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Insulin inhibits AMPK activity and phosphorylates AMPK Ser$^{485/491}$ through Akt in hepatocytes, myotubes and incubated rat skeletal muscle

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Abstract

Recent studies have highlighted the importance of an inhibitory phosphorylation site, Ser$^{485/491}$, on the α-subunit of AMP-activated protein kinase (AMPK); however, little is known about the regulation of this site in liver and skeletal muscle. We examined whether the inhibitory effects of insulin on AMPK activity may be mediated through the phosphorylation of this inhibitory Ser$^{485/491}$ site in hepatocytes, myotubes and incubated skeletal muscle. HepG2 and C2C12 cells were stimulated with or without insulin for 15-min. Similarly, rat extensor digitorum longus (EDL) muscles were treated +/− insulin for 10-min. Insulin significantly increased Ser$^{485/491}$ p-AMPK under all conditions, resulting in a subsequent reduction in AMPK activity, ranging from 40% to 70%, despite no change in p-AMPK Thr^{172}. Akt inhibition both attenuated the increase in Ser$^{485/491}$ p-AMPK caused by insulin, and prevented the decrease in AMPK activity. Similarly, the growth factor IGF-1 stimulated Ser$^{485/491}$ AMPK phosphorylation, and this too was blunted by inhibition of Akt. Inhibition of the mTOR pathway with rapamycin, however, had no effect on insulin-stimulated Ser$^{485/491}$ p-AMPK. These data suggest that insulin and IGF-1 diminish AMPK activity in hepatocytes and muscle, most likely through Akt activation and the inhibitory phosphorylation of Ser$^{485/491}$ on its α-subunit.

Keywords

Insulin; Growth factors; AMPK; PKB; Ser$^{485/491}$

Introduction

Activation of the fuel-sensing enzyme AMP-activated protein kinase (AMPK)[1] results in up-regulation of processes involved in energy generation, such as glucose transport and fatty acid oxidation. Importantly, in addition to this classical role, it is now clear that AMPK activation leads to a host of other benefits, including inhibition of lipid and protein synthesis, inflammation, ER and oxidative stress and increased mitochondrial biogenesis.

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[1]*Abbreviations used:* AMPK, AMP-activated protein kinase; mTOR, mammalian target of rapamycin.

Furthermore, it exerts these effects both by acute actions on certain molecules and by genetically regulating the expression of others [1,2].

Whereas there is ample evidence regarding physiological (starvation and exercise) and pharmacological (biguanides, thiazolidinediones, salicylate) activation of AMPK in skeletal muscle and liver [2,3], the mechanisms responsible for its physiological down-regulation in these tissues are less well understood. This is particularly relevant as AMPK activity is diminished in obese hyperinsulinemic rodents and humans (compared to their healthy counterparts) [4–6], and its inhibition is an early event in the development of insulin resistance in response to nutrient excess [7–9].

Insulin, in addition to other anabolic stimuli, has been reported to diminish AMPK activity, as assessed by the SAMS peptide assay, in liver and muscle [10,11]. Although Thr^{172} of the α-subunit is regarded as the main phosphorylation/activation site on AMPK, changes in AMPK activity are often observed in the absence of altered Thr^{172} phosphorylation. As such, AMPK activity may be impacted by one of several other phosphorylation sites with less defined functions [12]. For instance, the reduction in AMPK activity caused by anabolic signals can be due, at least in part, to the phosphorylation of the inhibitory $Ser^{485/491}$ site on α1/α2, in a variety of tissues, including cardiomyocytes/heart [13,14], adipocytes [15], and vascular smooth muscle cells [16]. However, data on $Ser^{485/491}$ p-AMPK in liver and skeletal muscle are limited. $Ser^{485/491}$ phosphorylation by insulin and IGF-1 appears to be primarily through Akt/PKB, as inhibition of Akt attenuates this phosphorylation and maintains AMPK activity in various cell types [14–17]. In addition to the role of Akt in phosphorylating $Ser^{485/491}$ AMPK in other tissues, it was recently shown that leptin signals through mTOR/p70S6K to directly phosphorylate α2 Ser^{491} AMPK and reduce AMPK activity independently of changes in Thr^{172} in the hypothalamus [18]. Despite these findings, the physiological regulation of AMPK $Ser^{485/491}$ phosphorylation under anabolic conditions has not been addressed in two of the primary insulin responsive tissues, skeletal muscle and liver.

In this study we investigated whether the inhibitory effects of insulin on AMPK activity may be mediated through the phosphorylation of its $Ser^{485/491}$ site using cultured hepatocytes and myotubes and incubated EDL muscle as models. We hypothesized that (1) insulin would stimulate phosphorylation of $Ser^{485/491}$ AMPK and reduce AMPK activity in these cells, and (2) inhibition of Akt would attenuate insulin-stimulated $Ser^{485/491}$ p-AMPK. In addition, we assessed whether mTOR inhibition would have the same effects.

