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**RESEARCH ARTICLE**

# Inhibition of AMPK activity in response to insulin in adipocytes: involvement of AMPK pS485, PDEs, and cellular energy levels

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**Kopietz F, Rupar K, Berggreen C, Säll J, Vertommen D, Degerman E, Rider MH, Göransson O.** Inhibition of AMPK activity in response to insulin in adipocytes: involvement of AMPK pS485, PDEs, and cellular energy levels. *Am J Physiol Endocrinol Metab* 319: E459–E471, 2020. First published July 14, 2020; doi:10.1152/ajpendo.00065.2020.—Insulin resistance in obesity and type 2 diabetes has been shown to be associated with decreased de novo fatty acid (FA) synthesis in adipose tissue. It is known that insulin can acutely stimulate FA synthesis in adipocytes; however, the mechanisms underlying this effect are unclear. The rate-limiting step in FA synthesis is catalyzed by acetyl-CoA carboxylase (ACC), known to be regulated through inhibitory phosphorylation at S79 by the AMP-activated protein kinase (AMPK). Previous results from our laboratory showed an inhibition of AMPK activity by insulin, which was accompanied by PKB-dependent phosphorylation of AMPK at S485. However, whether the S485 phosphorylation is required for insulin-induced inhibition of AMPK or other mechanisms underlie the reduced kinase activity is not known. To investigate this, primary rat adipocytes were transduced with a recombinant adenovirus encoding AMPK-WT or a nonphosphorylatable AMPK S485A mutant. AMPK activity measurements by Western blot analysis and in vitro kinase assay revealed that WT and S485A AMPK were inhibited to a similar degree by insulin, indicating that AMPK S485 phosphorylation is not required for insulin-induced AMPK inhibition. Further analysis suggested an involvement of decreased AMP-to-ATP ratios in the insulin-induced inhibition of AMPK activity, whereas a possible contribution of phosphodiesterases was excluded. Furthermore, we show that insulin-induced AMPK S485 phosphorylation also occurs in human adipocytes, suggesting it to be of an importance yet to be revealed. Altogether, this study increases our understanding of how insulin regulates AMPK activity, and with that, FA synthesis, in adipose tissue.

adipocytes; AMP-activated protein kinase; fatty acid synthesis; insulin; pS485

## INTRODUCTION

A major function of white adipose tissue is to store excess energy in the form of triglycerides, which can rapidly be hydrolyzed into fatty acids (FAs) in the event of energy deprivation (10). Adipose tissue dysfunction, for example, reduced de novo FA synthesis and inadequate storage of fat, is believed to cause insulin resistance, which is a strong predictor of type 2 diabetes (T2D) (22, 52).

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An emerging drug target for the treatment of insulin resistance and T2D is the AMP-activated protein kinase (AMPK) (9, 14). AMPK was shown to be activated by antidiabetic drugs like metformin and thiazolidinediones, and its activation is associated with reduced glucose production and lipid accumulation in the liver as well as increased glucose uptake in muscle and improved insulin sensitivity (3, 5, 15, 16, 32, 37, 39, 59). AMPK is a heterotrimeric protein consisting of a catalytic α-subunit and the regulatory β- and γ-subunits. It is described as a cellular fuel gauge, which senses and regulates cellular energy levels. A drop in the ratio of AMP/ADP to ATP (AMP/ATP ratio) induces AMPK activation by promoting net-phosphorylation of the activity-controlling site T172 in the α-subunit, as well as allosterically, by binding of AMP or ADP to the γ-subunit (21). Once activated, AMPK functions to restore cellular energy levels by switching on energy-generating processes like FA oxidation and shutting down energy-consuming processes like FA synthesis (25, 31, 37, 42, 45, 49).

The rate-limiting step in FA synthesis is the conversion of acetyl-CoA to the active FA precursor malonyl-CoA, which is catalyzed by acetyl-CoA carboxylase (ACC) (23, 40). Insulin was previously shown to promote ACC activation by reducing inhibitory phosphorylation of ACC on S79, a site known to be phosphorylated by AMPK in adipocytes (6, 18, 24). However, the mechanism underlying this insulin-induced dephosphorylation of ACC S79 remains unclear. One of our own studies in primary adipocytes revealed that the regulation of ACC activity by insulin is highly dependent on protein kinase B (PKB/Akt) activity (6). Pharmacological inhibition of PKB resulted in a reversal of the insulin-induced dephosphorylation of ACC, which was also accompanied by a reduction in insulin-induced lipogenesis (incorporation of glucose into lipids). Moreover, our study showed that AMPK is phosphorylated at S485 in a PKB-dependent manner in response to insulin, and this was associated with a reduction of AMPK activity. Whether the phosphorylation of AMPK S485 is required for the inhibition of AMPK by insulin and S485-mediated AMPK inhibition underlies the activating effect of insulin on ACC and FA synthesis remains unknown.

In addition to our observation in adipocytes, PKB-dependent AMPK S485 phosphorylation has also been shown in several other models. In 2006, two independent studies in rodent cardiac muscle reported PKB-induced AMPK S485 phosphorylation, suggested to antagonize AMPK T172 phosphorylation by liver kinase B1 (LKB1) and with that AMPK activation (30, 53). Another study demonstrated that AMPK S485 phosphor-

ylation inhibits subsequent activation of AMPK in different human tumor cell lines and suggested that this effect was due to conformational changes rendering AMPK T172 inaccessible to phosphorylation (26). Furthermore, inhibition of AMPK T172 phosphorylation in response to PKB-dependent S485 phosphorylation was also reported in vascular smooth muscle cells and was suggested to be involved in the downregulation of AMPK activity in human hepatoma cells infected with hepatitis C virus (36, 44).

Another way in which insulin can promote lipid storage is by, in a PKB-dependent manner, inducing phosphodiesterase 3B (PDE3B) activity, thereby causing inhibition of catecholamine-induced lipolysis (6, 34, 56). Previous studies in adipocytes showed that cAMP-elevating agents activate AMPK, an effect which was lost when cotreating cells with insulin, and reinstalled when pretreating with a PDE3B inhibitor (19, 47, 57). Furthermore, it has been demonstrated that stimulation of lipolysis results in an elevated cellular AMP/ATP ratio, which was suggested to be the mechanism whereby cAMP-elevating agents cause AMPK activation (19). Altogether, these findings suggest that insulin might cause an inactivation of AMPK by activating PDE3B and thereby reducing lipolysis and the cellular AMP/ATP ratio.

The aim of our study was to characterize the insulin-induced inhibition of AMPK in primary adipocytes and to identify mechanisms underlying this regulation. Together, this will provide a greater understanding of how lipid storage in adipocytes is regulated by insulin.

## MATERIALS AND METHODS

*Materials.* Insulin was purchased from Novo Nordisk (Copenhagen, Denmark). Complete protease-inhibitor cocktail was from Roche (Mannheim, Germany). Precast Novex SDS polyacrylamide bis-Tris gels, DTT, and lauryl dodecyl sulfate (LDS) sample buffer were purchased from Invitrogen (Carlsbad, CA). Dulbecco's modified Eagle's medium (DMEM), gentamicin, 1,4-bis(5-phenyl-2-oxazolyl) benzene, 2,2′-*p*-phenylene-bis(5-phenyloxazole) (POPOP), 2,5-diphenyloxazole (PPO), phenylisopropyl adenosine (PIA), anti-c-Myc-agarose, free glycerol reagent, and cytochalasin B (CytB) were from Sigma Aldrich (St. Louis, MO). p81 phosphocellulose cation-exchange paper was from Whatman (Dassel, Germany). $^{32}$Pγ-ATP and $^{3}$H-acetic acid were obtained from Perkin Elmer (Boston, MA). AMARA peptide (AMARAASAAALARRR) was synthesized by GL Biochem (Shanghai, China). Enhanced chemiluminescent (ECL) substrates SuperSignal West Pico and SuperSignal West Femto were obtained from Thermo Fisher Scientific (Rockford, IL). MK-2206 was from Active BioChem (Hong Kong). 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was purchased from Toronto Research Chemicals (Toronto, Canada) and A-769662 from Abcam (Cambridge, UK). OPC 3911 was obtained from Otsuka Pharmaceuticals (Tokyo, Japan) and Rolipram from Biomol International (Hamburg, Germany). Myc-AMPK constructs [wild type (WT), S485A, and S485D] were a kind gift from Grahame Hardie, and recombinant adenoviruses (Ad-Myc-WT AMPK, Ad-Myc-S485A AMPK, and Ad-Myc-S485D AMPK) were generated by Vector Biolabs (Malvern, PA).

*Antibodies.* The following primary antibodies were used for Western blots: anti-AMPK (no. 2603), anti-AMPK-pT172 (1:1,000; no. 2535), anti-AMPK-pS485 (1:1,000; no. 4185), anti-HSL (no. 4107), anti-HSL-pS563 (1:1,000; no. 4139), anti-Raptor (1:1,000; no. 2280), anti-Raptor-pS792 (1:1,000; no. 2083), anti-ACC (1:1,000; no. 3662), anti-ACC-pS79 (1:1,000; no. 3661), anti-PKB-pT308 (1:2,000; no. 9275), anti-GSK3α/β pS21/9 (1:1,000; no. 9331), and anti-Myc (1:1,000; no. 2276), and were all purchased from Cell Signaling Technology (Danvers, MA). Anti-β-actin (1:2,000; no. A5441) was from Sigma Aldrich (St. Louis, MO) and anti-AS160 (1:1,000; no. 07-741) from Merck. Anti-PKB-pS473 (1:5,000; no. 44-621G), anti-AS160-pT642 (1:1,000; no. 44-1071G), anti-GSK3α/β (1:2,000; no. 44-610), and anti-rabbit secondary antibodies conjugated to horseradish peroxidase (HRP) were from Thermo Fisher Scientific (Rockford, IL). Anti-mouse secondary conjugated to HRP was from GE Healthcare (Uppsala, Sweden).

