Protein Phosphatase 1α Regulates AS160 Ser\textsuperscript{588} and Thr\textsuperscript{642} Dephosphorylation in Skeletal Muscle

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ABSTRACT

Akt substrate of 160 kDa (AS160) phosphorylation on Thr$^{642}$ and Ser$^{588}$ by Akt is essential for insulin’s full-effect on glucose transport. However, protein phosphorylation is determined by the balance of actions by kinases and phosphatases, and the specific phosphatase(s) controlling AS160 dephosphorylation is(are) unknown. Accordingly, we assessed roles of highly expressed skeletal muscle serine/threonine-phosphatases (PP1, PP2A, PP2B and PP2C) on AS160 dephosphorylation. Preliminary screening of candidate phosphatases used an AS160 dephosphorylation assay. Lysates from insulin-stimulated skeletal muscle were treated with pharmacological phosphatase inhibitors and assessed for AS160 Ser$^{588}$ and Thr$^{642}$ dephosphorylation. AS160 dephosphorylation on both phospho-sites was unaltered by PP2B or PP2C inhibitors. Okadaic acid (low dose inhibits PP2A; high dose inhibits PP1) delayed AS160 Ser$^{588}$ (both doses) and Thr$^{642}$ (high dose only) dephosphorylation concomitant with greater Akt phosphorylation (both doses). AS160 was co-immunoprecipitated with PP1α, but not with PP1β, PP1γ1 or PP2A. Recombinant Inhibitor-2 protein (a selective PP1 inhibitor) delayed AS160 dephosphorylation on both phospho-sites without altering Akt phosphorylation. Furthermore, knockdown of PP1α, but not PP1β or PP1γ1, by siRNA caused greater AS160 Ser$^{588}$ and Thr$^{642}$ phosphorylation concomitant with unaltered Akt phosphorylation. Together, these results identified PP1α as a regulator of AS160 Thr$^{642}$ and Ser$^{588}$ dephosphorylation in skeletal muscle.
INTRODUCTION

Skeletal muscle accounts for the largest portion of insulin-mediated whole body glucose disposal, and skeletal muscle insulin resistance is crucial for both whole body insulin resistance and type 2 diabetes mellitus (1). Muscle insulin resistance is secondary, in large part, to defective GLUT4 glucose transporter translocation and glucose transport (2). Insulin’s stimulation of glucose transport is triggered by a complex insulin signaling pathway that begins with insulin’s binding to its receptor, leading to receptor autophosphorylation and activation of receptor tyrosine kinase (2). The insulin receptor kinase phosphorylates insulin receptor substrate (IRS) proteins on multiple tyrosine residues, resulting in IRS protein engagement with phosphatidylinositol 3-kinase (PI3K), that in turn, phosphorylates phosphatidylinositol (PI) 4, 5-bisphosphate to create 3, 4, 5-trisphosphate (PIP3). The serine/threonine-kinase Akt is recruited to bind PIP3 and become activated secondary to phosphorylation on Thr\(^{308}\) (via phosphoinositide-dependent kinase-1, PDK1) and Ser\(^{473}\) (via mTORC2). Akt phosphorylates many protein substrates, several of which have been implicated in insulin’s regulation of GLUT4 traffic to the cell surface membranes, including a Rab-GTPase activating protein known as Akt Substrate of 160 kDa (AS160; also known as TBC1D4) (3-5). Akt can phosphorylate several residues on AS160. Mutation of serine or threonine to alanine to prevent phosphorylation of either Ser\(^{588}\) or Thr\(^{642}\) resulted in attenuation of insulin-stimulated GLUT4 translocation, and mutation of several other Akt phosphomotifs did not produce any further effects on GLUT4 localization (6). It is essential to fully understand the regulation of AS160 phosphorylation given the crucial role that it plays in regulating insulin-stimulated glucose uptake by skeletal muscle.

The reversible serine/threonine phosphorylation of proteins is balanced by the opposing actions of kinases and phosphatases, but for most proteins, there has been an overwhelming bias to focus on serine/threonine kinases, with strikingly fewer studies assessing the role of serine/threonine phosphatases (7). Serine/threonine protein phosphatases regulate diverse aspects of growth, development and metabolism, but relatively few protein serine/threonine
phosphatases control the specific dephosphorylation of a much greater number of phosphoprotein substrates (8). With specific regard to AS160, many studies have analyzed Akt’s role in the insulin-stimulated phosphorylation of AS160 (9-13), but essentially nothing is known about the serine/threonine protein phosphatase(s) regulating AS160 dephosphorylation.

PP1, PP2A, PP2B and PP2C are among the most abundant serine/threonine protein phosphatases expressed by skeletal muscle (14), and we hypothesized that AS160 dephosphorylation on Thr<sup>642</sup> and Ser<sup>588</sup> would be regulated by one or more of these enzymes. We evaluated the hypothesis using multiple approaches, including assessment of: 1) the effects of several pharmacologic serine/threonine-protein phosphatase inhibitors on AS160 Ser<sup>588</sup> and Thr<sup>642</sup> dephosphorylation; 2) AS160’s physical association with serine/threonine-protein phosphatases; 3) the influence of a selective inhibitor of protein phosphatase 1 (PP1) known as Inhibitor-2 (15) on AS160 Ser<sup>588</sup> and Thr<sup>642</sup> phosphorylation; and 4) the consequences of knockdown of serine/threonine-protein phosphatases by small interfering RNA silencing on AS160 Ser<sup>588</sup> and Thr<sup>642</sup> phosphorylation. These experiments identified protein phosphatase 1α (PP1α) as a serine/threonine-protein phosphatase that regulates AS160 Ser<sup>588</sup> and Thr<sup>642</sup> dephosphorylation in skeletal muscle.