Materials and methods

Materials

HepG2 and C2C12 cells were purchased from ATCC (Manassas, VA). DMEM, Penicillin–Streptomycin (PS), fetal bovine serum (FBS) and horse serum (HS) were from Invitrogen (Grand Island, NY). D-(+)-Glucose solution, 45%, insulin, insulin-like growth factor-1 (IGF-1), and Akt inhibitor VIII were purchased from Sigma–Aldrich (St. Louis, MO). Rapamycin was purchased from LC Laboratories (Woburn, MA), and wortmannin was from

Adipogen (San Diego, CA). SAMS peptide was purchased from Abcam (Cambridge, MA) and P32 was from Perkin–Elmer (Boston, MA).

Cell culture studies

HepG2 cells were cultured in normal glucose (5.5 mM) DMEM supplemented with 10% FBS and 1% PS. Media was replaced every 24–48 h and cells were passaged upon reaching 80–90% confluence. Glucose and FBS-free DMEM, supplemented with 1% PS and glucose at a final concentration of 5.5 mM, was used for all experimental incubations. C2C12 myoblasts were cultured as described above, with the addition of 1% glutamine. At 80–90% confluence they were differentiated into myotubes in DMEM supplemented with 2% HS and 1% PS.

Muscle incubations

Protocols for animal use were reviewed and approved by the Institutional Animal Care and Use Committee of Boston University Medical Center and were in accordance with National Institutes of Health guidelines. Male Sprague–Dawley rats weighing 55–65 g were purchased from Charles River Breeding Laboratories (Wilmington, MA). Rats were maintained on a 12:12-h light–dark cycle in a temperature-controlled (19–21 °C) room and were fed standard rat chow and water *ad libitum*. Following an overnight fast, they were anesthetized with pentobarbital (6 mg/100 g body weight), and muscles were removed for incubation.

After their removal, rat extensor digitorum longus (EDL) muscles were first equilibrated for 20 min at 37 °C in oxygenated Krebs–Henseleit solution (95% O_2/5% CO_2) containing 5.5 mM glucose. They were then stimulated with or without insulin (10 mU/ml) for 10 min. Following incubations muscles were snap-frozen in liquid nitrogen, and stored at −80 °C until analysis.

Cell lysate preparation

Cells were washed once on ice with Dulbecco's PBS, lysed in buffer containing 20 mM Tris–HCl – pH 8.0, 1% IGEPAL, 1 mM EGTA, 10 mM nicotinamide, 1 μM trichostatin A, 10 mM sodium butyrate, 1 mM PMSF, 1 × phosphatase inhibitor cocktail 3 (Sigma), and 1 × protease inhibitor cocktail containing 1 mM EDTA (Complete Mini, Roche, Basel, Switzerland), and removed from wells using a cell scraper with a polyethylene copolymer blade (Fisher Scientific). Cell debris was removed by centrifugation at 13,200 g for 10 min at 4 °C, and the supernatant was removed and stored at −80 °C until analysis. Protein concentration was assessed by the bicinchoninic acid method (BCA; Pierce Biotechnology, Inc., Rockford, IL).

SDS–PAGE western blot analysis

Protein expression and phosphorylation were determined in 15–30 μg of protein lysate using SDS–PAGE gel electrophoresis and immunoblotting. Primary antibodies for Acetyl-CoA carboxylase (ACC), AMPK, Thr^{172} phospho-AMPKα, Ser^{485} phospho-AMPKα1, $Ser^{485/491}$ phospho-AMPKα1/α2, Ser^{2448} phospho-mTOR, mTOR, Thr^{389} phospho-p70S6K, Ser^{473} phospho-Akt, and Akt antibodies, as well as secondary horseradish peroxidase (HRP)-linked

antibodies were purchased from Cell Signaling Technology (Danvers, MA). Ser^{79} phospho-ACC antibody was from Upstate/Millipore (Temecula, CA). Anti-β-actin was from Sigma–Aldrich (St. Louis, MO). Following transfer onto a polyvinylidene difluoride membrane, membranes were blocked in Tris–buffered saline (pH 7.5) containing 0.05% Tween-20 (v/v; TBST) and 5% non-fat dry milk (w/v) for 1 h at room temperature, followed by incubation in primary antibodies (1:1000) at 4 °C overnight. After washing, membranes were incubated in a secondary antibody conjugated to horseradish peroxidase at a 1:5000 dilution for 1 h at room temperature. Bands were visualized using enhanced chemiluminescence solution (ECL; Pierce Biotechnology, Inc., Rockford, IL), and densitometry was performed with Scion Image software.

AMPK activity assay

AMPK activity was assessed as previously described [8,19]. Briefly, AMPK α1 or α2 was immunoprecipitated from 500 μg of protein from cell lysates by incubation at 4 °C overnight on a roller mixer using AMPK α1 or α2-specific antibodies (1:80; Santa Cruz Biotechnology, Inc.) and protein A/G agarose beads (1:10; Santa Cruz Biotechnology, Inc). Following several washes, activity was measured in the presence of 200 μM AMP and 80 μM [γ-^{32}P] ATP (2 μCi) using 200 μM SAMS peptide (Abcam) as a substrate. Label incorporation into the SAMS peptide was quantified using a Lab-Logic (Brandon, FL) scintillation counter.

Statistical analysis

Results are reported as means ± standard error of the mean (SEM). Statistical significance was determined by two-tailed unpaired Student's *t* tests or ANOVA with Tukey's post hoc test. A level of $p < 0.05$ was considered statistically significant.

