

## Akt Substrate of 160 kDa Dephosphorylation Rate Is Reduced in Insulin-Stimulated Rat Skeletal Muscle After Acute Exercise

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### 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 3 h earlier (3 h post-exercise; 3hPEX). Paired muscles were incubated with [<sup>3</sup>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<sup>Thr642</sup> and AS160<sup>Ser588</sup> dephosphorylation assays. Prior exercise led to greater 2-DG uptake concomitant with greater AS160<sup>Thr642</sup> phosphorylation and a non-significant trend (P=0.087) for greater AS160<sup>Ser588</sup>. Prior exercise also reduced AS160<sup>Thr642</sup> and AS160<sup>Ser588</sup> dephosphorylation rates. These results support the idea that attenuated AS160 dephosphorylation may favor greater AS160 phosphorylation post-exercise.

### Key words

Insulin sensitivity • Glucose transport • TBC1D4

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### 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<sup>642</sup> 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.

Memcode Reversible Protein Stain, bicinchoninic acid protein assay kits and Tissue Protein Extraction Reagent, T-PER were from Thermo Fisher (Pittsburgh, PA, USA). Luminata Forte Western HRP

Substrate was from EMD Millipore (Billerica, MA, USA). Anti-phospho AS160 Thr642 (pAS160<sup>Thr642</sup>), anti-phospho AS160 Ser588 (pAS160<sup>Ser588</sup>) and anti-rabbit IgG horseradish peroxidase conjugate were from Cell Signaling Technology (Danvers, MA, USA).

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 17 h on the night before the experiment. Exercised rats swam in a barrel filled with water (35 °C; ~45 cm depth; 6/barrel) for 4x30 min bouts with 5 min rest between bouts. Exercising rats were dried and returned to their cages without food for ~3 h, then anesthetized (intraperitoneal sodium pentobarbital, 50 mg/kg) at the same time as sedentary controls (SED). Epitrochlearis muscles from SED and exercised (3 h post-exercise, 3hPEX) rats were used for the analyses described below.

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

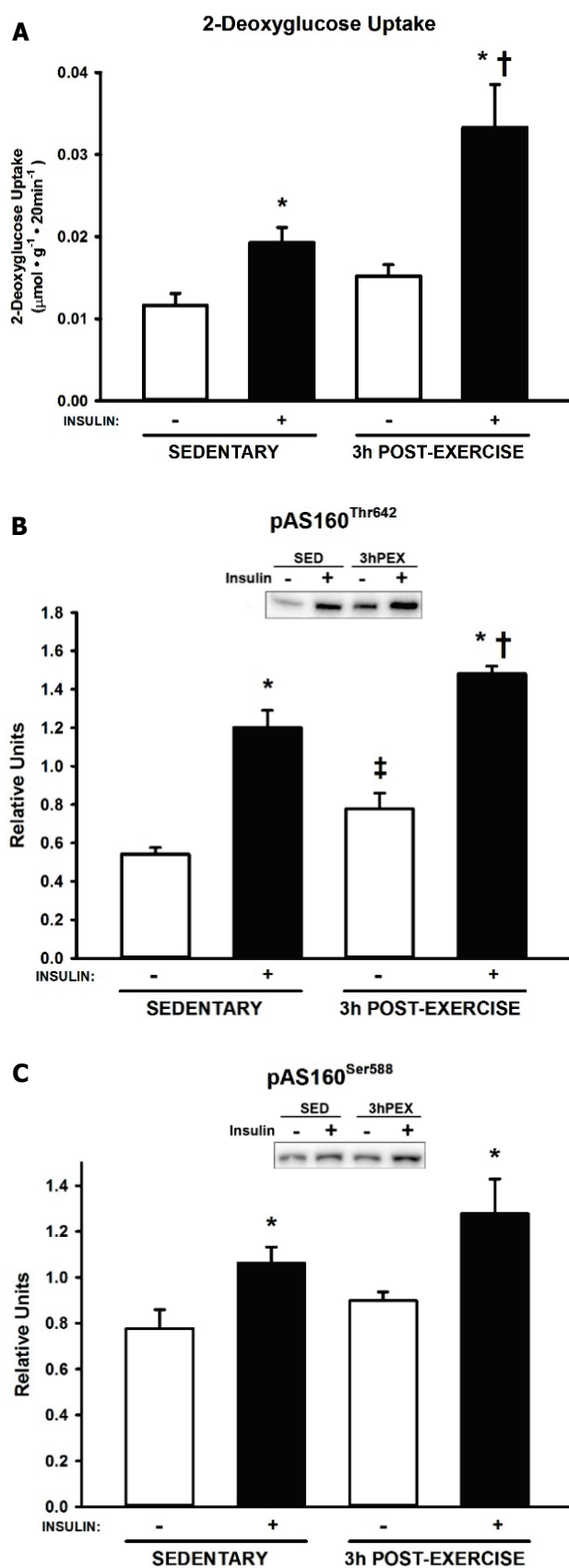
Laemmli buffer was added to equal amounts of lysate protein, boiled and subjected to SDS-PAGE. Proteins were transferred to PVDF. Equal loading was confirmed by MemCode protein stain. Membranes were blocked, washed, incubated with secondary antibody, washed and incubated with enhanced chemiluminescence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