**RESEARCH DESIGN AND METHODS**

**Materials**

The reagents and apparatus for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and non-fat dry milk (#170-6404XTU) were from Bio-Rad (Hercules, CA). MemCode Reversible Protein Stain (#24580) and Bicinchoninic acid (BCA; #23227) protein assay kits and tissue protein extraction reagent (T-PER; #78510) were from Thermo Fisher (Waltham, MA). Luminata Forte Western HRP Substrate (#WBLUF0100) were from EMD Millipore (Billerica, MA). Sanguinarine chloride (#ALX-350-076) was from Enzo Life Sciences (Farmingdale, NY). FK-506 (#3631) was purchased from Tocris (Bristol, U.K). Okadaic
acid (#459620) was purchased from Merck Millipore (Billerica, MA). Recombinant Protein Phosphatase Inhibitor-2 (#P0755) was from NEB Biolabs ( Ipswich, MA). Anti-pAkt\textsuperscript{Thr308} (#9275), anti-pAkt\textsuperscript{Ser473} (#9271), anti-Akt (#4691), anti-pAS160\textsuperscript{Thr642} (#8881), anti-pAS160\textsuperscript{Ser588} (#8730), anti-PP1\(\alpha\) (#2582), anti-spinophilin (#14136) and anti-rabbit IgG horseradish peroxidase conjugate (#7074) were from Cell Signaling Technology (Danvers, MA). Anti-AS160 (#ABS54), anti-PP1\(\beta\) (#07-1217), anti-PP1\(\gamma\)1 (#07-1218), anti-\(\alpha\)-Tubulin (#04-1117), Normal rabbit IgG polyclonal antibody control (#12-370) were purchased from EMD Millipore (Billerica, MA). Anti-PP2\(\alpha\) (#610556) was from BD Bioscience (San Jose, CA). PP1 Inhibitor-2 antibody (#AF4719) was from R&D Bioso systems (Minneapolis, MN). Anti-PP1\(\alpha\) (#sc-443), anti-GADD34 (#sc-8327), anti-goat IgG horseradish peroxidase conjugate (#sc-2020) and anti-mouse IgG horseradish peroxidase conjugate (#sc-2060), were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phostensin (#MB1057) was from BioWorld Technology, Inc. (St. Louis Park, MN). siRNA against rat protein phosphatase 1 catalytic subunit \(\alpha\) (#L-100270-02-0020), PP1 catalytic subunit \(\beta\) (#L-100263-02-0020), or PP1 catalytic subunit \(\gamma\)1 (#L-096319-02-0020) and RNA interference-negative control #/(D-001810-10-20) were purchased as smartpools from Dharmacon (Lafayette, CO). Magnetic Protein G beads (#10004D), RNAiMAX transfection reagent (#13778-150) and Dulbecco’s Modified Eagle’s Medium (DMEM; #11995) were from Life Technologies (Grand Island, NY).

Animal Treatment

Procedures for animal care were approved by the University of Michigan Committee on Use and Care of Animals. Male Wistar rats (aged 8–10 wk) were from Harlan (Indianapolis, IN). Lean (Fa/Fa) and obese (fa/fa) male Zucker rats (aged 7–8 wk) were from Charles River Laboratories (Wilmington, MA). Animals were provided with rodent chow (Lab Diet No. 5001; PMI Nutrition International, Brentwood, MO) ad libitum until 1700 h the night before the experiment when food was removed. On the next day at 1000 h to 1200 h, rats were
anesthetized (intraperitoneal injection of sodium pentobarbital), and both epitrochlearis muscles were isolated and treated as described below.

**Muscle Incubation**

Isolated epitrochlearis muscles were incubated in glass vials containing Krebs-Henseleit buffer (KHB), 0.1% bovine serum albumin (BSA), 2 mM sodium pyruvate, 6 mM mannitol, without (basal) or with insulin either 0.6 nM (for co-immunoprecipitation assays described below) or 30 nM (for dephosphorylation assays described below) for 30 min in a heated, shaking water bath at 35°C with continuous gassing (95% O\textsubscript{2}/5% CO\textsubscript{2}). Immediately after the incubation, muscles were blotted, rapidly trimmed of connective tissue, and freeze-clamped with liquid N\textsubscript{2}–cooled aluminum tongs. Frozen muscles were stored at −80°C until subsequent homogenization and analysis.

**L6 Cell Culture and Treatment**

L6 myoblasts were purchased from the American Type Culture Collection (Manassas, VA, USA). L6 cells were cultured in DMEM, supplemented with 10% (v/v) FBS, 1% penicillin, and 100 µg/mL streptomycin in a humidified atmosphere with 5% CO\textsubscript{2} at 37°C. Cells were washed twice with 1X PBS, and starved for 5 h in serum free DMEM medium before 20 min of incubation without insulin (basal) or with insulin (100 nM).

**Muscle and Cell Lysate Preparation**

Unless otherwise noted, epitrochlearis muscles were homogenized (TissueLyser II homogenizer; Qiagen Inc., Valencia, CA) using ice-cold tissue protein extraction reagent (T-PER) buffer supplemented with 2.5 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM β-glycerophosphate, 1 µg/mL leupeptin, 1 µg/mL pepstatin, 1 µg/mL aprotinin and 1 mM phenylmethylsulfonyl fluoride (PMSF). Homogenates were then transferred to microcentrifuge
tubes and rotated (1h, 4°C) before being centrifuged (15,000g, 20 min, 4°C). L6 cells were scraped in T-PER that was supplemented as described above, incubated (4°C, 20 min), and then centrifuged (15,000 g, 20 min, 4°C). Total protein in supernatants from muscle or cell lysates was measured by the BCA method.

Epitrochlearis muscles and L6 cells used for Co-IP were processed as described above using Co-IP buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1% IGEPAL, 10% glycerol) supplemented with 2.5 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM β-glycerophosphate, 1 µg/mL leupeptin, 1 µg/mL pepstatin, 1 µg/mL aprotinin and 1 mM PMSF.

