Effects of PKB/Akt inhibitors on insulin-stimulated lipogenesis and phosphorylation state of lipogenic enzymes in white adipose tissue

by

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Abstract

We investigated acute effects of two allosteric protein kinase B (PKB) inhibitors, MK-2206 and Akti-1/2, on insulin-stimulated lipogenesis in rat epididymal adipocytes incubated with fructose as carbohydrate substrate. In parallel, the phosphorylation state of lipogenic enzymes in adipocytes and incubated epididymal fat pads was monitored by immunoblotting. Preincubation of rat epididymal adipocytes with PKB inhibitors dose-dependently inhibited the following: insulin-stimulated lipogenesis, increased PKB Ser473 phosphorylation, increased PKB activity and decreased acetyl-CoA carboxylase (ACC) Ser79 phosphorylation. By contrast, the effect of insulin to decrease the phosphorylation of pyruvate dehydrogenase (PDH) at Ser293 and Ser300 was not abolished by PKB inhibition. Insulin treatment also induced ATP-citrate lyase (ACL) Ser454 phosphorylation, but this effect was less sensitive to PKB inhibition than ACC dephosphorylation by insulin. In incubated rat epididymal fat pads, Akti-1/2 treatment reversed insulin-induced ACC dephosphorylation, while ACL phosphorylation by insulin was maintained. ACL and ACC purified from white adipose tissue were poor substrates for PKBα in vitro. However, effects of wortmannin and torin, along with Akti-1/2 and MK-2206, on recognized PKB target phosphorylation by insulin were similar to their effects on insulin-induced ACL phosphorylation, suggesting that PKB could be the physiological kinase for ACL phosphorylation by insulin. In incubated epididymal fat pads from wild-type versus ACC1/2 S79A/S212A knockin mice, effects of insulin to increase lipogenesis from radioactive fructose or from radioactive acetate were reduced but not abolished. Together, the results support a key role for PKB in mediating insulin-stimulated lipogenesis by decreasing ACC phosphorylation, but not by decreasing PDH phosphorylation.

Key words: Lipogenesis, ACL, ACC, PDH, SGK3, PKB, Akti-1/2, MK-2206, AMPK
INTRODUCTION

The stimulation of de novo fatty acid synthesis (lipogenesis) in white adipose tissue is one of the best known short-term metabolic effects of insulin [1]. This effect has been attributed to the stimulation of glucose transport and activation of the regulatory enzymes pyruvate dehydrogenase (PDH) [2,3] and acetyl-CoA carboxylase (ACC) [4]. Although the effects of insulin on PDH and ACC were described more than 40 years ago, the exact mechanisms by which insulin affects the activity of these enzymes have never been fully elucidated. Insulin activates PDH by promoting its dephosphorylation via a persistent increase in PDH phosphatase activity [5]. The insulin-induced activation of ACC was proposed to be due to increased phosphorylation at the so-called "I-site" [6]. However, the stoichiometry of phosphorylation of the I-site peptide in 32P-labelled adipocytes after insulin treatment was very low and ACC activation by insulin did not persist after purification of the enzyme [7-9]. An insulin-stimulated protein kinase activity that phosphorylates ACC was partially purified from epididymal adipose tissue, but never characterized [10,11]. AMP-activated protein kinase (AMPK) was identified as the major regulatory kinase for ACC causing phosphorylation-induced ACC inactivation [12]. Witters and Kemp [13] made the important observation that AMPK was inhibited by insulin, associated with ACC activation, and it was proposed that AMPK might be the major target for insulin regulation of ACC. It was subsequently shown by our laboratory that insulin antagonized AMPK activation via AMPKα1/α2 Ser485/Ser491 phosphorylation involving protein kinase B (PKB, also called "AKT") [14]. Indeed in adipocytes, insulin-induced dephosphorylation of ACC at the inactivating Ser79 site was accompanied by an increase in AMPKα1 Ser485 phosphorylation and these effects were reversed by PKB inhibition [15].

ATP citrate-lyase (ACL) is a major phosphoprotein in white adipose tissue and its phosphorylation increases after insulin treatment [16,17]. Insulin increases the phosphorylation of ACL at Ser454 in rat adipocytes [18,19], a site that is also phosphorylated in vitro by an insulin-stimulated cytosolic protein kinase purified from adipocytes [20]. However, its physiological relevance is somewhat of an enigma, since changes in kinetic properties or function resulting from ACL phosphorylation have not been observed [21] or have been difficult to demonstrate [22,23]. Increased ACL phosphorylation by insulin in rat adipocytes was abrogated by wortmannin treatment, implicating the phosphatidylinositol 3-kinase (PI 3-kinase) signalling pathway [24]. Although in incubated fat pads insulin-induced PDH activation was insensitive to wortmannin [24], inhibition was seen in adipocytes [25]. PI 3-kinase activation generates phosphatidylinositol-3,4,5-trisphosphate (PIP3) in the plasma membrane [26]. PIP3 is a lipid second messenger that recruits two serine/threonine protein kinases, namely phosphoinositide-dependent protein kinase-1 (PDK1) and...
PKB (also called AKT), to the plasma membrane via their pleckstrin homology (PH) domains. Full activation of PKB requires phosphorylation of Thr308 by PDK1 [27] and Ser473 by mammalian target of rapamycin (mTOR) complex 2 (mTORC2) [28,29]. PKB exists as three isoforms (PKBα, PKBβ and PKBγ also called AKT 1, 2 and 3, respectively) and PKBβ is considered to mediate short-term metabolic effects of insulin [30]. Indeed, ACL was shown to be phosphorylated by PKB in insulin-treated adipocytes [19].

Pharmacological PKB inhibitors have been developed [31] with the advantage of inhibiting endogenous PKB acutely without unwanted compensatory effects that might occur in genetic models. Akti-1/2 prevents PKB activation by binding to the PH domain, interfering with activation by PDK1 and TORC2 [32]. Akti-1/2 has been used to study the role of PKB in the control of glucose uptake [32] and lipid metabolism in adipocytes [15]. However, the specificity of Akti-1/2 was questioned in 3T3-L1 adipocytes where it decreased insulin-stimulated glucose uptake independently of its effect on PKB activity [33]. MK-2206 is a newer compound related to Akti-1/2 and in 3T3-L1 adipocytes [34] and rat skeletal muscle [35] MK-2206 displayed improved specificity in inhibiting insulin-stimulated glucose transport.

In the present study, effects of PKB inhibitors on insulin-stimulated lipogenesis and phosphorylation state of key lipogenic enzymes were measured in incubated rat epididymal adipocytes and epididymal fat pads. To reduce or circumvent effects of insulin on substrate transport, lipogenesis was measured from radioactive fructose, lactate or acetate. Experiments were also performed on fat pads from wild-type versus ACC1/2 S79A/S212A double knockin mice to explore the role of ACC phosphorylation in the control of lipogenesis. Lastly, the in vitro phosphorylation of purified adipose tissue ACL and ACC by insulin-stimulated protein kinases was investigated.

