MECHANISMS FOR THE SUSTAINED EFFECTS OF PRIOR EXERCISE ON AS160 PHOSPHORYLATION AND INSULIN-STIMULATED GLUCOSE TRANSPORT IN RODENT SKELETAL MUSCLE

by

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Dedicated to the memory of my grandmother,

Mary G. Tourajigian Maksudian, 1928-2010

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As acquiring a doctorate is a result of many personal influences going back many years, I'll start from the past and work my way to the present. I want to first acknowledge my favorite science teacher from Oakville Senior High School, James Roble, who solidified my love for actual science previously only known from my childhood love of science fiction cinema and books. Mr. Roble was instrumental in my acceptance at my first choice undergraduate university, Washington University in St. Louis.

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Meanwhile, in addition to working in Dr. Unanue's lab, I was also taking post-baccalaureate classes from four different universities trying to find an area of science to be passionate about. In 2005-2006, I took a class at St. Louis University in the Department of Nutrition taught by adjunct professor Dr. Polly Hansen, a former post-doctorate researcher in the lab of the famous exercise physiologist, Dr. John Holloszy at Washington University. Polly's class on exercise metabolism and nutrition was the scientific topic I was looking for, and she invited me to attend talks given by her former mentor and member of his department. With her help, I applied to the only Master's exercise physiology program in the St. Louis metro area, Southern Illinois University of Edwardsville (SIUE).

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ABSTRACT

One bout of exercise can lead to a long-lasting increase in insulin-stimulated glucose transport and a sustained increase in the phosphorylation of Akt Substrate of 160 kDa (AS160), a signaling protein proposed to be important for the post-exercise improvement in insulin sensitivity. The aims of the studies in this dissertation were to evaluate the roles of: 1) the Kallikrein-Kininogen System (KKS) in the increase in post-exercise insulin-stimulated glucose transport in rodent skeletal muscle; and 2) specific protein kinases and phosphatases for the sustained AS160 phosphorylation after acute exercise. KKS, previously proposed to be necessary for increased insulin-stimulated glucose transport after *in vitro* contractions, was studied with two genetic rodent models with deficits in KKS proteins: 1) mice null for the B2 receptor of bradykinin (B2R), and 2) rats deficient for plasma kininogen. The results indicated that neither the B2R in mice nor normal plasma kininogen levels in rats was essential for the post-exercise increase in insulin-stimulated glucose transport, suggesting that an intact KKS is not essential for enhanced muscle insulin sensitivity after exercise. The second part of the dissertation evaluated proteins regulating sustained AS160 phosphorylation after exercise. Results from these experiments demonstrated sustained phosphorylation of the Thr642 and Ser588 phospho-sites of AS160 in rat skeletal muscle 3-4 hours post-exercise. Each of the four known AS160 kinases (AMPK, Akt, RSK, and SGK) were studied, but only AMPK had greater phosphorylation immediately after exercise, and none had greater sustained phosphorylation in 3-4 hours post-exercise. Each of the four most abundant skeletal muscle serine/threonine phosphatases (PP1, PP2A, PP2B, and PP2C) was found to dephosphorylate AS160 in a cell-free assay, but only PP1 and PP2C were associated with AS160 based on co-immunoprecipitation analysis. PP1 and PP2C association with

AS160 were unaltered post-exercise. The results suggest: 1) AMPK is likely to participate in the immediate post-exercise increase in AS160 phosphorylation; 2) sustained AS160^{Thr642} and AS160^{Ser588} phosphorylation 3-4 hours post-exercise is not attributable to greater phosphorylation of the known AS160 kinases; and 3) PP1 and PP2C may modulate AS160 phosphorylation in skeletal muscle because they are associated with AS160 and able to dephosphorylate AS160^{Thr642} and AS160^{Ser588}.

CHAPTER I

INTRODUCTION

It has been known for nearly 30 years that a single exercise bout can lead to a subsequent increase in insulin sensitivity for skeletal muscle glucose transport (11). However, the molecular mechanisms by which exercise leads to increases in post-exercise insulin-stimulated glucose transport in skeletal muscle are still not fully understood. Akt Substrate of 160kDa (AS160), a key signaling protein for regulating glucose transport (2, 7, 12, 13), has been shown to respond to acute exercise with sustained phosphorylation in skeletal muscle, and this persistent increase in AS160 phosphorylation tracks with the long-lasting increase in insulin-stimulated glucose transport (1).