Epitrochlearis muscles used for the dephosphorylation assay described below were homogenized for 2 min in cold T-PER buffer supplemented with 1 µg/mL leupeptin, 1 µg/mL pepstatin, 1 µg/mL aprotinin and 1 mM PMSF, but without phosphatase inhibitors (2.5 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM β-glycerophosphate). Lysates were centrifuged (2 min, 15,000g, 4°C), and the resultant supernatants were immediately used for the dephosphorylation assay described below.

AS160 Dephosphorylation Assay

Preliminary insights about the regulation of AS160 dephosphorylation were provided by testing the ability of several chemical phosphatase inhibitors, with differing specificity for inhibiting selected protein phosphatases, to delay the rate of AS160 dephosphorylation in lysates prepared from isolated epitrochlearis muscles. The AS160 dephosphorylation assay was a modification of a method previously used to identify the serine/threonine-phosphatase that dephosphorylates calcium-binding protein-4 (16). The muscles used for this AS160 dephosphorylation assay were first incubated with 30 nM insulin, to ensure initially high levels of AS160 phosphorylation. Freeze-clamped muscles were rapidly homogenized, in ice-cold buffer in the absence of protein phosphatase inhibitors. An initial aliquot (20 µL) was rapidly withdrawn from each muscle lysate, and immediately mixed with an equal volume of 2X SDS loading buffer.
and heated (95°C, 6 min). This initial aliquot was denoted as the 0 min time-point for the AS160 dephosphorylation assay. To the remaining lysate, either a chemical protein phosphatase inhibitor [5 nM okadaic acid for PP2A inhibition and 1000 nM okadaic acid for PP1 inhibition (17); 10 µM sanguinarine for PP2C inhibition (18); or 100 ng/mL FK-506 for PP2B inhibition (19)] or an equal volume of vehicle (dimethyl sulfoxide, DMSO) was rapidly added. The lysates were then incubated at 37°C, with aliquots (20 µL) withdrawn at subsequent time points (30, 60 and 120 min). Upon withdrawal, each aliquot was rapidly mixed with an equal volume of 2X SDS loading buffer, and heated to 95°C for 6 min. These aliquots were then subjected to SDS-PAGE and immunoblotting to assess the phosphorylation levels of AS160^{Ser588} and AS160^{Thr642}.

When using Inhibitor-2 protein, dephosphorylation assays were performed as described above for chemical inhibitors, except that epitrochlearis lysates were incubated with or without recombinant Inhibitor-2 (rInh-2) protein (50 µg/mL lysate) rather than a chemical inhibitor. Dephosphorylation assays in lean Zucker (LZ) and obese Zucker (OZ) rats were performed as described above, except lysates were incubated without any phosphatase inhibitors.

**Co-immunoprecipitation**

Lysates prepared from epitrochlearis muscles (300 µg total protein) and L6 cells (400 µg total protein) were subjected to immunoprecipitation using either AS160 antibody or normal rabbit IgG at 4°C overnight. On the next morning, the protein-antibody complex was incubated with 50 µL magnetic Protein G beads for 2 h at 4°C with gentle rotation. The Ab-protein-beads complex was washed three times with co-immunoprecipitation (Co-IP) buffer. The protein in the complex was then eluted with 30 µL of 2X SDS loading buffer and boiled before running on a polyacrylamide gel. Proteins were transferred to PVDF membranes, and AS160-associated proteins were immunoblotted using antibodies against PP1α, PP1β, PP1γ1 and PP2A. AS160’s association with PP1α was also assessed by immunoprecipitation using anti-PP1α followed by immunoblotting with anti-AS160. In addition, AS160’s association with several PP1α regulatory
subunits (20-22) was evaluated by immunoprecipitation using anti-AS160 followed by
immunoblotting with antibodies against: spinophilin (also known as PPP1R9B), GADD34 (also
known as PPP1R15A), phostensin (also known as PPP1R18) and Inhibitor-2 (also known as
PPP1R2).

Small Interfering RNA (siRNA) Silencing

L6 cells were transfected with 200 nM control scrambled siRNA, siPP1α, siPP1β, or
siPP1γ1 using RNAiMAX transfection reagent from Invitrogen using the manufacturer’s
instructions, with minor modifications. Briefly, cells were seeded in 60 mm plates at 50%
confluence in DMEM supplemented with 10% FBS. On day 2, myoblasts were transfected with
200 nM of siRNA in reduced serum Opti-MEM media without antibiotics, with the media
changed 48 h later to DMEM supplemented with 10% FBS. At 72 h of transfection, the cells
were treated without insulin (basal) or with 100 nM insulin in serum free medium for 20 min prior
to being harvested. The extent of PP1α, PP1β, or PP1γ1 knockdown was assessed by
immunoblot.

Immunoblotting

Equal amounts of protein from each sample were loaded on a Tris glycine acrylamide
gel and transferred to PVDF membrane. The membranes were incubated with appropriate
primary and secondary antibodies. Immunoreactive proteins were detected using Luminata
Forte Western HRP Substrate and quantified by densitometry (Alpha Innotech, San Leandro,
CA).

Statistical Analysis

Statistical analyses were performed using Prism 4.0 software (GraphPad Software, San
Diego, CA). Data are expressed as means ± SEM. Differences between two groups were
evaluated using a two-tailed $t$ test. Differences between more than two groups were evaluated using One-way ANOVA. The source of significant variance was identified using Tukey post hoc analysis. A $P$ value of $\leq 0.05$ was considered statistically significant.