Results and discussion

Insulin stimulates phosphorylation of $Ser^{485/491}$ AMPK and reduces AMPK activity

We first set out to ascertain whether $Ser^{485/491}$ AMPK was physiologically regulated in hepatocytes, myotubes, and incubated skeletal muscle. Of note, the predominant AMPK isoform in hepatocytes is the α1 subunit, whereas in skeletal muscle α2 predominates, and in C2C12 myotubes α1 and α2 are fairly equally expressed *in vitro.* For these reasons, we focused on the regulation of α1 Ser^{485} p-AMPK in HepG2 hepatocytes and α1/α2 $Ser^{485/491}$ p-AMPK in myotubes and EDL muscle.

As demonstrated previously in cardiomyocytes [14,17] and adipocytes [15], we found that insulin treatment acutely increased p-AMPK Ser^{485} in HepG2 hepatocytes (Fig. 1A) and p-AMPK $Ser^{485/491}$ in C2C12 myotubes (Fig. 1B) by >5-fold. Similarly, incubation of rat EDL muscle with insulin increased AMPK $Ser^{485/491}$ phosphorylation by over 60% (Fig. 1C). The reason for the quantitative difference between *in vitro* results and those observed *ex vivo* in the EDL muscle is not entirely clear and warrants further investigation. Interestingly, insulin also significantly diminished AMPK activity, as measured by the SAMS peptide assay, by 36–64% in HepG2 hepatocytes, C2C12 myotubes, and incubated EDL muscles (Fig. 3), despite no change in p-AMPK Thr^{172} (Fig. 1). The phosphorylation

of ACC Ser^{79} coincided with that of p-AMPK Thr^{172} *in vitro*, showing no change with insulin treatment, whereas in the incubated EDL muscle insulin diminished p-ACC Ser^{79} (Fig. 1).

Inhibition of Akt attenuates insulin-induced p-AMPK Ser$^{485/491}$ and partially prevents the reduction in AMPK activity

The Akt pathway is involved in the phosphorylation of Ser$^{485/491}$ in other tissues, including heart [13,14], brown adipose tissue [20], white adipocytes [15], and vascular smooth muscle [16] and tumor cells [21]. We tested the role of Akt on insulin's effect on p-AMPK Ser^{485} in hepatocytes and Ser$^{485/491}$ in myotubes. Pretreatment of the cells for 1 h with the Akt inhibitor, Akt inhibitor VIII, attenuated the insulin-stimulated increase in serine phosphorylation of AMPK in both cell types, despite no effect on p-AMPK Thr^{172} (Fig. 2A and B, respectively). This reduction in Ser^{485} in HepG2 cells or Ser$^{485/491}$ p-AMPK in C2C12 myotubes and EDL muscle by Akt inhibition was accompanied by a significant attenuation in insulin's effect on AMPK α1 and α2 activity. Taken together, insulin-induced phosphorylation of Ser$^{485/491}$ of AMPK and inhibition of AMPK activity are both partially prevented by Akt inhibition (Fig. 3).

These data suggest a dissociation between p-AMPK Thr^{172}, the commonly used surrogate for activity, and AMPK activity assessed via the SAMS peptide assay, under conditions of insulin-induced AMPK inactivation. Similar results were reported by Dagon and colleagues in the hypothalamus, in which they showed that AMPK activity decreased when Ser^{491} p-AMPK was elevated by leptin, whereas p-AMPK Thr^{172} did not change [18]. Of note, in our study, the decline in AMPK activity caused by insulin was proportional to the increase in p-AMPK Ser^{485} (HepG2) or Ser$^{485/491}$ (C2C12) in each case (Fig. 3B and D).

Wortmannin prevents insulin-induced p-AMPK Ser$^{485/491}$

Inhibition of PI3K signaling by wortmannin, and subsequent reduction in Akt signaling recapitulated the results observed with the Akt inhibitor VIII in both HepG2 and C2C12 cells. Specifically, wortmannin pre-treatment prevented insulin-induced p-AMPK Ser$^{485/491}$ without affecting Thr^{172} p-AMPK (Fig. 2C and D). Our results are limited to insulin's effect on basal AMPK activity; however, others have shown that insulin antagonizes the activation of AMPK by various stimuli, including anoxia in heart [22], palmitate in adipocytes [23], and AICAR in hepatocytes and myotubes (Valentine, unpublished data).

Interestingly, although wortmannin and Akt inhibitor VIII had similar effects to diminish p-Akt Ser^{473}, wortmannin also essentially eliminated the phosphorylation of AMPK at Ser^{485} in both cell types, whereas Akt inhibition only partially blunted this phosphorylation in HepG2 cells. This suggests a pathway independent of Akt may be involved in the phosphorylationof AMPK at Ser^{485}, atleast in cultured hepatocytes, however the mechanism(s) involved remain to be determined.

