Phosphatidylinositol 3-kinase p110 α mediates phosphorylation of AMP-Activated Protein

Kinase in myoblasts

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Running title: *PI3K p110 α regulates AMPK phosphorylation*

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Abstract

AMP-activated protein kinase (AMPK) is a serine/threonine kinase that functions as a sensor of intracellular energy. Activation of AMPK is associated with increased phosphorylation of the α -subunit at threonine 172 (T172) and decreased phosphorylation at serine 485 in AMPK α 1 and serine 491 in AMPK α 2 (S485/491). One potential mediator of AMPK phosphorylation is phosphatidylinositol 3-kinase (PI3K); however, the mechanism and the identities of the specific PI3K isoforms that regulate AMPK activation are not known. To determine whether PI3K p110 α regulated AMPK activation in muscle cells, C2C12 myoblasts were subjected to pharmacological inhibition of p110 α , siRNA directed against p110 α , or overexpression of constitutively-active or dominant negative p110 α . Chemical inhibition, siRNA, and expression of dominant-negative p110 α were all associated with increased AMPK T172 phosphorylation, whereas expression of constitutively-active p110 α reduced T172 phosphorylation. Conversely, pharmacological inhibition of p110 α reduced AMPK S485/491 phosphorylation, while constitutively-active p110 α increased AMPK S485/491 phosphorylation. This p110 α -mediated increase in AMPK S485/491 phosphorylation was eliminated in the presence of the Akt inhibitor MK2206, suggesting that p110 α -mediated phosphorylation of AMPK α at S485/491 is Akt-dependent. In response to oligomycin or serum-starvation, AMPK T172 phosphorylation was elevated in p110 α -deficient myoblasts compared to control myoblasts. Overall, our findings identify PI3K p110 α as a mediator of AMPK phosphorylation in myoblasts.

Key words: Phosphatidylinositol 3-kinase (PI3K), AMP-Activated Protein Kinase (AMPK), p110 α , Akt, myoblast

Abbreviations:

AMPK: Adenosine monophosphate-Activated Protein Kinase

PI3K: phosphatidylinositol 3-kinase

Akt: homologue to the oncogene in transforming retrovirus AKT-8

siRNA: small-interfering RNA

PRAS40: proline-rich Akt substrate of 40 kDa

Introduction

AMP-activated protein kinase (AMPK) is a heterotrimeric serine/threonine kinase that acts as a sensor and regulator of cellular energy status. In skeletal muscle, AMPK regulates multiple metabolic and energetic processes including glucose uptake, fatty acid oxidation, and mitochondrial biogenesis [1]. AMPK is activated in response to low cellular energy (high AMP:ATP ratio) and physical exercise [2,3,4] and this activation is generally associated with phosphorylation of the α -subunit at threonine 172 (T172). AMPK inhibition on the other hand, is associated with phosphorylation at serine 485 in AMPK α 1 and serine 491 in AMPK α 2 (S485/491) in several cell types, including skeletal muscle [5,6,7,8,9]. A number of upstream regulators of AMPK have been identified that either positively or negatively modify AMPK activity [10], and the coordination of these inputs maintains metabolic and energetic homeostasis.

It has recently been reported that insulin and IGF-I mediate the phosphorylation of AMPK S485/491 through a wortmannin-sensitive mechanism in skeletal muscle, suggesting the involvement of phosphoinositide 3-kinase (PI3K) [9]. PI3K is a heterodimeric protein and lipid kinase expressed in a number of tissues, including skeletal muscle. Class IA PI3K enzymes are composed of an 85 kDa regulatory subunit (p85) and a 110 kDa catalytic subunit (p110) and are recruited to the cell membrane following activation of receptor tyrosine kinases (RTK) and G protein-coupled receptors (GPCRs). In skeletal muscle, three Class IA PI3K catalytic isoforms are expressed (p110 α , p110 β , and p110 δ) [11,12]. While p110 α is the primary p110 isoform regulating cellular responses to RTK agonists such as insulin and IGF-I [13,14], p110 β is the primary p110 isoform regulating cellular responses to GPCR agonists such as lysophosphatidic acid [15,16]. However, the role of PI3K in regulating AMPK activation, and the identity of the catalytic subunits involved, are not known.

Given the importance of PI3K p110 α in regulating insulin/IGF signal transduction in skeletal muscle, and given that AMPK activation is sensitive to PI3K pharmacological inhibition, we performed a series of experiments in cultured myoblasts to determine whether p110 α regulated AMPK phosphorylation. Our findings demonstrate that PI3K p110 α mediates AMPK phosphorylation under a variety of experimental conditions.