*Isolation, stimulation, and lysis of primary adipocytes.* Rat adipocytes were isolated from epididymal adipose tissue of 36- to 38-day-old Sprague-Dawley rats (Taconic, Ejby, Denmark), as described in Berggreen et al. (6). Animal experiments were approved by the Regional Ethical Committee on Animal Experiments in Malmö/Lund (approval nos. M286-10 and 5.8.18-18569/2018). Human adipocytes were isolated from abdominal subcutaneous adipose tissue collected from female subjects who underwent reconstructive breast surgery [$n$ = 15 individuals, body mass index (BMI) 26.1 ± 2.4 kg/m$^2$ (means ± SD)]. All subjects gave their written informed consent, and the studies were approved by the Regional Ethical Review Board at Lund University (approval nos. 2013/298 and 2017/920). Adipocytes were isolated by collagenase (1 mg/mL) digestion in a shaking incubator at 37°C (28). Digests were filtered and washed with Krebs-Ringer medium containing 25 mM HEPES pH 7.4, 200 nM adenosine, 2 mM glucose, and 1% (wt/vol) BSA (KRH buffer). After isolation, human adipocytes were incubated overnight in DMEM containing 0.1 mg/mL gentamicin, 3.5% (wt/vol) BSA, and 200 nM PIA (overnight incubation medium) at 37°C under 5% $CO_2$. Subsequently, adipocytes were washed and resuspended in KRH buffer and treated as indicated in the figures. Rat adipocytes were stimulated directly after isolation. After stimulation, cells were washed in KRH buffer without BSA and lysed in lysis buffer containing 50 mM Tris·HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium-β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 mM DTT, 1% (wt/vol) NP40, and complete protease inhibitor (1 tablet/50 mL). Lysates were centrifuged at 13,000 *g* for 15 min at 4°C, and protein concentration in the supernatant was determined according to Bradford (7).

*Western blots.* Cell lysates were heated in LDS sample buffer, subjected to electrophoresis on precast Novex 4–12% bis-Tris gels, and subsequently electrotransferred to nitrocellulose membranes. Membranes were blocked for 30–60 min in 50 mM Tris·HCl pH 7.6, 137 mM NaCl, and 0.1% (wt/vol) Tween 20 (TBS-T) containing 10% (wt/vol) skimmed milk and then probed with primary antibodies in TBS-T containing 5% (wt/vol) BSA for 16 h at 4°C. Protein detection was performed with HRP-conjugated secondary antibodies and ECL substrate. Signals were visualized using a ChemiDoc XRS+ system, followed by analysis of band intensities with the software Image Laboratory 6.0 (both from Bio-Rad; Hercules, CA).

*Measurement of de novo fatty acid synthesis.* After isolation, 700 μL of 3–4% (vol/vol) packed primary rat adipocytes in KRH buffer (0.55 mM glucose, 3.5% BSA) were left untreated or stimulated with the PKB inhibitor MK-2206 or AMPK activator A-769662 for 1 h and subsequently incubated with or without insulin and 1 mM [$^3$H]-acetate (final concentration ≈ 0.8 μCi/mL) for an additional 2 h at 37°C, 120 rpm shaking. Each stimulation was performed in triplicates. As a blank, KRH buffer (without cells) was incubated with [$^3$H]-acetate for 2 h. All reactions, including the blank, were stopped with 3.5 mL of a toluene-based scintillation liquid containing 0.3 g/L POPOP and 5 g/L PPO. Samples were subjected to liquid scintillation counting, and data were expressed as percentage of a nonpretreated sample after subtraction of the blank value.

*Adenovirus-mediated Myc-AMPK expression.* Primary rat adipocytes isolated as described above were incubated in overnight incubation medium for 16–18 h at 37°C under 5% $CO_2$, with adenoviruses encoding Myc-AMPK-WT, Myc-AMPK-S485A, or Myc-AMPK-S485D, at a concentration of 50 × 10$^6$ plaque-forming units

virus/mL cell suspension. Cells were then washed in KRH buffer, stimulated as mentioned in the figure legends, and lysed as described above.

*Immunoprecipitation and in vitro assay of AMPK activity.* Lysates containing 5–10 μg of protein were incubated at 4°C for 1–2 h on a shaking platform with anti-c-Myc-agarose. The immunoprecipitates were washed twice with 500 μL lysis buffer, defined above, supplemented with 0.5 M NaCl and 1 mM DTT, and twice with 500 μL of 50 mM Tris·HCl pH 7.5, 0.1 mM EGTA, and 1 mM DTT. The kinase activity was measured in a volume of 50 μL containing 50 mM Tris·HCl pH 7.5, 0.1% 2-mercaptoethanol, 10 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM $^{32}$Pγ-ATP (final concentration ≈ 0.36 μCi/mL), and 200 μM AMARA peptide for 20 min at 30°C. The assay was terminated by applying 40 μL of the reaction mixture on p81 cation-exchange phosphocellulose paper, followed by immersion of the paper in 50 mM phosphoric acid and 5–6 subsequent washes in 50 mM phosphoric acid before liquid scintillation counting. Incorporation of $^{32}$P-phosphate was expressed as picomoles ATP incorporated per milligram protein per minute (mU/mg).

*Collection of medium for glycerol determination.* Adipocyte suspensions [300 μL of 5–10% (vol/vol) packed cells in KRH buffer] were stimulated at 37°C, in a 150-rpm shaking incubator, as described in the figure legends. When measuring glycerol release, cells were allowed to rest on ice for 10 min after stimulation and the medium was collected. Subsequently, cells were washed twice in KRH buffer (without BSA) and lysed as described above. The cell lysates were centrifuged for 15 min at 13,000 *g*, and the infranatant was collected. The amount of glycerol released into the medium was measured with an enzymatic method as previously described (12) or the help of the commercially available free glycerol reagent (Sigma) and presented as percentage of the basal sample.

*Nucleotide measurements.* Rat adipocyte suspensions [700 μL of 10% (vol/vol) packed cells in KRH buffer] were stimulated at 37°C with increasing doses of insulin or 100 nM isoproterenol or preincubated with MK-2206 (1 h) or cytochalasin B (30 min) and subsequently stimulated with insulin for 30 min as indicated in the figure. Incubations were stopped by the addition of ice-cold KRH buffer without BSA. After removal of the KRH buffer, cells were lysed in 400 μL 0.1 M perchloric acid in 40% methanol. Lysates were centrifuged at 4°C, 4,000 rpm for 10 min. Supernatants were transferred to new tubes and centrifuged one more time. The pH of the final supernatant was adjusted to 7 with 1.1 M $(NH_4)_2HPO_4$ and samples were dried in a vacuum concentrator. Dried pellets were resuspended in water and purine nucleotides were measured by HPLC as described in (48).

*Statistical analysis.* Results are presented as means +SD (± SD for glycerol release) of several independent experiments (number specified in figure legends). Western blot quantification data were normalized to a control sample (100%) to account for variations in absolute values in between experiments, which were analyzed on separate gels and occasions. Statistical analysis was performed using GraphPad Prism 8, and the respective tests are described in the figure legends. Differences between two groups were considered significant when $P < 0.05$ ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$).

