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Insulin signalling downstream of protein kinase B is potentiated by 5′AMP-activated protein kinase in rat hearts in vivo

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Abstract *Aims/hypothesis:* 5′AMP-activated protein kinase (AMPK) and insulin stimulate glucose transport in heart and muscle. AMPK acts in an additive manner with insulin to increase glucose uptake, thereby suggesting that AMPK activation may be a useful strategy for ameliorating glucose uptake, especially in cases of insulin resistance. In order to characterise interactions between the insulin- and AMPK-signalling pathways, we investigated the effects of AMPK activation on insulin signalling in the rat heart in vivo. *Methods:* Male rats (350–400 g) were injected with 1 g/kg 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) or 250 mg/kg metformin in order to activate AMPK. Rats were administered insulin 30 min later and after another 30 min their hearts were removed. The activities and phosphorylation levels of components of the insulin-signalling pathway were subsequently analysed in individual rat hearts. *Results:* AICAR and metformin administration activated AMPK and enhanced insulin signalling downstream of protein kinase B in rat hearts in vivo. Insulin-induced phosphorylation of glycogen synthase kinase 3 (GSK3) β, p70 S6 kinase ($p70^{S6K}$)(Thr389) and IRS1 (Ser636/639) were significantly increased following AMPK activation. To the best of our knowledge, this is the first report of heightened insulin responses of GSK3β and $p70^{S6K}$ following AMPK activation. In addition, we found that AMPK inhibits insulin stimulation of IRS1-associated phosphatidylinositol 3-kinase activity, and that AMPK activates atypical protein kinase C and extracellular signal-regulated kinase in the heart. *Conclusions/interpretations:* Our data are indicative of differential effects of AMPK on the activation of components in the cardiac insulin-signalling pathway. These intriguing observations are critical for characterisation of the crosstalk between AMPK and insulin signalling.

Keywords AICAR · AMPK · GSK3 · Heart · Insulin · Insulin signalling · Metformin · $p70^{S6K}$ · PI3-kinase · PKB

Abbreviations ACC: acetyl-CoA carboxylase · AICAR: 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside · AMPK: 5′AMP-activated protein kinase · aPKC: atypical protein kinase C · ERK: extracellular signal-regulated kinase · GSK3: glycogen synthase kinase 3 · IR: insulin receptor · mTOR: mammalian target of rapamycin · $p70^{S6K}$: p70 S6 kinase · $p90^{RSK}$: p90 ribosomal S6 kinase · PDK1: phosphoinositide-dependent protein kinase 1 · PI3-kinase: phosphatidylinositol 3-kinase · PKB: protein kinase B · TSC: tuberous sclerosis complex

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Introduction

5′AMP-activated protein kinase (AMPK), a heterotrimeric serine/threonine kinase composed of a catalytic (α) subunit and two regulatory (β and γ) subunits, phosphorylates and inactivates key enzymes involved in ATP-consuming pathways and activates ATP-producing pathways, thus acting as a sensor of the cellular energy state [1]. In the heart, AMPK stimulates fatty acid oxidation by phosphorylating and inactivating acetyl-CoA carboxylase (ACC), thereby decreasing the level of malonyl CoA [2] and relieving its inhibitory effect on long-chain fatty acid entry into the mitochondria. In addition, activated AMPK stimulates myocardial glucose uptake by increased SLC2A4 (also known as GLUT4) translocation [3] and glycolysis [4]. Several studies have implicated AMPK as an important protein involved in acute myocardial metabolism regulation [5]. Of particular interest, cardiac AMPK is essential in the

regulation of glucose uptake and glycolysis during ischaemia [6], in which the α2 catalytic subunit of AMPK has been reported to play a critical role [7]. In addition, there is growing evidence that AMPK may play an important role in regulating metabolic protein expression in response to chronic metabolic stress in the heart [5, 8].

Insulin signalling is initiated by hormone binding to the insulin receptor (IR), which activates IR tyrosine kinase leading to its autophosphorylation on multiple tyrosine residues and subsequent phosphorylation of IRS. Generally speaking, insulin signalling downstream of IRS is mediated by at least two pathways; that of mitogen-activated protein kinase and that of phosphatidylinositol 3-kinase (PI3-kinase). PI3-kinase produces phosphatidylinositol (PI) 3,4,5-P_3 and PI 3,4-P_2, which bind to pleckstrin-homology domains of at least two different serine/threonine protein kinases, namely phosphoinositide-dependent protein kinase 1 (PDK1) and protein kinase B (PKB). PDK1 participates in the phosphorylation and activation of several downstream protein kinases including PKB, p70 S6 kinase ($p70^{S6K}$), and atypical protein kinase C (aPKC, λ and ζ). The mammalian target of rapamycin (mTOR) is a downstream target of PKB and, when stimulated, promotes $p70^{S6K}$ phosphorylation. Glycogen synthase kinase 3 (GSK3) is another of the many targets of PKB, but may also be phosphorylated and inhibited by p90 ribosomal S6 kinase ($p90^{RSK}$), which lies downstream of extracellular signal-regulated kinase (ERK) [9]. Following PI3-kinase stimulation by insulin, glucose uptake is increased in the heart.

AMPK activation by 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) stimulates glucose transport in skeletal muscle [10–12] and in heart [3] in the absence of PI3-kinase activation, suggesting that this action is not dependent upon the insulin-signalling pathway, at least at or above the level of PI3-kinase. The mechanisms responsible for AMPK stimulation of glucose transport are uncertain, but may involve aPKC and the ERK pathway [13] and the nitric oxide pathway [14]. Thus, AMPK may be an important target for regulating cardiac glucose metabolism, especially under conditions in which insulin-stimulated glucose use is inhibited, such as poorly controlled diabetes.

