporation was measured by liquid scintillation counting. One unit of enzyme activity corresponds to the formation of 1 μmol (PFK-2 or LDH) or 1 nmol (protein kinase) of product per min under the assay conditions.

Immunoblotting

For HEK-293T cells, extracts (30 μg of protein for PKB and SGK3 detection; 5 μg of protein for PFK-2 detection) were subjected to SDS/PAGE (10 % gels). Proteins were electro-eluted on to PVDF 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-knockin mice or perfused rat hearts, 100–130 μg of extracts were loaded on to 10 % (w/v) acrylamide or 7.5 % (w/v) acrylamide pre-cast gels respectively, for SDS/PAGE, electro-elution [blocking of PVDF membranes was with 5 % (w/v) BSA in Tris-buffered saline containing 0.1 % Tween 20]. Membranes were probed with the anti-phospho-Thr^{320} SGK3 antibody (1:100 dilution for a first purification of antibody on extracts from control/PDK-1-knockin mice and 1:1000 dilution of a second purification of antibody on extracts from perfused hearts) or the anti-total SGK3 antibody (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 μg) was incubated with activated GST–SGK3 (12 m-units) purified from insulin-stimulated transfected HEK-293T cells (see above) and 0.1 mM [γ-^{32}P]ATP (1000 c.p.m./pmol) in a final volume of 100 μl of phosphorylation buffer [20]. At various times, 15 μl aliquots were taken for SDS/PAGE (10 % acrylamide mini-gels). The stoichiometry of ^{32}P incorporation was calculated as described previously [26]. To measure the effects of phosphorylation on PFK-2 activity, 1 μg of recombinant heart enzyme was incubated with 12 m-units of PKB or 10 m-units of SGK3 in a final volume of 20 μl of phosphorylation buffer [20] and 1 mM Mg-ATP at 30 °C, prior to dilution for the PFK-2 assay [26].

Phosphorylation site identification by MS

Recombinant heart PFK-2 (5 μg) was incubated as described above with 50 m-units of SGK3 and 0.1 mM [γ-^{32}P]ATP (specific radioactivity 2000 c.p.m./pmol) for 60 min at 30 °C. After SDS/PAGE, bands corresponding to phosphorylated PFK-2 were excised from the gel and digested with trypsin [35]. Peptides were separated by reverse-phase narrow-bore HPLC at a flow rate of 200 μl/min, and radioactive peaks were detected by Cerenkov counting and analysed by nano-ESI (electrospray ionization) tandem MS in a LCQ Deca XP Plus ion-trap mass spectrometer (ThermoScientific).

Other methods

Protein concentration was estimated [36] with γ-globulin as a standard. PFK-2 activities were expressed as specific activities

(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 PEG fractions with the anti-BH PFK-2 antibody and quantification of blots by scanning densitometry. The results are expressed as the means ± S.E.M. for the indicated number of individual experiments. The statistical significance of the results was assessed by a two-sided unpaired or paired Student's *t* test as indicated. Band intensities of immunoblots were quantified by scanning films and processing the image intensities with the program Image J (version 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), 0.1 μM insulin led to a 2-fold activation of SGK3 after 5 min and to a parallel increase in the phosphorylation state of the activation loop Thr^{320} residue of SGK3 (Supplementary Figure S1 at http://www.BiochemJ.org/bj/431/bj4310267add.htm). Recombinant GST–SGK3 purified from insulin-treated HEK-293T cells phosphorylated BH PFK-2 in a time-dependent manner (Figure 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 (Figure 1B). For phosphorylation site identification by MS, heart PFK-2 was maximally phosphorylated by activated GST–SGK3, digested with trypsin and the radioactive peptides were separated by HPLC (Figure 1C). The first peak contained a peptide (N^{465}SFTPLSSSNTIR^{477}) in which Ser^{466} (underlined) was phosphorylated, as determined by tandem MS, whereas the second peak contained a peptide (N^{481}YSVGSRPLQPLSPLR^{496}) in which Ser^{483} (underlined) was phosphorylated. These phosphorylation sites are identical with the *in vitro* sites for PKB [25,26] and with those identified in intact HEK-293 cells transfected with heart PFK-2 and incubated with insulin [23].

As 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 that 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 (Figure 2A). In non-transfected cells, immunoblotting with anti-phospho-Thr^{320} SGK3 antibody revealed a 25-fold increase in band intensity in response to insulin treatment (results not shown). In addition, insulin activated endogenous immunoprecipitated PKB (Figure 2B) and p70^{S6K} (Figure 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 (Figure 2D).