Materials and Methods

Materials and Reagents. C2C12 cells and DMSO were purchased from American Type Culture Collection (ATCC, Manassas, VA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, trypsin, Lipofectamine 2000, and Opti-mem

medium were purchased from Life Technologies (Carlsbad, CA). MK2206 was from Selleck Chemicals (Houston, TX). PI3-K alpha inhibitor 2 (cat # B-0304, corresponds to Figure 15e in reference [17] and referred to as p110 α i in this manuscript) was purchased from Echelon Biosciences (Salt Lake City, UT). Antibodies for p-AMPK α Thr 172 (#2535), p-AMPK α Ser 485/491 (#4185), total AMPK α (#2603), p-Akt Ser 473 (#4060), total (pan) Akt (#4685), p-PRAS40 Thr 246 (#2997), total PRAS40 (#2691), p110 α (#4249), p-S6 Ser 240/244 (#4838), total S6 (#2217), and oligomycin (#9996) were purchased from Cell Signaling Technologies (Danvers, MA). HRP-linked secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals and reagents were purchased from Sigma (St. Louis, MO).

Cell culture and pharmacological inhibitors. C2C12 were grown and passaged in high-glucose DMEM containing 10% FBS and 1% antibiotics at 37° C in 5% CO₂. All experiments were performed within six passages of receipt from the vendor (ATCC). Following reconstitution, cells were expanded for a minimum of five days prior to any experimental manipulations. Chemical inhibitors p110 α i and MK2206 were dissolved in DMSO and diluted in culture medium such that cells were exposed to a final DMSO concentration of 0.02%. Control cells received an equivalent concentration of DMSO.

Small-interfering RNA (siRNA). Non-targeting control siRNA (#4390483) and siRNA directed against p110 α (#s71604) were purchased from Life Technologies. Cells were reverse-transfected in DMEM containing 10% FBS without antibiotics at a density of 0.95×10^4 cells/cm² using 0.2% (vol/vol) Lipofectamine 2000 and a 5 nM final concentration for siRNAs. Twenty four hours following seeding, medium was replaced with DMEM + 10% FBS with antibiotics for another 24 hours whereupon cells were used for experiments.

Baculoviruses and transductions. For delivery and expression of genes in cultured cells, we utilized non-replicating baculoviruses that had been modified by insertion of a mammalian expression cassette (Life Technologies). Open reading frame (ORF) clone encoding human p110 α (*PIK3CA*; #IOH3620) in the pENTR221 vector was purchased from Life Technologies. The PIK3CA-E545K and PIK3CA-K802R point mutations were introduced by standard PCR-based site-directed mutagenesis. Sequence-verified clones were subcloned into a pDEST8-CMV vector as described previously [18]. These expression constructs were transformed into DH10BAC bacteria cells to generate and isolate bacmid DNA. This PCR-qualified bacmid DNA was transfected into SF9 insect cells for BacMam virus production according to Bac-to-Bac® Baculovirus Expression Systems manual [19,20] (Life Technologies). Viral transductions were performed at the time of seeding using 10% (v/v) virus in antibiotic-free DMEM + 10% FBS containing 0.02% enhancer solution (#PV5835, Life Technologies). Medium was replaced 16-hours following transduction and cells were maintained for an additional 24-hours before experiments.

Protein extraction and immunoblotting. Extraction of protein from cells was performed as described [21], and protein concentrations were determined by the method of Bradford [22]. Western immunoblotting was performed exactly as described previously [23], and densitometric analysis was performed on scanned images converted to 8-bit TIFF using NIH Image J 1.60 (<http://rsbweb.nih.gov/ij/>).

Statistics. Data are presented as mean $\pm$ S.E.M. Statistics were performed using Student's t-test, one-way ANOVA with Dunnett test performed *post hoc*, or two-way ANOVA with Bonferroni test performed *post hoc* as indicated in figure legends. A P-value < 0.05 was considered significant.