## RESULTS

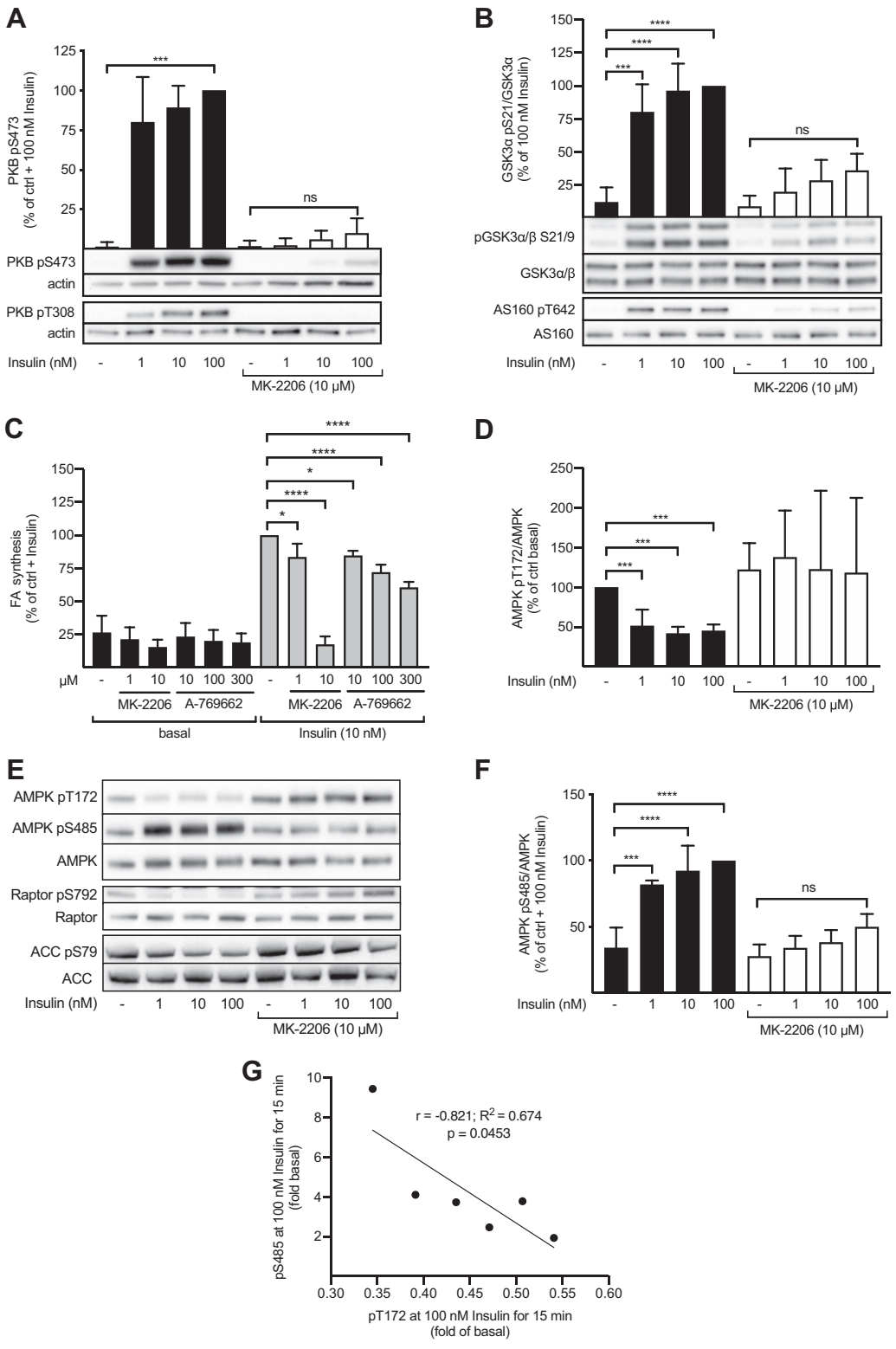
*Insulin induces fatty acid synthesis and inhibits AMPK in a PKB-dependent manner.* For detailed characterization of the involvement of PKB in the regulation of FA synthesis as well as AMPK phosphorylation and activity, the PKB inhibitor MK-2206 was employed in primary rat adipocytes. To verify the potency of MK-2206, we monitored the phosphorylation of PKB at the activity-controlling sites S473 and T308, which was reduced in the presence of the inhibitor (Fig. 1*A*). Additionally, inhibition of PKB activity was also confirmed by impaired phosphorylation of the downstream targets GSK3 and AS160 in the presence of MK-2206 (Fig. 1*B*). Subsequently, the effect of insulin, in the absence or presence of MK-2206, on FA synthesis (incorporation of acetate into lipids) and AMPK phosphorylation was investigated. As shown in Fig. 1*C*, PKB inhibition greatly reduced insulin-stimulated FA synthesis in primary rat adipocytes, whereas in the basal state no significant change was observed. The phosphorylation of AMPK on the activity-promoting site T172 was significantly decreased in a dose-dependent manner in response to insulin, an effect that was abolished when inhibiting PKB (Fig. 1, *D* and *E*). This effect of MK-2206, indicating a PKB-dependent inactivation of AMPK, was confirmed by monitoring the downstream AMPK substrates Raptor S792 and ACC S79. The phosphorylation of these sites was reduced in response to insulin, in the absence, but not in the presence, of MK-2206 (Fig. 1*E*). The insulin-induced decrease in AMPK activity was accompanied by an increase in S485 phosphorylation, which was also greatly reduced in the presence of MK-2206 (Fig. 1, *E* and *F*). Furthermore, correlation analysis of fold changes in AMPK pT172 versus pS485 at 100 nM insulin revealed a significant correlation between decreased pT172 and increased pS485 phosphorylation (Fig. 1*G*). In addition to the inverse association between AMPK activity and S485 phosphorylation, the data in Fig. 1 also demonstrate an association between low AMPK activity and high levels of FA synthesis. In line with this, we also observed the opposite: AMPK activation by the compound A-769662 (8) significantly inhibited the insulin-induced increase in FA synthesis in adipocytes (Fig. 1*C*).

To explore whether the time frame for insulin-induced changes in AMPK phosphorylation is compatible with a causal role for S485 in the inactivation of the kinase, adipocytes were treated with insulin for increasing time periods. Western blot analysis of AMPK pT172 and pS485 demonstrated a significant decrease and increase, respectively, already after 5 min, reaching the maximum of these effects after 60 min (Fig. 2, *A–C*). These results further indicate an inverse correlation between the phosphorylation of these two sites on AMPK. The time-dependent, insulin-stimulated inactivation of AMPK was again verified by a decreased phosphorylation of Raptor at S792 and ACC at S79 (Fig. 2*C*).

*Insulin-induced inactivation of AMPK is not dependent on S485 phosphorylation.* To investigate whether AMPK S485 phosphorylation is required for the insulin-induced inhibition of kinase activity, Myc-tagged AMPKα-WT or a nonphosphorylatable AMPKα-S485A mutant were expressed in primary rat adipocytes via adenoviral transduction. Cells transduced with green fluorescent protein (GFP) served as a control. Subsequently, the cells were stimulated with increasing doses of insulin and AMPK phosphorylation and activity were monitored. Western blot analysis showed that S485 phosphorylation was indeed prevented in cells expressing AMPKα-S485A (Fig. 3*A*). However, AMPK T172 phosphorylation was significantly reduced by insulin in both AMPKα-WT and AMPKα-S485A-expressing cells (Fig. 3*A*). Measurement of Myc-AMPKα1 kinase activity in vitro (Fig. 3*B*) revealed a comparable significant decrease in activity of both wild-type (WT) and S485A AMPK in response to increasing doses of insulin. Insulin-induced reduction in Raptor S792 and ACC S79 phos-

phorylation (Fig. 3*C*) also confirmed that AMPK was inhibited to a similar extent in cells expressing the WT and the S485A mutant.

To further examine a possible role of S485 phosphorylation in the regulation of AMPK activity, an AMPKα-S485D mutant, mimicking phosphorylation at S485, was expressed and Myc-AMPKα1 activity was measured. After normalizing Myc-AMPKα1 activity to the varying transduction/expression efficiencies of the different constructs (Myc-AMPKα1-WT, S485A, S485D), basal (without insulin) kinase activities were compared. The results showed no significant differences in basal kinase activity; however, the S485A and S485D mutants



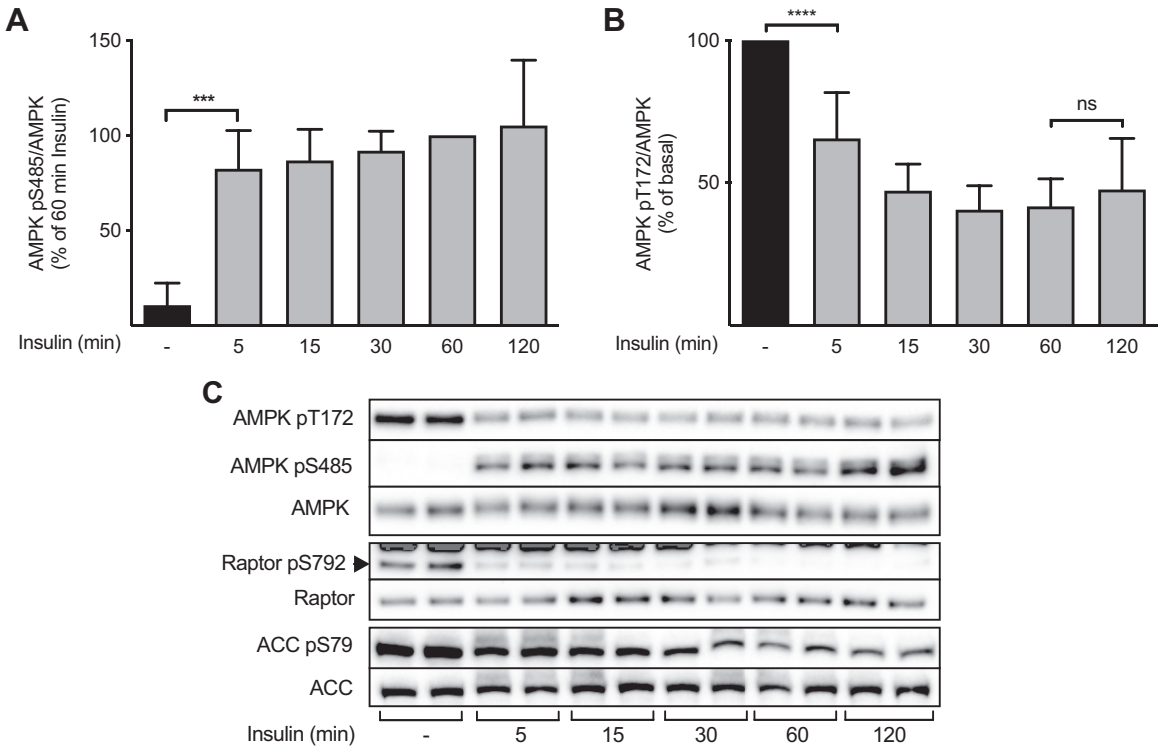



Fig. 2. Insulin-induced increase of AMP-activated protein kinase (AMPK) S485 phosphorylation correlates in time with decreased activity. Isolated primary rat adipocytes were stimulated with 100 nM insulin for an increasing period of time or left untreated, as indicated in the figure. Phosphorylation of AMPK S485 (*A* and *C*) and T172 (*B* and *C*), Raptor S792 (*C*), and acetyl-CoA carboxylase (ACC) S79 (*C*) was analyzed by Western blot using phospho-specific antibodies. Blots are representative of 2–9 independent experiments. *A* and *B*: pAMPK Western blot signals were normalized to total AMPK levels, expressed as % of the 60-min insulin-treated sample (pS485) or the basal sample (pT172), in each experiment and are presented as means + SD. Statistical significance was determined by unpaired two-tailed Student's *t* test (*A*) or one-way ANOVA followed by Holm–Sidak's multiple comparison test (*B*). ***$P < 0.001$, ****$P < 0.0001$; ns, nonsignificant.

displayed tendencies to be more and less active than the WT, respectively (Fig. 3*D*).

