Akt Substrate of 160 kDa Dephosphorylation Rate Is Reduced in Insulin-stimulated Rat Skeletal Muscle after Acute Exercise

Edward B. Arias[1], Haiyan Wang[1] and Gregory D. Cartee[1,2,3]

[1]Muscle Biology Laboratory, School of Kinesiology, University of Michigan, Ann Arbor, MI, USA

[2]Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI, USA

[3]Institute of Gerontology, University of Michigan, Ann Arbor, MI, USA

Corresponding Author:

Gregory D. Cartee, Ph.D.

University of Michigan, School of Kinesiology

401 Washtenaw Avenue, Ann Arbor, MI 48109-2214

Phone: (734)615-3458

email: gcartee@umich.edu

Short Title: Exercise and AS160 dephosphorylation

SUMMARY

Because greater Akt substrate of 160 kDa (AS160) phosphorylation has been reported in insulin-stimulated skeletal muscles without improved Akt activation several hours post-exercise, we hypothesized that prior exercise would result in attenuated AS160 dephosphorylation in insulin-stimulated rat skeletal muscle. Epitrochlearis muscles were isolated from rats that were sedentary (SED) or exercised 3h earlier (3h post-exercise; 3hPEX). Paired muscles were incubated with [^{3}H]-2-deoxyglucose (2-DG) without insulin or with insulin. Lysates from other insulin-stimulated muscles from SED or 3hPEX rats were evaluated using AS160^{Thr642} and AS160^{Ser588} dephosphorylation assays. Prior exercise led to greater 2-DG uptake concomitant with greater AS160^{Thr642} phosphorylation and a non-significant trend (P=0.087) for greater AS160^{Ser588}. Prior exercise also reduced AS160^{Thr642} and AS160^{Ser588} dephosphorylation rates. These results support the idea that attenuated AS160 dephosphorylation may favor greater AS160 phosphorylation post-exercise.

INTRODUCTION

One exercise session can substantially elevate subsequent insulin-stimulated glucose uptake by skeletal muscle (Cartee 2015a, Wojtaszewski *et al.* 2003). Earlier research indicates this improvement is not caused by elevated insulin signaling at proximal steps from insulin receptor binding to stimulation of the serine/threonine protein kinase Akt (Cartee 2015a, Castorena *et al.* 2014, Funai *et al.* 2009, Pehmoller *et al.* 2012) suggesting the mechanism may involve events distal to Akt. Akt substrate of 160 kDa (AS160; also called TBC1D4) phosphorylation on Thr^{642} by Akt is crucial for insulin-stimulated glucose transport (Cartee 2015b, Chen *et al.* 2011). Earlier research demonstrated that exercise can induce greater AS160 phosphorylation for hours post-exercise, and greater AS160 phosphorylation is implicated in the exercise-induced improvement of insulin sensitivity (Arias *et al.* 2007, Cartee 2015b, Castorena *et al.* 2014, Funai *et al.* 2009, Pehmoller *et al.* 2012). Given the evidence that exercise does not lead to subsequently elevated Akt activity in insulin-stimulated muscle, we hypothesized that exercise producing greater insulin-stimulated glucose uptake and AS160 phosphorylation would also attenuate AS160 dephosphorylation in rat muscle.

METHODS

Memcode Reversible Protein Stain, bicinchoninic acid protein assay kits and Tissue Protein Extraction Reagent, T-PER were from ThermoFisher (Pittsburgh, PA). Luminata Forte Western HRP Substrate was from EMD Millipore (Billerica, MA). Anti-phospho AS160 Thr642 ($pAS160^{Thr642}$), anti-phospho AS160 Ser588 ($pAS160^{Ser588}$) and anti-rabbit IgG horseradish peroxidase conjugate were from Cell Signaling Technology (Danvers, MA).