Results

PI3K p110 α regulates AMPK phosphorylation at Thr 172 and Ser 485/491

To determine whether PI3K p110 α regulated AMPK activation in muscle cells, we first treated C2C12 myoblasts with increasing concentrations of a pharmacological inhibitor of p110 α (“p110 α i”) [17]. Following 4 hours of treatment, we observed a dose-dependent increase in AMPK phosphorylation at T172, and a decrease in phosphorylation of AMPK at S485/481 (Figure 1A). Decreased phosphorylation of Akt (S473) and S6 (S240/244) verified the efficacy of p110 α inhibition. We next tested whether siRNA-mediated reduction of p110 α produced similar results. In p110 α -deficient myoblasts, phosphorylation of AMPK was increased by ~3.6-fold as compared to myoblasts treated with control siRNA, whereas no differences in phosphorylation of AMPK at S485/491 were observed (Figure 1B and 1C). We also observed an ~44% reduction in phosphorylated Akt at S473 in p110 α -deficient myoblasts compared to control myoblasts. Finally, we asked whether forced expression of a constitutively-active form of p110 α (“p110 α -E545K”) produced the opposite effect; that is, whether p110 α inhibited AMPK phosphorylation at T172. We found that, indeed, phosphorylation of AMPK was reduced by ~54% in cells expressing constitutively-active p110 α . Additionally, myoblasts expressing p110 α -E545K showed significantly increased (~2.1-fold) phosphorylation of AMPK at S485/491 (Figure 1D and 1E). In myoblasts expressing a kinase-inactive form of p110 α (“p110 α -K802R”), phosphorylation of AMPK T172 was increased ~50%, although no change in phosphorylation of AMPK S485/491 was observed (Figure 1C and 1D). Collectively, these data reveal that p110 α negatively regulates phosphorylation of AMPK at T172, and that under

conditions of p110 α hyper-activation, p110 α positively regulates phosphorylation of AMPK at S485/491.

Akt is necessary for p110 α -mediated reduction in AMPK phosphorylation

The serine-threonine kinase Akt lies downstream of p110 α and regulates multiple cellular functions including growth factor signaling, survival, and metabolism [24]. With regard to AMPK, Akt has been shown to negatively regulate AMPK activation in cardiac myocytes [8], MCF-7 breast cancer cells [25], and vascular smooth muscle cells [7]. Akt has also been shown to regulate phosphorylation of AMPK α at S485/491 both in vitro [6] and in vivo [26]. Given these reports, we performed a series of experiments designed to determine whether Akt mediated AMPK phosphorylation in myoblasts.

We first established whether pharmacological inhibition of Akt affected phosphorylation of AMPK by using the Akt inhibitor MK2206 [27]. Phosphorylation of AMPK S485/491 was decreased in a dose-dependent fashion in the presence of MK2206; however, MK2206 had no effect on phosphorylation of AMPK T172 (Figure 2A). We next asked whether inhibition of Akt altered p110 α -mediated phosphorylation of AMPK. Control myoblasts or myoblasts expressing p110 α -E545K were treated with inhibitor vehicle (DMSO) or 125 nM MK2206, a concentration sufficient to inhibit phosphorylation Akt and the Akt substrate PRAS40 (FIG 2A). Expression of p110 α -E545K decreased AMPK phosphorylation at T172 and increased phosphorylation of AMPK at S485/491, whereas treatment with MK2206 alone had no statistical effect on AMPK phosphorylation at either residue (Figure 2B and 2C). In the presence of MK2206 however, the p110 α -E545K-induced decrease in p-AMPK T172, and the p110 α -E545K-induced increase in p-

AMPK 485/491, were restored to levels comparable to control cells. These data indicate that Akt mediates p110 α -induced AMPK phosphorylation in C2C12 myoblasts.

p110 α deficiency modifies AMPK responses to cellular stress

Given our observations that p110 α regulated AMPK under basal conditions, we next asked whether p110 α also regulated AMPK under conditions of cellular stress. We first treated p110 α -sufficient or p110 α -deficient myoblasts with oligomycin, a known activator of AMPK [28], and analyzed phosphorylation of AMPK. Administration of oligomycin increased phosphorylation of AMPK at T172 in myoblasts treated with control siRNA by ~2.1-fold. In p110 α -deficient myoblasts, oligomycin promoted AMPK T172 phosphorylation beyond levels observed in p110 α -deficient cells treated with vehicle (Figure 3A and 3B). We also observed an ~2.2-fold increase in AMPK α S485/491 phosphorylation in response to oligomycin treatment in siRNA control myoblasts, although this increase was not evident in p110 α -deficient myoblasts (Figure 3A and 3B). Oligomycin increased Akt phosphorylation by ~2.5-fold in control cells, but this increase was eliminated in p110 α -deficient myoblasts. These data reveal an additive effect of oligomycin and p110 α deficiency on phosphorylation of AMPK at T172 and suggest that p110 α mediates oligomycin-induced phosphorylation of AMPK at S485/491.