Interactions between the insulin signalling and AMPK pathways have yet to be completely characterised. Stimulation of AMPK has been reported to increase insulin sensitivity at the level of glucose transport [15–17] and to correlate with an increase in insulin-stimulated IRS1-associated PI3-kinase activity [18]. However, effects of the AMPK activators metformin and thiazolidinediones on insulin sensitivity remain unclear. In skeletal muscle metformin has been reported both to enhance insulin action [19], and not to alter it [20, 21]. Discrepancies in the effects of metformin on insulin signalling may result from differences in metformin concentration or extracellular accumulation [22]. Similarly, thiazolidinediones have been demonstrated not to alter insulin sensitivity [21], or to enhance it [23–25]. Thus, the role of AMPK in enhanced insulin sensitivity remains to be characterised. AMPK may also inhibit mTOR signalling by enhancing its suppression by the tuberous sclerosis complex (TSC)1/2 [26]. TSC2 possesses a GTPase-activating protein for the small G-protein Rheb, which mediates mTOR signalling, although the precise mechanisms remain unclear [27]. Alternatively, PI3-kinase/PKB or ERK activation may promote mTOR activation through the reduced inhibition of TSC1/2 [27]. Further, in the heart, insulin may reduce AMPK activity [28, 29]. Interestingly, this inhibitory effect was found to be sensitive to wortmannin [29], consistent with the report that PKB activation leads to decreased AMPK activity [30]. Thus, AMPK has been reported to both promote and suppress components of insulin signalling. In addition, the insulin regulation of AMPK activity further complicates the interactions between these pathways. Moreover, the use of therapies such as the AMPK activator metformin in the treatment of diabetes highlights the importance of understanding the effects of AMPK activation on insulin signalling. In this study, we investigate the effects of AMPK activation on the insulin response in the rat heart in vivo.

Materials and methods

Materials AICAR was obtained from Toronto Research Chemicals (North York, ON, Canada). Insulin was purchased from Novo-Nordisk (Bagsvaerd, Denmark) and radioisotopes from Amersham (Buckinghamshire, UK). ACC, phospho-ACC(Ser79), phospho-tyrosine, IRS1, p85 and ERK antibodies were from Upstate Biotechnology (Lake Placid, NY, USA). AMPK-α, phospho-PKB(Ser473), phospho-PKB(Thr308), PKB, phospho-$p70^{S6K}$ and phospho-GSK3α/β(Ser21/9), phospho-ERK and phospho-IRS1 (Ser636/639) antibodies and the SAMS peptide were from Cell Signaling Technology (Beverly, MA, USA). IR β-subunit, GSK3β and $p70^{S6K}$ antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Unless stated otherwise, all other chemicals were obtained from Sigma (St Louis, MO, USA).

Experimental approach Male Sprague-Dawley rats (350–400 g) were fasted overnight and given either an s.c. injection of AICAR (1 g/kg body weight), metformin (250 mg/kg) or vehicle (0.9% NaCl). We chose to use 250 mg/kg metformin as this dosage has been reported to increase AMPK activity in mouse heart [31]. As we aimed to produce an acute stimulation of AMPK activity in the heart, we chose to inject metformin, rather than to administer it in the drinking water. For this acute stimulation, s.c. injections were used for metformin in order to maintain consistency with AICAR, which was injected as previously described [17, 32–34]. Thirty minutes later, rats were injected i.p. with 50 U/kg insulin or vehicle (PBS). This dose of insulin has been reported to provoke a maximal response in rats [35]. Thirty minutes following insulin injection, rats were anaesthetised with sodium pentobarbital (100 mg/kg) and hearts were excised and immediately frozen in liquid nitrogen. As expected, AICAR, metformin and insulin reduced the glycaemia, and thus glucose (4 mmol/l in sterile 0.9% saline solution) was administered through i.p.

Table 1 Measured blood glucose levels

	Glycaemia (mmol/l, mean±SEM)	
	At time of insulin injection	At time of heart removal
Control	4.6±0.3	5.1±0.2
Insulin	4.7±0.2	3.2±0.1*,#
AICAR	4.4±0.3	4.5±0.9
AICAR + insulin	4.8±0.5	3.3±0.2*
Metformin	5.5±0.5	4.5±0.4
Metformin+insulin	5.6±0.4	2.7±0.3*,#

*Significantly different from corresponding value at the time of insulin injection; #, significantly different from corresponding value without insulin; $p<0.05$, n=4–8 hearts

injections in order to eliminate any differences in glycaemia between treatment groups. Blood glucose was measured using a Glucotrend Blood Glucose Monitor (Boehringer Mannheim; Mannheim, Germany). Serum was prepared from blood samples taken at the time of heart excision and used to measure AICAR levels with a previously described protocol [36]. Insulin, glucagon, NEFA, corticosterone and adrenaline were also measured in the serum. Commercial kits for these measurements were purchased as follows: insulin and glucagon from Linco (St Charles, MO, USA), NEFAs from Wako (Osaka, Japan), corticosterone from Diagnostic Systems Laboratories (Webster, TX, USA) and adrenaline from IBL (Hamburg, Germany). All assays were performed following the manufacturers' instructions. This investigation conforms to the current Guidelines for the Care and Use of Laboratory Animals of the National Institute of Health and Medical Research of France (INSERM, France).

Western blot analysis Heart lysates were obtained as previously described [37]. Heart lysates (30–50 μg) or immunoprecipitates (from 0.5-1.0 mg protein of heart lysate) were subjected to SDS-PAGE, and transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA) using standard procedures. Each gel lane contains protein from only one heart, and each heart sample is from a different individual rat. Blots were probed with the appropriate horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (Jackson Immunoresearch Laboratories, West Grove, PA, USA) and visualised by the enhanced chemiluminescence system (Amersham).

Enzyme activity measurements AMPK activity in heart lysates was measured following immunoprecipitation as previously described [38, 39]. PI3-kinase lipid kinase assays were carried out as reported [40]. As previously described, aPKCs (PKC-λ and PKC-ζ) were immunoprecipitated from cell lysates with a rabbit polyclonal antiserum (Santa Cruz Biotechnology) that recognises the C-termini of both PKC-λ and PKC-ζ, collected on Sepharose-AG beads, and incubated for 8 min at 30°C in 100 μl buffer containing 50 mmol/l Tris/HCl (pH 7.5), 100 μmol/l Na_3VO_4, 100 μmol/l $Na_4P_2O_7$, 1 mmol/l NaF, 100 μmol/l phenylmethylsulphonylfluoride, 4 μg phosphatidylserine, 50 μmol/l [γ-^{32}P]ATP (NEN Life Science Products, Boston, MA, USA), 5 mmol/l $MgCl_2$, and, as substrate, 40 μmol/l serine analogue of the PKC-ε pseudosubstrate (BioSource, Camarillo, CA, USA) [41]. After incubation, ^{32}P-labelled substrate was trapped on P-81 filter papers and counted.