To investigate and compare the response of WT and S485A AMPK to insulin in more detail, transduced cells were stimulated with insulin for varying time periods (Fig. 4, *A–C*). Although S485 phosphorylation was almost completely lost in cells expressing the mutant, we did not observe any significant differences regarding the reduction in T172 phosphorylation (Fig. 4*A*) or Myc-AMPKα1 kinase activity (Fig. 4*B*) at any of the insulin time points between WT and the S485A mutant. The similar behavior of the two constructs was also demonstrated by a comparable reduction in Raptor S792 and ACC S79 phosphorylation (Fig. 4*C*) at different time points after insulin stimulation of AMPK-WT and S485A-expressing cells.

*Effect of insulin on activation of AMPK in response to pharmacological AMPK activators.* For further characterization of the inactivation of AMPK by insulin, the effect of pharmacological AMPK activators after treatment with insulin was monitored. Primary rat adipocytes were preincubated with or without insulin and subsequently stimulated with either of the two AMPK activators, AICAR or A-769662 (20). Western blot analysis demonstrated that both compounds were able to activate AMPK, shown as increased AMPK T172 (Fig. 5*A*) as well as Raptor S792 (Fig. 5*B*) and ACC S79 phosphorylation (Fig. 5*C*). After preincubation with insulin, AICAR retained the ability to increase AMPK activity, whereas the ability of A-769662 to do so was abolished (Fig. 5, *A–C*). AMPK S485 phosphor-

Fig. 1. PKB-dependent AMP-activated protein kinase (AMPK) inactivation by insulin is accompanied by increased AMPK S485 phosphorylation. *A*, *B*, and *D–F*: primary rat adipocytes were pretreated with (white bars) or without (black bars) 10 μM MK-2206 for 1 h, followed by stimulation with increasing doses of insulin for 15 min as indicated. Phosphorylation of PKB S473 and T308 (*A*), GSK3α/β S21/9 (*B*), AS160 T642 (*B*), AMPK T172 (*D* and *E*), and S485 (*E* and *F*), as well as Raptor S792 (*E*) and acetyl-CoA carboxylase (ACC) S79 (*E*), was analyzed by Western blot with phospho-specific antibodies and normalized to total protein levels where indicated. Blots shown are representative of 3 independent experiments, and data are expressed as % of the nonpretreated, 100-nM insulin-stimulated sample (*A*, *B*, and *F*) or the nonpretreated basal (without insulin) (*D*) in each experiment. *C*: primary adipocytes isolated from rat epididymal adipose tissue were pretreated with the PKB inhibitor MK-2206 or AMPK activator A-769662 for 1 h, as indicated, and subsequently stimulated with 10 nM insulin (gray bars) for another 2 h or left untreated (black bars). De novo fatty acid (FA) synthesis was measured as the incorporation of $^3$H-acetate into total lipids in $n = 4$ independent experiments and expressed as % of the nonpretreated insulin stimulated sample. *G*: fold changes of AMPK T172 and S485 phosphorylation at 100 nM insulin were plotted against each other ($R^2 = 0.674$, $P = 0.0453$, $n = 6$). Correlations were made using Pearson correlation test. *A–D* and *F*: data are presented as means + SD, and statistical significance was determined by one-way ANOVA followed by Holm–Sidak's multiple comparison test. *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$; ns, nonsignificant.

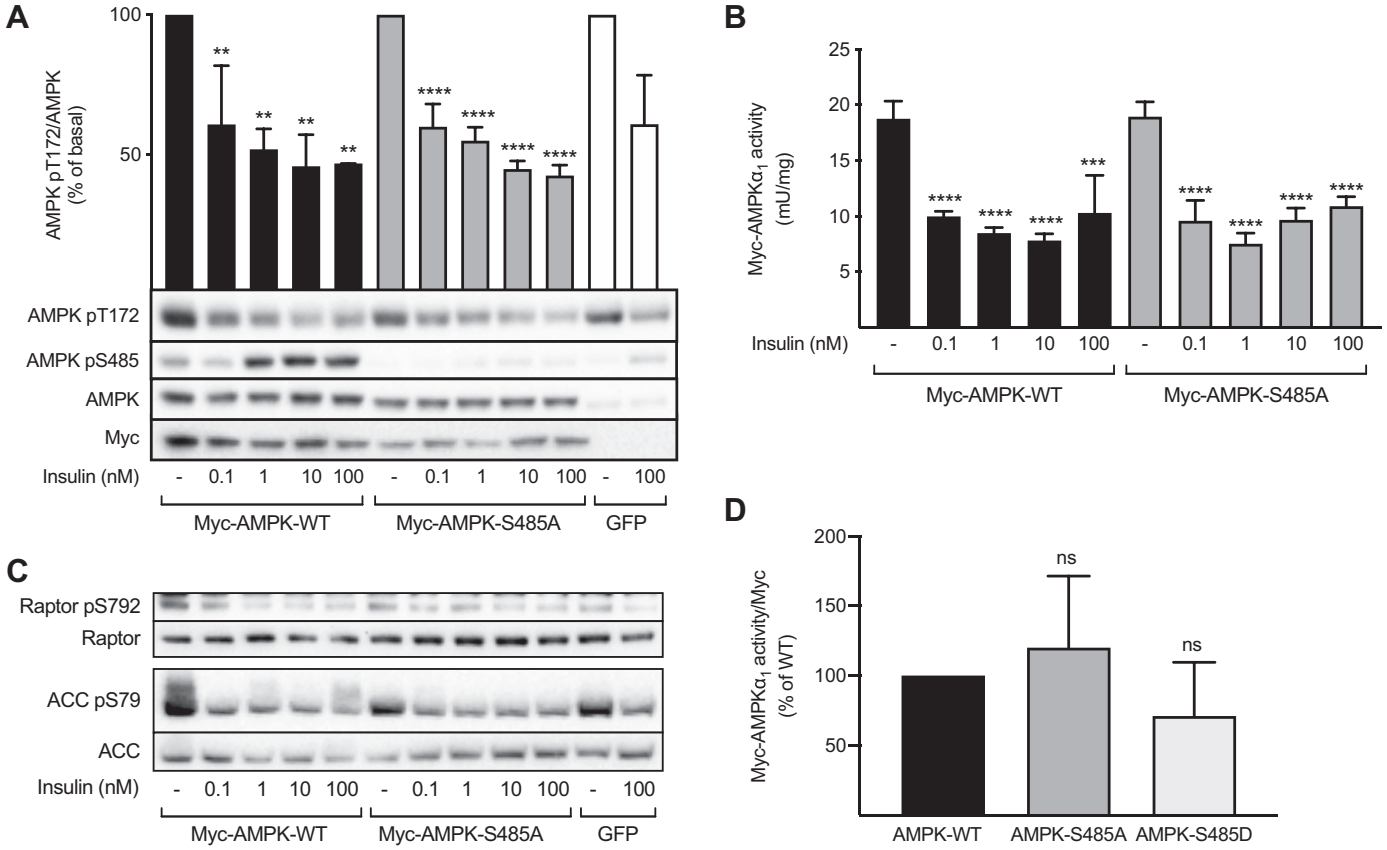



Fig. 3. AMP-activated protein kinase (AMPK) S485 phosphorylation is not required for insulin-induced inhibition of kinase activity. *A–D*: primary rat adipocytes expressing Myc-tagged AMPKα1-WT or AMPKα1-S485A were stimulated for 30 min with increasing doses of insulin as indicated. Green fluorescent protein (GFP)-expressing cells were used as control and stimulated with the highest dose of insulin. Phosphorylation of AMPK T172 (*A*) and S485 (*A*), as well as Raptor S792 (*C*) and acetyl-CoA carboxylase (ACC) S79 (*C*), was analyzed by Western blot using phospho-specific antibodies. AMPK pT172 signals were normalized to total AMPK levels and expressed as % of the basal for each construct (AMPK pT172/AMPK as % of WT basal: WT = 100%, S485A = 113.3 ± 22.1%, and GFP = 337.5 ± 132.6%) in each experiment. Blots shown are representative of 3 independent experiments. *B*: in vitro kinase activity against the peptide substrate AMARA was measured in anti-Myc immunoprecipitates ($n = 3$ independent experiments). *D*: in vitro kinase activity measured in anti-Myc immunoprecipitates from nontreated cells expressing Myc-tagged AMPK-WT, AMPK-S485A, or AMPK-S484D was normalized to Myc expression levels and expressed as % of WT-AMPK in each experiment ($n = 3$–7 independent experiments). Kinase activity is shown in *D*, expressed as mU/mg: WT = 15.6 ± 6.1, S485A = 17.5 ± 4.37, and S485D = 12.5 ± 11.0. All graphs show means + SD. Statistical significance was determined by one-way ANOVA followed by Holm–Sidak's multiple comparison test. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; ns, nonsignificant.

ylation was induced to a comparable degree before stimulating with either of the two activators (Fig. 5*A*).