IGF-1 mimics the effect of insulin on p-AMPK Ser$^{485/491}$ and is attenuated by Akt inhibition

In addition to insulin, the anabolic factor insulin-like growth factor 1 (IGF-1) signals through the Akt pathway. Ning and colleagues recently demonstrated that IGF-1 dose-

dependently increases Ser^{485} p-AMPK on the α1 AMPK subunit in vascular smooth muscle cells (VSMCs), an effect mediated by Akt [16]. Using a phosphodefective S485A mutant, the authors also demonstrated an inability of IGF-1 to diminish Thr^{172} p-AMPK, suggesting the involvement of Ser^{485} in AMPK downregulation by IGF-1, at least in VSMCs. In the present study we show for the first time that IGF-1 also increases the phosphorylation of $Ser^{485/491}$ AMPK, in both hepatocytes (Fig. 4A and B) and myotubes (Fig. 4C and D). The effect of IGF-1 was dose-dependent, and the increase in $Ser^{485/491}$ p-AMPK mirrored that for p-Akt Ser^{473} (data not shown). In addition, similar to its effect on insulin stimulation, inhibition of Akt attenuated the effect of IGF-1 on $Ser^{485/491}$ p-AMPK (Fig. 4).

mTOR inhibition by rapamycin did not effect insulin-stimulated p-AMPK $Ser^{485/491}$

As previously noted, it was shown that leptin can cause phosphorylation of p-AMPK $Ser^{485/491}$ in the hypothalamus, through the mTOR/p70S6Kinase (p70S6K) pathway [18]. It is well established that insulin leads to similar increases in p70S6K (activity and p-p70S6K Thr^{389} in hepatocytes and myotubes [24,25] Fig. 5). Thus, we examined whether this pathway might also mediate the effect of insulin on $Ser^{485/491}$ p-AMPK. Cells were pre-treated in the presence or absence of the mTOR inhibitor rapamycin for 1 h and then stimulated with insulin. As shown in Fig. 5, rapamycin inhibited the phosphorylation of mTOR and p70S6K by insulin; however, it had no effect on the increase in $Ser^{485/491}$ p-AMPK caused by insulin (Fig. 5) or IGF-1 (data not shown). In keeping with these data, Beauloye and colleagues have demonstrated in heart the ability of insulin to inhibit AMPK activation by anoxia is prevented by pre-treatment with wortmannin, but not by rapamycin [22].

Summary and conclusions

In this study, we show for the first time that insulin and IGF-1 can regulate the inhibitory phosphorylation of AMPK on its $Ser^{485/491}$ of its α1/α2 site in both cultured hepatocytes and myotubes, and incubated EDL muscle. Our results also indicate that this effect of insulin is mediated through the Akt pathway, and not mTOR/p70S6K Similar findings were observed when these cells were stimulated with IGF-1.

It is well accepted that activation of AMPK enhances insulin sensitivity in a variety of tissues, including heart, liver, adipose tissue and skeletal muscle [26,27], whereas loss of AMPK contributes to insulin resistance [28,29]. Our data also highlight a potential mechanism by which hyperinsulinemia downregulates AMPK, through phosphorylation of $Ser^{485/491}$ on its α-subunit, leading to diminished glucose uptake and exacerbation of insulin resistance. Furthermore, our data, along with those of others, suggest that there may be discordance between the commonly used surrogate measure of AMPK activity, its phosphorylation on Thr^{172}, and AMPK activity measured by the SAMS peptide assay, at least under certain conditions in which $Ser^{485/491}$ appears to play an inhibitory role [18].

These findings, along with previous results of others in heart [14] and adipocytes [15], suggest that insulin inhibits AMPK activity rapidly, with a peak in $Ser^{485/491}$ AMPK phosphorylation occurring within minutes. Under conditions such as high glucose this event appears to take longer, and may occur through a different mechanism. Other kinases

involved in the phosphorylation of AMPK Ser$^{485/491}$ have been identified under various conditions, including the mTOR/p70S6K pathway [18] and protein kinase A (PKA) [30]. Likewise, our laboratory recently found that activation of protein kinase C (PKC) can cause phosphorylation of AMPK Ser$^{485/491}$ and diminish its activity in myotubes[31]. Strategies to prevent this inhibitory phosphorylation may provide a novel approach to maintain AMPK function in the setting of hyperinsulinemia. Still to be determined is whether downregulation of AMPK by this mechanism occurs in states of chronic insulin resistance and what are the physiological and pathophysiological implications of this change.