Statistical analysis Results are presented as the means± SEM. n represents the number of hearts, each from a different individual rat, used in each measurement. Differences between the groups were compared with the two-tailed unpaired Student's t-test. The Bonferonni correction was applied to the p values obtained to correct for multiple comparisons. A corrected p value <0.05 was considered significant.

Results

No differences in glycaemia were measured among the different groups at the time of insulin injection (30 min following AICAR/metformin/vehicle injection). At the time of killing, glycaemia within each treatment group was significantly reduced by insulin. No differences were observed between the different treatment groups without insulin, i.e. control, AICAR and metformin. Similarly, no differences were observed between the different treatment

Table 2 Serum levels of AICAR, insulin, glucagon, NEFA, corticosterone and adrenaline

	Control		AICAR		Metformin	
	No insulin	Insulin	No insulin	Insulin	No insulin	Insulin
AICAR (μg/ml)	8.0±0.8	8.5±0.8	30.3±5.1#,§	41.0±7.6#,§	9.0±1.7	8.8±2.8
Insulin (ng/ml)	1.6±0.3	1186±89*	2.2±0.8	1347±208*	1.7±0.1	942.8±135*
Glucagon (pg/ml)	27±2.1	55±6.5*	41±2.8#	76±6.8*	30±5.0	66±8.0*
NEFAs (mmol/l)	0.252±0.014	0.038±0.003*	0.041±0.006#,§	0.019±0.002*,#	0.293±0.037	0.031±0.004*
Corticosterone (mg/ml)	722±132	604±57	630±74	899±85#	383±73	887±90*
Adrenaline† (ng/ml)	0.056±0.009	0.496±0.052	0.138±0.021	0.222±0.050	0.063±0.016	0.091±0.016

Values are (means±SEM)
*Significantly different from corresponding value without insulin; #, significantly different from corresponding value without AICAR or metformin; §, significantly different from corresponding value with metformin; $p<0.05$, n=4–7 hearts except for adrenaline values (†) for which n=2–3

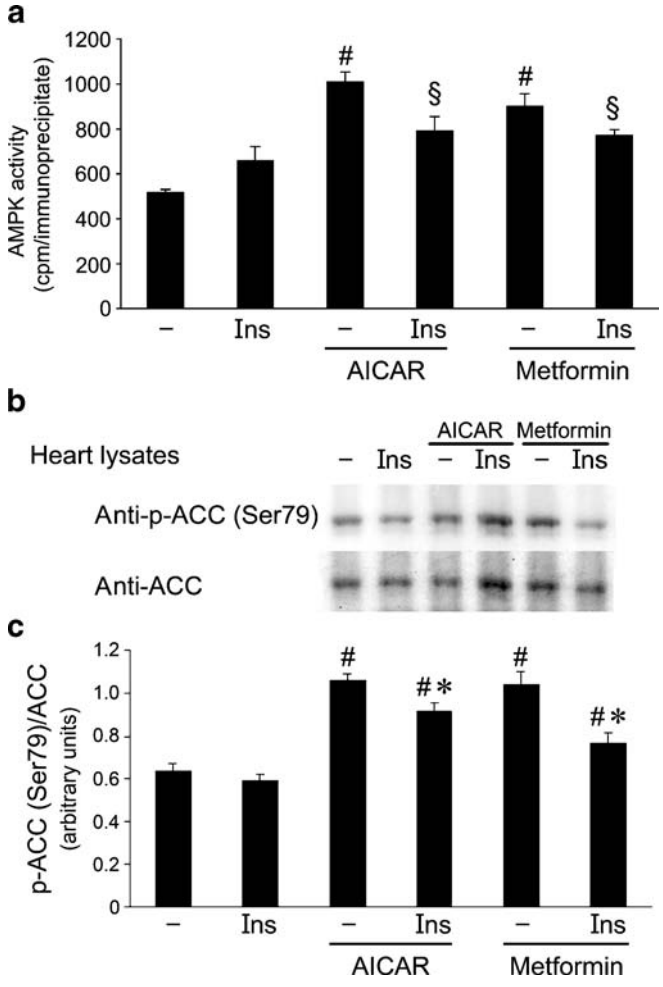

Fig. 1 AMPK activity and ACC phosphorylation. Heart lysates from rats injected with vehicle (−) or insulin (Ins), with and without AICAR or metformin were used for activity measurements and western blot analysis. Membranes were probed with an antibody to phospho-ACC(Ser79), stripped and re-probed with an antibody to ACC (**b**). Results were quantified, normalised to total levels and plotted (**c**). Values are means±SEM. *, significantly different from corresponding value without insulin; #, significantly different from corresponding value without AICAR or metformin; §, significantly different from value without insulin, AICAR or metformin; $p<0.05$, n=4–9 hearts

groups following insulin injection, i.e. control+insulin, AICAR+insulin and metformin+insulin (Table 1). Serum AICAR levels were significantly elevated in groups receiving AICAR compared with control or metformin groups (Table 2). Serum insulin levels correspond well with previously reported basal values [42] and were significantly increased in rats given insulin (Table 2). Glucagon levels corresponded well with previously published values [43] and were significantly increased with insulin, as expected [44]. Glucagon levels were also increased to similar