Isolated muscles from other rats were used for a dephosphorylation assay. Muscles were incubated in vials containing KHB/BSA with 2 mM sodium pyruvate, 6 mM mannitol and 0.6 or 30 nM insulin (30 min with shaking and gassing, 35 °C). Immediately post-incubation, muscles 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 (1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) but without protein phosphatase inhibitors. An initial aliquot (20 µl; 0 min time-point) was rapidly removed from each sample, immediately mixed with an equal volume of 2X SDS loading buffer, and heated (95 °C, 3 min). The remaining samples were incubated (37 °C) and aliquots (20 µl) were removed (5, 10, 20, 30, 40 and 50 min), rapidly mixed with an equal volume of 2X SDS loading buffer and heated (95 °C, 3 min). Samples were subjected to SDS-PAGE and immunoblotting for AS160<sup>Thr642</sup> and AS160<sup>Ser588</sup> 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.

There were significant main effects of insulin (P<0.001) and exercise (P<0.01) on 2-DG uptake (Fig. 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<sup>Thr642</sup> phosphorylation (Fig. 1B). *Post-hoc* analysis detected a significant insulin effect on AS160<sup>Thr642</sup> phosphorylation in SED (P<0.001) and 3hPEX (P<0.001) muscles. AS160<sup>Thr642</sup> 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<sup>Ser588</sup> phosphorylation (Fig. 1C). *Post-hoc* analysis detected a significant insulin effect on AS160<sup>Ser588</sup> phosphorylation in SED (P<0.05) and 3hPEX (P<0.01) muscles. The dephosphorylation assay results from muscles incubated