MATERIALS AND METHODS

Materials. MK-2206 was kindly provided by Dario Alessi (Dundee University, UK). Akti-1/2, Wortmannin and torin were purchased from Calbiochem. Insulin (Actrapid) was from Novo Nordisk. D-[U-¹⁴C] fructose, D-[U-¹⁴C] glucose, L-[U-¹⁴C] lactic acid and [¹⁴C] sodium acetate were from PerkinElmer. All commercial antibodies were produced in rabbit whereas the anti-PDH antibodies were raised in sheep. Anti-phospho-PKB Thr308, anti-phospho-PKB Ser473, anti-total PKB, anti-phosphoglycogen synthase kinase-3 (GSK3)α/β Ser21/Ser9, anti-total GSK-3, anti-phospho-Akt substrate of 160 kDa (AS160) Thr649, anti-total AS160, anti-phospho-proline-rich Akt substrate of 40 kDa (PRAS40) Thr246, anti-total PRAS40 and anti-phospho ACL Ser454 antibodies were from Cell Signaling. Anti-PKB PH domain, anti-total ACC and anti-phospho ACC1 Ser79 antibodies were from Millipore. Anti-phospho PDH E1α Ser293 (site 1), anti-phospho PDH E1α Ser300 (site 2) and anti-total PDH E1α subunit antibodies were kindly donated by Prof. D.G. Hardie (Dundee University, UK). Anti-total ACL antibody was from Abcam or Cell Signaling. Goat anti-rabbit IgG (H+L) dylight™ 800 conjugate was from Thermo Scientific. Alexa Fluor® 680 donkey anti-sheep IgG (H+L) and Alexa Fluor® 680 donkey anti-goat IgG (H+L) conjugated antibodies were from Invitrogen. Bovine heart catalytic subunits of cyclic-3',5'-AMP-dependent protein kinase (PKA, 800 units/ml), catalytic subunits of protein phosphatase 2A (PP2A, 8800 units/ml) and bacterially expressed recombinant His-tagged bovine heart 6-phosphofructo-2-kinase (PFK-2) (BH1(His)₆) were purified as described [36]. Purified activated GST-PKBα (6 units/ml), activated p70 ribosomal protein S6 kinase (p70S6K, 14 units/ml), and activated mitogen-activated protein
kinase-activated protein kinase-1 (MAPKAPK1, 350 units/ml) [36] were kindly provided by Prof. D. Alessi (University of Dundee). Activated mitogen-activated protein kinase (MAPK, 11 units/ml) was donated by Prof. J. Goris (KULeuven). Purified recombinant activated GST-tagged human serum- and glucocorticoid-inducible protein kinase-3 (SGK3) was purchased from Sigma. Liver AMPK (17 units/ml) was purified as described [37]. “SAMS” peptide (for AMPK assay) and peptides RPRAATF (for PKB and SGK3 assay [38]) and the PKA inhibitor peptide (PKI; TYADFIASGRTGRRNAIHD) were kindly synthesized by Dr. V. Stroobant (Ludwig Institute, Brussels). Other chemicals were of standard or analytical grade obtained from MP Biomedicals, Thermo Fisher Scientific, Sigma, or Merck.

Animals. All animal experiments were approved by the Université catholique de Louvain Brussels local ethics committee (reference number 2017/UCL/MD/016) and conducted within the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. Male Wistar rats (about 170 g of body weight) were obtained from the faculty animal house maintained at a 12:12h light-dark cycle with free access to food and water. Wild-type and ACC1/2 S79A/S212A knockin mice generated as described [39] were maintained in the laboratory of Cardiovascular Research, Institute of Clinical and Experimental Research, UCLouvain, Brussels. All animal experiments were carried out in the Protein Phosphorylation (PHOS) laboratory of the de Duve Institute, Université catholique de Louvain, Brussels.

Preparation and incubation of rat epididymal adipocytes. Adipocytes were prepared from the epididymal fat pads of fed rats and incubated essentially as described [40]. Briefly, adipocytes were suspended in Krebs-Ringer Bicarbonate buffer (KRB) containing 1 % (w/v) defatted albumin at a cell density equivalent to 1/2 fat pad/ml. Aliquots of cell suspension (1 ml) were incubated in a final volume of 4 ml of KRB containing 1 % (w/v) defatted albumin with 1/1000 dilutions of Akti-1/2, MK-2206, wortmannin or torin in DMSO to give the final concentrations indicated in the Figures. Control incubations contained 0.1 % (v/v) DMSO as vehicle. After 15 min at 37 °C, insulin (10 nM) was added and incubation was continued for a further 30 min under an atmosphere of 95% O₂/5% CO₂. Following incubation, the cells were harvested by centrifugation (200 g x 20 s), freeze-stopped on carboglace and homogenized in 0.5 - 1 ml of 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 5 mM sodium pyrophosphate, 5 mM β-glycerol phosphate, 15 mM 1 mM 2-mercaptoethanol, 0.1 % (w/v) Triton X-100, 20 mM Hepes, pH 7.4, and protease inhibitor cocktail (Roche) (“sucrose buffer”) on ice using an Ultra-Turrax tissue disintegrator. After centrifugation (3000 g x 10 min at 4 °C) the infranatants were removed and stored at -80 °C prior to enzyme assay and immunoblotting.

Lipogenesis was measured by incubating 1 ml aliquots of adipocytes in a final volume of 4 ml of KRB buffer containing 1 % (w/v) defatted albumin in the presence of 5 mM glucose, fructose or lactate and 1/1000 dilutions of Akti-1/2 or MK-2206 to give the final concentrations indicated in the Figures. After 15 min, the appropriate [U-14C] tracer was added (0.6 mCi/cell incubation) followed by the addition of 10 nM insulin and incubation was continued for a further 30 min under an atmosphere of 95% O₂/ 5% CO₂. The cells were then harvested by centrifugation (200 g x 20 s), washed once with 4 ml of KRB buffer containing 1 % (w/v) defatted albumin and freeze-stopped. After thawing, the cells were solubilized by vortexing the samples in 6 ml of Insta-Fluor toluene based scintillant for organic (non-aqueous) samples.
(Packard, Ltd.) which allows radioactivity in the lipid phase to be counted directly [41]. Duplicates containing tracer, incubation medium and 1 ml of unincubated cells were stopped immediately for each experiment and the counts were subtracted as a blank.

Epididymal fat pad incubations. Epididymal fat pads from fed rats were collected in warm (37 °C) KRB and the major blood vessel was removed. Paired fat pads were minced into small pieces with scissors and pre-incubated with 10 μM Akti-1/2 or 0.1 % (v/v) DMSO in 6 ml of KRB containing 1 % (w/v) defatted albumin with shaking (70 cycles/min). After 20 min, incubation was continued for a further 30 min, with or without 100 nM insulin, under an atmosphere of 95 % O₂/ 5% CO₂. The fat pad pieces were collected by floatation (200 g x 20 s) and freeze-stopped. For immunoblotting, pads were homogenized (Ultra-Turrax) in 1-2 ml of sucrose buffer containing protein phosphatase inhibitors but without Triton X-100 described above for adipocytes. Following centrifugation (3000 g x 10 min at 4 °C), the infranatants were removed and stored at -80 °C prior to enzyme assay and immunoblotting.