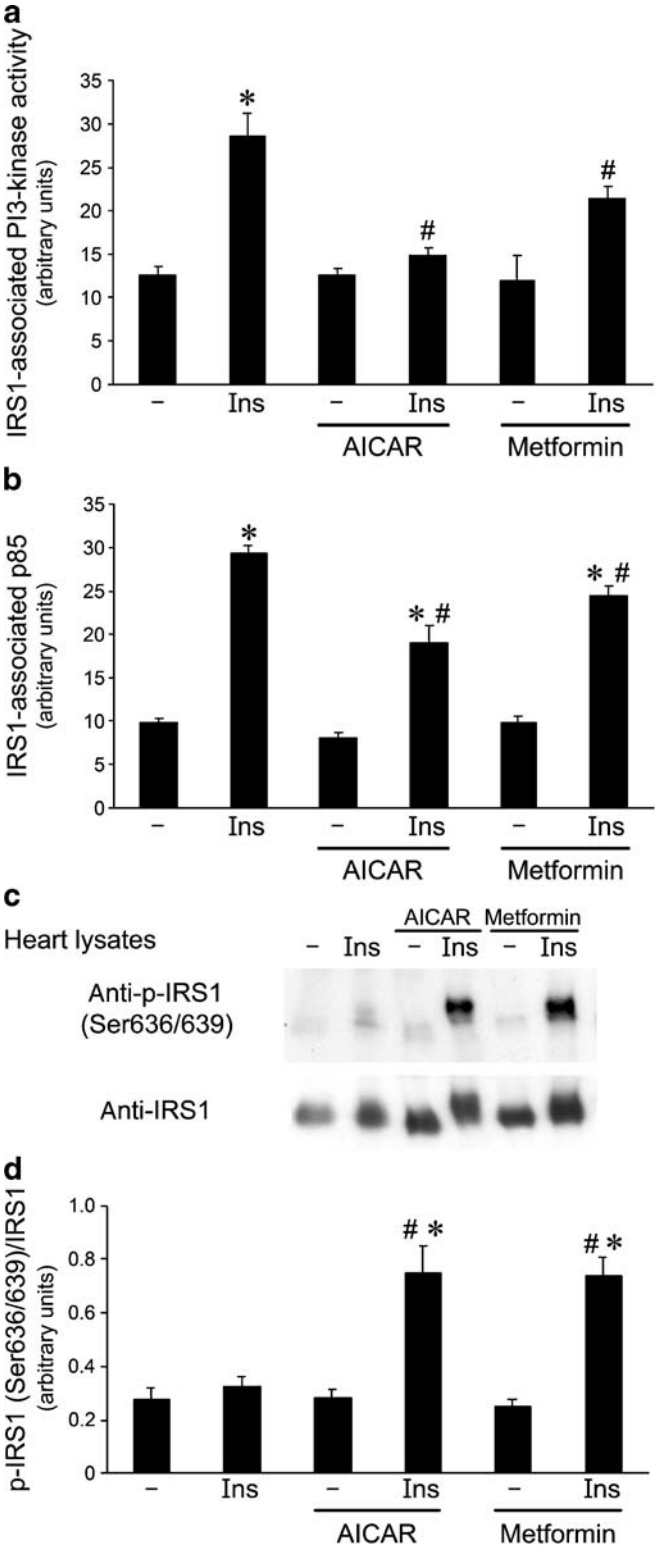

Fig. 2 IRS1-associated PI3-kinase activity and phosphorylation. IRS1-associated PI3-kinase activity (**a**) or p85 levels (**b**) were measured in immunoprecipitates from heart lysates using IRS1 antibodies from rats injected with vehicle (−) or insulin (Ins), with and without AICAR or metformin. Heart lysates from rats injected with vehicle (−) or insulin (Ins), with and without AICAR or metformin were used for western blot analysis. Membranes were probed with an antibody to phospho-IRS1(Ser636/639), stripped and re-probed with an antibody to IRS1 (**c**). Results were quantified, normalised to total levels and plotted (**d**). Values are means±SEM. *, significantly different from corresponding value without insulin; #, significantly different from corresponding value without AICAR or metformin; $p<0.05$, n=3–9 hearts

levels with AICAR alone, but were not altered with metformin. Serum NEFAs were significantly reduced in rats receiving insulin, and in those receiving AICAR (Table 2). Our NEFA levels in control rats correspond well with published values [42]. We measured reductions in NEFA levels in response to both insulin and AICAR as previously reported [42, 45]. The increased reduction in NEFA levels that we observed probably reflects the higher doses of AICAR and insulin used in our study. Metformin did not alter NEFA levels, in agreement with previous reports [46–48]. Generally, serum corticosterone levels were elevated in all groups, probably due to sodium pentobarbitol administration and by handling-induced stress during the experimental procedure, both of which have been demonstrated to increase circulating corticosterone [49]. Serum adrenaline levels are presented in Table 2 and are in the range of previously published basal values [50].

We used both AICAR and metformin to stimulate cardiac AMPK. The use of these structurally unrelated activators of AMPK ensures that we measure effects resulting from the activation of AMPK, and not from indirect effects of either AICAR or metformin. AMPK activity was significantly increased by AICAR and metformin compared with controls (Fig. 1a). No alterations in AMPK activity were measured in response to insulin. As AMPK is activated both allosterically and by increased phosphorylation on its Thr172 residue, and as we are able only to measure changes in activity relative to its level of phosphorylation, we chose to monitor the level of AMPK activation through its target enzyme ACC. As expected, we found a significant increase in ACC phosphorylation on the residue phosphorylated by AMPK following AICAR or metformin administration (Fig. 1b,c). Despite an insulin-induced decrease in ACC phosphorylation following stimulation by AICAR or metformin, treatment with these AMPK activators resulted in a significant increase in ACC phosphorylation.