Previous research has shown that incubation of C2C12 cells in serum-free media resulted in increased AMPK T172 phosphorylation [29]. To determine whether p110 α mediated AMPK phosphorylation during serum-starvation, control myoblasts or myoblasts deficient in p110 α were exposed to serum-free media for periods up to two hours. Western blotting revealed that phosphorylation of AMPK at T172 was increased by serum starvation in a time-dependent

manner; furthermore, AMPK T172 phosphorylation was significantly increased in p110 α -deficient cells relative to control myoblasts at all time points examined (Figure 3C and 3D). Exposure to serum-free media decreased AMPK S485/491 phosphorylation by 68 – 74% at all times in both control and p110 α -deficient myoblasts, while expression of phosphorylated Akt at S473 was completely eliminated in serum-free media (Figure 3C and 3D). These data demonstrate that during serum-starvation, loss of p110 α increases phosphorylation of AMPK at T172 to levels beyond those induced by serum-free media alone.

Discussion

In this study, we found that PI3K p110 α mediated AMPK phosphorylation in C2C12 myoblasts. In myoblasts treated with a pharmacological inhibitor of p110 α , treated with siRNA directed against p110 α , or over-expressing kinase-inactive p110 α , levels of phosphorylated AMPK at T172 were markedly increased (Figure 1 and Figure 3), whereas in cells expressing constitutively-active p110 α , levels of phosphorylated AMPK at T172 were significantly decreased. These findings provide convincing evidence that p110 α negatively regulates AMPK T172 phosphorylation; however, the mechanism through which this occurs is less clear. While it has previously been shown that phosphorylation of AMPK at S485/491 inhibits AMPK T172 phosphorylation [6,7], we found that p110 α -deficient myoblasts (or myoblasts expressing p110 α -K802R) possessed increased AMPK phosphorylation at T172 without a corresponding change in phosphorylation at S485/491 (Figure 1). Thus, it appears that phosphorylation of AMPK at T172 may be regulated through a mechanism other than AMPK S485/491 phosphorylation, at least under these conditions. In support of this, it was notable that in both p110 α -deficient myoblasts

and in myoblasts expressing p110 α -K802R, phosphorylation of Akt was reduced, but not completely eliminated. Since Akt is a downstream mediator of p110 α action, and since Akt has been shown to phosphorylate AMPK at S485/491 [30], it would be expected that a reduction in Akt activation would translate to reduced phosphorylation of AMPK at S485/491. It is possible however, that the residual, active Akt may have been sufficient to maintain levels of phosphorylated AMPK S485/491 in p110 α -deficient and p110 α -K802R expressing myoblasts at levels comparable to control myoblasts. Another possibility is that molecules other than Akt act to sustain AMPK S485/491 phosphorylation in p110 α -deficient myoblasts and in myoblasts expressing p110 α -K802R. For example, in non-muscle cells, protein kinase A [30] and p70S6 kinase [31] have both been shown to regulate AMPK phosphorylation at S485/491 under various conditions. Finally, it is possible that loss of p110 α activity results in inhibition of an unidentified AMPK phosphatase. In any case, it is clear that the increased phosphorylation of AMPK at T172 observed in p110 α -deficient myoblasts, or in myoblasts expressing p110 α -K802R, was not associated with decreased AMPK S485/491 phosphorylation under all experimental conditions (Figure 4).

The role of p110 α in regulating phosphorylation of AMPK at S485/491 however, appears more straightforward. Indeed, myoblasts expressing p110 α -E545K displayed robust induction of AMPK S485/491 phosphorylation, an effect that was sensitive to pharmacological inhibition of Akt (Figure 2). These data thus support the existence of a p110 α /Akt/AMPK pathway in C2C12 myoblasts. In contrast to p110 α -deficient myoblasts (which showed induction of AMPK phosphorylation at T172 but no change in AMPK phosphorylation at S485/491 compared to control cells (Figure 1)), myoblasts expressing p110 α -E545K exhibited reduced phosphorylation of AMPK at T172. This finding is in agreement with previous research reporting that AMPK

S485/491 inhibits phosphorylation of AMPK at T172 in response to insulin or IGF-I [6,7,9], suggesting that this phenomenon also occurs in C2C12 myoblasts expressing constitutively-active p110 α (Figure 4).

Interestingly, both AMPK T172 and S485/491 were elevated in response to oligomycin alone, indicating that the inverse relationship between phosphorylation of AMPK T172 and S485/491 observed in response to p110 α expression (Figure 1D and 1E) was not manifest in response to oligomycin. The simplest explanation for this finding is that oligomycin and p110 α act through separate mechanisms to mediate phosphorylation of AMPK at T172. We also found that phosphorylation of Akt S473 was elevated in response to oligomycin alone. While this observation supports that Akt acts to phosphorylate AMPK at S485/491, it does not support that AMPK T172 necessarily decreases in response to increased AMPK at S485/491 phosphorylation.