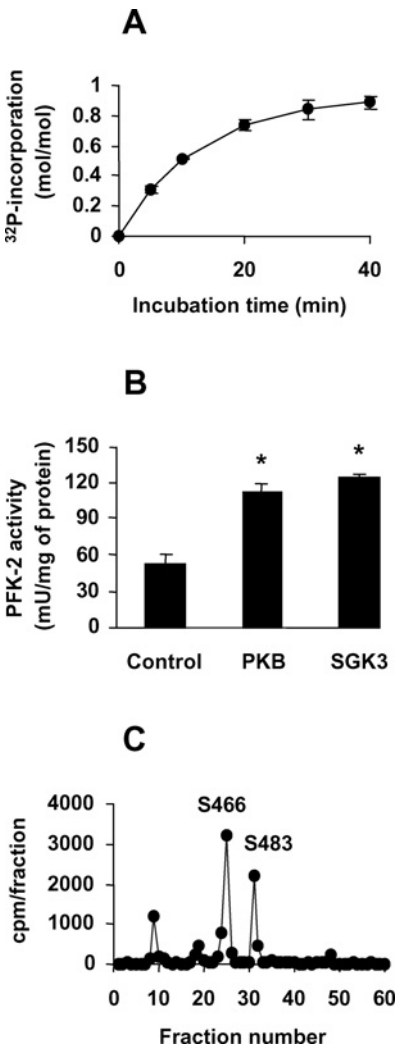

Figure 1 Phosphorylation and activation of heart PFK-2 by SGK3 *in vitro*

(**A**) Recombinant heart PFK-2 was incubated with activated SGK3 as described in the Experimental section. At the indicated times, ^{32}P incorporation (results are means ± S.E.M. for three separate experiments) was measured by phosphoimaging after SDS/PAGE. (**B**) PFK-2 activity was measured under V_{max} conditions (results are means ± S.E.M. for three separate experiments) after phosphorylation of heart PFK-2 with SGK3 or PKB as described in the Experimental section. *$P < 0.05$ compared with the control (paired *t* test). (**C**) Recombinant heart PFK-2 was incubated with SGK3 and 0.1 mM [γ-^{32}P]ATP for SDS/PAGE and in-gel trypsin digestion. Peptides were separated by reverse-phase narrow-bore HPLC and eluting fractions were collected and the radiation measured. The major radiolabelled peaks were analysed by tandem MS to identify the phosphorylation sites.

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 knockin mouse model in which muscle and heart PDK1 harboured a mutation of the key residue Leu^{155} to glutamate in the so-called PIF pocket responsible for binding its downstream targets [38]. Following phosphorylation of the

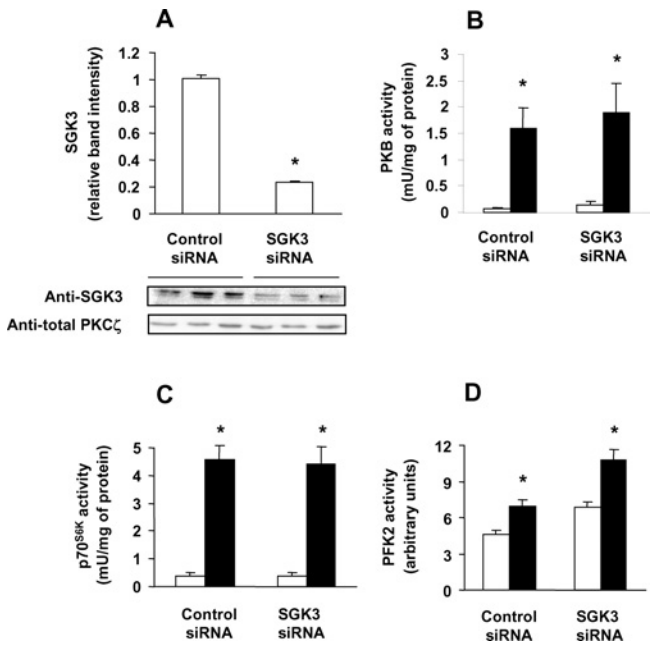

Figure 2 Effect of SGK3 knockdown by siRNA on insulin-induced PFK-2 activation in transfected HEK-293T cells

(**A**) HEK-293T cells were co-transfected with vectors (see the Experimental section) as indicated. Cell extracts were immunoblotted with the anti-total SGK3 and anti-total PKCζ (as a 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 (a represent gel is shown in the lower panel). Following transfection, the cells were incubated with (black bars) or without (white bars) 0.1 μM insulin for 10 min. *$P < 0.05$ for the effect of transfection of SGK3 siRNA compared with the control (paired *t*-test) (**B**) PKB and (**C**) p70^{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 levels, by immunoblotting as described in the Experimental section. The results are means $\pm$ S.E.M. for three separate experiments. *$P < 0.05$ for the effect of insulin compared with the control (paired *t* test).