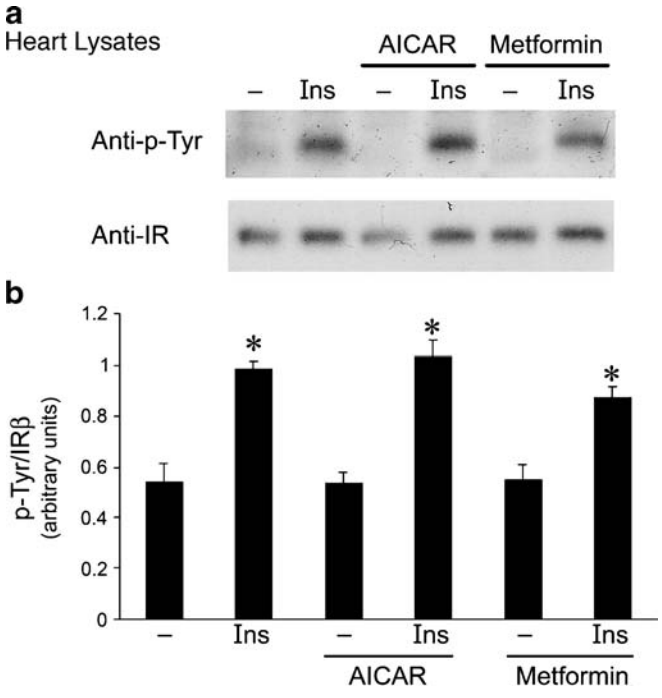

Fig. 3 Tyrosine phosphorylation of the IRβ-subunit. Representative western blots of heart lysates from rats injected with vehicle (−) or insulin (Ins), with and without AICAR or metformin. Membranes were probed with an antibody to phosphotyrosine (p-Tyr), stripped and reprobed with IRβ antibody (**a**). Results were quantified, normalised by total levels and plotted (**b**). Values are means ± SEM. *, significantly different from corresponding value without insulin, $p<0.05$, $n=4–7$ hearts

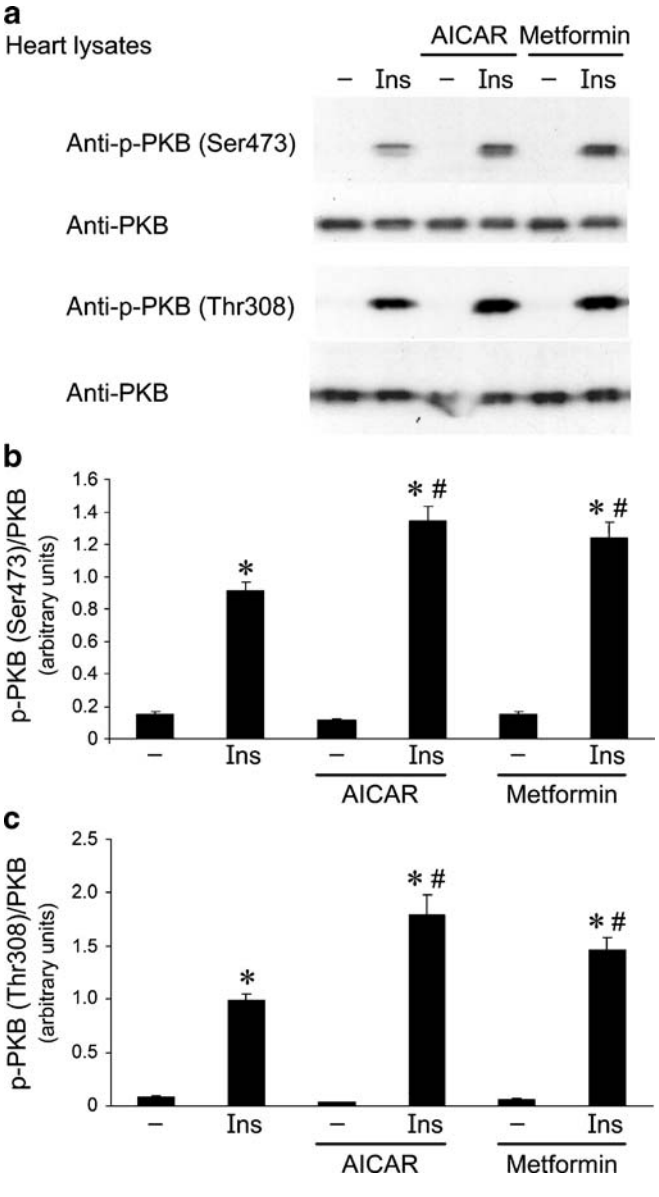

Fig. 4 PKB phosphorylation. Representative western blots of heart lysates from rats injected with vehicle (−) or insulin (Ins), with and without AICAR or metformin. Blots were probed with phospho-PKB (Ser473) or phospho-PKB (Thr308) antibodies, stripped and reprobed with PKB antibody (**a**). Results were quantified, normalised by total levels and plotted (**b**, **c**). Values are means±SEM. *, significantly different from corresponding value without insulin; #, significantly different from corresponding value without AICAR or metformin, $p<0.05$, $n=4–8$ hearts

To investigate the effects of AMPK activation on insulin signalling, we began by measuring one of the major targets of insulin, PI3-kinase. Insulin increased cardiac PI3-kinase activity in agreement with previous reports [51]. IRS1-associated PI3-kinase activation in response to insulin was dramatically reduced in the presence of AICAR and metformin, while basal levels remained unchanged (Fig. 2a). Since PI3-kinase activation by insulin is accomplished following the recruitment of PI3-kinase to the tyrosine-phosphorylated IRS proteins via its adapter subunit, p85, we determined whether the reduced PI3-kinase activity was associated with reduced p85 recruitment. Insulin stimulated the p85-IRS1 association in all groups. However, we observed a significant reduction in the amount of p85 associated with IRS1 in response to insulin with both AICAR and metformin pretreatment (Fig. 2b). In general, reduced IRS 'activity' is associated with reduced tyrosine phosphorylation and/or increased serine phosphorylation. We thus determined levels of IRS1 phosphorylation on Ser636/639. Insulin alone did not alter Ser636/639 phosphorylation, however, following AMPK activation insulin treatment resulted in significantly increased Ser636/639 phosphorylation of IRS1 (Fig. 2c,d). Importantly, total levels of IRS1 protein were not different among the groups.

As reduced insulin stimulation of PI3-kinase in the presence of AICAR and metformin could result from reduced IR activation, we measured the level of IR tyrosine phosphorylation (Fig. 3a,b). IR β-subunit tyrosine phosphorylation was increased in response to insulin, and this response was not significantly altered by AICAR or metformin. Since reduced PI3-kinase activity does not occur with reduced IR phosphorylation, AMPK-induced inhibition of IRS1-associated PI3-kinase probably acts at the level of the IRS proteins.