Epididymal fat pads from wild-type versus ACC1/2 S79A/S212A knockin mice were weighed, minced with scissors and incubated either with 5 mM [U-¹⁴C] fructose (0.6 mCi) or with 5 mM glucose plus 5 mM [¹³C] sodium acetate (3 mCi) with or without 100 nM insulin in 3 ml of KRB containing 1 % (w/v) defatted albumin to measure rates of lipogenesis. After 30 min (fructose incubations) or 60 min (glucose plus sodium acetate incubations) at 37 °C, the fat pad pieces were harvested by floatation in glass tubes and the incubation media were removed. A solution of 4 ml of 1:2 (v/v) CHCl₃:CH₃OH was added and the tubes were left overnight at 4 °C. The fat pad pieces were homogenized with a Dounce homogenizer, then 1.33 ml of CHCl₃ was added followed by 2.4 ml of water for phase separation. Following centrifugation (1000 g x 10 min), the upper layer was removed and the lower layer was washed twice with 5 ml of 50 mM NaCl by vortexing/centrifugation. Finally, 1 ml of CHCl₃ layer was taken for counting in 5 ml of toluene based scintillant for organic (non-aqueous) samples (Insta-Fluor™ Plus, Perkin Elmer).

Enzyme assays. Lactate dehydrogenase (LDH) [40] and ACC [4] were assayed as described. ACL was assayed in a final volume of 1 ml of 50 mM Hepes, pH 7.5, 100 mM KCl, 15 mM 2-mercaptoethanol, 0.15 mM NADH containing malate dehydrogenase (1 unit), 5 mM magnesium citrate and 0.2 mM CoA at 25°C. The reactions were started by the addition of 5 mM MgATP and NADH utilization was monitored spectrophotometrically at 340 nm. For PKB assay, adipocyte/fat pad lysates were immunoprecipitated with 2 µg of anti-PH domain PKB antibody pre-bound to 20 µl of Protein G-Sepharose. Immunoprecipitates were washed with 0.5 ml of buffer containing 50 mM Tris-HCl pH 7.5, 0.1 % (w/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM sodium pyrophosphate, 15 mM 2-mercaptoethanol and 0.5 M NaCl followed by two washes with buffer containing 50 mM Tris-HCl pH 7, 0.03 % (w/v) Brij-35, 0.1 mM EGTA and 15 mM 2-mercaptoethanol. Protein kinase activity was measured in a final volume of 50 µl of 10 mM Mops pH 7, 0.5 mM EDTA, 10 mM magnesium acetate, 15 mM 2-mercaptoethanol (phosphorylation buffer), 200 µM substrate peptide and 0.1 mM [γ-³²P] ATP (specific radioactivity 200-1000 cpm/pmol). After 20 min of incubation at 30°C with continual gentle shaking, aliquots of supernatant (20 µl) were taken and spotted onto Whatman P81 papers for the determination of ³²P-incorporation [42] by liquid scintillation counting. Purified PKB, SGK3 and AMPK were assayed as described above in 50 µl of phosphorylation
buffer with the appropriate substrate peptide. Purified PKA and PP2A were assayed with histone IIA or p-nitrophenyl phosphate as substrate, respectively [36].

Immunoblotting. Equal volumes of lysates (10-20 µl) were loaded into each well of polyacrylamide gels (7.5 % (w/v) for ACC, ACL and PKB and 12 % (w/v) for PDH) for SDS-PAGE using separate gels for detection with anti-phospho- versus anti-total protein antibodies. Proteins were transferred to PVDF membranes and blocked in StartingBlock™ (TBS) Blocking Buffer (Thermo Scientific for 2 h at room temperature). After blocking, membranes were incubated overnight at 4 °C with primary antibodies diluted 1:1000 in Blocking Buffer supplemented with 0.1 % (v/v) Tween-20. After washing with TBS-T (Tris-buffer saline-Tween-20) containing 10 mM Tris/HCl pH 7.4, 0.15 M NaCl and 0.1 % (v/v) Tween-20 (3 x 10 min), membranes were incubated with appropriate fluorescent secondary antibodies diluted 1:10000 in blocking buffer supplemented with 0.1 % (v/v) Tween-20 and 0.01 % (w/v) SDS for 1 h at room temperature and then washed again (3 x 10 min) with TBS-T. Immunoreactive bands were visualized by fluorescence and quantified by Odyssey imaging. Alternatively, following SDS-PAGE and transfer, PVDF membranes were blocked in TBS-T buffer containing 5 % (w/v) non-fat dry milk in for 1 h. After washing with TBS-T (3 x 10 min), the membranes were incubated with primary antibodies diluted 1:2000 in TBS-T containing 3 % (w/v) bovine serum albumin overnight at 4°C. The membranes were washed with TBS-T (3 x 10 min) and incubated with secondary antibodies (Goat anti-rabbit IgG H&L (HRP), Abcam) diluted 1:20000 at room temperature. After washing three times with TBS-T, immunoreactive bands were visualized with Immobilon® classico western HRP substrate (Millipore) using the Fusion Solo S (Vilber) imaging system.

Purification of ACC and ACL from white adipose tissue. Pig perirenal adipose tissue obtained from a local slaughterhouse (500 g) was homogenized in a Waring Blender (3 x 30 s) in 1.5 l of 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, 5 mM potassium phosphate, 15 mM 2-mercaptoethanol, 20 mM Tris-Cl pH 7.5 plus protease inhibitor cocktail comprising 1 mM phenylmethanesulphonyl fluoride, 2 mM benzamidine-Cl, and 1 mg/ml each of leupeptin, pepstatin and aprotinin. The homogenate was centrifuged (2000g x 20 min at 4 °C) and the resulting infranatant was centrifuged at 10000g for 30 min. The supernatant was filtered through glass wool and loaded onto a column of DEAE-Trisacryl (2.5 x 20 cm) equilibrated with 50 mM Tris-Cl pH 7.5, 1 mM EDTA, 1 mM EGTA, 5 mM potassium phosphate, 15 mM 2-mercaptoethanol, 20 % (v/v) glycerol plus protease inhibitor cocktail (buffer A). After thorough washing, the column was eluted with a linear gradient (0-0.5 M NaCl in 400 ml of buffer A). Fractions (7.5 ml) containing ACC activity were pooled and loaded onto a column of Avidin-Sepharose (1 x 12 cm) equilibrated in buffer A. The column was extensively washed with buffer A containing 150 mM NaCl and eluted with the same buffer supplemented with 2 mM biotin. Fractions (5 ml) containing ACC activity were pooled, concentrated to 2 ml, dialysed against 300 ml of buffer A containing 100 mM KCl and 20 % (v/v) glycerol (buffer B) and stored at -80 °C.