The primary goals of this research were 1) to evaluate the idea that elements of the Kallikrein-Kininogen System (KKS) may participate in the events that trigger the post-exercise increase in insulin-stimulated glucose transport, and 2) to characterize the post-exercise activation states of several kinases and the regulation of serine/threonine phosphatases that can contribute to increased AS160 phosphorylation on specific phospho-sites after exercise which is concomitant with the persistent post-exercise increase in insulin-stimulated glucose transport.

The first two studies used genetic models to probe the potential relationship of the KKS and post-exercise insulin sensitivity. Exercise has been shown to increase blood levels of bradykinin, a nonapeptide which is produced from the enzymatic lysis of kininogen, a plasma protein that is secreted by the liver. Several lines of evidence suggest that the KKS can influence glucoregulation and insulin action, at least under some conditions. For example, inhibition of kininases which degrade bradykinin can improve insulin sensitivity (4, 9, 15, 16), mice that are null for the B2 receptor of bradykinin (B2R) are insulin resistant (6), and rats with a congenital deficiency of plasma kininogen are characterized by moderate insulin resistance (3). Therefore, it seemed possible that some effects of exercise on muscle glucose uptake may involve elements of the KKS, including the B2R and circulating kininogen.

Study 1: *The B2 receptor of bradykinin is not essential for the post-exercise increase in glucose uptake by insulin-stimulated mouse skeletal muscle*

In order to understand the role of the B2 receptor of bradykinin, Study 1 used genetically modified mice null for the B2R to test the idea that this receptor may be important for the post-exercise increase in insulin action.

Study 2: *Post-exercise skeletal muscle glucose transport is normal in kininogen-deficient rats*

To more directly assess the role of kininogen in post-exercise increases in insulin sensitivity in skeletal muscle, Study 2 used rats deficient in plasma kininogen to determine if normal circulating kininogen levels are required for the sustained increase in

insulin-stimulated glucose transport in skeletal muscle after exercise. Furthermore, this study evaluated the insulin-independent effect of exercise on skeletal muscle glucose transport as it has been suggested that activation of the KKS may play a role in the exercise-induced increase in insulin-independent glucose transport (8, 14)

Study 3: *Mechanisms for the prolonged post-exercise increase in AS160 phosphorylation: Roles of protein kinases and phosphatases*

Sustained AS160 phosphorylation in rat skeletal muscle after exercise was previously found on the Thr642 site, a site known to be important for insulin-stimulated GLUT4 translocation. However, the other important site, Ser588, had not been investigated in rat epitrochlearis muscle under similar conditions. Accordingly, Study 3 evaluated the Ser588 phospho-site of AS160 to determine if a single exercise session results in sustained phosphorylation of this site in skeletal muscle. Additionally, Study 3 investigated the mechanisms that account for this sustained increase in AS160 phosphorylation post-exercise. The four known kinases of AS160 (Akt, AMPK, RSK, and SGK) as well as p70S6K, a kinase whose consensus motif implicates AS160 as a possible substrate (5, 10), were evaluated to determine if they have greater phosphorylation on phospho-sites essential to their activity three hours post-exercise versus sedentary controls concomitant with elevated AS160 phosphorylation. Finally, as there are no known phosphatases of AS160, Study 3 evaluated the four most abundant skeletal muscle serine/threonine phosphatases (PP1, PP2A, PP2B, and PP2C) on their ability to dephosphorylate AS160, their association with AS160, and if their association with AS160 is altered by prior exercise.