To determine if the inhibitory effect of AMPK activation on the insulin-stimulated IRS1-associated PI3-kinase activity is propagated downstream, we measured the response to insulin in several downstream targets of PI3-kinase including PKB, $p70^{S6K}$, GSK3β (the predominant isoform in the heart) and aPKC. PKB phosphorylation on Ser473 and Thr308 was significantly increased in response to insulin, as expected, and interestingly, this response was further increased following AMPK activation (Fig. 4a–c). Insulin stimulates $p70^{S6K}$ phosphorylation as shown by a shift in electrophoretic mobility (Fig. 5a). Interestingly, AMPK activation did not alter basal levels (without insulin), but further increased $p70^{S6K}$ phosphorylation in response to insulin, as demonstrated by the elevated proportion of shifted protein (Fig. 5a, bottom panel). We also measured the level of $p70^{S6K}$ phosphorylation on Thr389, a residue for which the phosphorylation has been shown to be rapamycin-sensitive [52, 53]. Interestingly, we observed a significant increase in $p70^{S6K}$ phosphorylation on Thr389 in response to insulin that was further significantly elevated by prior AMPK activation (Fig. 5b). We observed a similar pattern, i.e. an increase with insulin and greater insulin response following AMPK activation, with our measurements of GSK3β phosphorylation (Fig. 6a,b). In contrast, aPKC ac-

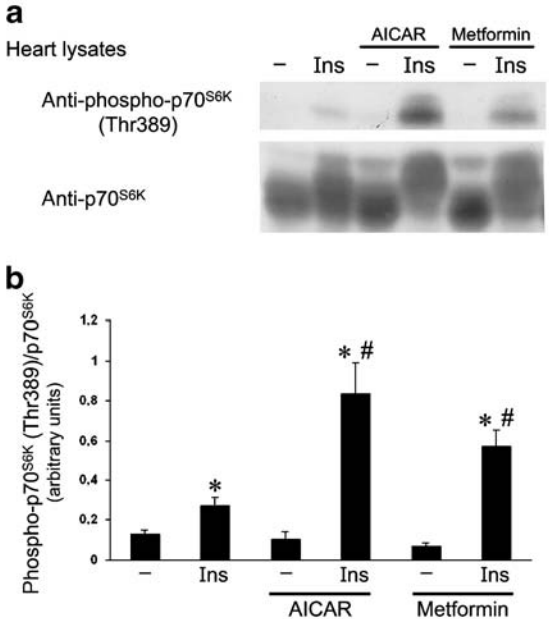

Fig. 5 $p70^{S6K}$ phosphorylation. Representative western blots of heart lysates from rats injected with vehicle (−) or insulin (Ins), with and without AICAR or metformin. Blots were probed with an antibody to phospho-$p70^{S6K}$ (Thr389), stripped and reprobed with $p70^{S6K}$ antibody (**a**). Results were quantified, normalised by total levels and plotted (**b**). Values are means±SEM. *, significantly different from corresponding value without insulin; #, significantly different from corresponding value without AICAR or metformin; $p<0.05$, n=5–6 hearts

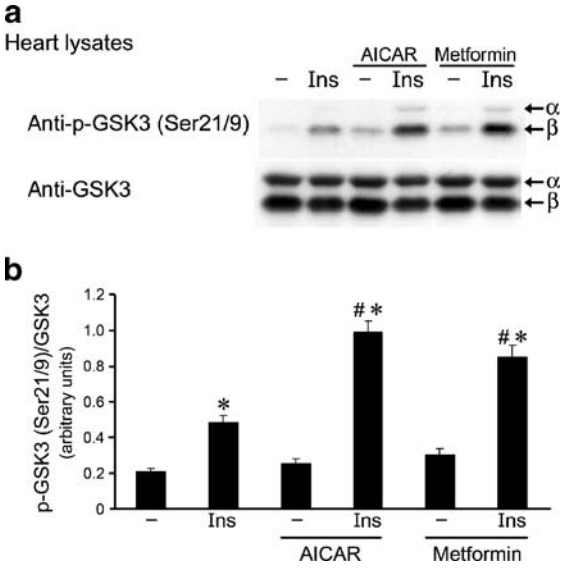

Fig. 6 GSK3β phosphorylation. Representative western blots of heart lysates from rats injected with vehicle (−) or insulin (Ins), with and without AICAR or metformin. Blots were probed with an antibody to phospho-GSK3α/β(Ser21/9), stripped and reprobed with GSK3α/β antibody (**a**). Results for the predominant form, GSK3β, were quantified, normalised by total levels and plotted (**b**). Values are means±SEM. *, significantly different from corresponding value without insulin, #, significantly different from corresponding value without AICAR or metformin; $p<0.05$, n=4–9 hearts

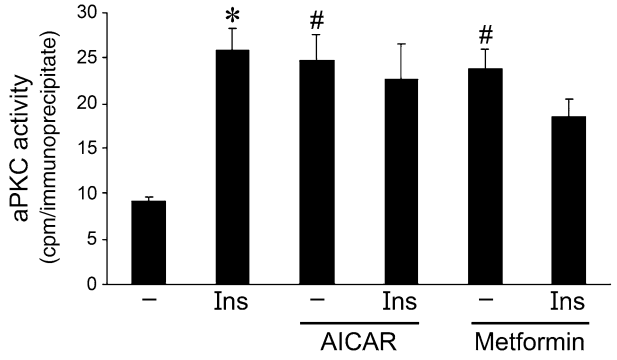

Fig. 7 aPKC activity. aPKC activity measured in hearts from rats injected with vehicle (−) or insulin (Ins), with and without AICAR or metformin. Values are means±SEM. *, significantly different from corresponding value without insulin; #, significantly different from corresponding value without AICAR or metformin; $p<0.05$, n=4–8 hearts

tivity was significantly increased by insulin, AICAR and metformin, but was not further elevated by insulin following AMPK activation (Fig. 7). As aPKCs may be activated by AICAR via ERK, we measured ERK1/2 phosphorylation on Thr202/Tyr204 (Fig. 8a). We observed a significant increase in ERK1/2 phosphorylation (Thr202/Tyr204) in response to both AICAR and metformin (Fig. 8b). Alone, insulin resulted in a small, but significant, increase in ERK1/2 phosphorylation of Thr202/Tyr204. Other reports have not