Epididymal fat pads (about 50 g) from fed male Wistar rats were extracted in homogenization buffer as described above. The supernatant was loaded on a Q-Sepharose column (1.5 x 6.5 cm) equilibrated in buffer A for elution in a linear gradient (0-0.5 M NaCl in 150 ml of buffer A). Fractions (3 ml) containing co-eluting ACL and ACC activity were applied on an Avidin-Sepharose column (1 x 7 cm)
equilibrated with buffer A. The flow-through fractions containing ACL activity were applied on a column of Blue Sepharose (1 x 5 cm) equilibrated in buffer B. The column was washed with buffer B containing 1 mM Mg ATP and 1 mM magnesium citrate and eluted with buffer B containing 1 mM Mg ATP, 1 mM Mg citrate and 200 mM CoA. Fractions (4 ml) containing ACL activity were pooled, concentrated to 1 ml, dialysed against buffer B and stored at -80 °C.

In vitro protein phosphorylation. Purified preparations of ACL or ACC (1.5–2 mg of protein/ml) were first incubated with catalytic subunits of PP2A (60 units/ml) and 5 mM MnCl$_2$ for 30 min at 30 °C. The reactions were stopped by the addition of 1 μM microcystin. Purified dephosphorylated ACC (0.39 mg/ml), purified dephosphorylated ACL (0.28 mg/ml) and bacterially expressed recombinant bovine heart PFK-2 as a positive control (0.24 mg/ml) were then incubated with 2.5 μM PKI (unless PKA was under study) and [γ-32P]ATP (500 cpm/pmol) and the various protein kinases at the following final concentrations: PKA (1.3 units/ml), p70S6K (0.56 units/ml), MAPKAPK1 (0.35 units/ml), MAPK (1.1 units/ml), PKB (0.12 units/ml) and AMPK (0.34 units/ml in the presence and absence of 0.2 mM AMP). After 30 min of incubation at 30°C, the reactions were stopped by the addition of sample buffer for SDS-PAGE in gels containing 10 % (w/v) acrylamide (ACL and PFK-2) or 7.5 % (w/v) acrylamide (ACC). 32P-incorporation was visualized by SDS-PAGE, gel drying and phosphorimaging [36].

Other methods. Protein concentrations of purified enzyme preparations were estimated by the Bradford method [43] with rabbit γ-globulin as a standard. The data are presented as means ± S.E.M. Statistical analysis was assessed using a Student’s paired or unpaired t-test or by two-way ANOVA in the software package “GraphPad Prism 5” with a Bonferroni post-hoc test. P < 0.05 was judged significant. One unit of protein kinase/protein phosphatase represents the amount that catalyses the phosphorylation/dephosphorylation of 1 nmol of substrate under the assay conditions. Otherwise, 1 unit of enzyme is the amount that catalyses the formation of 1 μmol of product under the conditions of the assay.

RESULTS

Effects of Akti-1/2 on insulin-stimulated rates of lipogenesis in adipocytes incubated with glucose, fructose or lactate as substrate.

It was previously shown that pre-incubation of primary rat and mouse 3T3-L1 adipocytes with Akti-1/2 dose-dependently inhibited insulin-induced PKB phosphorylation and reduced insulin-stimulated [3H] glucose incorporation into total lipids [15]. However, reduced insulin-stimulated lipogenesis could have been partly due to an effect on glucose transport, which is inhibited by Akti-1/2 [32]. Therefore, we first compared effects of Akti-1/2 on insulin-stimulated lipogenesis in rat epididymal adipocytes incubated with 5 mM [13C] glucose, 5 mM [13C] fructose or 5 mM [13C] lactate as substrate within the same experiment. Measurements of rates of lipogenesis with fructose and lactate as substrate were undertaken to minimize effects of insulin on uptake. Indeed in adipocytes, insulin effects on lactate metabolism are slight [44] and insulin only modestly stimulates fructose uptake [45]. With radioactive glucose as substrate, insulin (10 nM) stimulated the rate of lipogenesis ~3-fold and the increase was abrogated in cells that had been preincubated with 1 μM Akti-1/2 (Fig. 1A), in agreement with previous observations
on primary and 3T3-L1 adipocytes [15]. In adipocytes incubated with radioactive fructose, basal rates of lipogenesis were ~3-fold less than seen from radioactive glucose and ~4-fold less than observed with radioactive lactate, indicating that fructose is a poorer substrate for lipogenesis than either glucose or lactate. However, insulin also stimulated the rate of lipogenesis ~3-fold from fructose and preincubation with Akti-1/2 completely abolished the effect (Fig. 1A). In the presence of radioactive lactate, the basal rate of lipogenesis was about 50 % higher than seen with glucose (Fig. 1A). Moreover, incubation with insulin further increased lipogenesis with radioactive lactate as substrate by ~50 % and this effect was abolished by preincubation with Akti-1/2 (Fig. 1A).

**MK-2206 and Akti-1/2 dose-dependently inhibited insulin-stimulated lipogenesis, insulin-induced PKB Ser473 phosphorylation and PKB activation in adipocytes incubated with fructose.**

To investigate the relationship between signalling and lipogenesis, dose-response experiments were conducted. Preincubation of adipocytes with Akti-1/2 inhibited insulin-stimulated lipogenesis from radioactive fructose dose-dependently with an IC$_{50}$ value of 50 ± 10 nM (Table 1). Likewise in adipocytes preincubated with MK-2206, insulin-stimulated lipogenesis from radioactive fructose was inhibited in a dose-dependent manner (Fig. 1B) with an IC$_{50}$ value of 70 ± 40 nM (Table 1). In the presence of 5 mM unlabelled fructose, preincubation of adipocytes with Akti-1/2 inhibited insulin-induced PKB activation, measured in extracts (Fig. 2A), with an IC$_{50}$ value of 1.6 ± 0.2 µM (Table 1). Preincubation with MK-2206 likewise inhibited insulin-induced PKB activation (Supplementary Figure S1A) with an IC$_{50}$ value of 1.1 ± 0.7 µM (Table 1). Preincubation of adipocytes with MK-2206 also inhibited the increase in PKB Ser473 phosphorylation induced by insulin, measured by immunoblotting extracts (Fig. 2B) with an IC$_{50}$ value of 1.7 ± 0.2 µM (Table 1). Similarly, preincubation with Akti-1/2 inhibited insulin-induced PKB Ser473 phosphorylation (Fig. S2B) with an IC$_{50}$ value of 2.2 ± 0.6 µM (Table 1). These values for the inhibition of increased PKB activity/phosphorylation induced by insulin are notably about 15 to 30-fold higher than the IC$_{50}$ values obtained for the inhibition of insulin-stimulated lipogenesis (Table 1).

**Akti-1/2 and MK-2206 dose-dependently reversed insulin-induced ACC Ser79 dephosphorylation in adipocytes incubated with fructose.**

In adipocytes incubated with fructose, insulin treatment led to an ~50 % decrease in ACC Ser79 phosphorylation, measured by immunoblotting extracts, and preincubation with Akti-1/2 (Fig. 3A) or MK-2206 (Fig. 3B) dose-dependently reversed this effect, consistent with previous observations [15]. For MK-2206, the IC$_{50}$ value (0.3 ± 0.2 µM) was between the values for the inhibition of insulin-stimulated PKB phosphorylation/activation and lipogenesis (Table 1).