Animal care procedures were approved by the University of Michigan Committee on Use and Care of Animals. Methods were performed in accordance with the guidelines from the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, USA. Male Wistar rats (~250 g) had unlimited access to rodent chow until they were fasted at 1700h

64 on the night before the experiment. Exercised rats swam in a barrel filled with water (35°C; ~45
65 cm depth; 6/barrel) for 4x30min bouts with 5min rest between bouts. Exercising rats were dried
66 and returned to their cages without food for ~3h, then anesthetized (intraperitoneal sodium
67 pentobarbital, 50mg/kg) at the same time as sedentary controls (SED). Epitrochlearis muscles
68 from SED and exercised (3h post-exercise, 3hPEX) rats were used for the analyses described
69 below.

70 Isolated muscles underwent a two-step incubation in vials containing 2ml of media
71 (gassed with 95% O_2-5% CO_2) in a shaking water-bath (35°C). Incubation step 1 (30min) was
72 in KHB/BSA (Krebs Henseleit Buffer, KHB, with 0.1% bovine serum albumin, BSA)
73 supplemented with 2mM sodium pyruvate and 6mM mannitol with or without 0.6nM insulin.
74 Incubation step 2 (20min) was in KHB/BSA with 0.1mM 2-deoxy-D-glucose, 2-DG,
75 (2.25mCi/mmol ^{3}H-2-DG) and 9.9mM mannitol (0.022mCi/mmol ^{14}C-mannitol), and the same
76 insulin concentration as step 1. Muscles were freeze-clamped and stored at -80^{o}C until
77 homogenized. A portion of muscle lysate was used to determine ^{3}H-2-DG accumulation by
78 liquid scintillation counting (Hansen *et al.* 1994). Another portion of the lysate was used to
79 determine AS160^{Thr642} and AS160^{Ser588} phosphorylation by immunoblotting as previously
80 described (Castorena *et al.* 2014, Sharma *et al.* 2016) and summarized below.

81 Laemmli buffer was added to equal amounts of lysate protein, boiled and subjected to
82 SDS-PAGE. Proteins were transferred to PVDF. Equal loading was confirmed by MemCode
83 protein stain. Membranes were blocked, washed, incubated with secondary antibody, washed
84 and incubated with enhanced chemiluminescence reagent. Protein bands quantified by
85 densitometry were expressed relative to the normalized average of all samples on the blot.

86 Isolated muscles from other rats were used for a dephosphorylation assay. Muscles
87 were incubated in vials containing KHB/BSA with 2mM sodium pyruvate, 6mM mannitol and 0.6
88 or 30nM insulin (30min with shaking and gassing, 35°C). Immediately post-incubation, muscles
89 were freeze-clamped and stored at −80°C until processed.

The AS160 dephosphorylation assay has been described (Sharma *et al.* 2016). Frozen muscles were rapidly homogenized in ice-cold buffer including protease inhibitors (1µg/mL leupeptin, 1µg/mL pepstatin, 1µg/mL aprotinin, and 1mM phenylmethyl sulfonyl fluoride) but without protein phosphatase inhibitors. An initial aliquot (20µL; 0min time-point) was rapidly removed from each sample, immediately mixed with an equal volume of 2X SDS loading buffer, and heated (95^{o}C, 3 min). The remaining samples were incubated (37^{o}C) and aliquots (20µL) were removed (5, 10, 20, 30, 40 and 50min), rapidly mixed with an equal volume of 2X SDS loading buffer and heated (95^{o}C, 3min). Samples were subjected to SDS-PAGE and immunoblotting for AS160^{Thr642} and AS160^{Ser588} phosphorylation.

For 2-DG uptake and AS160 phosphorylation of muscles incubated ±insulin, two-way ANOVA was used to identify significant main effects. Holm-Sidak post-hoc analysis was used to identify the source of significant variance. For the dephosphorylation assay, differences were evaluated by two-tailed t-test.