RESULTS

Neither FKO506 nor Sanguinarine Delay AS160 Dephosphorylation

Neither the PP2B inhibitor FKO506 nor the PP2C inhibitor sanguinarine differed from the vehicle for either $p\text{AS160}^{\text{Thr642}}$ (Fig. 1A and 1C) or $p\text{AS160}^{\text{Ser588}}$ (Fig 1B and 1C) in epitrochlearis muscle lysates. Similarly, neither inhibitor differed from vehicle for either $p\text{Akt}^{\text{Thr308}}$ (Fig. 1D and 1F) or $p\text{Akt}^{\text{Ser473}}$ (Fig. 1E and 1F). These results provide no evidence that either PP2B or PP2C regulates AS160 dephosphorylation.

Dose-dependent Effects of Okadaic Acid (OA) on AS160 Dephosphorylation

There were no differences for the phosphorylation of $p\text{AS160}^{\text{Thr642}}$ of epitrochlearis muscle lysates treated with vehicle compared with 5 nM OA, a dose sufficient to inhibit PP2A, but not PP1 (23) at any time-point. In contrast, for lysates treated with 1000 nM OA, a dose sufficient to inhibit PP1 (23), the phosphorylation of $p\text{AS160}^{\text{Thr642}}$ was significantly ($P<0.05$) greater than the vehicle at 30, 60 and 120 min and significantly ($P<0.05$) greater than the 5 nM OA values at 30 and 60 min (Fig. 2A and 2C). For phosphorylation of $p\text{AS160}^{\text{Ser588}}$, the values with 5 nM OA significantly ($P<0.05$) exceeded the vehicle at 30 and 120 min, and the 1000 nM values were significantly ($P<0.05$) greater than vehicle at 30, 60 and 120 min (Fig. 2B and 2C). For phosphorylation of $p\text{Akt}^{\text{Thr308}}$, there were no significant differences between the 5 nM OA values and vehicle at any time-point, but the 1000 nM values significantly ($P<0.05$) exceeded both vehicle and 5 nM OA values at 30, 60 and 120 min (Fig. 2D and 2F). For phosphorylation of $p\text{Akt}^{\text{Ser473}}$, the values with 5 nM OA were significantly ($P<0.05$) greater than vehicle at 30 min, and the 1000 nM OA values significantly ($P<0.05$) exceeded vehicle and 5 nM values at 30, 60
and 120 min (Fig. 2E and 2F). Taken together, these results suggest that AS160 dephosphorylation is influenced by OA in a dose-dependent and site-selective manner. Only the higher OA dose delayed dephosphorylation of AS160<sup>Thr642</sup>, which would be consistent with an effect related to inhibition of PP1. In contrast, both OA doses delayed dephosphorylation of AS160<sup>Ser588</sup>, suggesting possible roles of PP2A and/or PP1.

**Selective AS160 Co-Immunoprecipitation with PP1α, but not PP1β, PP1γ1 or PP2A**

Because the OA results were not definitive, we used additional approaches to probe AS160’s relationship with PP1 and PP2A in skeletal muscle. Specific interaction between PP1α and AS160 in epitrochlearis was indicated by the significantly ($P<0.05$) greater amount of PP1α that co-immunoprecipitated with AS160 compared to normal IgG control regardless of insulin concentration (Fig. 3A and 3B). The specific association between AS160 and PP1α was confirmed by immunoprecipitation using anti-PP1α followed by immunoblotting with anti-AS160 compared to normal IgG control (data not shown). In contrast, there was no evidence that AS160 had any specific association with PPβ1 (Fig. 3C and 3D), PP1γ1 (Fig. 3E and 3F) or PP2A (Fig. 3G and 3H). Similar results were obtained for L6 cells in which specific interaction was detected for AS160 with PP1α, but not for AS160 with PP1β, PP1γ1 or PP2A (data not shown). The selective association of AS160 with PP1α supports the idea that PP1α may regulate AS160 dephosphorylation. The association of AS160 with several PP1α regulatory subunits (spinophilin, GADD34, phostensin and Inhibitor-2) was evaluated in epitrochlearis lysates immunoprecipitated using anti-AS160 followed by immunoblotting with antibodies against the respective regulatory subunits. However, this analysis did not detect evidence of selective association of these proteins with AS160 (data not shown).

**Recombinant Inhibitor-2 Protein Delays AS160 Dephosphorylation**
Inhibitor-2 was studied because it is a selective biological inhibitor of PP1 (15). Epitrochlearis lysates incubated with recombinant Inhibitor-2 (rInh-2) protein compared to control lysates had greater ($P<0.05$) phosphorylation of AS160$^{Thr642}$ at 15, 30, 45 and 60 min (Fig. 4A and 4C). Furthermore, rInh-2 treatment versus controls produced greater ($P<0.05$) phosphorylation of AS160$^{Ser588}$ at 15, 30 and 45 min (Fig. 4B and 4C). Greater AS160 phosphorylation was not accompanied by any significant effects of rInh-2 on the phosphorylation of either Akt$^{Thr308}$ (Fig. 4D and 4F) or Akt$^{Ser473}$ (Fig. 4E and 4F). The delayed AS160 dephosphorylation on both Thr$^{642}$ and Ser$^{588}$ with unaltered Akt phosphorylation is consistent with PP1 being a modulator of AS160 dephosphorylation.

Silencing PP1α, but not Other PP1 Isoforms, Leads to Greater AS160 Phosphorylation

Because PP1α was selectively associated with AS160 based on Co-IP, it was also critical to perform a functional assessment of the roles of PP1 isoforms on AS160’s phosphorylation status in intact muscle cells. L6 cells transfected with siPP1α had a significant ($P<0.05$) reduction in PP1α protein abundance compared to cells transfected with scrambled control siRNA (Fig. 5A). The specificity of the PP1α knockdown in the siPP1α-transfected cells was evidenced by the lack of changes in PP1β or PP1γ1 protein levels (Fig. 5B). Lower PP1α abundance led to significantly ($P<0.05$) greater phosphorylation of AS160$^{Thr642}$ (Fig. 5C) and AS160$^{Ser588}$ (Fig. 5D) in the insulin-stimulated siPP1α transfected cells compared to insulin stimulated control cells. Greater AS160 phosphorylation was not attributable to any effect of siPP1α transfection on the phosphorylation of either Akt$^{Thr308}$ (Fig. 5F) or Akt$^{Ser473}$ (Fig. 5G).