**Effects of Akti-1/2 and MK-2206 on the insulin-induced increase in ACL phosphorylation and insulin-induced PDH dephosphorylation in adipocytes incubated with fructose.**

In adipocytes incubated with fructose, insulin increased ACL Ser454 phosphorylation ~3-fold, measured in extracts by immunoblotting, but this effect was not dose-dependently inhibited in cells that had been preincubated with increasing concentrations of Akti-1/2 (Fig. 4A). Also in adipocytes preincubated with increasing concentrations of MK-2206, insulin-induced ACL Ser454 phosphorylation seemed to
be maintained (Fig. S2). However, at a concentration of 10 µM Akti-1/2 or 10 µM MK-2206, the increase in ACL phosphorylation by insulin was not statistically significant (Fig. 4A, Fig. S2). Insulin induced a decrease in PDH Ser293 phosphorylation of ~50% that was not dose-dependently inhibited either in cells that had been preincubated with increasing concentrations of MK-2206 (Fig. 4B).

**Effects of Akti-1/2, MK-2206 and other inhibitors on insulin-induced PKB phosphorylation along with downstream target and ACL phosphorylation in adipocytes incubated with fructose**

Adipocytes were preincubated with fructose and DMSO or maximal doses of inhibitors prior to incubation with and without insulin for 30 min. Treatment with insulin resulted in a dramatic increase in PKB Thr308 phosphorylation that was largely abrogated by the PI 3-kinase inhibitor, wortmannin, and MK-2206 and to a somewhat lesser extent by Akti-1/2 and mTORC2 inhibitor, torin (Fig. 5A). Insulin also caused a robust rise in PKB Ser473 phosphorylation that was almost completely abrogated by torin and MK-2206, but not by wortmannin or Akti-1/2 (Fig. 5B). Preincubation with wortmannin or MK-2206 largely abrogated the insulin-induced increases in GSK3, PRAS40, AS160 and ACL phosphorylation (Fig. 6A-D). In the presence of torin, the residual PKB Thr308 phosphorylation seen in the presence of insulin (Fig. 5A) was presumably sufficient for insulin-induced PKB activation and downstream GSK3, AS160 and ACL phosphorylation (Fig. 6A,C,D). Also, preincubation with 10 µM Akti-1/2 was not sufficient to suppress insulin-induced ACL phosphorylation (Fig. 6D).

**Lack of effect of Akti-1/2 on decreased ACC, increased ACL and decreased PDH phosphorylation by insulin in epididymal fat pads incubated with fructose.**

Experiments were carried out on epididymal fat pads to see if effects of PKB inhibition on the changes in phosphorylation state of lipogenic enzymes could be reproduced, as fat pads are easier to manipulate for extending to mouse genetic models. In rat epididymal fat pads incubated with fructose, insulin increased PKB activity more than 3-fold, measured in extracts, and PKB activation by insulin was largely abrogated in fat pads that had been preincubated with Akti-1/2 (Fig. S3A). By immunoblotting extracts, ACC Ser79 phosphorylation decreased by ~40% in fat pads incubated with insulin and this decrease was reversed in fat pads that had been preincubated with Akti-1/2 (Fig. S3B), as seen in adipocytes (Fig. 3). Also as seen in adipocytes (Fig. 6D), insulin treatment increased ACL Ser454 phosphorylation more than 2-fold, and increased ACL phosphorylation was still observed in fat pads that had been preincubated with Akti-1/2 (Fig. S3C). Moreover in fat pads incubated with fructose, insulin decreased PDH Ser293 and PDH Ser300 phosphorylation and these effects were maintained in fat pads that had been preincubated with Akti-1/2 (Fig. 7).

**Effect of insulin on the rates of lipogenesis from radioactive fructose and radioactive acetate in mouse epididymal fat pads from wild-type and ACC1/2 S79A/S212A double knockin mice**

To further investigate the role of ACC phosphorylation in the control of lipogenesis, experiments were performed on fat pads from wild-type versus ACC1/2 S79A/S212A double knockin mice. In mouse epididymal fat pads from wild-type mice, incubation with insulin increased the rate of lipogenesis from radioactive fructose by ~70% (Fig. 8A). In incubated epididymal fat pads from ACC1/2 S79A/S212A double knockin versus wild-type mice, basal lipogenesis was increased by ~70%, consistent with
removal of the inactivating phosphorylation sites by mutation. However, insulin treatment still led to an increase (~25%) in the rate of lipogenesis from radioactive fructose in fat pads from ACC1/2 S79A/S212A double knockin mice (Fig. 8A). In fat pads from wild-type mice, incubation with insulin increased the rate of lipogenesis from cold glucose plus radioactive acetate ~4-fold (Fig. 8B). In incubated fat pads from ACC1/2 S79A/S212A double knockin mice, insulin treatment increased the rate of lipogenesis from radioactive acetate ~2-fold (Fig. 8B).

In vitro phosphorylation of purified preparations of ACL and ACC
We tested whether purified adipose tissue ACL and ACC were substrates for insulin-stimulated protein kinases with PKA as a positive control. Purified dephosphorylated ACC was phosphorylated when incubated with PKA and [γ-32P]ATP (Fig. S4A), as described previously [46]. ACC was also phosphorylated by AMPK, as expected, and phosphorylation increased in the presence of AMP (Fig. S4A). As positive controls, the insulin-stimulated protein kinases p70S6K, MAPKAPK1 and PKBα along with PKA phosphorylated recombinant bovine heart PFK-2 [36] (Fig. S4B). However, only MAPKAPK1 appreciably phosphorylated ACC (Fig. S4A). Purified dephosphorylated ACL was phosphorylated by PKA and [γ-32P]ATP (Fig. S4B), as described previously [47]. Indeed PKA was shown to phosphorylate ACL stoichiometrically to about 0.7 mol/mol of enzyme subunit [47]. In fact PKA phosphorylates ACL on Ser454, the same site phosphorylated by both insulin and glucagon in incubated hepatocytes [48]. However, purified ACL was poorly phosphorylated by PKBα, p70S6K and MAPKAPK1 in vitro (Fig. S4B).