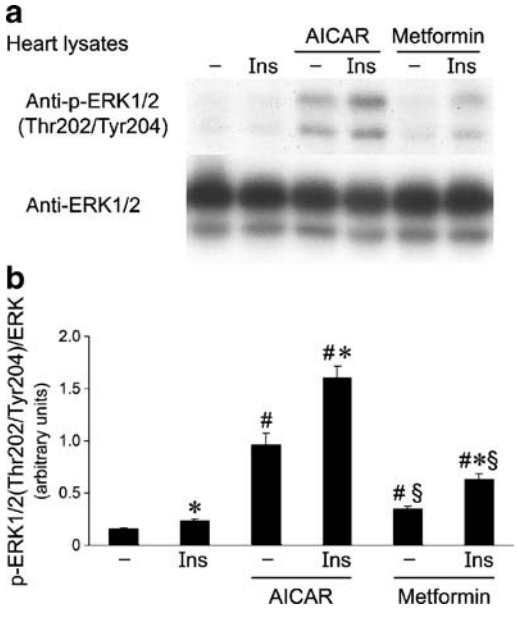

Fig. 8 ERK1/2 phosphorylation. Western blot analysis of heart lysates from rats injected with vehicle (−) or insulin (Ins), with and without AICAR or metformin. Blots were probed with an antibody to phospho-ERK (Thr202/Tyr204), stripped and reprobed with ERK1/2 antibody (**a**). Results were quantified, normalised by total levels and plotted (**b**). Values are means±SEM. *, significantly different from corresponding value without insulin; #, significantly different from corresponding value without AICAR or metformin; §, significantly different from corresponding AICAR value; $p<0.05$, n=4–6 hearts

measured a significant increase in ERK1/2 Thr202/Tyr204 phosphorylation under similar conditions [51, 54], probably because it was measured at least 30 min following insulin administration. ERK phosphorylation has been demonstrated to peak at approximately 5 min of insulin exposure and to return to basal levels by 30 min in cardiomyocytes [55]. Interestingly, following AMPK activation, insulin-induced ERK1/2 phosphorylation of Thr202/Tyr204 was significantly elevated. Further, in the presence of AICAR, ERK1/2 Thr202/Tyr204 phosphorylation was elevated compared with control or metformin-treated rats.

Discussion

We report here, for the first time to the best of our knowledge, that the insulin response in elements downstream of PKB is potentiated following AMPK activation in the heart in vivo. We also show that the insulin-induced stimulation of PI3-kinase associated with IRS1 is reduced with AMPK activation. In addition, we show that ERK1/2 phosphorylation is increased with AMPK activation and that insulin and the AMPK activators, AICAR and metformin, all stimulate aPKC activity to a similar level in the rat heart in vivo.

Following AMPK activation, PKB phosphorylation on Ser473 and Thr308 is significantly increased in response to insulin. The mechanism of PKB-Ser473 phosphorylation remains contentious, with evidence for autophosphorylation and for phosphorylation by exogenous kinase(s) [56]. However, a recent report indicates that the rictor/GβL/mTOR complex directly phosphorylates Ser473 in vitro [57]. Thus, the increase in insulin-induced Ser473 phosphorylation following AMPK activation may be mediated by mTOR, which indicates increased mTOR activity, in agreement with potentiation of insulin action downstream of PKB. PKB is phosphorylated on Thr308 by PDK1 [58]. However, PDK1 is a constitutively active enzyme, thus elevated levels of Thr308 phosphorylation may be reflective of: (1) increased levels of PI-P_3 and thereby increased recruitment of PKB to the membrane where it is phosphorylated; and/or (2) reduced activity of the Thr308 phosphatase, protein phosphatase 2A (PP2A) [59]. The inhibited insulin stimulation of PI3-kinase following AMPK activation is not transmitted to PKB phosphorylation. This dissociation between PI3-kinase and PKB is similar to that described in previous reports [18, 60]. Interestingly, it has been demonstrated that the IRS1/SHP2 association may be important for PKB phosphorylation [61]. This concept may explain our results; however, the precise mechanisms of AMPK-PKB interactions remain to be characterised.

AMPK activation enhances insulin's stimulatory effects on $p70^{S6K}$ phosphorylation. In contrast, several studies have reported that AMPK activation is associated with reduced $p70^{S6K}$ phosphorylation [62–66]. However, this inhibitory effect of AMPK has consistently been reported following $p70^{S6K}$ stimulation by various agents. We examine the effects of AMPK activation prior to insulin stimulation of $p70^{S6K}$, which is highly physiologically relevant since

therapies, such as metformin, that activate AMPK are used in combination with insulin. Clearly, the timing of input signals is critical for the understanding of crosstalk between insulin- and AMPK-signalling and, thus, the characterisation of both patterns of stimulation is essential.

The activation of $p70^{S6K}$ involves its phosphorylation at multiple sites, although the precise mechanisms are not yet completely understood. $p70^{S6K}$ phosphorylation may result from signalling through: (1) PI3-kinase/PDK1 and potentially PKB; and (2) mTOR [55]. The role of the: MAPK/ERK kinase cascade remains contentious; however, it may activate $p70^{S6K}$ in a mTOR-dependent manner [67, 68]. Further, aPKC may contribute to $p70^{S6K}$ phosphorylation [69, 70]. mTOR is believed to phosphorylate $p70^{S6K}$ on Thr389 [52, 53], and phosphorylation of this residue most closely correlates with $p70^{S6K}$ activity in vivo [71]. Here, AMPK-activated ERK may play a role in stimulating mTOR, as ERK has been reported to be involved in mTOR activation [68, 72]. Inputs from PDK1, aPKCs and PKB have been reported to cooperate to increase the phosphorylation of Thr389 [69, 70]. Thus, in our study, increased insulin-induced Thr389 phosphorylation following AMPK activation probably resulted from increased mTOR signalling, and may have been further promoted by PDK1, aPKC and PKB.

In the heart, both insulin and AMPK activators stimulated aPKC. Our data are consistent with the concept that insulin and AMPK activate aPKCs through different mechanisms; insulin acts via PI3-kinase/PDK1 [73] and AMPK via ERK [13]. Interestingly, no additional increase in aPKC activity was observed in the presence of both AMPK activation and insulin. The AMPK-induced reduction in IRS1-associated PI3-kinase activity may result in the lack of further stimulation of aPKC by insulin under these conditions. Alternatively, aPKC may already be maximally activated by AMPK or, once activated, aPKC may no longer be accessible for further stimulation.