REFERENCES

1. **Arias EB, Kim J, Funai K, and Cartee GD.** Prior exercise increases phosphorylation of Akt substrate of 160 kDa (AS160) in rat skeletal muscle. *American journal of physiology* 292: E1191-1200, 2007.
2. **Cartee GD and Wojtaszewski JF.** Role of Akt substrate of 160 kDa in insulin-stimulated and contraction-stimulated glucose transport. *Appl Physiol Nutr Metab* 32: 557-566, 2007.
3. **Damas J, Bourdon V, and Lefebvre PJ.** Insulin sensitivity, clearance and release in kininogen-deficient rats. *Exp Physiol* 84: 549-557, 1999.
4. **Dragovic T, Minshall R, Jackman HL, Wang LX, and Erdos EG.** Kininase II-type enzymes. Their putative role in muscle energy metabolism. *Diabetes* 45 Suppl 1: S34-37, 1996.
5. **Dubois F, Vandermoere F, Gernez A, Murphy J, Toth R, Chen S, Geraghty KM, Morrice NA, and Mackintosh C.** Differential 14-3-3-affinity capture reveals new downstream targets of PI 3-kinase signaling. *Mol Cell Proteomics*, 2009.
6. **Duka I, Shenouda S, Johns C, Kintsurashvili E, Gavras I, and Gavras H.** Role of the B(2) receptor of bradykinin in insulin sensitivity. *Hypertension* 38: 1355-1360, 2001.
7. **Kane S, Sano H, Liu SC, Asara JM, Lane WS, Garner CC, and Lienhard GE.** A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain. *The Journal of biological chemistry* 277: 22115-22118, 2002.
8. **Kishi K, Muromoto N, Nakaya Y, Miyata I, Hagi A, Hayashi H, and Ebina Y.** Bradykinin directly triggers GLUT4 translocation via an insulin-independent pathway. *Diabetes* 47: 550-558, 1998.
9. **Margolius HS.** Kallikreins and kinins. Molecular characteristics and cellular and tissue responses. *Diabetes* 45 Suppl 1: S14-19, 1996.
10. **Pearce LR, Komander D, and Alessi DR.** The nuts and bolts of AGC protein kinases. *Nature reviews* 11: 9-22, 2010.
11. **Richter EA, Garetto LP, Goodman MN, and Ruderman NB.** Muscle glucose metabolism following exercise in the rat: increased sensitivity to insulin. *J Clin Invest* 69: 785-793, 1982.
12. **Sakamoto K and Holman GD.** Emerging role for AS160/TBC1D4 and TBC1D1 in the regulation of GLUT4 traffic. *American journal of physiology* 295: E29-37, 2008.
13. **Sano H, Kane S, Sano E, Miinea CP, Asara JM, Lane WS, Garner CW, and Lienhard GE.** Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *The Journal of biological chemistry* 278: 14599-14602, 2003.
14. **Taguchi T, Kishikawa H, Motoshima H, Sakai K, Nishiyama T, Yoshizato K, Shirakami A, Toyonaga T, Shirontani T, Araki E, and Shichiri M.** Involvement of bradykinin in acute exercise-induced increase of glucose uptake and GLUT-4 translocation in skeletal muscle: studies in normal and diabetic humans and rats. *Metabolism* 49: 920-930, 2000.
15. **Tomiyama H, Kushiro T, Abeta H, Ishii T, Takahashi A, Furukawa L, Asagami T, Hino T, Saito F, Otsuka Y, and et al.** Kinins contribute to the improvement

of insulin sensitivity during treatment with angiotensin converting enzyme inhibitor. *Hypertension* 23: 450-455, 1994.

16. **Uehara M, Kishikawa H, Isami S, Kisanuki K, Ohkubo Y, Miyamura N, Miyata T, Yano T, and Shichiri M.** Effect on insulin sensitivity of angiotensin converting enzyme inhibitors with or without a sulphydryl group: bradykinin may improve insulin resistance in dogs and humans. *Diabetologia* 37: 300-307, 1994.

CHAPTER II

REVIEW OF LITERATURE

Importance of Skeletal Muscle Glucose Transport

Insulin resistance (a subnormal physiologic response to normal insulin concentration) by skeletal muscle is a primary defect in the progression of type 2 diabetes mellitus (non-insulin dependent diabetes mellitus, NIDDM). In 2007, Type-2 diabetes mellitus represented 90 to 95% of all diabetes cases in the United States, totaling to more than 17.5 million Americans, increasing at a rate of ~1 million new cases each year, and costing $174 billion (1, 149). Insulin resistance and hyperinsulinemia in people who do not become diabetic are linked to a myriad of pathologies such as atherogenesis, hypertension, cardiovascular disease, cancer, and cognitive dysfunction (54, 70, 105). Skeletal muscle accounts for 85% of insulin-stimulated blood glucose disposal (46), and glucose transport is a rate-limiting step in muscle glucose metabolism (185). Additionally, exercise can positively regulate glucose homeostasis by increased glucose transport independent of insulin and enhance insulin's ability to increase glucose transport. The ability of exercise to enhance insulin sensitivity is often suggested to be a major factor supporting epidemiological evidence showing regular exercise prevents or delays type 2 diabetes onset (99, 163).