DISCUSSION
Insulin stimulates lipogenesis in white adipose tissue by increasing glucose transport and by activating PDH and ACC [1]. There is strong evidence that the stimulation of glucose transport by insulin involves increased GLUT4 trafficking to the plasma membrane mediated by PKB activation (for a review see [49]). Moreover, PKB inhibitors block insulin-stimulated glucose uptake in adipocytes [32,33,34]. Therefore, to limit or circumvent effects of insulin on substrate transport, lipogenesis was measured from radioactive fructose, lactate or acetate. Adipose tissue was shown to be an important site of fructose metabolism and fructose was proposed to enter adipose tissue cells in two ways, one of which is independent of glucose and insulin (probably via mainly GLUT5) the other being insulin-dependent and inhibited by glucose (probably via GLUT4) [50]. Moreover, rat adipocytes express the GLUT5 fructose transporter responsible for about 80% of total fructose uptake that is insensitive to insulin [51]. Fructose metabolism in adipose tissue involves hexokinase whereas in liver fructokinase is involved, bypassing the regulatory step at phosphofructo-1-kinase, such that diets rich in fructose cause fat accumulation in the liver [52]. Fructose metabolism in extra-hepatic tissues has not been well studied in vivo but increased visceral adiposity and weight gain was seen upon fructose feeding in rats [53]. In adipocytes pre-incubated with Akti-1/2, insulin-stimulated lipogenesis from radioactive fructose or radioactive lactate was abrogated (Fig. 1A). However, only a small proportion of the insulin-stimulated lipogenic flux from fructose transported via GLUT4 would have been expected to be affected by PKB inhibition with Akti-1/2. PKB inhibitors dose-dependently inhibited insulin-stimulated lipogenesis from radioactive fructose along with PKB activation and insulin-induced PKB Ser473 phosphorylation (Fig. 1B, Fig. 2, Fig. S1), although there was a 15- to 30-fold difference in IC50 values obtained for the inhibition of these parameters (Table 1).
suggesting possible off-target effects. As noted previously with Akti-1/2 [15], both MK-2206 and Akti-1/2 dose-dependently reversed insulin-induced ACC Ser79 dephosphorylation (Fig. 3). This would explain the old observation of Halestrap and Denton that insulin activates ACC in white adipose tissue [4]. It also implies that there would be basal AMPK activity in adipocytes that would be inactivated by insulin via PKB, since AMPK is the only kinase known to phosphorylate ACC at the Ser79 inactivating site. Interestingly, based on AMPKα Thr172 phosphorylation, AMPK activity was found to be higher in several adipose tissue depots of insulin-sensitive compared with insulin-resistant humans [54]. Insulin was shown to increase AMPKα1 Ser485 phosphorylation, associated with a decrease in AMPK activity and these effects were reversed by Akti-1/2 [15]. However, we showed that AMPKα1 Ser485 phosphorylation decreased AMPK activation by LKB1 without affecting basal AMPKα Thr172 phosphorylation [14]. Further studies are required to substantiate the precise mechanism by which insulin, via PKB, reduces AMPK activity in adipose tissue, for example by using AMPKα1 S485A knockin mice. In incubated epididymal fat pads from ACC1/2 S79A/S212A double knockin mice, lipogenesis from radioactive fructose could still be increased by insulin, albeit to a lesser extent than in incubated fat pads from wild-type mice (Fig. 8A), suggesting targets of insulin other than ACC in the lipogenic pathway. From radioactive fructose, insulin-stimulated lipogenesis that was seen in ACC1/2 S79A/S212A double knockin mice could have been due to PDH dephosphorylation (Fig. 7) and/or increased ACL phosphorylation (Fig. S3C). From radioactive acetate plus cold glucose, insulin increased the rate of lipogenesis ~2-fold in incubated epididymal fat pads from ACC1/2 S79A/S212A double knockin mice (Fig. 8B). Lipogenesis from radioactive acetate would bypass PDH and ACL due to the direct formation of acetyl-CoA in the cytosol by acetyl-CoA synthetase and the presence of cold glucose would allow sn-glycerol-3-phosphate production for esterification. In the presence of glucose, insulin indeed increased lipogenesis measured from radioactive acetate [55]. In incubated epididymal fat pads from ACC1/2 S79A/S212A double knockin mice, the increase in lipogenesis from radioactive acetate plus glucose by insulin could have been due to a rise in citrate, an allosteric stimulator of ACC, and/or an increase in sn-glycerol-3-phosphate concentration [56,57] secondary to the stimulation of glucose transport by insulin, and/or effects of insulin on enzymes of the esterification pathway [58].

By contrast with the effect of PKB inhibitors to reverse insulin-induced ACC Ser79 dephosphorylation (Fig. 3, Fig. S3B), the effects of insulin to decrease phosphorylation of PDH at Ser293 and Ser300 (Fig. 4B, Fig. 7) were not abolished by PKB inhibition, suggesting that PKB was not involved. The lack of effect of PKB inhibition on PDH dephosphorylation at Ser293 and Ser300 by insulin is perhaps not surprising since PDH is a mitochondrial enzyme and PDH activation by insulin was reported to be insensitive to wortmannin [24].

Concerning insulin-induced ACL Ser454 phosphorylation, this effect was less sensitive to inhibition by Akti-1/2 (Fig. 4A) or MK-2206 (Fig. S2) than ACC dephosphorylation by insulin (Fig. 3). Also, after preincubation of fat pads with Akti-1/2, which reversed insulin-induced ACC dephosphorylation, ACL phosphorylation by insulin was maintained (Fig. S3). However, the effects of wortmannin and torin, along with Akti-1/2 and MK-2206, on recognized PKB downstream target phosphorylation by insulin were similar to their effects on insulin-induced ACL Ser454 phosphorylation although there were differences in levels of phosphorylation compared to ACL in the presence of insulin and inhibitors, suggesting that PKB has different substrate affinities for its targets (Fig. 6). ACL phosphorylation by insulin in adipocytes was...
shown to be wortmannin-sensitive [24] and PKB was implicated in ACL phosphorylation in rat primary adipocytes [19] and in 3T3-L1 adipocytes [59]. PKBγ expression in human adipose tissue is "low" (https://www.proteinatlas.org/search/AKT3) while PKBβ (and PKBα) expression is "medium" (https://www.proteinatlas.org/search/AKT2) and it is PKBβ that seems to mediate short-term metabolic effects of insulin [30,49]. Akt-1/2 is selective for PKBα and PKBβ with little effect on PKBγ [60], while MK-2206 seems to inhibit all three PKB isoforms, [61]. Therefore, based on the use of Akt-1/2, the effects of insulin on lipogenesis and ACC/ACL phosphorylation probably do not involve PKBγ. In vitro, purified ACL was a poor substrate for PKBα (Fig. S4) and based on knockouts in mice, the PKB isoenzymes are functionally different [30,49]. Therefore, the physiological kinase for ACL phosphorylation might well be PKBβ, proposed to mediate metabolic effects of insulin in adipose tissue [30]. SGK3 is another insulin-stimulated protein kinase [36]. Although bacterially expressed purified human recombinant ACL with 6 C-terminal His residues was phosphorylated by SGK3 in vitro, albeit to a low stoichiometry of about 0.2 mol/mol of enzyme subunit (data not shown), SGK3 does not appear to be expressed in human adipose tissue (https://www.proteinatlas.org/search/SGK3) and we were unable to detect the protein in a tryptic digest of a rat epididymal fat pad cytosol after elution from Blue Sepharose with KCl using the Oribtrap Lumos mass spectrometer. In addition to increasing the phosphorylation of ACL at Ser454, insulin decreases the phosphorylation of Ser450 and Thr446 that are targeted by glycogen synthase kinase-3 (GSK3) in the sequence GSTST$_{446}$PAPS$_{450}$RTAS$_{454}$F [62,63]. The sequence surrounding Ser454 is well conserved in ACL of vertebrates, suggesting its importance for insulin-stimulated lipogenesis. Phosphorylation of ACL Ser454 by insulin could affect its subcellular localization [64] or be important for protein-protein interaction with other enzymes of the lipogenic pathway.