RESULTS

There were significant main effects of insulin ($P<0.001$) and exercise ($P<0.01$) on 2-DG uptake (Figure 1A). Post-hoc analysis identified a significant effect of insulin versus no insulin on 2-DG uptake in SED ($P<0.05$) and 3hPEX ($P<0.001$) groups, and 2-DG uptake in insulin-stimulated muscles was greater for 3hPEX versus SED ($P<0.01$). There were significant main effects of insulin ($P<0.001$) and exercise ($P<0.001$) for AS160^{Thr642} phosphorylation (Figure 1B). Post-hoc analysis detected a significant insulin effect on AS160^{Thr642} phosphorylation in SED ($P<0.001$) and 3hPEX ($P<0.001$) muscles. AS160^{Thr642} phosphorylation was significantly greater in 3hPEX versus SED muscles without insulin ($P<0.05$) or with insulin ($P<0.05$). There was a significant main effect of insulin ($P<0.005$) and a trend for a main effect of exercise ($P=0.087$) for AS160^{Ser588} phosphorylation (Figure 1C). Post-hoc analysis detected a significant insulin

115 effect on AS160^{Ser588} phosphorylation in SED (P<0.05) and 3hPEX (P<0.01) muscles. The
116 dephosphorylation assay results from muscles incubated with 0.6nM insulin demonstrated
117 AS160^{Thr642} (P<0.001 at 5 and 10min; P<0.005 at 20min; P<0.05 at 30, 40 and 50min) and
118 AS160^{Ser588} phosphorylation for 3hPEX significantly exceeded SED values (P<0.005 at 5min;
119 P<0.05 at 10, 20 and 30min). However, using muscles incubated with 30nM insulin, there were
120 no significant differences between 3hPEX versus SED for AS160^{Thr642} or AS160^{Ser588}
121 phosphorylation at any time-point (results not shown).
122
123 **DISCUSSION**
124 Because greater AS160 phosphorylation has been reported in insulin-stimulated
125 muscles without greater Akt activation several hours post-exercise (Castorena *et al.* 2014, Funai
126 *et al.* 2009, Pehmoller *et al.* 2012), we hypothesized that prior exercise would attenuate AS160
127 dephosphorylation in insulin-stimulated rat muscle. The dephosphorylation assay results for
128 muscles stimulated with a physiologic insulin dose supported the hypothesis. Exercise reduced
129 AS160^{Thr642} and AS160^{Ser588} dephosphorylation in muscles that had been stimulated with the
130 same insulin dose (0.6nM) as was used for glucose uptake assessment. Interestingly, no
131 exercise-effect occurred in muscles stimulated with 30nM insulin. Perhaps this
132 supraphysiologic insulin dose resulted in modifications in AS160 and/or phosphatases that
133 masked the normal exercise-effect that was found in muscles exposed to a physiologic insulin
134 concentration.
135 Consistent with previous studies (Cartee and Holloszy 1990, Castorena *et al.* 2014,
136 Funai *et al.* 2009), prior exercise resulted in greater insulin-stimulated glucose uptake several
137 hours after acute exercise versus SED. AS160^{Thr642} phosphorylation was also increased in
138 insulin-stimulated muscles at 3hPEX versus SED, consistent with earlier research (Cartee
139 2015a, Castorena *et al.* 2014, Funai *et al.* 2009). Previous research detected greater muscle

AS160^{Ser588} phosphorylation at 3hPEX versus SED (Castorena *et al.* 2014), and there was a trend for an exercise-effect on AS160^{Ser588} in this study.

What are possible mechanisms for the exercise-related attenuation of AS160 dephosphorylation? Protein phosphorylation depends on the balance between the opposing activities of protein kinases and protein phosphatases, but a disproportionate amount of prior research has focused on kinases rather than phosphatases. Notably, prior studies reporting exercise does not increase Akt activity in insulin-stimulated muscles employed standard Akt enzyme activity assays using muscle lysates (Castorena *et al.* 2014, Funai *et al.* 2009). It seems reasonable to suspect that the exercise-related differences in AS160's dephosphorylation in our assay may involve changes in the phosphatase and/or AS160's susceptibility to being dephosphorylated. The dephosphorylation assay used muscle lysates, so the results are probably not attributable to exercise-effects on subcellular localization of AS160 or phosphatases. Because protein phosphatase 1α (PP1α) regulates AS160^{Thr642} and AS160^{Ser588} dephosphorylation in muscle (Sharma *et al.* 2016), it will be important to determine if prior exercise alters PP1α activity.