Transfection of L6 cells with siPP1β produced a robust and significant ($P<0.05$) reduction in PP1β protein abundance compared to cells transfected with scrambled control siRNA (Fig. 6A). The specificity of the PP1β knockdown was confirmed by the absence of changes in PP1α or PP1γ1 abundance (Fig. 6B). The lower PP1β content had no detectable
effects on the phosphorylation of AS160\textsuperscript{Thr642} (Fig. 6C), AS160\textsuperscript{Ser588} (Fig. 6D), Akt\textsuperscript{Thr308} (Fig. 6F) or Akt\textsuperscript{Ser473} (Fig. 6G) in siPP1\textbeta transfected cells compared to control cells.

Transfection of L6 cells with siPP1\textgamma caused a substantial and significant ($P<0.05$) reduction in PP1\textgamma protein abundance compared to cells transfected with the scrambled control siRNA (Fig. 7A). The specificity of the PP1\textgamma knockdown was demonstrated by unchanged PP1\textalpha or PP1\textbeta levels (Fig. 7B). The lower PP1\textgamma abundance resulted in no changes in the phosphorylation of AS160\textsuperscript{Thr642} (Fig. 7C), AS160\textsuperscript{Ser588} (Fig. 7D), Akt\textsuperscript{Thr308} (Fig. 7F) or Akt\textsuperscript{Ser473} (Fig. 7G) in siPP1\textgamma transfected cells versus control cells.

**AS160 Dephosphorylation in Muscles from Lean Zucker versus Obese Zucker Rats**

The relative phosphorylation of AS160\textsuperscript{Thr642} was significantly ($P<0.05$) lower for epitrochlearis muscle lysates from OZ versus LZ rats at 5 min (Fig. 8A and 8C). The relative phosphorylation of AS160\textsuperscript{Ser588} was significantly ($P<0.05$) lower for OZ versus LZ at 30 and 40 min (Fig. 8B and 8C). In contrast to AS160 phosphorylation, there were no significant differences between LZ and OZ rats for phosphorylation of either Akt\textsuperscript{Thr308} (Fig. 8D and 8F) or Akt\textsuperscript{Ser473} (Fig. 8E and 8F).

Eptrochlearis from LZ versus OZ did not differ for protein abundance of AS160 ($P$ value 0.42), PP1\textalpha ($P$ value 0.78) or Inhibitor-2 ($P$ value 0.27) (data not shown). Neither did epitrochlearis from LZ versus OZ differ with regard to the amount of AS160-associated PP1\textalpha ($P$ value 0.40) determined by Co-IP (data not shown).

**DISCUSSION**

Protein phosphorylation status reflects the balance of phosphorylation by kinases and dephosphorylation by phosphatases. However, there is an extreme disparity in the level of knowledge about the roles of specific kinases compared to phosphatases in the regulation of protein phosphorylation. Although AS160 phosphorylation is a key determinant of insulin-
stimulated glucose transport, prior research had not identified the serine/threonine protein phosphatases that regulate AS160 dephosphorylation. The current study tackled this important problem using a series of different experimental approaches leading to the discovery that PP1α modulates AS160 dephosphorylation on Ser$^{588}$ and Thr$^{642}$ in skeletal muscle.

PP1, PP2A, PP2B and PP2C are four of the most highly expressed serine/threonine phosphatases in skeletal muscle (14). We initially tested AS160 dephosphorylation in muscle using several pharmacologic inhibitors that are commonly employed to block each of these phosphatases. This first screening step provided no evidence that PP2B or PP2C inhibitors delayed AS160 dephosphorylation on either AS160 phosphosite. Okadaic acid was tested at two doses because it has differing potency for inhibiting PP2A (requiring a lower dose) compared to PP1 (requiring a higher dose) (23). The higher okadaic acid dose, but not the lower okadaic dose, delayed AS160 dephosphorylation on Thr$^{642}$. This result is consistent with the possibility that PP1 regulated AS160 dephosphorylation on this site, but a caveat is that this okadaic acid dose also produced greater Akt phosphorylation. Both okadaic acid doses delayed AS160 dephosphorylation on Ser$^{588}$ concomitant with increased Akt phosphorylation. Earlier research has indicated that okadaic acid can attenuate Akt dephosphorylation (24-26). The okadaic acid results did not conclusively isolate the possible roles of PP1 or PP2A for controlling AS160 dephosphorylation, so we subsequently used additional experimental approaches to gain more specific insights.

For our first alternative approach, we used Inhibitor-2 to specifically assess PP1’s role in regulating AS160 dephosphorylation. The results from the recombinant Inhibitor-2 experiment were simpler to interpret because Inhibitor-2 selectively inhibits PP1 without inhibiting PP2A (27), and because Inhibitor-2 caused greater phosphorylation on both AS160 sites in the absence of altered Akt phosphorylation. These observations implicated PP1 in the regulation of AS160 dephosphorylation on both Ser$^{588}$ and Thr$^{642}$. However, because skeletal muscle expresses three PP1 isoforms (PP1α, PP1β and PP1γ1), and Inhibitor-2 can bind and inhibit
each of these PP1 isoforms (28), it was necessary to next address the possibility of PP1 isoform selectivity.

We used two distinct approaches to address the role of different PP1 isoforms. We first performed Co-IP analysis to evaluate the physical association between AS160 and candidate phosphatases (29). We found that in both rat skeletal muscle and L6 myocytes AS160 was selectively associated with PP1α, and not PP1β or PP1γ1. There was also no evidence for specific interaction between AS160 and PP2A in either rat skeletal muscle or L6 cells.