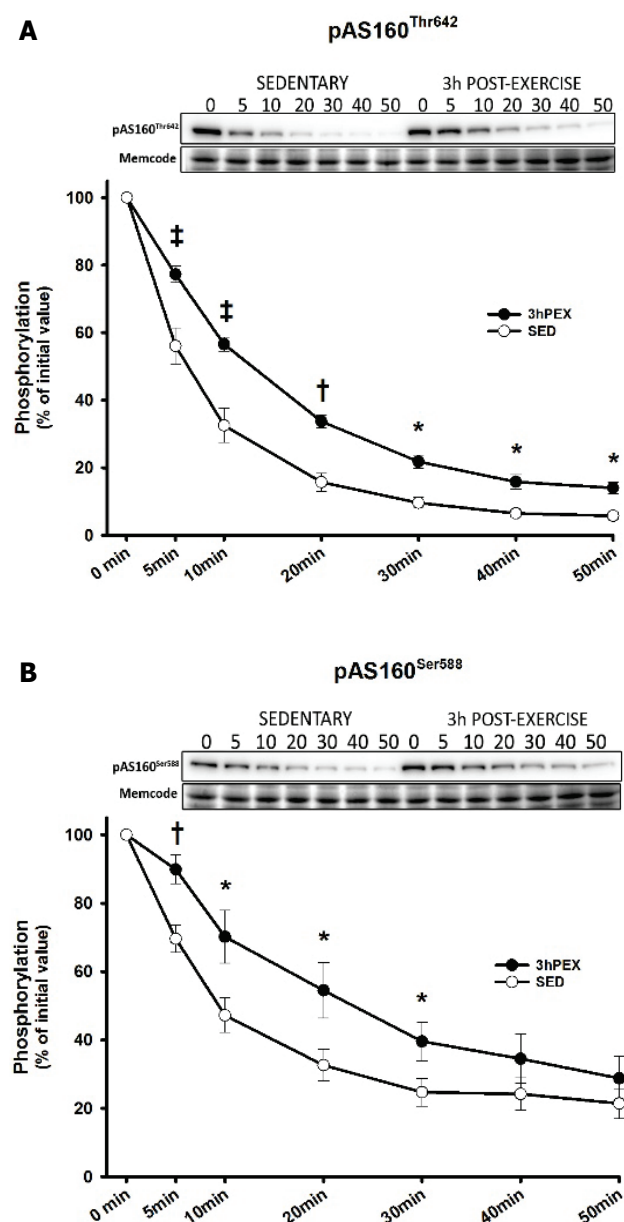
**Fig. 1.** (A) There were significant main effects of insulin ( $P < 0.001$ ) and exercise ( $P < 0.01$ ) on 2-DG uptake. \*  $P < 0.05$ , Sedentary or 3hPEX muscles with insulin versus muscles without insulin; †  $P < 0.05$ , 3hPEX muscles with insulin versus Sedentary muscles without insulin. (B) There were significant main effects of insulin ( $P < 0.001$ ) and exercise ( $P < 0.001$ ) on AS160<sup>Thr642</sup> 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. (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<sup>Ser588</sup> 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  $\pm$  SEM;  $n = 6-9$  per treatment.

3hPEX significantly exceeded SED values ( $P < 0.005$  at 5 min;  $P < 0.05$  at 10, 20 and 30 min; Fig. 2B). However, using muscles incubated with 30 nM insulin, there were no significant differences between 3hPEX versus SED for AS160<sup>Thr642</sup> or AS160<sup>Ser588</sup> phosphorylation at any time-point (results not shown).

Because greater AS160 phosphorylation has been reported in insulin-stimulated muscles without greater Akt activation several hours post-exercise (Castorena *et al.* 2014, Funai *et al.* 2009, Pehmoller *et al.* 2012), we hypothesized that prior exercise would attenuate AS160 dephosphorylation in insulin-stimulated rat muscle. The dephosphorylation assay results for muscles stimulated with a physiologic insulin dose supported the hypothesis. Exercise reduced AS160<sup>Thr642</sup> and AS160<sup>Ser588</sup> dephosphorylation in muscles that had been stimulated with the same insulin dose (0.6 nM) as was used for glucose uptake assessment. Interestingly, no exercise-effect occurred in muscles stimulated with 30 nM insulin. Perhaps this supraphysiologic insulin dose resulted in modifications in AS160 and/or phosphatases that masked the normal exercise-effect that was found in muscles exposed to a physiologic insulin concentration.

Consistent with previous studies (Cartee and Holloszy 1990, Castorena *et al.* 2014, Funai *et al.* 2009), prior exercise resulted in greater insulin-stimulated glucose uptake several hours after acute exercise versus SED. AS160<sup>Thr642</sup> phosphorylation was also increased in insulin-stimulated muscles at 3hPEX versus SED, consistent with earlier research (Cartee 2015a, Castorena *et al.* 2014, Funai *et al.* 2009). Previous research detected greater muscle AS160<sup>Ser588</sup> phosphorylation at 3hPEX versus SED (Castorena *et al.* 2014), and there was a trend for an exercise-effect on AS160<sup>Ser588</sup> in this study.

with 0.6 nM insulin demonstrated AS160<sup>Thr642</sup> ( $P < 0.001$  at 5 and 10 min;  $P < 0.005$  at 20 min;  $P < 0.05$  at 30, 40 and 50 min; Fig. 2A) and AS160<sup>Ser588</sup> phosphorylation for



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

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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 1 $\alpha$  (PP1 $\alpha$ ) regulates AS160<sup>Thr642</sup> and AS160<sup>Ser588</sup> dephosphorylation in muscle (Sharma *et al.* 2016), it will be important to determine if prior exercise alters PP1 $\alpha$  activity.

## Conflict of Interest

There is no conflict of interest.

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