REFERENCES

ARIAS EB, KIM J, FUNAI K, CARTEE GD: Prior exercise increases phosphorylation of Akt substrate of 160 kDa (AS160) in rat skeletal muscle. *Am J Physiol Endocrinol Metab* **292**: E1191-1200, 2007.

CARTEE GD: Mechanisms for greater insulin-stimulated glucose uptake in normal and insulin-resistant skeletal muscle after acute exercise. *Am J Physiol Endocrinol Metab* **309**: E949-959, 2015a.

CARTEE GD: Roles of TBC1D1 and TBC1D4 in insulin- and exercise-stimulated glucose transport of skeletal muscle. *Diabetologia* **58**: 19-30, 2015b.

CARTEE GD, HOLLOSZY JO: Exercise increases susceptibility of muscle glucose transport to activation by various stimuli. *Am J Physiol* **258**: E390-393, 1990.

CASTORENA CM, ARIAS EB, SHARMA N, CARTEE GD: Postexercise improvement in insulin-stimulated glucose uptake occurs concomitant with greater AS160 phosphorylation in muscle from normal and insulin-resistant rats. *Diabetes* **63**: 2297-2308, 2014.

CHEN S, WASSERMAN DH, MACKINTOSH C, SAKAMOTO K: Mice with AS160/TBC1D4-Thr649Ala knockin mutation are glucose intolerant with reduced insulin sensitivity and altered GLUT4 trafficking. *Cell Metab* **13**: 68-79, 2011.

FUNAI K, SCHWEITZER GG, SHARMA N, KANZAKI M, CARTEE GD: Increased AS160 phosphorylation, but not TBC1D1 phosphorylation, with increased postexercise insulin sensitivity in rat skeletal muscle. *Am J Physiol Endocrinol Metab* **297**: E242-251, 2009.
HANSEN PA, GULVE EA, HOLLOSZY JO: Suitability of 2-deoxyglucose for in vitro measurement of glucose transport activity in skeletal muscle. *J Appl Physiol* **76**: 979-985, 1994.
PEHMOLLER C, BRANDT N, BIRK JB, HOEG LD, SJOBERG KA, GOODYEAR LJ, KIENS B, RICHTER EA, WOJTASZEWSKI JF: Exercise alleviates lipid-induced insulin resistance in human skeletal muscle-signaling interaction at the level of TBC1 domain family member 4. *Diabetes* **61**: 2743-2752, 2012.
SHARMA P, ARIAS EB, CARTEE GD: Protein Phosphatase 1-alpha Regulates AS160 Ser588 and Thr642 Dephosphorylation in Skeletal Muscle. *Diabetes* **65**: 2606-2617, 2016.
WOJTASZEWSKI JF, JORGENSEN SB, FROSIG C, MACDONALD C, BIRK JB, RICHTER EA: Insulin signalling: effects of prior exercise. *Acta Physiol Scand* **178**: 321-328, 2003.

Acknowledgements

Yilin Nie and Carmen Yu provided valuable technical assistance. Supported by a grant from the National Institutes of Health (DK71771).