Acknowledgments

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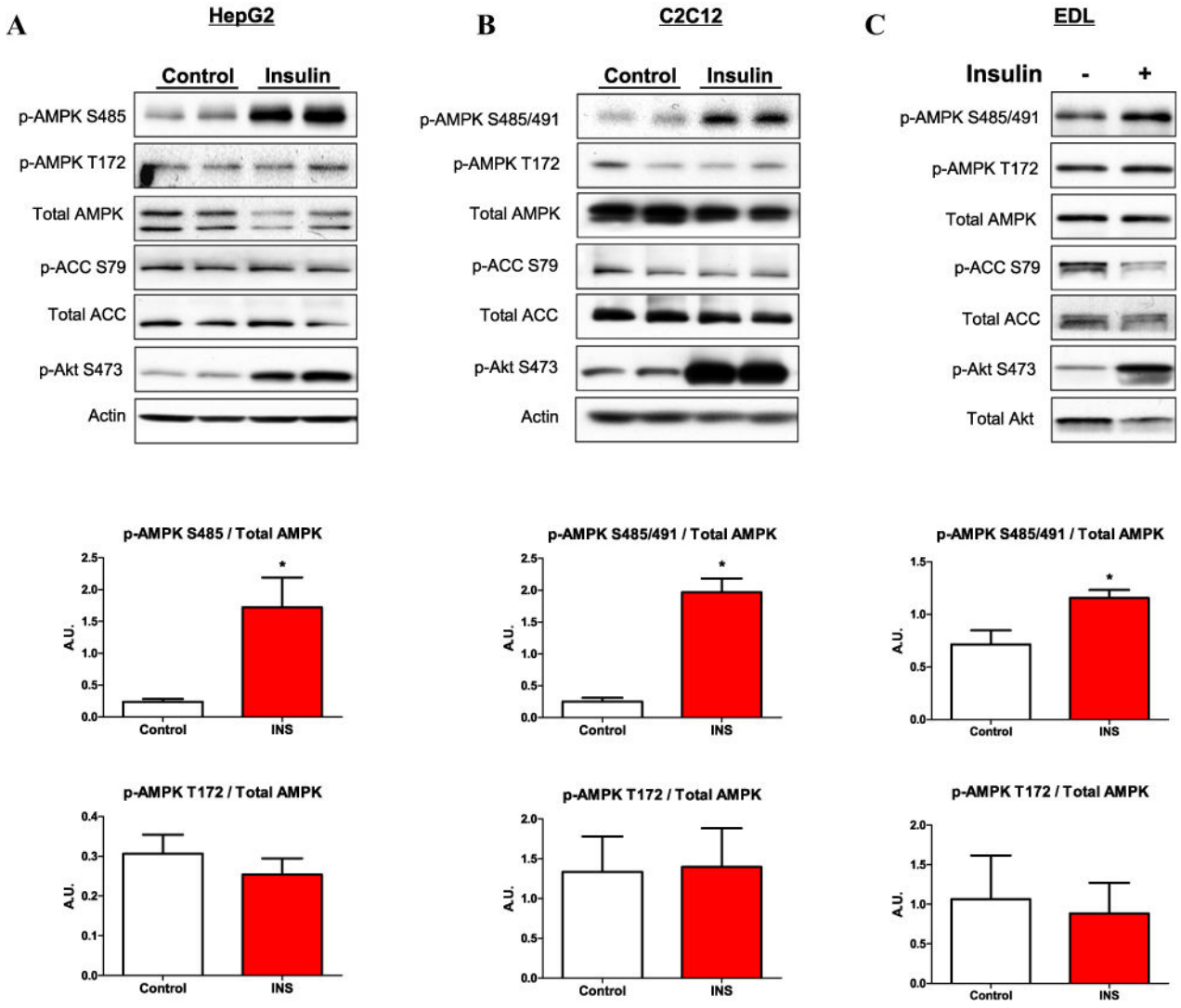

Fig. 1.

Insulin stimulates phosphorylation of AMPK Ser^{485} in HepG2 hepatocytes and $Ser^{485/491}$ in C2C12 myotubes and incubated EDL muscle. HepG2 cells (A) and C2C12 myotubes (B) were cultured in normal glucose (5.5 mM), serum starved overnight, and stimulated with insulin (100 nM) for 15 min. Rat extensor digitorum longus (EDL) muscles were removed and equilibrated in Krebs–Henseleit buffer for 20 min, then stimulated with insulin (10 mU/ml) for 10 min (C). Following cell/tissue lysis, western blot analyses were performed, and representative blots are shown. Densitometry was used to quantify western blots. Phosphorylation of AMPK was normalized to total AMPK, and normalized data are displayed (shown in line with the corresponding cell type/tissue). Results are means ± SE (n = 3–6 per treatment). All experiments were performed in triplicate. *$p < 0.05$ vs. control.