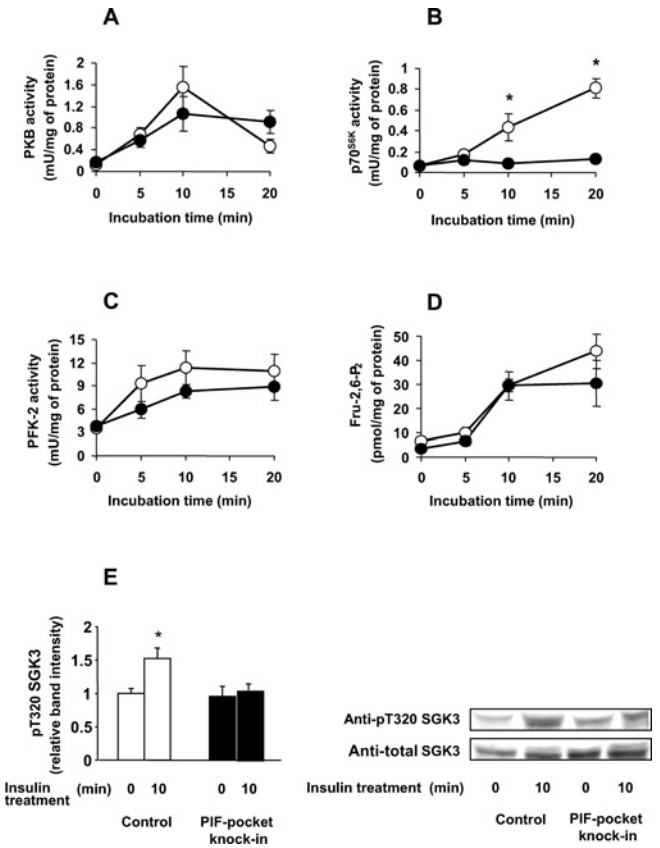

Figure 3 Effect of insulin on the activities of PKB, p70^{S6K} and PFK-2, and the effect on Fru-2,6-P_2 content and SGK3 Thr^{320} phosphorylation in hearts of transgenic mice bearing a muscle-specific mutation in the PDK1-substrate-docking site

PDK1$^{fl/L155E}$MckCre$^{-/-}$ (control, white symbols) mice and PDK1$^{fl/L155E}$MckCre$^{+/-}$ (knockin, black symbols) mice bearing the PIF-pocket mutation were starved for 16 h prior to intravenous injection with insulin. At the indicated times, the hearts were rapidly excised and freeze-clamped. (**A**) PKB and (**B**) p70^{S6K} were immunoprecipitated from extracts and assayed by phosphorylation of synthetic peptides. *$P < 0.05$ for the effect effect of insulin on p70^{S6K} activity in heart extracts from control mice compared with extracts from knockin mice (unpaired *t* test). Extracts were also prepared for the (**C**) PFK-2 assay and (**D**) Fru-2,6-P_2 measurement. The results are the means $\pm$ S.E.M. of measurements on at least four different animals. (**E**) Heart extracts from control PDK1$^{fl/L155E}$MckCre$^{-/-}$ (white bars) and PDK1$^{fl/L155E}$MckCre$^{+/-}$ knockin mice (black bars) after 0 and 10 min of treatment with insulin were blotted with the anti-phospho-Thr^{320} and anti-total SGK3 (loading control) antibodies (a representative blot is shown in the right-hand panel). Following scanning densitometry, band intensities obtained with the anti-phospho -Thr^{320} SGK3 antibody were expressed relative to band intensities of the loading control (left-hand panel). *$P < 0.05$ for the effect of insulin on SGK3 Thr^{320} phosphorylation compared with the 0 min values (unpaired *t* test). The results are means $\pm$ S.E.M. of measurements on three different animals.

hydrophobic motif serine/threonine residue of SGK1, and p70^{S6K} and p90^{RSK} (p90 ribosomal S6 kinase) 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 threonine residue. Mutation of Leu^{155} to glutamate 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 PIP_3 thereby inducing a conformational change allowing PDK1 to phosphorylate its activation loop Thr^{308} residue. Mice were obtained in which the wild-type PDK1 allele had been flanked with LoxP sites (PDK1$^{fl/fl}$) and bred with PDK1$^{L155E/+}$-knockin mice to generate PDK1$^{fl/L155E}$ animals, which were in turn crossed with transgenic mice expressing the Cre recombinase under the *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 knockin 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 (Figure 3A) and activated p70^{S6K} almost 13-fold after 20 min (Figure 3B). In hearts from mice bearing the PIF-pocket mutation (PDK1$^{fl/L155E}$MckCre$^{+/-}$), PKB was activated 4-fold by insulin after 5 min (Figure 3A), whereas p70^{S6K} activation by insulin was completely ablated (Figure 3B). In heart extracts from PDK1$^{fl/L155E}$MckCre$^{-/-}$ and PDK1$^{fl/L155E}$MckCre$^{+/-}$ mice, the insulin-induced increase in GSK3β Ser^{9} phosphorylation was comparable (results not shown), indicating that the PDK1 PIF-pocket mutation did not affect the phosphorylation of PKB targets. Indeed, the time courses of PFK-2 (Figure 3C) activation and the subsequent rise in Fru-2,6-P_2 (Figure 3D) were similar in hearts from PDK1$^{fl/L155E}$MckCre$^{-/-}$ and PDK1$^{fl/L155E}$MckCre$^{+/-}$ mice. In contrast, the increase in SGK3 activation loop Thr^{320} 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 (Figure 3E). Together these 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