The insulin-induced increase in GSK3β phosphorylation was further elevated by AMPK activation. GSK3 may be phosphorylated by PKB and by $p90^{RSK}$, which lies downstream of ERK [9]. In the present study, AMPK stimulated phosphorylation of PKB and ERK, both of which may contribute to the elevated phosphorylation of GSK3β observed in response to insulin.

We report that AMPK inhibits insulin-stimulation of IRS1-associated PI3-kinase activity. The effects of AICAR on PI3-kinase are controversial; it has been reported not to alter its activity [16] and to enhance its stimulation [18]. Further, IRS1-associated PI3-kinase activity may be inhibited by aPKC [74, 75] or ERK [76]. AMPK directly phosphorylates IRS1 on Ser-789 [18]. However, this phosphorylation has been reported both to promote [18] and to inhibit [77] downstream responses. mTOR phosphorylates IRS1 on Ser636/639 [78], thereby promoting reduced IRS1-associated PI3-kinase activity. Here, AMPK activation enhanced insulin-induced IRS1 Ser636/639 phosphorylation and reduced IRS1-associated PI3-kinase activity, consistent with potentialisation of insulin signalling downstream of PKB by AMPK. Taken together, mTOR, aPKC and ERK may all have contributed to reduced insulin-stimulated IRS1 activity following AMPK activation. The precise mechanism of IRS regulation by AMPK remains to be determined, as do possible tissue- and/or species-related variations.

Increased levels of circulating fatty acids are known to inhibit the insulin response. Fatty acid oxidation is stimulated by AMPK in the heart through the phosphorylation and inhibition of ACC, leading to the relieved inhibition of mitochondrial fatty acid uptake [2]. In isolated hearts, increased exogenous fatty acid levels are associated with diminished insulin-induced phosphorylation of PKB (Ser473 and Thr308) and GSK3 [54]. We do not believe that fatty acid-induced alterations are responsible for the AMPK alterations of insulin action in this study, as we observe increased, rather than decreased, phosphorylation of PKB and GSK3β, and circulating levels of NEFAs were not increased. Nonetheless, AMPK-induced modifications of fatty acid metabolism may contribute to our reduced insulin stimulation of IRS1-associated PI3-kinase in the heart.

TSC1/2 has been shown to tightly control mTOR. AMPK enhances the ability of the TSC1/2 complex to suppress mTOR signalling towards $p70^{S6K}$, while ERK signalling and PKB reduce the ability of the complex to inhibit $p70^{S6K}$ [27]. We simultaneously stimulated all three TSC1/2 modulators in the heart in vivo. Under these conditions, the stimulatory effects of AMPK on TSC1/2 are

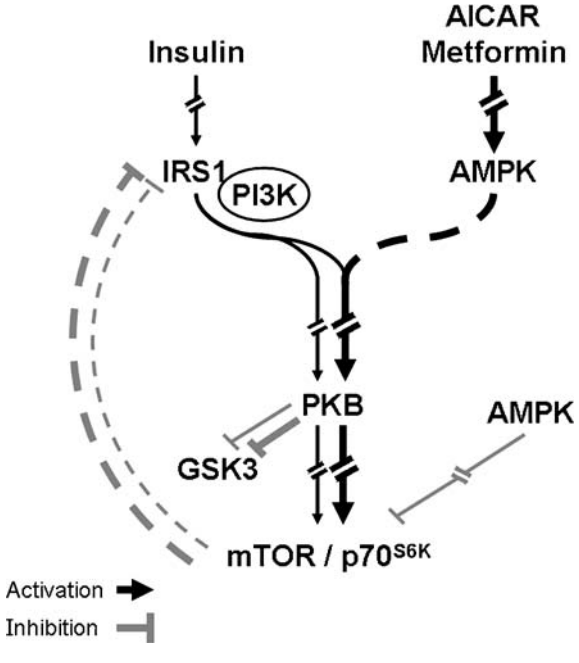

Fig. 9 Crosstalk between insulin- and AMPK-signalling pathways. AMPK has been reported to inhibit mTOR by promoting TSC1/2 activity [26]. However, we demonstrate here (represented by thick lines) that the dominant effect of AMPK activation in the rat heart in vivo is to potentiate the insulin response downstream of PKB. Heightened mTOR signalling may then, in turn, feedback to inhibit IRS1-associated PI3-kinase (PI3K) activity stimulated by insulin. Dotted lines indicate that the precise mechanism remains unclear

overridden by the inhibitory effects of ERK signalling and PKB. PKB and AMPK may also directly regulate mTOR in an antagonistic manner through the phosphorylation of mutually exclusive sites [63]. However, alterations in mTOR activity in response to phosphorylation remain unclear. Interestingly, the elevation in mTOR signalling by AMPK requires insulin stimulation, thereby implying hierarchical regulation of mTOR. This dominant effect is intriguing in light of the data demonstrating that AMPK inhibits prior stimulation of $p70^{S6K}$. Thus, it appears that the final outcome on TSC1/2 and mTOR activities may depend on the timing of the signalling inputs. Interestingly, mTOR inhibits insulin signalling by phosphorylating IRS1 on Ser636/639 [78] and we observed that this feedback is heightened following AMPK activation (Fig. 9). On the other hand, insulin reduces AMPK activity [28–30]. Thus, the integrated control of these two pathways is complex and the outcome of multiple signalling inputs is highly likely to vary with time.

In summary, we report that AMPK activation in rat hearts in vivo enhances insulin signalling downstream of PKB. To the best of our knowledge, this is the first report of heightened insulin responses of GSK3β and $p70^{S6K}$ following AMPK activation. In addition, we report that AMPK inhibits insulin signalling at the level of IRS1-associated PI3-kinase activity, and that AMPK activates aPKC and ERK in the heart. These novel data are indicative of differential effects of AMPK on the activation of components in the cardiac insulin-signalling pathway. These intriguing observations are critical for characterisation of the crosstalk between AMPK and insulin signalling.

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