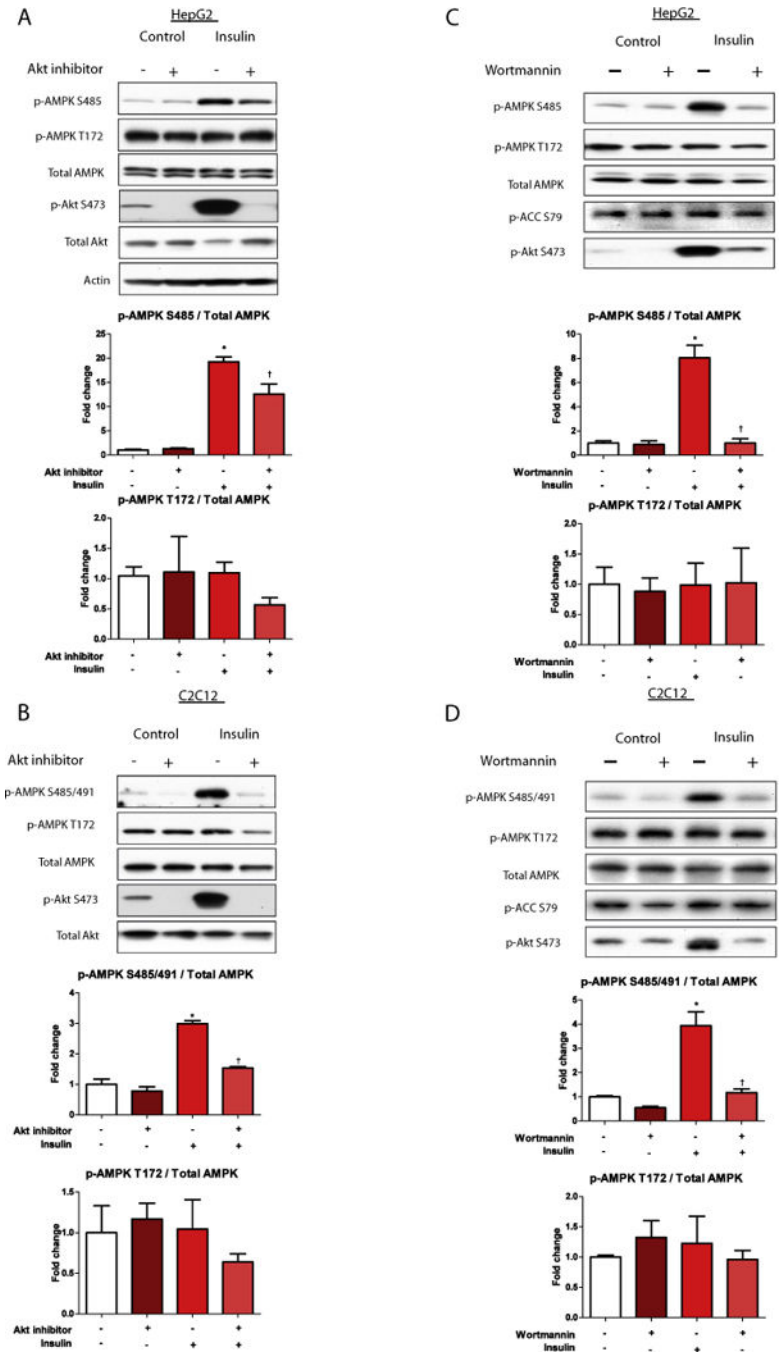

Fig. 2.

Inhibition of Akt attenuates insulin stimulated phosphorylation of AMPK Ser$^{485/491}$. HepG2 (A) and C2C12 cells (B) were treated with or without Akt inhibitor VIII (250 μM) for 2 h, then stimulated with insulin (100 nM) for 15 min. Cells were lysed and subjected to western blot analysis. Representative western blots are shown. Quantification of western blots was performed using densitometry, and AMPK phosphorylation was normalized to total AMPK. Results are presented as fold-change in AMPK phosphorylation vs. control in HepG2 cells (A) and C2C12 cells (B). In Figures C (HepG2) and D (C2C12) cells were pre-treated with

the PI3-kinase inhibitor wortmannin (10 nM) for 1 h, then stimulated with insulin (100 nM) for 10 min. Cells were processed and protein expression and phosphorylation were quantified as described in (A) and (B). Results are means ± SE (n = 3–6 per treatment). All experiments were performed in triplicate. *$p < 0.05$ indicates a significant effect of insulin vs. control, $^{\dagger}p < 0.05$ vs. insulin treatment alone.