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AUTHOR CONTRIBUTIONS

N.H. carried out protein kinase assays, immunoblotting and data analyses. S.-J.C. carried out experiments on fat pads and adipocytes and performed immunoblotting and data analysis. M.J. and N.H. cloned, expressed and purified recombinant ACL. G.R.S. and B.E.K. generated and provided ACC1/2 S79A/S212A knockin mice. D.V. performed analyses by mass spectrometry. M.H.R. carried out adipocyte and adipose tissue incubations, purified ACC and ACL and performed in vitro phosphorylation.
experiments. M.H.R. was involved in conception, design, interpretation of the data and drafted the article.

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**Figure legends**

Fig. 1 Effect of PKB inhibitors on insulin-stimulated lipogenesis in adipocytes incubated with different substrates. In (A) adipocytes were incubated with 5 mM glucose, fructose or lactate and either 0.1% (v/v) DMSO (vehicle control) or 1 μM Akti-1/2 for 15 min prior to incubation without (white bars) or with 10 nM insulin (black bars) and [U-14C] glucose, [U-14C] fructose or [U-14C] lactate for a further 30 min of incubation to measure rates of lipogenesis. The values are means ± S.E.M., n = 4 separate experiments except for the control glucose + Akti-1/2 condition in (A) where n = 2. */# Indicates a significant difference between with and without insulin/with and without Akti-1/2, respectively (P < 0.05, two-way ANOVA). ¶ Indicates P < 0.05 (paired Student’s t-test). In (B) adipocytes were incubated with 5 mM fructose and either 0.1% (v/v) DMSO (vehicle control) or with the indicated doses of MK-2206 for 15 min prior to incubation without (open symbols) or with 10 nM insulin (filled symbols) and [U-14C] fructose for a further 30 min for measurements of rates of lipogenesis. The values are means ± S.E.M., n = 4 separate experiments. By two way ANOVA, P < 0.05 for the interaction between insulin-stimulated lipogenesis and inhibition by increasing concentrations of MK-2206. */# Indicates a significant difference between with and without insulin/with and without MK-2206, respectively (P < 0.05). ¶ Indicates P = 0.08 (paired Student’s t-test).

Fig. 2 Effects of Akti-1/2 and MK-2206 on insulin-induced PKB activation and PKB Ser473 phosphorylation in adipocytes incubated with fructose. Adipocytes were incubated with 5 mM fructose and either 0.1% (v/v) DMSO (vehicle control) or with the indicated doses of Akti-1/2 (A) or MK-2206 (B) for 15 min prior to incubation without (open symbols) or with 10 nM insulin (filled symbols). After 30 min of incubation, the adipocytes were harvested and freeze-stopped. Extracts were assayed for PKB normalized to PKB band intensity by immunoblotting (A) or immunoblotted with anti-phospho Ser473 and anti-total PKB antibodies followed by blot quantification by Odyssey imaging (a representative blot is shown in the upper panel) (B). The values are means ± S.E.M., n = 4 separate experiments. By two way ANOVA, P < 0.05 for the interaction between insulin-induced PKB activation/phosphorylation and inhibition of activation by increasing concentrations of PKB inhibitors. */# Indicates a significant difference between with and without insulin/with and without Akti-1/2 or MK-2206, respectively (P < 0.05).

Fig. 3 Effects of Akti-1/2 and MK-2206 on insulin-induced ACC dephosphorylation in adipocytes incubated with fructose. Adipocytes were
incubated with Akti-1/2 (A) or MK-2206 (B) and insulin as described in the legend to Figure 2. Extracts were immunoblotted with anti-phospho Ser79 ACC and anti-total ACC antibodies followed by blot quantification by Odyssey imaging (representative blots are shown in the upper panels). The values are means ± S.E.M., n = 4 separate experiments. By two way ANOVA, \( P < 0.05 \) for the interaction between insulin-induced ACC dephosphorylation and inhibition of ACC dephosphorylation by increasing concentrations of PKB inhibitors. */# Indicates a significant difference between with and without insulin/with and without Akti-1/2 or MK-2206, respectively (\( P < 0.05 \)).

Fig. 4 Effects of Akti-1/2 on insulin-induced ACL phosphorylation and MK-2206 on insulin-induced PDH dephosphorylation adipocytes incubated with fructose. Adipocytes were incubated with Akti-1/2 (A) or MK-2206 (B) and insulin as described in the legend to Figure 2. Extracts were immunoblotted with anti-phospho Ser454 ACL and anti-total ACL antibodies (A) or with anti-phospho Ser293 (site 1) PDH and total PDH antibodies (B) followed by blot quantification by Odyssey imaging (representative blots are shown in the upper panels). The values are means ± S.E.M., n = 6 (A) or n = 3 (B) separate experiments. By two way ANOVA, there was no significant interaction between insulin-induced ACL phosphorylation or insulin-induced PDH dephosphorylation and effects of increasing concentrations of PKB inhibitors on these parameters. * Indicates a significant difference between with and without insulin, respectively (\( P < 0.05 \)).

Fig. 5 Effects of wortmannin, torin, Akti-1/2 and MK-2206 on insulin-induced PKB phosphorylation in adipocytes incubated with fructose. Adipocytes were incubated with 5 mM fructose and either 0.1% (v/v) DMSO (vehicle control) or with the indicated concentrations of wortmannin, torin, Akti-1/2 or MK-2206 for 15 min prior to incubation without (open bars) or with 10 nM insulin for 30 min (black bars). Extracts were immunoblotted with anti-phospho Thr308 and anti-total PKB antibodies (A), anti-phospho Ser473 and anti-total PKB antibodies (B), followed by blot quantification using the Fusion Solo S (Vilber) imaging system (representative blots are shown in the upper panels). The values are means ± S.E.M., n = 4 separate experiments. */# Indicates a significant difference respectively between with and without inhibitors or versus the controls, respectively (\( P < 0.05 \), two way ANOVA).

Fig. 6 Effects of wortmannin, torin, Akti-1/2 and MK-2206 on insulin-induced downstream PKB target and ACL phosphorylation in adipocytes incubated with fructose. Adipocytes were incubated with and without inhibitors and insulin as described in the legend to Figure 5. Extracts were immunoblotted with anti-phospho Ser21/Ser9 GSK3 and anti-total GSK3 antibodies (A), anti-phospho Thr246 PRAS 40 and anti-total PRAS 40 antibodies (B), anti-phosphoThr649 AS160 and anti-total AS160 antibodies (C) and anti-phospho Ser454 ACL and anti-total ACL antibodies (D) followed by blot quantification using the Fusion Solo S (Vilber) imaging system (representative blots are shown in the upper panels). The values are means ± S.E.M., n = 4 separate experiments. */# Indicates a significant difference respectively between with and without insulin/with and without inhibitors or versus the controls, respectively (\( P < 0.05 \), two way ANOVA).
Fig. 7 **Effects of Akti-1/2 on insulin-induced PDH dephosphorylation in epididymal fat pads incubated with fructose.** Rat epididymal fat pads were incubated as described in the legend to Figure 5. Extracts were immunoblotted with anti-phospho-Ser293 PDH (site 1), anti-phospho-Ser300 PDH (site 2) and anti-total PDH antibodies for blot quantification by Odyssey imaging (representative blots are shown in the upper panel). The values are means ± S.E.M., n = 10 separate experiments. */# Indicates a significant difference between with and without insulin, respectively (P < 0.05, two-way ANOVA).