Phosphorylation of AMPK at S485/491 and phosphorylation of Akt at S473 were significantly reduced in serum-free media. While these latter observations are consistent with the idea that low levels of phosphorylated AMPK at 485/491 are permissive for high levels of phosphorylated AMPK at T172 [7], the extent of reduction in AMPK S485/491 phosphorylation did not correlate with the extent of induction of AMPK T172 phosphorylation. Indeed, phosphorylation of AMPK at T172 was increased to various degrees in response to serum-free media and p110 α deficiency, whereas phosphorylation of AMPK at S485/491 was decreased by a relatively constant ~70% in both p110 α -sufficient and p110 α -deficient cells throughout the entire time in serum-free media (Figure 3C and 3D). This finding suggests that reductions in phosphorylation of AMPK at S485/491 do not proportionally translate to increased AMPK T172 phosphorylation under these conditions.

In summary, we identified PI3K p110 α as a principal regulator of AMPK phosphorylation in myoblasts. Actions of p110 α on AMPK were found to be sensitive to pharmacological inhibition of Akt, suggesting a key role for Akt in p110 α -AMPK signal transduction. In p110 α -deficient myoblasts, phosphorylation of AMPK at T172 was further altered under conditions of cellular stress, demonstrating that p110 α acts as a negative regulator of T172 phosphorylation under a variety of experimental conditions. The precise role of p110 α in mediating phosphorylation of AMPK at S485/491, as well as a potential relationship between phosphorylation of T172 and S485/491, remains to be established in this model system.

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Figure Legends

Figure 1. PI3K p110 α regulates AMPK phosphorylation in C2C12 myoblast. (A) Myoblasts were exposed to the indicated concentrations of p110 α inhibitor (p110 α i) for 4-hours. Lysates were immunoblotted with the indicated antibodies. (B) Myoblasts were treated with 5 nM control siRNA (si-Con) or 5 nM siRNA directed against p110 α (si-p110 α) as described in Materials and Methods. Lysates were immunoblotted with the indicated antibodies. Blots are representative of three independent experiments. (C) Quantification of blots shown in panel B (means $\pm$ SEMs; n = 3 independent experiments; Student's t-test; **, $P < 0.01$). (D) Myoblasts were transduced with vector control (Vector), constitutively-active p110 α (p110 α -E545K) or kinase-dead p110 α (p110 α -K802R) as described in Materials and Methods. Lysates were immunoblotted with the indicated antibodies. Blots are representative of three independent experiments. (E) Quantification of blots shown in panel D (means $\pm$ SEMs; n = 3 independent experiments; one-way ANOVA with Dunnett test post hoc; **, $P < 0.01$; ***, $P < 0.001$).

Figure 2. Akt regulates p110 α -mediated AMPK phosphorylation. (A) Myoblasts were exposed to the indicated concentrations of MK2206 for 4-hours. Lysates were immunoblotted with the indicated antibodies. (B) Myoblasts transduced with vector control or p110 α -E545K were treated with MK2206 or DMSO for 4-hours prior to harvest for protein lysates. Blots are representative of three independent experiments. (C) Quantification of blots shown in panel B (means $\pm$ SEMs; n = 3 independent experiments; one-way ANOVA with Dunnett test post hoc; ***, $P < 0.001$).

Figure 3. Effects of oligomycin and serum starvation on p110 α -deficient myoblasts. (A)

Control or p110 α -deficient myoblasts were treated with 500 nM oligomycin for 30-minutes and then harvested for protein lysates. Blots are representative of at least four independent experiments. (B) Quantification of blots shown in panel A (means $\pm$ SEMs; $n = 4 - 6$ independent experiments; one-way ANOVA with Dunnett test post hoc; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (C) Control or p110 α -deficient myoblasts were exposed to serum-free (SF) media for the indicated times and then harvested for protein lysates. Blots are representative of three independent experiments. (D) Quantification of blots shown in panel C (means $\pm$ SEMs; $n = 3$ independent experiments; two-way ANOVA with Bonferroni test post hoc; *, $P < 0.05$; **, $P < 0.01$).

Figure 4. Model of p110 α -mediated AMPK phosphorylation. p110 α negatively regulates AMPK T172 phosphorylation independent of Akt through a yet-unidentified mechanism. p110 α also regulates AMPK S485/491 phosphorylation in an Akt-dependent manner. AMPK phosphorylation at S485/491 was associated with decreased AMPK T172 phosphorylation under conditions of p110 α hyper-activation, but this association was not observed in p110 α deficient myoblasts, suggesting that AMPK S485/491-mediated inhibition of T172 does not occur under all conditions (dashed line).

Figure 1

A

B

C

D

E

Figure 2

A

B

C

Figure 3

Figure 4

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