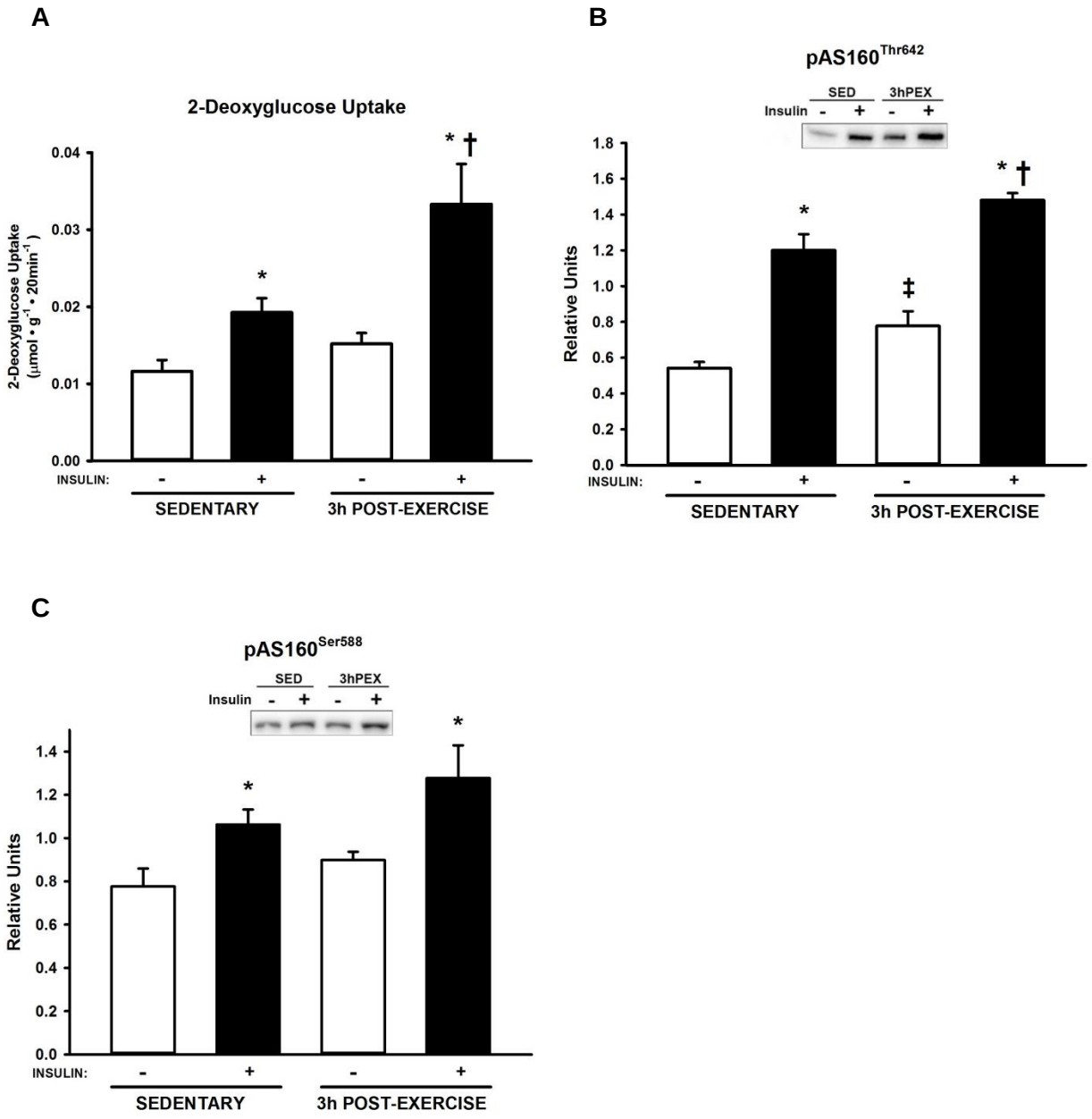

Figure 1. (A) There were significant main effects of insulin (P<0.001) and exercise (P<0.01) on 2-DG uptake. *P<0.05, Sedentary or 3hPEX muscles with insulin versus muscles without insulin; †P<0.05, 3hPEX muscles with insulin versus Sedentary muscles with insulin. (B) There were significant main effects of insulin (P<0.001) and exercise (P<0.001) on AS160^{Thr642} phosphorylation. *P<0.001, Sedentary muscles with insulin versus Sedentary muscles without insulin and 3hPEX muscles with insulin versus 3hPEX muscles without insulin; ‡P<0.05, 3hPEX