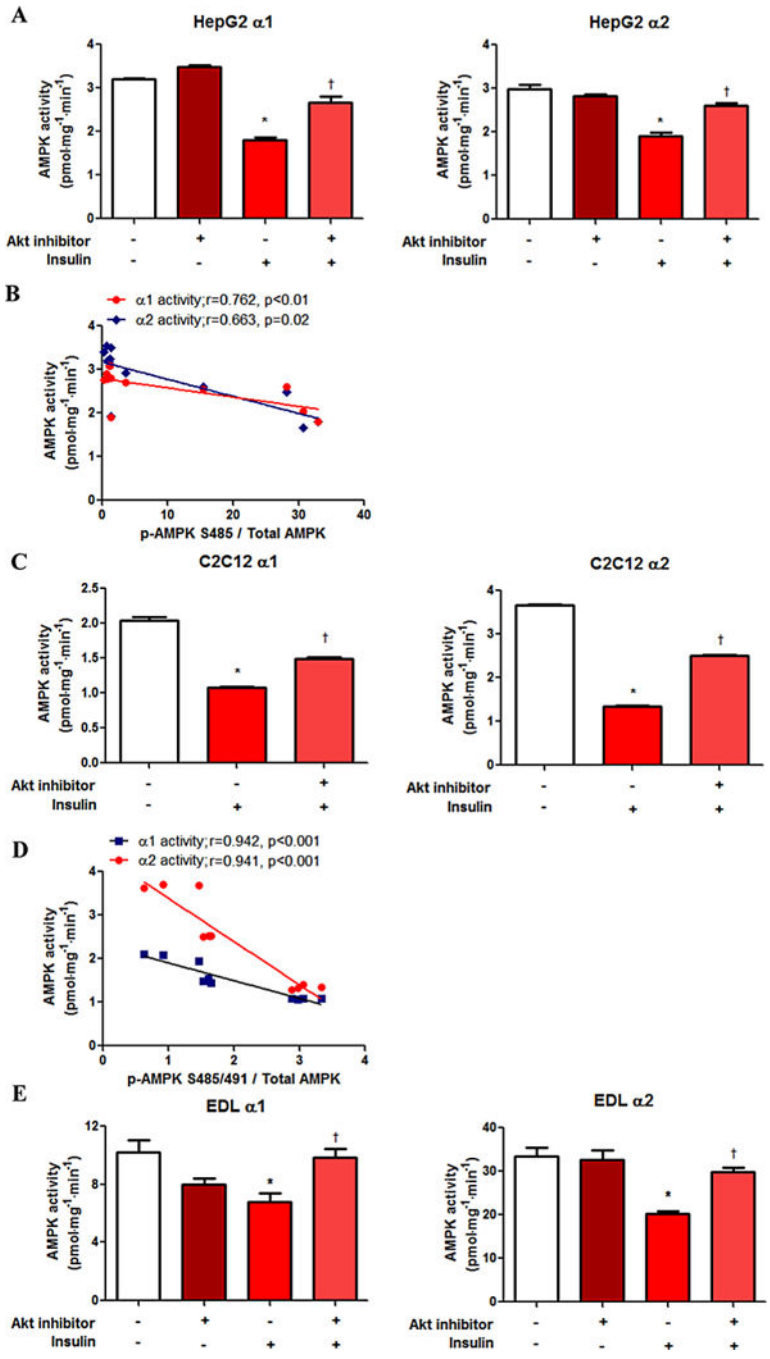

Fig. 3.

Insulin-induced inhibition of AMPK activity is partially prevented by Akt inhibition. AMPK α1 and α2 activities were assessed using the SAMS peptide assay, as described in the materials and methods section. HepG2 (A), C2C12 (C) and EDL (E) AMPK activity data are presented. Correlations between $Ser^{485/491}$ AMPK phosphorylation data from western blot analysis (Fig. 2) and AMPK activity are shown (B and D).

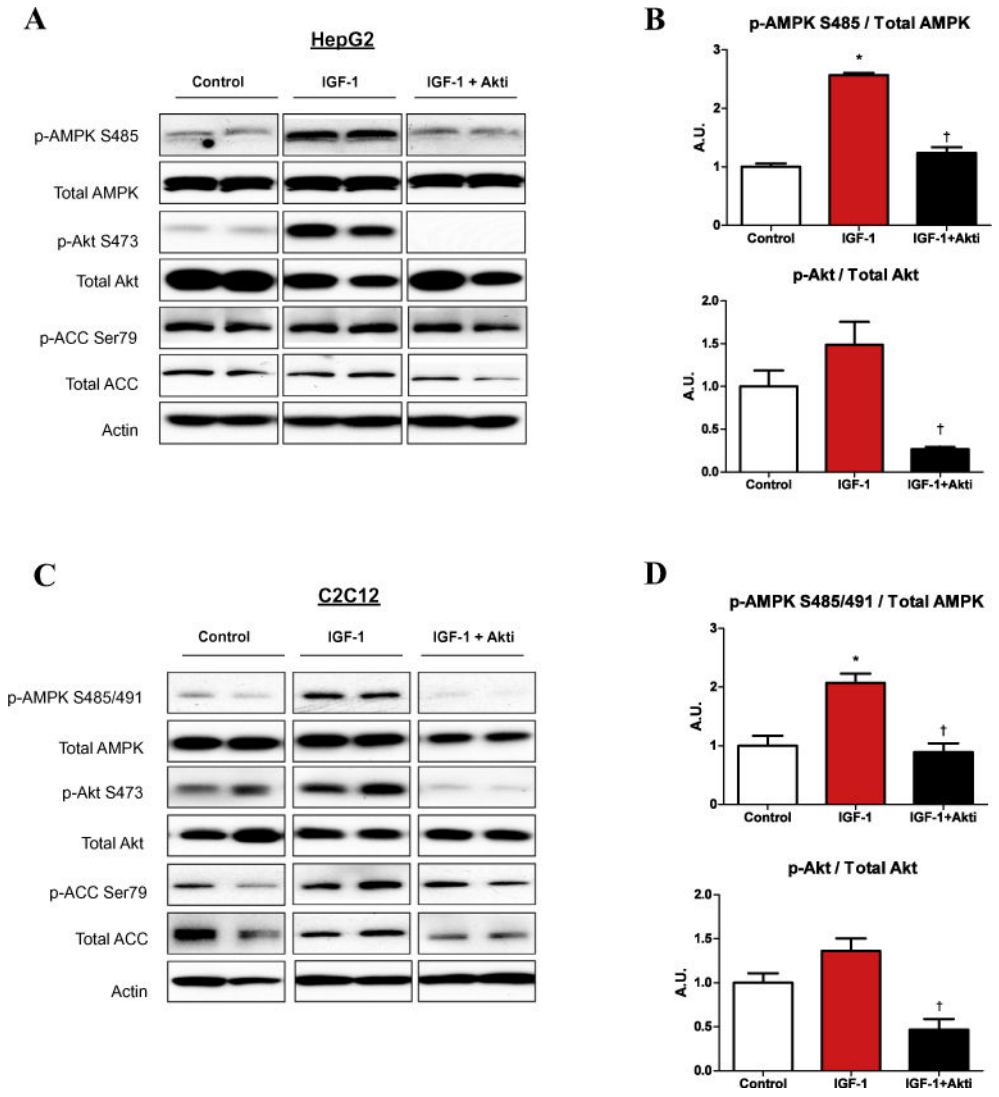

Fig. 4.