The Co-IP analysis demonstrated that PP1α and AS160 are binding partners, but this approach cannot establish if there is a functional relationship between the two proteins. Accordingly, we next turned to siRNA silencing to test for PP1 isoform specific effects. The results of this experiment were invaluable because they clearly revealed that a selective reduction in PP1α protein abundance produced greater AS160 phosphorylation on both Ser\(^{588}\) and Thr\(^{642}\). Importantly, Akt phosphorylation was unaltered by PP1α knockdown, so the greater AS160 phosphorylation was not an indirect consequence of greater Akt phosphorylation.

Furthermore, PP1α knockdown was specific, as there were no changes in the abundance of the other PP1 isoforms. In contrast to the significant effects of PP1α knockdown, neither PP1β nor PP1γ1 knockdown altered AS160 phosphorylation.

It seems possible that altered serine/threonine dephosphorylation of AS160 may contribute to attenuated AS160 phosphorylation that has been reported in insulin resistant muscles. For example, OZ rats compared to LZ rats are characterized by reductions in both insulin-stimulated AS160 phosphorylation and glucose uptake (30-33). The current study included the first evaluation of the potential role of accelerated AS160 dephosphorylation in insulin resistant muscles. Results from the AS160 dephosphorylation assay revealed modestly faster dephosphorylation of AS160 on Ser\(^{588}\) and Thr\(^{642}\) in muscle lysates from insulin resistant OZ rats versus LZ controls. These differences were not explained by disparities in the abundance of AS160, PP1α or Inhibitor-2 protein or in the association between PP1α and
AS160. However, these results do not eliminate the possibility of a role for PP1\(\alpha\) in the dysregulation of AS160 phosphorylation in insulin resistant muscle.

In conclusion, AS160 plays a pivotal role in insulin’s regulation of glucose transport in skeletal muscle. Many previous studies have focused on Akt’s role in the insulin-stimulated phosphorylation of AS160. In contrast, the current study was the first to focus on identifying the serine/threonine phosphatases that control the dephosphorylation of AS160. We discovered that PP1\(\alpha\) regulates AS160 dephosphorylation on Ser\(^{588}\) and Thr\(^{642}\), two key sites that control insulin-stimulated glucose transport. This knowledge represents an essential building block for fully understanding the processes that control this key insulin signaling protein that is a crucial regulator of insulin-stimulated glucose transport.

ARTICLE INFORMATION

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Author Contributions. P.S. performed the experiments, analyzed the data, designed the experiments, discussed the manuscript, developed the hypothesis, and wrote the manuscript. E.B.A. performed the experiments, analyzed the data, and discussed the manuscript. G.D.C. designed the experiments, coordinated and directed the project, developed the hypothesis, discussed the manuscript, and wrote the manuscript. G.D.C. is the guarantor of this work and,
as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

FIGURE LEGENDS

Figure 1: Effects of the PP2B inhibitor FK-506 (100 ng/mL) and the PP2C inhibitor sanguinarine (10 µM) on AS160 phosphorylation in epitrochlearis muscles. The time-course for AS160 dephosphorylation was assessed using isolated muscles that were incubated with insulin (30 nM) before being rapidly homogenized. To determine initial AS160 phosphorylation, an aliquot from each lysate was immediately combined with 2X SDS buffer to stop dephosphorylation (denoted on the figure as 0 min). Aliquots from the remaining lysate were incubated (30, 60 or 120 minutes at 37°C) in the presence of vehicle, FK-506 [denoted in the figure as PP2B (I)], or the sanguinarine denoted in the figure as PP2C (I). After each incubation time-point, lysates were combined with 2X SDS buffer before being subjected to immunoblotting. A) pAS160^{Thr642}; B) AS160^{Ser588}; C) representative immunoblots (pAS160^{Thr642}, pAS160^{Ser588} and total AS160); pAkt^{Ser473}; D) pAkt^{Thr308}; E) pAkt^{Ser473}; F) representative immunoblots (pAkt^{Thr308}, pAkt^{Ser473} and total Akt). Data were analyzed using 1-way ANOVA. Values are expressed as mean ± SEM; n = 4 muscle lysates per treatment.

Figure 2: Effects of okadaic acid (OA; 5 nM for PP2A inhibition; or 1000 nM for PP1 inhibition) on AS160 dephosphorylation in epitrochlearis muscles. The procedure described for Figure 1 was used, except that OA was the phosphatase inhibitor used rather than FK-506 or sanguinarine. A) pAS160^{Thr642}; B) AS160^{Ser588}; C) representative immunoblots (pAS160^{Thr642}, pAS160^{Ser588} and total AS160); D) pAkt^{Thr308}; E) pAkt^{Ser473}; F) representative immunoblots (pAkt^{Thr308}, pAkt^{Ser473} and total Akt). Data were analyzed using 1-way ANOVA. Tukey post hoc analysis was performed to identify the source of significant variance. *P<0.05, 1000 nM OA
exceeds both 0 and 5 nM OA at the corresponding time-point. *P<0.05, 1000 nM OA exceeds 0 nM OA at the corresponding time-point. **P<0.05, 5 nM exceeds 0 nM OA at the corresponding time-point. Values are expressed as mean ± SEM; n = 4 muscle lysates per treatment.