Fig. 8 **Insulin-stimulated lipogenesis measured with fructose or acetate as substrate in epididymal fat pads from wild-type versus ACC1/2 S79A/S212A knockin mice.** Epididymal fat pads from wild-type (WT) versus ACC1/2 S79A/S212A knockin mice were weighed and incubated either with 5 mM [U-14C] fructose (A) or 5 mM non-radioactive glucose plus 5mM [1-14C] sodium acetate (B) without (white bars) or with 100 nM insulin (black bars) as described under “Materials and Methods” for measurements of rates of lipogenesis. The results are means ± S.E.M., n = 5 (fructose) or n = 4 (acetate) separate experiments. */# Indicates a significant difference between with and without insulin/with and knockin versus wild-type, respectively (P < 0.05, two-way ANOVA). ¶ Indicates significance (P < 0.05) for the effect of insulin (paired Student’s t-test) and for the effect of ACC knockin versus the wild-type (unpaired Student’s t-test).
Table 1 IC\textsubscript{50} values for the effects of MK-2206 and Akti-1/2 on insulin-stimulated lipogenesis from fructose, PKB activation/phosphorylation and ACC phosphorylation in incubations of adipocytes with fructose.

|                | MK-2206 IC\textsubscript{50} (µM) | Akti-1/2 IC\textsubscript{50} (µM) |
|----------------|----------------------------------|----------------------------------|
| PKB Ser473     | 2.3 ± 0.6 (3)                    | 1.7 ± 0.2 (3)                    |
| phosphorylation|                                  |                                  |
| PKB activation | 1.1 ± 0.7 (3)                    | 1.6 ± 0.2 (4)                    |
| Lipogenesis    | 0.07 ± 0.04 (4)                  | 0.05 ± 0.01 (3)                  |
| ACC Ser79      | 0.3 ± 0.2 (4)                    | N.D.                             |
| phosphorylation|                                  |                                  |

IC\textsubscript{50} values were calculated using the software package GraphPad Prism 5. The values are means ± S.E.M. for the number of separate experiments indicated in parentheses. N.D. – not determined.
Rate of lipogenesis (n mol of [U-14C] substrate incorporated/30 min/Unit of LDH)

A

Rate of lipogenesis (n mol of [U-14C] fructose incorporated/30 min/Unit of LDH)

B

MK-2206 concentration (µM)
Fig. 2

A) PKB activity (pmol/min/PKB band intensity) as a function of Akti-1/2 concentration (µM).

B) Relative band intensity (p-PKB/Total PKB) as a function of MK-2206 concentration (µM).

- Insulin
+ Insulin

0 0.01 0.1 1 10

0 0.01 0.03 0.1 3 10

pSer473 PKB
Total PKB
Fig. 3

A

| Akti-1/2 concentration (µM) | - Insulin | + Insulin |
|---------------------------|----------|-----------|
| µM                        |          |           |
| 0                         |          |           |
| 0.1                       |          |           |
| 1                         |          |           |
| 10                        |          |           |

B

| MK-2206 concentration (µM) | - Insulin | + Insulin |
|---------------------------|----------|-----------|
| µM                        |          |           |
| 0                         |          |           |
| 0.01                      |          |           |
| 0.1                       |          |           |
| 1                         |          |           |

**Relative band intensity (p-ACC/Total ACC)**

- **Akti-1/2**
  - - Insulin: Control without insulin.
  - + Insulin: Treatment with insulin.
  - Concentration levels: 0, 0.1, 1, 10 µM.

- **MK-2206**
  - - Insulin: Control without MK-2206.
  - + Insulin: Treatment with MK-2206.
  - Concentration levels: 0, 0.01, 0.1, 1, 10 µM.

Significance markers:
- *: Significant difference compared to the control.
- #: Significant difference between treatments.

Graphs show the relative band intensity of pSer79 ACC and Total ACC under different concentrations of Akti-1/2 and MK-2206 with and without insulin.
Fig. 4

A

- Insulin
+ Insulin

| µM Akti-1/2 | pSer454 ACL | Total ACL |
|-------------|-------------|-----------|
| 0           |            |           |
| 0.1         |            |           |
| 1           |            |           |
| 10          |            |           |

| µM Akti-1/2 | pSer293 PDH | Total PDH |
|-------------|-------------|-----------|
| 0           |            |           |
| 0.1         |            |           |
| 1           |            |           |
| 10          |            |           |

B

- Insulin
+ Insulin

| µM MK-2206 | pSer454 ACL | Total ACL |
|------------|-------------|-----------|
| 0          |            |           |
| 0.01       |            |           |
| 0.1        |            |           |
| 1          |            |           |
| 10         |            |           |

| µM MK-2206 | pSer293 PDH | Total PDH |
|------------|-------------|-----------|
| 0          |            |           |
| 0.01       |            |           |
| 0.1        |            |           |
| 1          |            |           |
| 10         |            |           |
Fig. 5

A

pThr308 PKB

Total PKB

Relative band intensity (p-PKB/Total PKB)

0.1% DMSO
100 nM wortmannin
0.1% Akti-1/2
10 μM MK-2206
2 μM torin
100 nM wortmannin
10 μM Akti-1/2
10 μM MK-2206
2 μM torin

+10 nM insulin

B

pSer473 PKB

Total PKB

Relative band intensity (p-PKB/Total PKB)

0.1% DMSO
100 nM wortmannin
0.1% Akti-1/2
10 μM MK-2206
2 μM torin
100 nM wortmannin
10 μM Akti-1/2
10 μM MK-2206
2 μM torin

+10 nM insulin

*p < 0.05

# p < 0.01

**p < 0.001
Fig. 7

| Akti-1/2 | - | + |
|----------|---|---|
| Insulin  | - | + |

- pSer293 PDH
- pSer300 PDH
- Total PDH

**Graph:**

- pSer293 PDH (site 1)
- pSer300 PDH (site 2)

**Relative band intensity (p-PDH/Total PDH):**

- - Akti-1/2
- + Akti-1/2

* indicates statistical significance.
Rate of lipogenesis (nmol of [U-^{14}C] fructose incorporated 60 min/g wet weight)

**A**

| Treatment          | WT       | ACC1/2 Ser7/9 Ala/Ser212 Ala |
|--------------------|----------|-----------------------------|
| 0                  | ![Bar](#) | ![Bar](#)                   |
| 50                 | ![Bar](#) | ![Bar](#)                   |
| 100                | ![Bar](#) | ![Bar](#)                   |
| 150                | ![Bar](#) | ![Bar](#)                   |

Rate of lipogenesis (nmol of [U-^{14}C] acetate incorporated 60 min/g wet weight)

**B**

| Treatment          | WT       | ACC1/2 Ser7/9 Ala/Ser212 Ala |
|--------------------|----------|-----------------------------|
| 0                  | ![Bar](#) | ![Bar](#)                   |
| 50                 | ![Bar](#) | ![Bar](#)                   |
| 100                | ![Bar](#) | ![Bar](#)                   |
| 150                | ![Bar](#) | ![Bar](#)                   |

*Significant difference compared to control (p < 0.05).*

**Fig. 8**

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