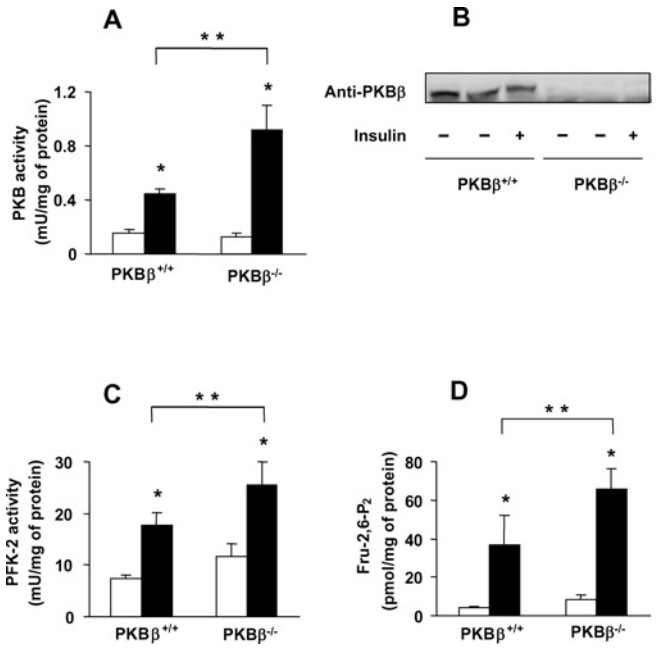

Figure 4 Effect of insulin on PKB and PFK-2 activity and Fru-2,6-P_2 content in hearts of PKBβ-deficient mice

Wild-type (PKB$\beta^{+/+}$) and PKBβ-deficient (PKB$\beta^{-/-}$) mice were starved for 16 h prior to intravenous injection of insulin (black bars) or were untreated (white bars). After 10 min, the hearts were rapidly excised and freeze-clamped. (**A**) Extracts were immunoprecipitated for PKB assay, immunoblotted with anti-PKBβ antibody [(**B**) shows a representative blots from three wild-type and three PKB$\beta^{-/-}$ mice] and also assayed for (**C**) PFK-2 or (**D**) Fru-2,6-P_2 content. The results are the means $\pm$ S.E.M. of measurements on at least four different animals. $^{*}P < 0.05$ for the effect of insulin compared with the controls (unpaired *t* test). $^{**}P < 0.05$ for the effect of insulin in PKB$\beta^{-/-}$ mice compared with wild-type mice (unpaired *t* test).

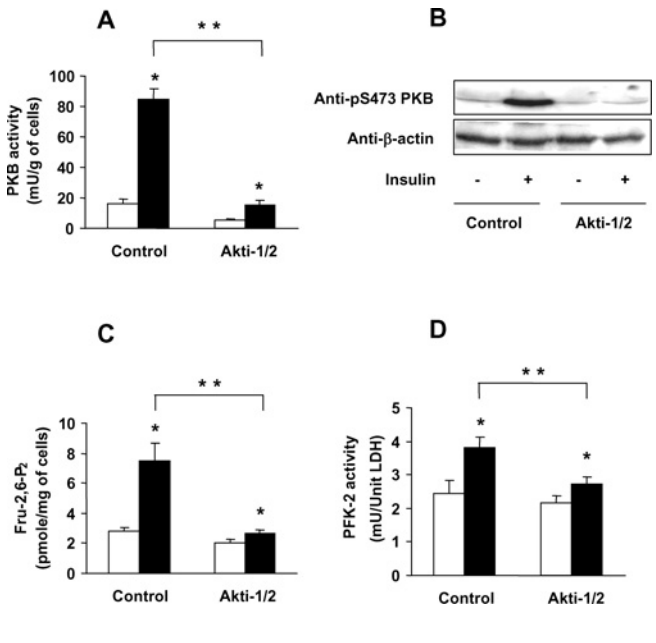

Figure 5 Effect of insulin on PKB and PFK-2 activity, and Fru-2,6-P_2 content in rat cardiomyocytes treated with Akti-1/2 PKB inhibitor

Freshly isolated rat cardiomyocytes were pre-incubated with 10 μM Akti-1/2 inhibitor or DMSO vehicle for 15 min prior to treatment with (black bars) or without (white bars) insulin (0.1 μM) for 5 min. (**A**) Extracts were immunoprecipitated for the PKB assay, (**B**) immunoblotted with the anti-phospho-Ser^{473} PKB and anti-β-actin (loading control) antibody (a representative blot is shown), and assayed for (**C**) Fru-2,6-P_2 content and (**D**) PFK-2 activity. The results are the means $\pm$ S.E.M. of measurements on four different cell preparations. $^{*}P < 0.05$ for the effect of insulin compared with the controls (paired *t* test). $^{**}P < 0.05$ for the effect of insulin in cells treated with Akti-1/2 compared with control incubations (paired *t* test).