*Alternative mechanism for insulin-induced AMPK inhibition: the role of cAMP signaling/lipolysis and cellular energy levels.* Considering the previously reported role of lipolysis in the regulation of AMPK activity via changes in the AMP/ATP ratio, as well as the known antilipolytic effect of insulin, we wanted to investigate whether changes in basal (unstimulated) lipolysis could possibly be involved in the regulation of AMPK activity by insulin (19, 43, 54). Primary adipocytes were incubated with increasing doses of insulin and lipolysis was measured as the release of glycerol. As shown in Fig. 6*A*, insulin did not induce any significant lowering of lipolysis under conditions where we observe AMPK inhibition (Fig. 1). However, taking compartmentalization of cAMP into account, it is still possible that AMPK regulation by insulin involves cAMP in a PKA-dependent or -independent manner (1). Thus, adipocytes were treated with a PDE3 inhibitor (OPC 3911) and a PDE4 inhibitor (Rolipram) in the absence and presence of insulin. Isoproterenol was used as a control. The results show that both PDE inhibitors induced, in comparison to isoproterenol, a small increase in lipolysis (Fig. 6*B*, *top*). When adding insulin in the presence of PDE inhibitors, no further changes in lipolysis were detected (Fig. 6*B*, *top*). However, when analyzing hormone-sensitive lipase (HSL) S563 phosphorylation, we observed increased phosphorylation in the presence of the PDE inhibitors, which in the case of Rolipram was counteracted by insulin (Fig. 6*B*, *bottom*). This latter observation is in line with the involvement of PDE3B, but not PDE4, in the ability of insulin to inhibit catecholamine-induced lipolysis (13, 60). When monitoring the effect of PDE inhibition on the ability of insulin to reduce AMPK activity, measured as a decrease in AMPK T172 as well as pACC S79 and Raptor S792 phosphorylation, neither OPC 3911 (Fig. 6, *C* and *E*) nor Rolipram (Fig. 6, *D* and *E*) induced any significant changes compared with the noninhibitor-treated cells.

With the finding that changes in basal lipolysis or other cAMP-regulated processes are not likely to be involved in the effect of insulin on AMPK activity, we wanted to investigate whether insulin stimulation of primary adipocytes induces a decrease in the cellular AMP/ATP ratio regardless of lipolysis, which could account for the decreased AMPK activity. For this

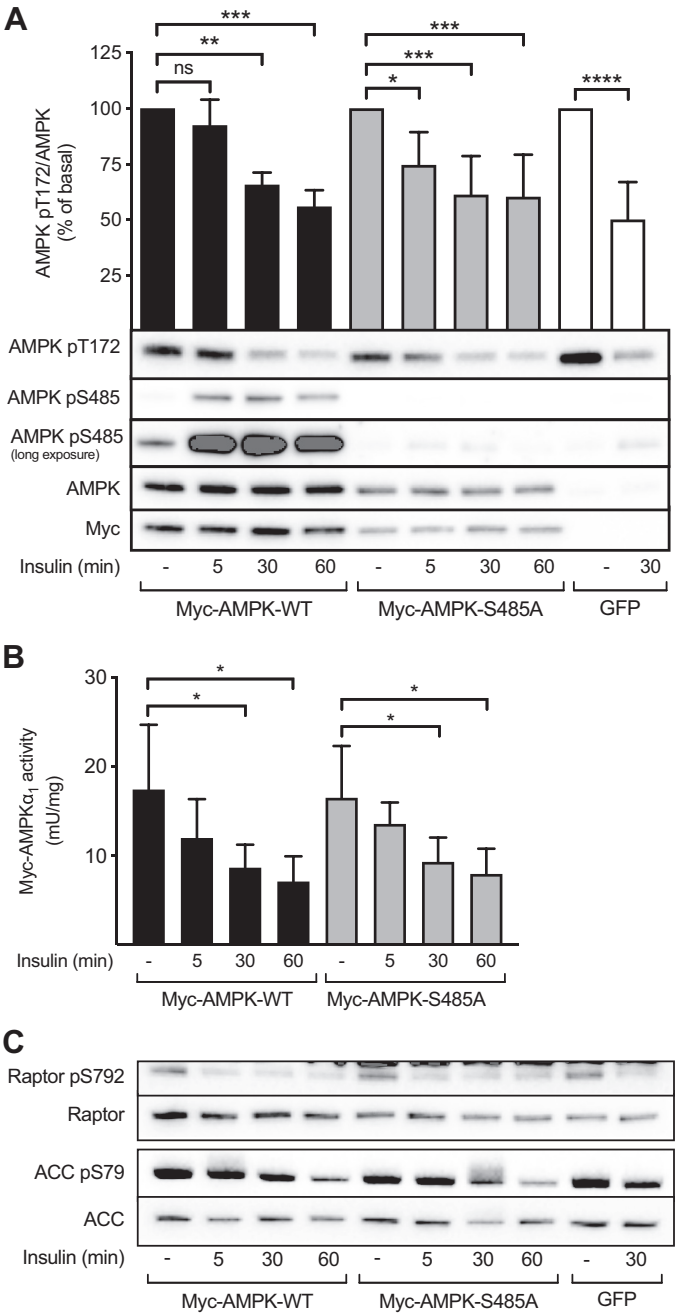



Fig. 4. AMP-activated protein kinase (AMPK)-S485A is inhibited by insulin in a time-dependent manner comparable to AMPK-WT. Primary rat adipocytes overexpressing Myc-tagged AMPK-WT or AMPK-S485A were stimulated with 100 nM of insulin for different time periods as indicated. Green fluorescent protein (GFP)-expressing cells were used as a control and stimulated with insulin for 30 min. Phosphorylation of AMPK T172 and S485 (*A*), as well as Raptor S792 and acetyl-CoA carboxylase (ACC) S79 (*C*), was analyzed by Western blot using phospho-specific antibodies. AMPK pT172 signals were normalized to total AMPK levels and expressed as % of the basal for each construct (AMPK pT172/AMPK as % of WT basal: WT = 100%, S485A = 105.3 ± 14.4%, and GFP = 441.0 ± 242.6%). Blots shown are representative of 4 independent experiments. *B*: in vitro kinase activity against the peptide substrate AMARA was measured in anti-Myc immunoprecipitates ($n = 4$ independent experiments). All graphs show means + SD. Statistical significance was determined by one-way ANOVA followed by Holm–Sidak's multiple comparison test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; ns, nonsignificant.

purpose, adipocytes were stimulated with increasing doses of insulin as well as isoproterenol, known to increase the AMP/ATP ratio, as a control (19). Cell extracts were then analyzed by HPLC. Analysis of the nucleotide content revealed that isoproterenol induced a robust increase in the AMP/ATP ratio, whereas insulin stimulation indeed resulted in a dose-dependent, significant decrease in the cellular AMP/ATP ratio (Fig. 7*A*, *top*). Furthermore, the results show that this observed decrease in the AMP/ATP ratio by insulin was caused by an increase in ATP levels and an even stronger decrease in AMP levels (Fig. 7*A bottom*). With this result suggesting an insulin-induced decrease in cellular AMP/ATP ratio as a possible cause for reduced AMPK activity in response to insulin, we wanted to investigate the involvement of PKB in this mechanism. For this purpose, nucleotides were analyzed after treatment of adipocytes with the PKB inhibitor MK-2206 before insulin stimulation. The results demonstrated that PKB inhibition prevented the insulin-induced decrease in AMP/ATP ratio (Fig. 7*B*). To address whether the observed decrease in AMP/ATP ratio was due to increased glucose uptake in response to insulin stimulation, nucleotides were also analyzed in adipocytes pretreated with cytochalasin B (CytB), an inhibitor of glucose transporters (27, 46). The results showed a comparable insulin-induced decrease in AMP/ATP in nonpretreated and CytB-treated cells (Fig. 7*B*).

*Insulin-induced regulation of AMPK activity in human adipocytes.* To explore whether the regulation of AMPK activity by insulin is also of relevance in humans, primary adipocytes isolated from subcutaneous human adipose tissue were treated with increasing doses of insulin and AMPK phosphorylation at T172 and S485 and ACC S79 phosphorylation was monitored. Similar to what we observed in rat adipocytes, AMPK S485 phosphorylation increased in a dose-dependent manner in response to insulin (Fig. 8*A*). However, although we observed a decrease in AMPK pT172 phosphorylation at 10 nM insulin in the majority of the experiments (Fig. 8*B*), the variation was large and no significant changes in the average response to insulin were detected—a result that was also reflected in the level of ACC S79 phosphorylation (Fig. 8*C*). Analysis of fold changes in AMPK pT172 versus pS485 at 10 nM insulin showed no significant correlation between these two phosphorylations (Fig. 8*D*).