**Figure 3:** Co-immunoprecipitation (Co-IP) of PP1α, PP1β, PP1γ1 and PP2A with AS160 in epitrochlearis muscles. Anti-AS160 antibody was used to precipitate AS160 in lysates prepared from isolated muscles that had been incubated with or without insulin, and IgG was used as a control for non-specific Co-IP. Immune-complexes were immunoblotted for AS160, PP1α, PP1β, PP1γ1 and PP2A. A) Representative immunoblots and B) data for the Co-IP of PP1α with AS160. C) Representative immunoblots and D) data for the Co-IP of PP1β with AS160. E) Representative immunoblots and F) data for the Co-IP of PP1γ1 with AS160. G) Representative immunoblots and H) data for the Co-IP of PP2A with AS160. Data were analyzed using a two-tailed t test. *P<0.05, IP using anti-AS160 exceeds IP using IgG control for lysates from muscles incubated without insulin. **P<0.05, IP using anti-AS160 exceeds IP using IgG control for lysates from muscles incubated with insulin. Values are expressed as mean ± SEM; n = 3-4 per treatment.

**Figure 4:** Effects of recombinant Inhibitor-2 protein (rInh-2) on AS160 dephosphorylation in epitrochlearis muscles. The procedure described for Figure 1 was used, except that the specific PP1 inhibitor protein rInh-2 was assessed rather than pharmacologic inhibitors. A) pAS160<sup>Thr642</sup>; B) AS160<sup>Ser588</sup>; C) representative immunoblots (pAS160<sup>Thr642</sup>, pAS160<sup>Ser588</sup> and total AS160); pAkt<sup>Ser473</sup>; D) pAkt<sup>Thr308</sup>; E) pAkt<sup>Ser473</sup>; F) representative immunoblots (pAkt<sup>Thr308</sup>, pAkt<sup>Ser473</sup> and total Akt). Data were analyzed using a two-tailed t test. *P<0.05, plus rInh-2 exceeds minus rInh-2. Values are expressed as mean ± SEM; n = 4 muscle lysates per treatment.
Figure 5: Effects of silencing the PP1α on AS160 phosphorylation in L6 myocytes. Cells were transfected with siPP1α or scrambled (Scr) siRNA. At 72 h post-transfection, cells were incubated without or with insulin (100 nM) for 20 min. Protein abundance and phosphorylation were assessed by immunoblotting. A) PP1α; B) representative immunoblots (PP1α, PP1β, PP1γ1 and α-Tubulin); C) pAS160\textsuperscript{Thr642}; D) pAS160\textsuperscript{Ser588}; E) representative immunoblots (pAS160\textsuperscript{Thr642}, pAS160\textsuperscript{Ser588} and AS160); F) pAkt\textsuperscript{Thr308}; G) pAkt\textsuperscript{Ser473}; H) representative immunoblots (pAkt\textsuperscript{Thr308}, pAkt\textsuperscript{Ser473} and Akt). Data were analyzed using a two-tailed t test. #P<0.05, siPP1α is less than Scr for cells incubated without insulin; ψP<0.05, siPP1α is less than Scr for cells incubated with insulin; *P<0.05, siPP1α exceeds Scr for cells incubated with insulin. Values are expressed as mean ± SEM, n = 5-6 experiments.

Figure 6: Effects of silencing the PP1β on AS160 phosphorylation in L6 myocytes. L6 cells were transfected either with siPP1β or scrambled (Scr) siRNA and treated with insulin as described for Figure 5. Protein abundance and phosphorylation were assessed by immunoblotting. A) PP1β; B) representative immunoblots (PP1β, PP1α, PP1γ1 and α-Tubulin); C) pAS160\textsuperscript{Thr642}; D) pAS160\textsuperscript{Ser588}; E) representative immunoblots (pAS160\textsuperscript{Thr642}, pAS160\textsuperscript{Ser588} and total AS160); F) pAkt\textsuperscript{Thr308}; G) pAkt\textsuperscript{Ser473}; H) representative immunoblots (pAkt\textsuperscript{Thr308}, pAkt\textsuperscript{Ser473} and total Akt). Data were analyzed using a two-tailed t test. #P<0.05, siPP1β is less than Scr for cells incubated without insulin; ψP<0.05, siPP1β is less than Scr for cells incubated with insulin; Values are expressed as mean ± SEM, n = 4 experiments.

Figure 7: Effects of silencing PP1γ1 on AS160 phosphorylation in L6 myocytes. Cells were transfected with siPP1γ1 or scrambled (Scr) siRNA and treated with insulin as described for Figure 5. Knockdown was confirmed by measuring the protein abundance of PP1γ1. Protein abundance and phosphorylation were assessed by immunoblotting. A) PP1γ1; B) representative immunoblots (PP1γ1, PP1α, PP1β and α-Tubulin); C) pAS160\textsuperscript{Thr642}; D) pAS160\textsuperscript{Ser588}; E)
representative immunoblots (pAS160\textsuperscript{Thr642}, pAS160\textsuperscript{Ser588} and AS160); F) pAkt\textsuperscript{Thr308}, G) pAkt\textsuperscript{Ser473}, H) representative immunoblots (pAkt\textsuperscript{Thr308}, pAkt\textsuperscript{Ser473} and Akt). Data were analyzed using a two-tailed t test. \#P<0.05, siPP1γ1 is less than Scr for cells treated without insulin; ψP<0.05, siPP1γ1 is less than Scr for cells treated with insulin; Values are expressed as mean ± SEM, n = 4 experiments.

Figure 8: Comparison of AS160 dephosphorylation in epitrochlearis muscle lysates from lean Zucker versus obese Zucker rats. Isolated muscles from lean and obese rats were incubated with insulin (30 nM) for 30 min. The dephosphorylation assay was as described for Figure 1, except that lysates were incubated at 37°C for 5, 10, 20, 30 and 40 min without addition of any phosphatase inhibitors. The initial phosphorylation levels for AS160 and Akt were determined for each sample and denoted as the 0 min time-point with a value equal to 100%. The relative phosphorylation values for AS160 and Akt were plotted at each time point. A) pAS160\textsuperscript{Thr642}; B) pAS160\textsuperscript{Ser588}; C) representative immunoblots (pAS160\textsuperscript{Thr642}, pAS160\textsuperscript{Ser588}, and total AS160); D) pAkt\textsuperscript{Thr308}; E) pAkt\textsuperscript{Ser473}; F) representative immunoblots (pAkt\textsuperscript{Thr308}, pAkt\textsuperscript{Ser473} and total Akt). Data were analyzed using a two-tailed t test. Values are expressed as mean ± SEM (n = 7 animals for each group). *P<0.05 indicates lean exceeds obese at the corresponding time points.