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 PKBβ-deficient mice

The studies on insulin-induced PFK-2 activation in hearts from mice bearing the PIF-pocket mutation strongly suggests that PKB, and not SGK3, is mediating the effect of insulin. Therefore we examined PFK-2 activation by insulin in hearts from PKBβ-deficient mice. These mice are viable, but insulin-resistant, whereas mice deficient in PKBα 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 PKBβ-deficient mice, the hearts were freeze-clamped and cell extracts were obtained for PKB activity measurement after immunoprecipitation with the anti-PH-domain PKB antibody (which recognizes PKBα and PKBβ). Insulin induced a 3-fold activation of PKB in hearts from wild-type mice but surprisingly, in hearts from PKBβ-deficient mice, a 7-fold activation of PKB by insulin was seen (Figure 4A). This increase in PKB activity in hearts from the knockout mice suggests that the deletion of PKBβ results in over-compensation by PKBα. The absence of PKBβ in heart extracts from PKBβ-deficient mice was apparent after immunoblotting with the isoenzyme-specific antibody (Figure 4B). However, owing to the fact that the available anti-PKBα antibody was not specific and cross-reacts with PKBβ, we could not test for over-compensation by PKBα either by immunoblotting or by the immunoprecipitation/PKB assay. In parallel with the rise in insulin-induced PKB activity in hearts from PKBβ-deficient mice, the increase in PFK-2 activity (Figure 4C) and Fru-2,6-P_2 content (Figure 4D) was greater in hearts from PKBβ-deficient mice. This correlation supports the implication that PKB mediates insulin-induced heart activation and indicates that this effect is not mediated by PKBβ.

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

Cultured cardiomyocytes are difficult to transfect for PKB isoform expression knockdown by RNA interference. Therefore freshly isolated rat cardiomyocytes were pre-incubated with or without the highly selective Akti-1/2 inhibitor, then with or 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 pre-incubation with the Akti-1/2 inhibitor (results not shown). Insulin treatment activated PKB approx. 4-fold after immunoprecipitation from cardiomyocyte extracts, the effect being drastically reduced by pre-incubation with the Akti-1/2 inhibitor (Figure 5A). Likewise, immunoblotting revealed that the Akt-1/2 inhibitor blocked insulin-induced PKB Ser^{473} phosphorylation (Figure 5B). Moreover, the insulin-induced rise in Fru-2,6-P_2 (Figure 5C) and PFK-2 activation (Figure 5D) were abrogated in cardiomyocytes pre-incubated

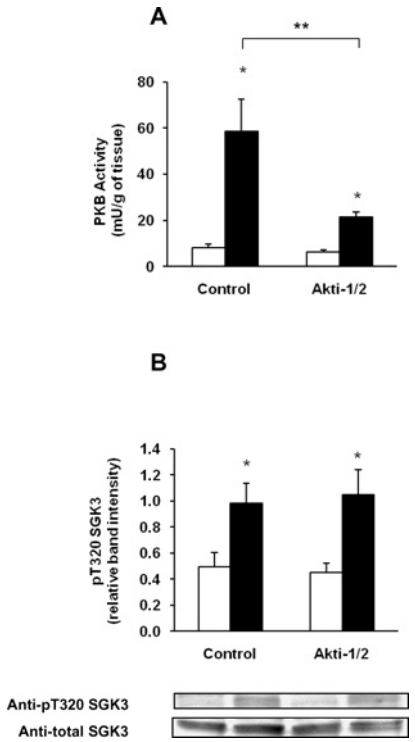

Figure 6 Effect of insulin on PKB activity and SGK3 Thr^{320} phosphorylation in rat hearts perfused with Akti-1/2 PKB inhibitor

Rat hearts were perfused with 5 mM glucose, and DMSO vehicle or 10 μM Akti-1/2 inhibitor for 15 min prior to perfusion with (black bars) or without (white bars) 0.1 μM insulin for 10 min then freeze-clamped and homogenized. (**A**) Extracts were immunprecipitated for the PKB assay or (**B**) immunoblotted with anti-phospho-Thr^{320} 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-Thr^{320} SGK3 antibody were expressed relative to band intensities of the loading controls (upper panel). The results are the means $\pm$ S.E.M. for four separate experiments. *$P < 0.05$ for the effect of insulin compared with the controls (unpaired t test). **$P < 0.05$ for the effect of insulin in hearts treated with Akti-1/2 compared with the controls (unpaired t test).