IGF-1 phosphorylation of AMPK Ser$^{485/491}$ is prevented by Akt inhibition. HepG2 (A) and C2C12 cells (C) were serum starved overnight and treated with or without Akt inhibitor VIII (Akti; 250 μM) for 2 h, then stimulated with IGF-1 (20 ng/ml) for 15 min followed by whole cell lysis and analyzed by western blot. Representative western blots are shown (A and C). Densitometric analysis of western blots was used to quantify protein expression. Phosphorylation of proteins of interest were normalized to the corresponding total protein, and results are presented as fold-change compared to control (B and D). Results are means ± SE (n = 3–6 per treatment). All experiments were performed in triplicate. *$p < 0.05$ indicates a significant effect of IGF-1, $^{\dagger}p < 0.05$ for the effect of AktiVIII.

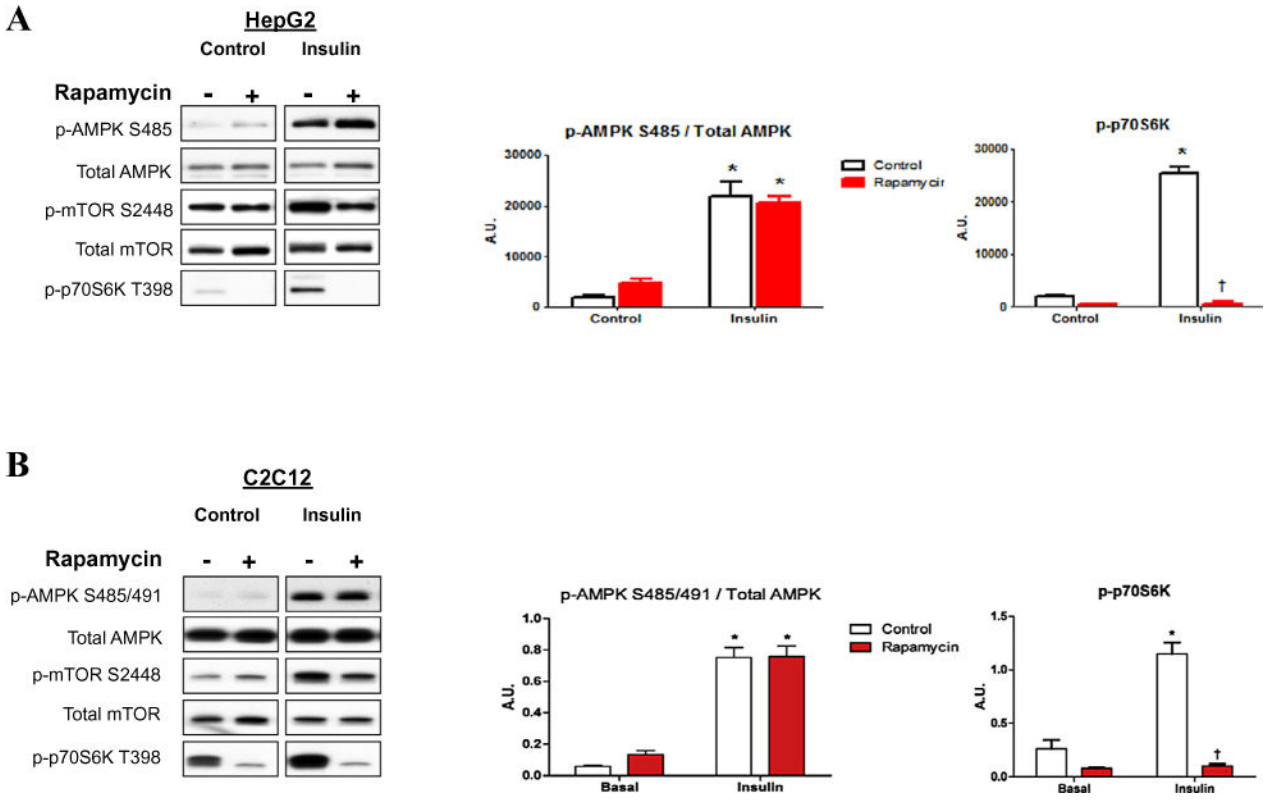

Fig. 5.

Insulin-stimulation of p-AMPK Ser$^{485/491}$ is not affected by the mTOR inhibitor rapamycin. HepG2 (A) and C2C12 cells (B) were treated with or without rapamycin (50 nM) for 2 h, then stimulated with insulin for 15 min. Cell lysates were run on western blots and protein expression and phosphorylation were quantified using densitometry. Representative western blots are shown. Densitometry results are presented in graphs. Results are means ± SE (n = 3–6 per treatment). All experiments were performed in triplicate. *$p < 0.05$ indicates a significant effect of insulin, $^{\dagger}p < 0.05$ for the effect of rapamycin treatment.