## DISCUSSION

In our previous study (6), we demonstrated that insulin stimulation of primary rat adipocytes induces a PKB-dependent AMPK S485 phosphorylation, which is associated with decreased AMPK T172 phosphorylation and kinase activity. In our present study, we showed that AMPK S485 phosphorylation is no requirement for reduced kinase activity. However, we have identified alternative mechanisms involving reduced cellular energy levels and PDEs, which could be responsible for mediating the insulin-induced inhibition of AMPK.

In the current study, we used the pharmacological PKB inhibitor MK-2206 to show that insulin-induced S485 phosphorylation and inhibition of AMPK is dependent on active PKB, which is in line with our previous result obtained with the less-specific inhibitor Akti1/2 (6). This observation is also in agreement with other studies, showing PKB-dependent AMPKα1-S485 phosphorylation in different cell models, as

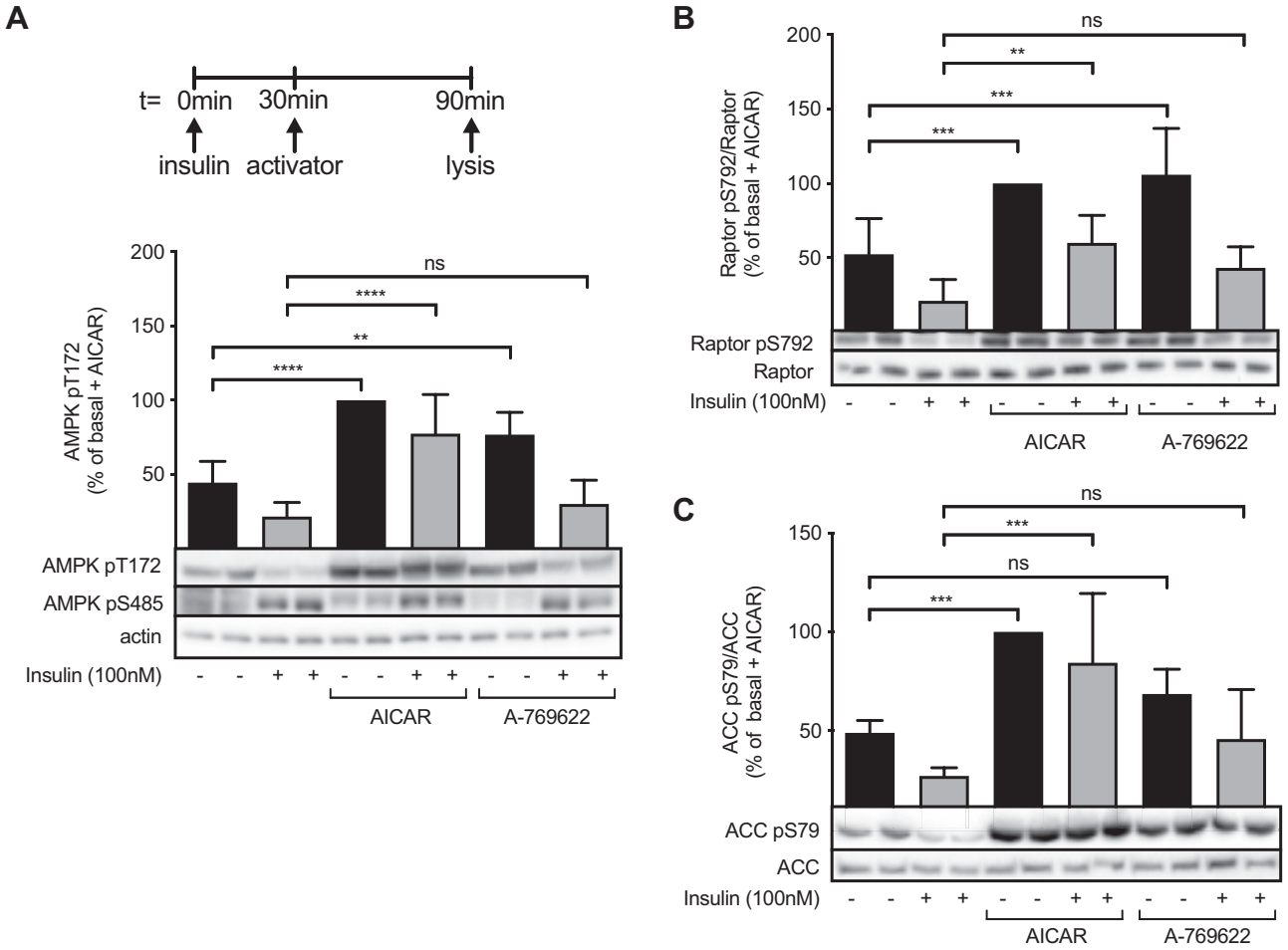



Fig. 5. AMP-activated protein kinase (AMPK) activation by exogenous activators is impaired by prior insulin treatment. Isolated primary rat adipocytes were pretreated with or without 100 nM insulin for 30 min and subsequently stimulated with 2 mM 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) or 300 μM A-769662 for 1 h (*A*, *top*). AMPK T172 and S485 (*A*, *bottom*), as well as Raptor S792 (*B*) and acetyl-CoA carboxylase (ACC) S79 phosphorylation (*C*) was analyzed by Western blot using phospho-specific antibodies. Western blot signals were normalized to total protein levels where indicated. Graphs show means + SD of 5–6 independent experiments, each expressed as % of the nonpretreated, AICAR-stimulated sample. Statistical significance was determined by one-way ANOVA followed by Holm–Sidak's multiple comparison test. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; ns, nonsignificant.

well as in vitro—the latter suggesting that S485 is a direct target for PKB (26, 30, 44, 53).

A key finding in this paper is that the activity of the nonphosphorylatable AMPK S485A mutant was still inhibited by insulin, down to a level similar to that of WT AMPK. This clearly demonstrates that S485 phosphorylation is not a prerequisite for the ability of insulin to inhibit AMPK in adipocytes. Nevertheless, our results suggest that S485 phosphorylation might, under some conditions, contribute to the regulation of AMPK activity because when normalizing to expression levels (Myc), S485A AMPK showed a tendency to be more active, whereas the phospho-mimetic S485D mutant appeared to be less active than WT AMPK, at least under basal conditions.

In contrast to our results, previous studies using the AMPK S485A mutant demonstrated a requirement of Akt/PKB-dependent S485 phosphorylation for the inhibition of AMPK activity in rat hearts as well as the human-derived HEK-293 cell line (26, 30). However, it is important to mention that both of these studies focused on the role of S485 phosphorylation in the prevention of subsequent AMPK activation by LKB1 in vitro or by AMPK activators in cells. In contrast, the aim of our study was to test the requirement of insulin-induced S485 phosphorylation for inhibition of basal AMPK activity, i.e., in the absence of exogenous AMP activators, with the ultimate goal to determine the mechanism by which insulin increases FA synthesis under physiological conditions.

Furthermore, we found that insulin pretreatment impaired the ability of the pharmaceutical activator A-769662 to activate AMPK. AICAR, however, was able to activate AMPK despite prior insulin treatment. The differences in the ability of AICAR and A-769662 to activate AMPK in the presence of insulin might be due to their differential mechanisms of action. Whereas AICAR acts as an AMP-mimetic, which, like AMP, binds to the γ-subunit (33), A-769662 binds to the so-called ADaM site located at an interface between the α- and β-subunit (20, 50, 51). Considering the different binding sites, one way to interpret the observation that insulin seems to affect AMPK activation by