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Figure 1

(A) AS160<sup>Thr642</sup>

Relative units

0 min  Vehicle  PP2B (I)  PP2C (I)
30 min  +  +  +  +  +
60 min  +  +  +  +  +
120 min +  +  +  +  +

PP2B Inhibitor = PP2B(I)
PP2C Inhibitor = PP2C(I)

(B) pAS160<sup>Ser588</sup>

Relative units

0 min  Vehicle  PP2B (I)  PP2C (I)
30 min  +  +  +  +  +
60 min  +  +  +  +  +
120 min +  +  +  +  +

(C) Summary table

| Time   | Vehicle | PP2B(I) | PP2C(I) |
|--------|---------|---------|---------|
| 0 min  | +       | -       | -       |
| 30 min | -       | +       | +       |
| 60 min | +       | +       | +       |
| 120 min| +       | +       | +       |

(D) pAkt<sup>Thr308</sup>

Relative units

0 min  Vehicle  PP2B (I)  PP2C (I)
30 min  +  +  +  +  +
60 min  +  +  +  +  +
120 min +  +  +  +  +

(E) pAkt<sup>Ser473</sup>

Relative units

0 min  Vehicle  PP2B (I)  PP2C (I)
30 min  +  +  +  +  +
60 min  +  +  +  +  +
120 min +  +  +  +  +

(F) Western blotting

pAS160<sup>Thr642</sup>
pAS160<sup>Ser588</sup>
AS160

pAkt<sup>Thr308</sup>
pAkt<sup>Ser473</sup>
Akt
Figure 2

Diabetes

A

Relative units

0.0

0.5

1.0

1.5

2.0

2.5

0 min

OA (nM) 0 5 1000 0 5 1000 0 5 1000

30 min

60 min

120 min

pAS160<sup>Thr642</sup>

B

Relative units

0.0

0.5

1.0

1.5

2.0

2.5

0 min

OA (nM) 0 5 1000 0 5 1000 0 5 1000

30 min

60 min

120 min

pAS160<sup>Ser588</sup>

C

OA (nM) 0 5 1000 0 5 1000 0 5 1000

0 min 30 min 60 min 120 min

pAS160<sup>Thr642</sup>

pAS160<sup>Ser588</sup>

AS160

D

Relative units

0.0

0.5

1.0

1.5

2.0

2.5

0 min

OA (nM) 0 5 1000 0 5 1000 0 5 1000

30 min

60 min

120 min

pAkt<sup>Thr308</sup>

E

Relative units

0.0

0.5

1.0

1.5

2.0

2.5

0 min

OA (nM) 0 5 1000 0 5 1000 0 5 1000

30 min

60 min

120 min

pAkt<sup>Ser473</sup>

F

OA (nM) 0 5 1000 0 5 1000 0 5 1000

0 min 30 min 60 min 120 min

pAkt<sup>Thr308</sup>

pAkt<sup>Ser473</sup>

Akt
Figure 3

A

Insulin

AS160

PP1α

B

Diabetes Post IP Supernatant

AS160 IP

IgG IP

C

Insulin

AS160

PP1β

D

E

Insulin

AS160

PP1γ1

F

G

Insulin

AS160

PP2A

H

Relative units

IP: AS160

IP: IgG

IB: AS160

IB: PP1α

IB: PP1γ1

IB: PP2A

Figure 3
Figure 4

A. pAS160^{Thr642}

B. pAS160^{Ser588}

C. 

D. pAkt^{Thr308}

E. pAkt^{Ser473}

F. 

Diabetes

Recombinant Inhibitor-2 = rInh-2

Relative units

0 min  rInh-2  -  +  -  +  -  +  -  +  -  +

15 min  30 min  45 min  60 min

0.0  0.5  1.0  1.5  2.0  2.5  3.0

0 min  rInh-2  -  +  -  +  -  +  -  +  -  +

15 min  30 min  45 min  60 min

0.0  0.5  1.0  1.5  2.0  2.5  3.0

0 min  rInh-2  -  +  -  +  -  +  -  +  -  +

15 min  30 min  45 min  60 min

0.0  0.5  1.0  1.5  2.0  2.5  3.0

0 min  rInh-2  -  +  -  +  -  +  -  +  -  +

15 min  30 min  45 min  60 min

0.0  0.5  1.0  1.5  2.0  2.5  3.0

0 min  rInh-2  -  +  -  +  -  +  -  +  -  +

15 min  30 min  45 min  60 min

0.0  0.5  1.0  1.5  2.0  2.5  3.0

0 min  rInh-2  -  +  -  +  -  +  -  +  -  +

15 min  30 min  45 min  60 min

0.0  0.5  1.0  1.5  2.0  2.5  3.0

0 min  rInh-2  -  +  -  +  -  +  -  +  -  +

15 min  30 min  45 min  60 min

0.0  0.5  1.0  1.5  2.0  2.5  3.0

0 min  rInh-2  -  +  -  +  -  +  -  +  -  +

15 min  30 min  45 min  60 min

0.0  0.5  1.0  1.5  2.0  2.5  3.0

0 min  rInh-2  -  +  -  +  -  +  -  +  -  +

15 min  30 min  45 min  60 min

0.0  0.5  1.0  1.5  2.0  2.5  3.0

0 min  rInh-2  -  +  -  +  -  +  -  +  -  +

15 min  30 min  45 min  60 min

0.0  0.5  1.0  1.5  2.0  2.5  3.0
Diabetes