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. As in cardiomyocytes (Figure 5A), insulin induced a robust (6-fold) activation of PKB in perfused hearts, which was markedly decreased by pre-treatment with Akti-1/2 (Figure 6A). As observed in control PDK1$^{fl/L155E}$MckCre$^{-/-}$ mice injected with insulin (Figure 3E), perfusion of rat hearts with insulin led to an increase in SGK3 Thr^{320} activation loop phosphorylation (Figure 6B). However, the insulin-induced increase in SGK3 Thr^{320} phosphorylation was unaffected by pre-treatment with Akti-1/2 (Figure 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 His$_6$-tagged recombinant BH PFK-2 [23] and either control siRNA or PKBα/β siRNA, to knockdown the expression of both PKB isoforms. Immunoblotting with the anti-total PH-domain PKB antibody indicated that the PKBα/β siRNA treatment decreased the PKB content of the cells by more than 80 % without affecting PKCζ expression (used as a loading control) (Figure 7A). In order to evaluate the efficiency of PKB

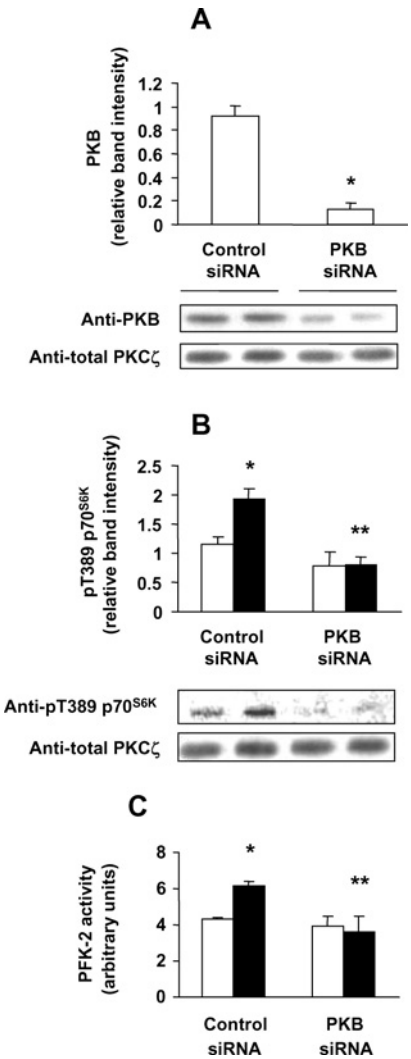

Figure 7 Effect of PKBα/β knockdown by siRNA on insulin-induced PFK-2 activation in transfected HEK-293T cells

HEK-293T cells were co-transfected with siRNA vectors (see the Experimental section) as indicated. (**A**) Extracts were immunoblotted with anti-total PH-domain PKB and anti-total PKCζ (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 (white bars) 0.1 μM insulin for 10 min. (**B**) Extracts were immunoblotted with anti-phospho-Thr^{389} p70^{S6K} and anti- PKCζ (loading control) antibodies for quantification of p70^{S6K} phosphorylation relative to the loading control as described for (**A**) (a representative blot 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 levels, by immunoblotting as described in the Experimental section. The results are the means $\pm$ S.E.M. for three separate experiments. *$P < 0.05$ for the effect of insulin compared with the control (paired t test). **$P < 0.05$ for the effect of insulin in cells transfected with PKB siRNA compared with cells transfected with control siRNA (paired t test).

knockdown, the phosphorylation state of Thr^{389} of p70^{S6K}, an indirect downstream target, was measured by immunoblotting. In cells treated with control siRNA, insulin increased p70^{S6K} Thr^{389} phosphorylation by approx. 50 %, which was completely abrogated by transfection with PKBα/β siRNA (Figure 7B). Moreover, in PKBα/β siRNA-treated HEK-293T cells, heart PFK-2 activation by insulin was likewise abolished (Figure 7C). The results suggest that PKBα and/or PKBβ is involved in insulin-induced heart PFK-2 activation.