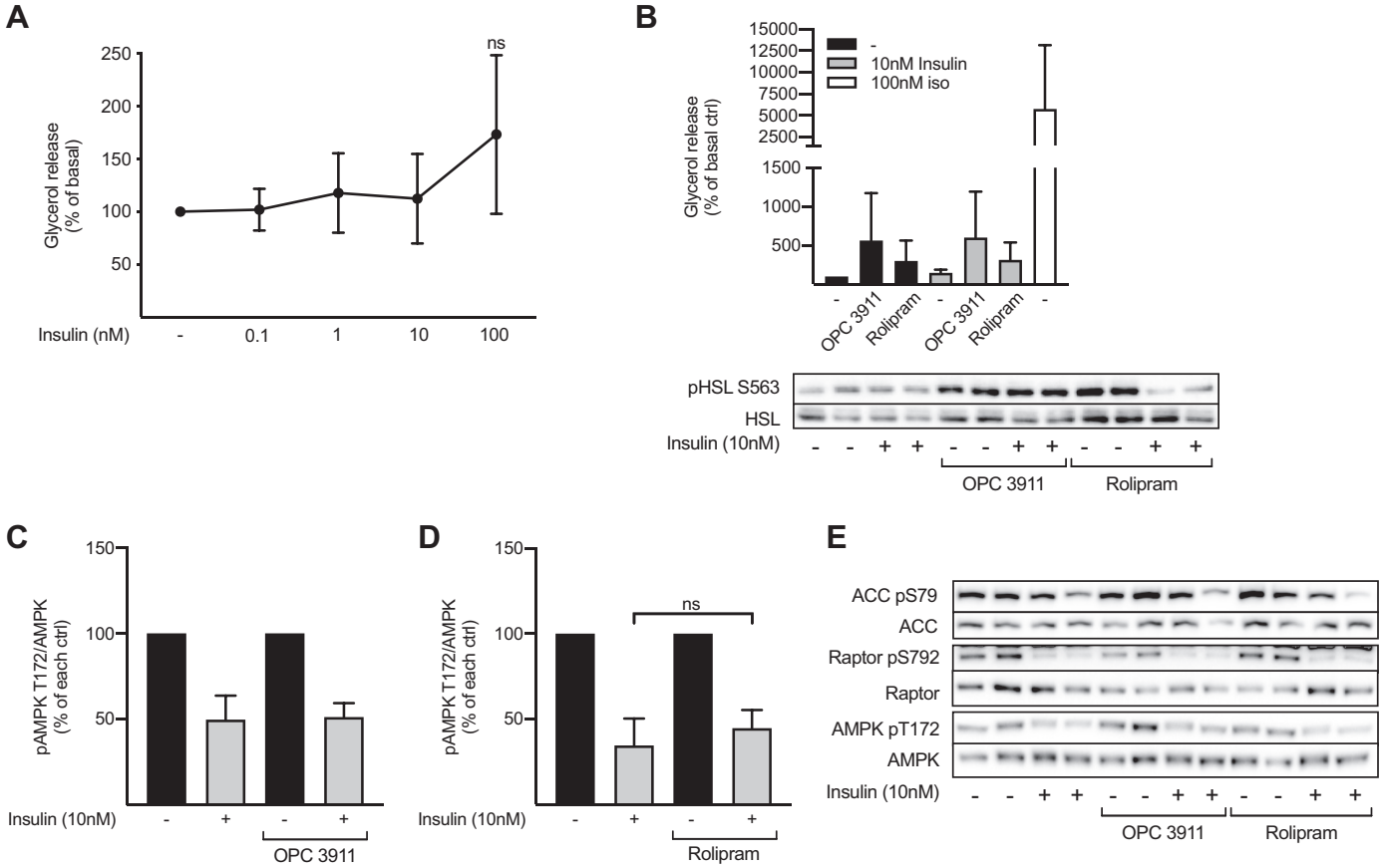



Fig. 6. Changes in lipolysis and cellular cAMP levels are not likely to be involved in the insulin-induced inhibition of AMP-activated protein kinase (AMPK) activity. *A*: lipolysis from adipocytes stimulated with increasing concentrations of insulin was measured as glycerol release into the medium and expressed as % of the basal sample. The graph shows means ± SD of 3 independent experiments. *B–E*: isolated primary rat adipocytes were pretreated with or without 10 μM OPC 3911 (PDE3 inhibitor) or 10 μM Rolipram (PDE4 inhibitor) for 10 min and subsequently stimulated with (gray bars) or without (black bars) 10 nM insulin for 30 min. Lipolysis (*B*, *top*) was measured as the release of glycerol into the medium and expressed as % of the nonpretreated, basal sample ($n = 3$ independent experiments). Cell lysates were subjected to Western blot, and phosphorylation of hormone-sensitive lipase (HSL) S563 (*B*, *bottom*), AMPK T172 (*C–E*), AMPK S485 (*E*), Raptor S792 (*E*), and acetyl-CoA carboxylase (ACC) S79 (*E*) was analyzed with phospho-specific antibodies. Blots are representative of 4–5 independent experiments. *C* and *D*: AMPK pT172 signals were normalized to total AMPK levels and expressed as % of each basal sample [AMPK pT172/AMPK as % of the nonpretreated basal sample: OPC 3911 (*C*) = 131.26 ± 23.5% and Rolipram (*D*) = 84.9 ± 26.1%]. Graphs display means ± SD (*A*) or + SD (*B–D*). Statistical significance was determined by unpaired two-tailed Student's *t* test; ns, nonsignificant. iso, isoproterenol.

A-769662 more than that by AICAR, which binds much closer to the affected phosphorylation sites, is that S485 does not play an important role in insulin-induced inhibition of AMPK. In the future, it would be interesting to further investigate the mechanism by which insulin affects AMPK activation by these two activators.

Having observed that S485 phosphorylation is dispensable, we investigated additional mechanisms that might be involved in insulin-induced AMPK inhibition, leading to decreased ACC S79 phosphorylation and subsequently increased FA synthesis. It has previously been demonstrated by us and others that AMPK is activated in a cAMP-dependent manner, likely through reduced lipolysis and cellular AMP/ATP ratio, as a result of energy-consuming re-esterification of FAs (19, 47, 57). Therefore, we investigated the role of cAMP signaling/lipolysis in the insulin-induced inhibition of AMPK activity. The results showed that increasing doses of insulin did not result in significant changes in glycerol release, indicating that lipolysis is not likely to be involved in the inhibition of AMPK activity by insulin. To determine whether other cAMP-dependent pathways could possibly be involved in the regulation of AMPK activity by insulin, we employed the two PDE inhibitors OPC 3911 and Rolipram. Although both PDE inhibitors induced an increase in cAMP levels, measured as an increase in HSL S563 phosphorylation and glycerol release, neither of the inhibitors prevented the inhibition of AMPK by insulin. Of note here is that the increase in basal lipolysis observed when inhibiting PDEs was only marginal compared with the catecholamine-induced lipolysis, indicating low basal cAMP production/lipolysis. Therefore, we were not surprised that insulin-induced effects on basal lipolysis could not be detected. Furthermore, responses induced by this minor change are not likely to be of physiological relevance.

As AMPK activity is highly dependent on cellular energy levels, we investigated the possibility that insulin might decrease the overall cellular AMP/ATP ratio, which could account for the inhibition of AMPK activity. Indeed, we observed a dose-dependent, insulin-induced decrease in the cellular AMP/ATP ratio, which was dependent on active PKB. This is in line with the requirement of PKB for the insulin-induced inhibition of AMPK activity and stimulation of FA synthesis. Additionally, as the AMP/ATP ratio-lowering effect of insulin

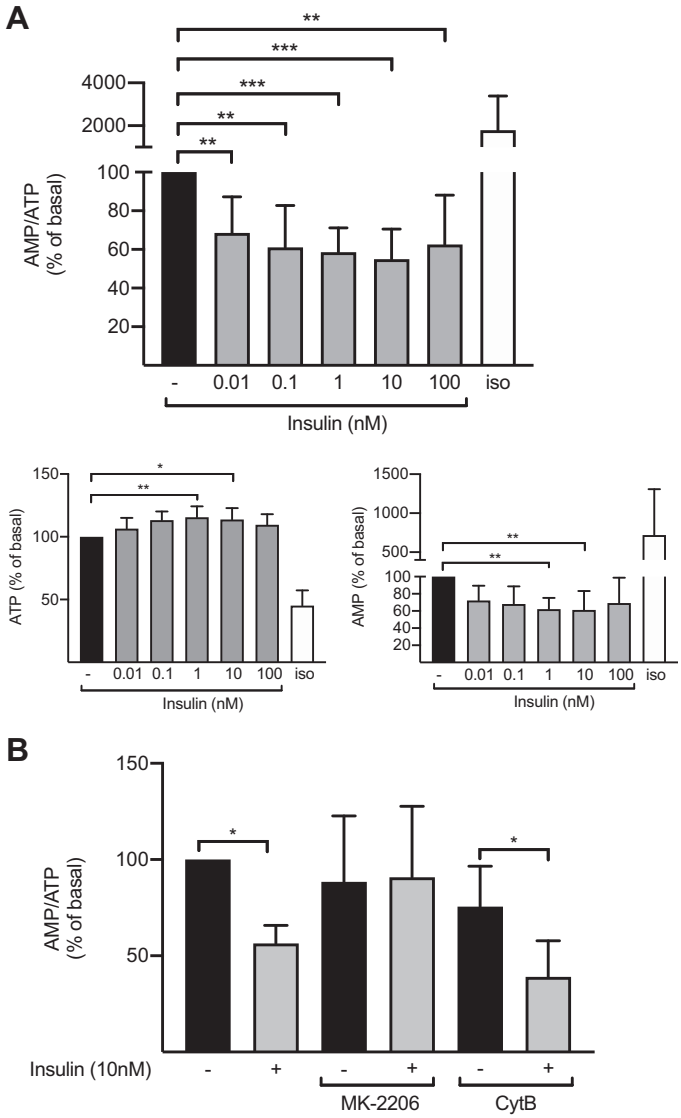



Fig. 7. Insulin stimulation induces a reduction in the cellular AMP-to-ATP ratio (AMP/ATP ratio) in primary adipocytes. *A*: isolated primary rat adipocytes were stimulated with different concentrations of insulin as indicated or 100 nM isoproterenol (iso) for 30 min. Cell extracts were prepared and subjected to HPLC for nucleotide measurement. AMP/ATP ratios (*top*) as well as the respective ATP (*bottom left*) and AMP (*bottom right*) levels are presented as % of the basal sample ($n = 3$ independent experiments). Average AMP/ATP ratio at basal level was 0.067 ± 0.040, and average ATP and AMP levels at basal were 0.021 nmol/μL ± 0.007 and $1.2 \times 10^{-3}$ nmol/μL ± $0.43 \times 10^{-3}$. *B*: isolated primary rat adipocytes were pretreated with 10 μM MK-2206 for 1 h or 10 μM cytochalasin B (CytB) for 30 min and subsequently stimulated with 10 nM insulin for 30 min where indicated. AMP/ATP ratios are presented as % of the nonpretreated basal sample ($n = 3$–4 independent experiments). Average AMP/ATP ratio at basal level (means ± SD) was 0.094 ± 0.045. Graphs display means + SD. Statistical significance was determined by one-way ANOVA followed by Holm–Sidak's multiple comparison test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

was maintained even though glucose uptake was inhibited, we believe that insulin induces this effect independently of increased glucose uptake. Together, these results indicate that the reduction of AMPK activity in response to insulin does not likely involve changes in cAMP levels or lipolysis but a PKB-dependent decrease in the cellular AMP/ATP ratio. The mechanism underlying this decrease in AMP/ATP ratio is still to be elucidated.