muscles without insulin versus Sedentary muscles without insulin; †P<0.05, 3hPEX muscles with insulin versus Sedentary muscles with insulin. (C) There was a significant main effect of insulin (P<0.005) and a trend for a main effect of exercise (P=0.087) on AS160^{Ser588} phosphorylation. *P<0.05, Sedentary muscles with insulin versus Sedentary muscles without insulin; *P<0.01, 3hPEX muscles with insulin versus 3hPEX muscles without insulin. Values are expressed as mean ±SEM; n=6-9 per treatment.

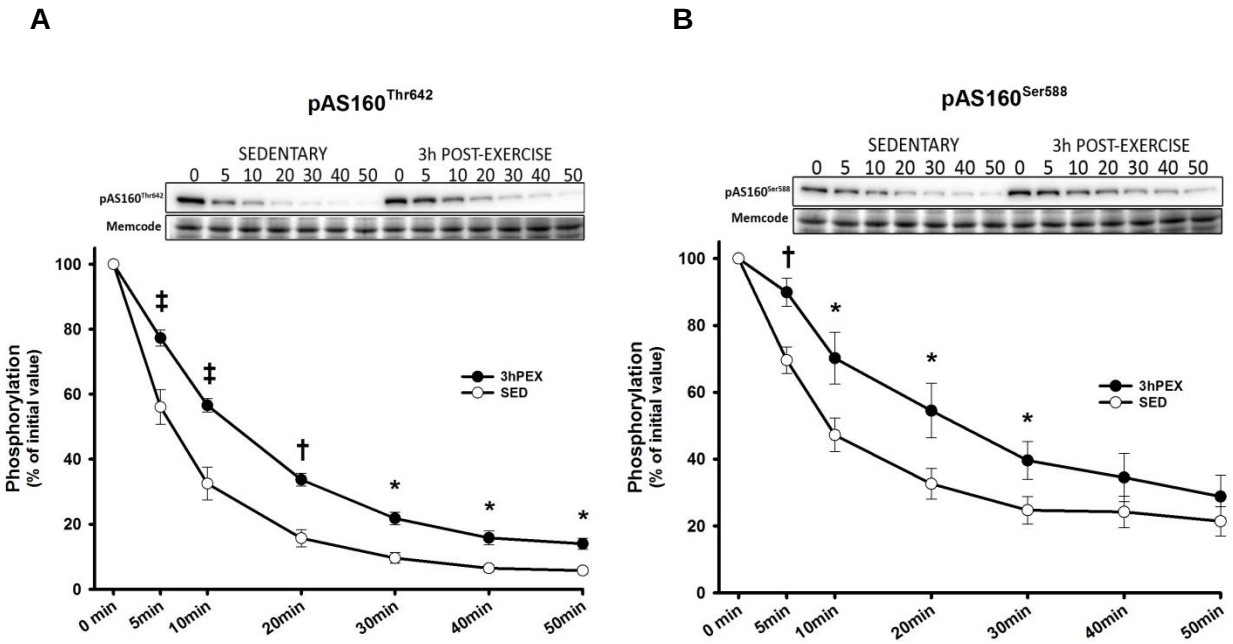

Figure 2. Lysates prepared from insulin-stimulated (0.6nM) muscles dissected from Sedentary or 3h Post-exercise (3hPEX) rats were incubated for various time-points to AS160 dephosphorylation. (A) AS160^{Thr642} phosphorylation differed significantly between Sedentary and 3hPEX groups at 5 and 10min (‡P<0.001), 20min (†P<0.005) and 30, 40 and 50min (*P<0.05). (B) AS160^{Ser588} differed significantly between Sedentary and 3hPEX groups at 5min (†P<0.005) and 10, 20 and 30min (*P<0.05). Values are expressed as mean ±SEM; n=11-12 per treatment.