In HEK-293T cells co-transfected with His_6-tagged BH PFK-2 and PKBα siRNA, a PKBα knockdown of at least 50 % was achieved after 96 h of transfection (Supplementary Figure S2A at http://www.BiochemJ.org/bj/431/bj4310267add.htm); however, the anti-PKBα antibody used for immunoblotting was not totally specific and cross-reacts to some extent with PKBβ. Total PKB expression was unchanged (Supplementary Figure S2A) and there was no effect of PKBα knockdown on PKB Ser^{473} phosphorylation (Supplementary Figure S2B). In addition, by immunoblotting with the specific antibodies, we were unable to detect any change in expression of PKBβ or PKBγ following PKBα knockdown (results not shown). The 96 h time frame of transfection needed to achieve a reasonable knockdown in the PKBα 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. PKBα siRNA transfection abrogated insulin-induced PFK-2 phosphorylation (Supplementary Figure 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 [40] 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. 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 [40] 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 cAMP. 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 selective kinase inhibitors, by transfecting cells with vectors overexpressing constitutively active and/or dominant-negative forms of the kinase, by knockdown of the kinase by transfecting siRNAs, or by using mouse knockout or transgenic models.

In the present study 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 Ser^{466} and Ser^{483}, the same sites phosphorylated by PKB *in vitro*. As 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 knockin mice and use of the Akti-1/2 selective PKB inhibitor (see below). Our previous conclusions that PKB was not involved in 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 and 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 are 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 knockdown by siRNA. The fact that insulin-induced heart PFK-2 activation was still observed in conditional knockin mice bearing a mutation in the PDK1 substrate-binding motif (Figures 3C and 3D) argues against the involvement of kinases other than PKB (those containing a hydrophobic phosphorylation site motif but without a PH domain) for insulin-induced PFK-2 activation. Indeed, the increase in SGK3 activation loop Thr^{320} phosphorylation induced by insulin injection in control mice was abolished in the PDK1 PIF-pocket knockin mice (Figure 3E). In freshly isolated rat cardiomyocytes incubated with the Akti-1/2 inhibitor, PFK-2 activation by insulin was almost ablated (Figure 5D) and in hearts perfused with Akti-1/2 insulin-induced PKB activation markedly decreased, whereas the increase in SGK3 Thr^{320} phosphorylation was unaffected (Figure 6). Thus, whereas the PDK1 PIF-pocket knockin mouse model can differentiate between insulin effects on PKB activity and SGK3 Thr^{320} phosphorylation *in vivo* (Figure 3), the use of Akti-1/2 in perfused hearts can distinguish between PKB activation and SGK3 Thr^{320} phosphorylation by insulin *ex vivo* (Figure 6). Experiments in HEK-293T cells after PKBα/β siRNA knockdown clearly implicated either PKBα or PKBβ in heart PFK-2 activation by insulin (Figure 7). Unexpectedly, in hearts from PKBβ-deficient mice, insulin-induced PFK-2 activation was not abolished and was even greater than in wild-type mice, presumably due to overcompensation by PKBα in hearts of the knockout animals (Figure 4). Hence, we conclude that PKBβ does not mediate heart PFK-2 activation by insulin, which we propose rather involves PKBα, as supported by the fact that siRNA knockdown of this PKB isoenzyme in HEK-293T cells ablated heart PFK-2 phosphorylation (Supplementary Figure S2).

AUTHOR CONTRIBUTION

Véronique Mouton and Louise Toussaint contributed equally and performed most of the experimental work. Didier Vertommen performed the phosphorylation site identification by MS. Marie-Agnès Gueuning performed the immunoblot SGK3 phosphorylation analyses on hearts and cardiomyocytes. Liliane Maisin conducted the experiments on perfused rat hearts and isolated rat cardiomyocytes. Xavier Havaux, Cossette Sanchez-Canedo and Luc Bertrand carried out the animal experiments on control/PDK1-knockin mice and wild-type/PKBβ-deficient mice injected with insulin. Reagents for studying phosphorylation by purified recombinant SGK3 were processed in the laboratory of Franck Dequiedt. Wild-type and PKBβ-deficient mice were generated and bred by Brian Hemmings. Louis Hue participated in conception and design, and analysis and interpretation of the results. Mark Rider was involved in conception and design, analysis and interpretation of the results and writing the article.

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online data

SUPPLEMENTARY ONLINE DATA

Heart 6-phosphofructo-2-kinase activation by insulin requires PKB (protein kinase B), but not SGK3 (serum- and glucocorticoid-induced protein kinase 3)

Véronique MOUTON*[1], Louise TOUSSAINT*†[1], Didier VERTOMMEN*, Marie-Agnès GUEUNING*, Liliane MAISIN*, Xavier HAVAUX‡, Cossette SANCHEZ-CANEDO‡, Luc BERTRAND‡, Franck DEQUIEDT†, Brian A. HEMMINGS§, Louis HUE* and Mark H. RIDER*[2]

*Université catholique de Louvain and de Duve Institute, 75 Avenue Hippocrate, B-1200 Brussels, Belgium, †Cellular and Molecular Biology Unit, FUSAGx, 13 Avenue Marechal Juin, 5030 Gembloux, Belgium, ‡Université catholique de Louvain Division of Cardiology, 55 Avenue Hippocrate, CARD5550, B-1200 Brussels, Belgium, and §Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, 4058 Basel, Switzerland