Of note, a previous study performed in 3T3-L1 adipocytes reported the opposite of our findings, namely an induction of T172 phosphorylation and an increase in the cellular AMP/ATP in response to insulin (35). Furthermore, whereas we observed that the reduction of AMPK pT172 in response to insulin was accompanied by reduced ACC S79, Liu et al. (35) reported an increase in pT172 with no effect on ACC phosphorylation in 3T3-L1 adipocytes. This apparent contradiction might indicate a specific difference in the insulin response between cultured and primary adipocytes.

In addition to primary rat adipocytes, we demonstrated that insulin treatment induces increased AMPK S485 phosphorylation in primary human adipocytes: an observation in line with that by Albers et al. (2) in adipose tissue from subjects during euglycemic clamp. In that study, a simultaneous decrease in AMPK T172 phosphorylation in response to insulin was also observed, although a strong decrease in AMPK pT172 was not always associated with an increase in pS485. Conversely, in our experiments, the phosphorylation of S485 in response to insulin was evident in all subjects; however, there was a large interindividual variation of the effect of insulin on AMPK pT172 or ACC pS79, and thus no significant change was detected. However, it is worth mentioning that in the majority of the subjects that we analyzed, a decreased AMPK T172 and ACC S79 phosphorylation was detected and there was a trend toward an inverse correlation between pS485 and pT172. Collectively, the data from human adipose tissue and adipocytes suggest that insulin, like in rodent cells, induces S485 phosphorylation, which is possibly associated with AMPK inhibition, at least in some subjects. However, as we observed increased AMPK S485 phosphorylation but no change (or in some individuals, even increased) AMPK T172 phosphorylation, this supports the previous conclusion from rat adipocytes of S485 being negligible in the regulation of AMPK kinase activity by insulin.

An ultimate goal of our investigations is to increase the understanding of how insulin stimulates FA synthesis. De novo lipogenesis only accounts for a small portion of the FAs that, together with glycerol, make up adipose tissue triglyceride stores (55). Nevertheless, previous studies show an inverse correlation between insulin sensitivity in obesity and de novo lipogenesis (4, 29). Additionally, recent studies identified a new group of lipids, the FA esters of hydroxy FAs (FAHFAs), which are synthesized as a result of de novo lipogenesis in white but also in brown adipose tissue (52, 58). These lipids are suggested to improve metabolic status, including promotion of insulin-stimulated glucose uptake as well as a reduction in proinflammatory responses (58). In insulin-resistant obesity, the abundance of these lipids was shown to be greatly reduced (58). Taken together, this highlights the importance of increasing our understanding of how FA synthesis is regulated (58).

It has been demonstrated that the main phosphorylation event required for regulating ACC activity is phosphorylation at S79 (11, 41). Furthermore, ACC S79 clearly becomes dephosphorylated following insulin stimulation, as shown in both our current and previous work (6). So far, AMPK is the only kinase shown to be required for S79 phosphorylation (11), strongly suggesting an involvement of AMPK in the insulin-induced increase in FA synthesis, which is also further under-

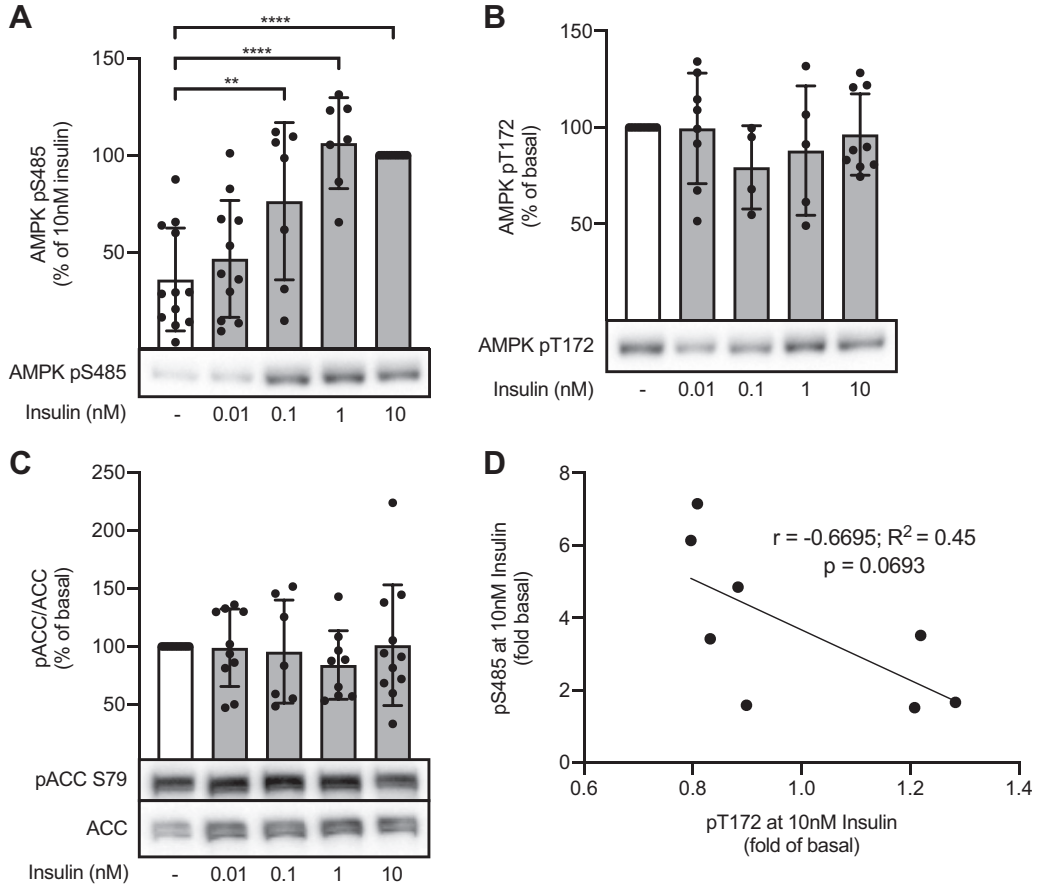



Fig. 8. Association of insulin-induced AMP-activated protein kinase (AMPK) S485 phosphorylation and kinase activity in human adipocytes. Primary adipocytes isolated from human subcutaneous adipose tissue were stimulated with different doses of insulin as indicated for 10 min. Phosphorylation of AMPK at S485 (*A*) and T172 (*B*), as well as acetyl-CoA carboxylase (ACC) S79 (*C*), was analyzed by Western blot with phospho-specific antibodies. Blots shown are representative of $n = 4–12$ independent experiments, and data were expressed as % of the 10 nM insulin-stimulated sample (*A*) or the basal sample (*B* and *C*). *D*: fold changes of AMPK T172 and S485 phosphorylation at 10 nM insulin were plotted against each other ($r = -0.670$, $P = 0.069$, $n = 8$). Correlations were made using Pearson correlation test. Statistical significance was determined by one-way ANOVA followed by Holm–Sidak's multiple comparison test. $^{**}P < 0.01$, $^{****}P < 0.0001$.

lined by our observation of decreased insulin-induced FA synthesis in response to AMPK activation by A-769662. Additionally, with the help of a nonphosphorylatable ACC S79A mutant, it was demonstrated that ACC S79 is the main ACC site required to inhibit FA synthesis in response to AMPK activation (17). To further delineate the mechanisms underlying insulin-induced activation of ACC and FA synthesis, it would be interesting to employ adipocytes isolated from the ACC S79A knockin model previously described (38).

In summary, in this study we showed that AMPK S485 phosphorylation is not required for insulin-induced inhibition of AMPK in adipocytes. Moreover, we demonstrated that insulin-induced S485 phosphorylation also takes place in human adipocytes, making further investigations of the importance of S485 phosphorylation warranted. Additionally, we reveal a decrease in cellular energy levels as an alternative mechanism that might be involved in the regulation of AMPK by insulin.

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## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

## AUTHOR CONTRIBUTIONS

F.K., C.B., E.D., M.H.R., and O.G. conceived and designed research; F.K., K.R., J.S., and D.V. performed experiments; F.K., K.R., C.B., D.V., E.D., M.H.R., and O.G. analyzed data; F.K., K.R., C.B., D.V., E.D., M.H.R., and O.G. interpreted results of experiments; F.K. prepared figures; F.K. drafted manuscript; K.R., J.S., D.V., E.D., M.H.R., and O.G. edited and revised

manuscript; F.K., K.R., C.B., J.S., D.V., E.D., M.H.R., and O.G. approved final version of manuscript.

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