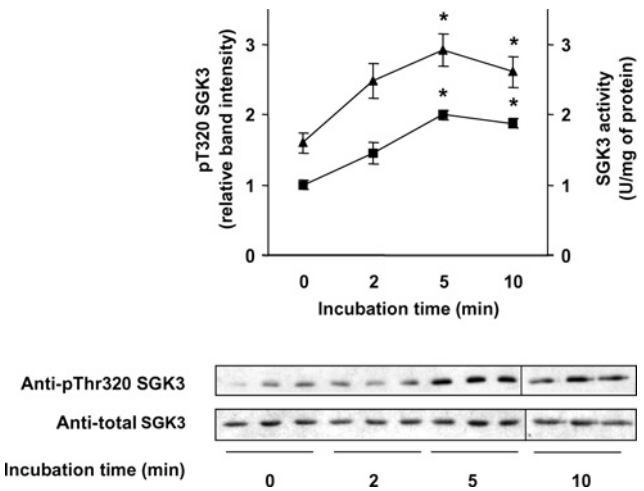

Figure S1 Effect of insulin on GST–SGK3 activation and phosphorylation in transfected HEK-293T cells

HEK-293T cells expressing the wild-type SGK3 GST-fusion construct were treated with 0.1 μM insulin for the indicated times. GST–SGK3 activity was measured in extracts after immunoprecipitation and extracts were immunoblotted with anti-phospho-Thr^{320} SGK3 and anti-total SGK3 antibodies as a loading control (a representative blot is shown in the lower panel). Following scanning densitometry, band intensities obtained with anti-phospho-Thr^{320} SGK3 antibody were calculated relative to band intensities for the loading control (upper panel). The results are means $\pm$ S.E.M. for three separate experiments. *$P < 0.05$ for the effect of insulin compared with the controls (paired *t* test).

[1] These authors contributed equally to this work.
[2] To whom correspondence should be addressed (email mark.rider@uclouvain.be).

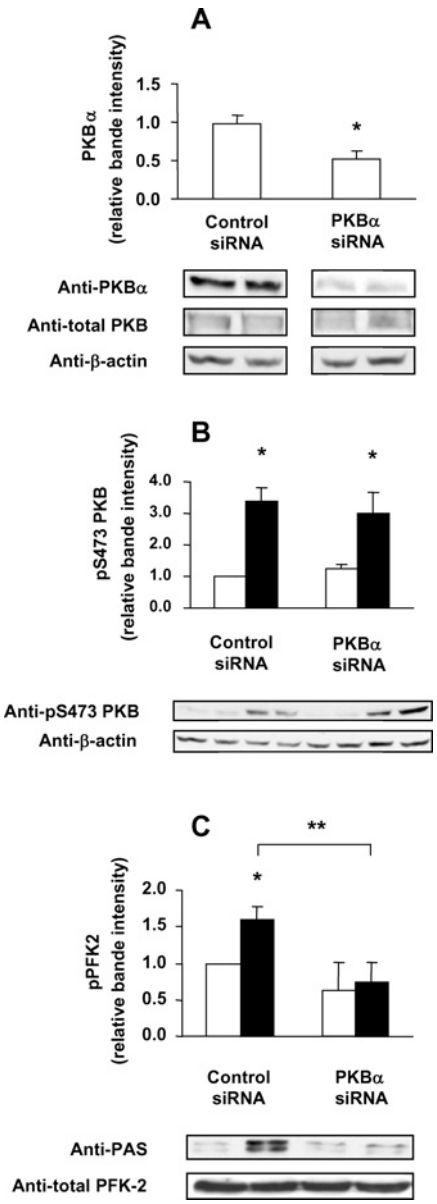

Figure S2 Effect of PKBα knockdown by siRNA on insulin-induced PFK-2 activation in transfected HEK-293T cells

HEK-293T cells were co-transfected with siRNA vectors (see the Experimental section in the main paper) as indicated. (**A**) Extracts were immunoblotted with the anti-PKBα, anti-total PH-domain PKB and anti-β-actin (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 PKBα 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 (white bars) 0.1 μM insulin for 10 min. (**B**) Extracts were immunoblotted with anti-phospho Ser^{473} and anti-β-actin (loading control) antibodies for quantification of PKB phosphorylation as described for (**A**) (a representative blot is shown below the histogram). (**C**) Extracts were immunoblotted with anti-PAS and anti-full-length heart PFK-2 (loading control) antibodies for quantification of heart PFK-2 phosphorylation as described above (a representative blot is shown below the histogram). The results are the means $\pm$ S.E.M. for three separate experiments. *$P < 0.05$ for the effect of insulin compared with the control (paired t test). **$P < 0.05$ for the effect of insulin in cells transfected with PKBα siRNA compared with cells transfected with control siRNA (paired t test).

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