In addition to the health benefits, increased skeletal muscle glucose transport during exercise provides a means for blood glucose to contribute as an energy source as well as aid in blood glucose entering the muscle cells to be stored as glycogen after exercise has ceased. Because repeated bouts of exercise and/or strenuous exercise can deplete muscle glycogen, rapid muscle glycogen replenishment from increased skeletal muscle glucose uptake is especially important for athletes who wish to maintain performance (36, 92).

Effect of Insulin on Glucose Transport

Nearly 60 years ago, Levine and colleagues first showed that insulin's action, which leads to cellular glucose uptake, occurs at the cell membrane rather than on intracellular enzymes (112). Subsequently, insulin has been shown to increase glucose transport by increasing glucose transporter 4 (GLUT4) protein translocation to the cell surface membranes (11, 67, 110, 169, 180). In muscle fibers, these cell-surface membranes are the sarcolemma and T-tubules. As shown in Figure 2.1, insulin signaling begins when insulin binds to the insulin receptor (IR) and causes receptor autophosphorylation on multiple tyrosine residues (21, 157, 173, 181). The increased tyrosine kinase receptor activity of IR leads to docking of insulin receptor substrate (IRS) proteins (IRS1 and IRS2 in skeletal muscle), which are also phosphorylated on specific tyrosine residues (123, 141, 155, 172). Phosphatidylinositol 3-kinase (PI3K) binds to tyrosine phosphorylated IRS proteins, inducing a conformational change in PI3K and increasing the kinase activity of PI3K (22). PI3K phosphorylates phosphatidylinositol-(4,5)-bisphosphate (PIP2) in the phospholipid bilayer to produce phosphatidylinositol-

(3,4,5)-trisphosphate (PIP3) (164, 165). PIP3 recruits proteins, including (phosphoinositide-dependent kinases-1) PDK1 and Akt, through binding of their pleckstrin homology (PH) domains to PIP3 (4, 111). Akt is phosphorylated and activated by PDK1 at Thr308 within the T loop of the catalytic domain (2, 3, 152) and by mammalian target of rapaymycin complex-2 (mTORC-2) at Ser 473 in a noncatalytic hydrophobic motif (144, 145). Activated Akt phosphorylates many substrates, including Akt substrate of 160 kDa (AS160) also known as tre-2/USP6, BUB2, cdc16 domain family, member 4 (TBC1D4). AS160 phosphorylation has been implicated in insulin-stimulated GLUT4 translocation and glucose transport (94, 143).

Kallikrein-Kininogen System: Exercise and Glucose Metabolism

A number of studies have demonstrated that the Kallikrein-Kininogen System (KKS) can benefit glucoregulation and insulin sensitivity. Bradykinin, a 9-amino peptide product of the KKS, has been shown to be able to induce an increase in GLUT4 translocation and glucose uptake of primary adipocytes, L6 myotubes, and cardiac muscle cells (89, 98, 133). Angiotensin-converting enzyme (ACE) inhibitors that antagonize the enzymes that degrade bradykinin and reduce antiogensin II concentration are able to improve insulin sensitivity (76). The benefits of ACE inhibitors for glucoregulation may be partly the result of hemodynamic mechanisms, but there is also evidence for direct effects of bradykinin on isolated cell or muscle glucose uptake (8, 79, 89, 103, 118). Bradykinin binding to its receptor, bradykinin 2 receptor (B2R), which is expressed on skeletal muscle fibers (131), has been implicated in increased glucose uptake and improved insulin sensitivity (28, 48, 73, 77, 78, 118). Mice that are null for

the bradykinin 2 receptor (B2R) are characterized by insulin resistance during a euglycemic-hyperinsulinemic clamp (51). Furthermore, rats that are deficient in plasma kininogen, a protein that is a precursor for bradykinin, are also insulin resistant during a euglycemic-hyperinsulinemic clamp (41).

It has also been documented that exercise or muscle contraction can activate the KKS (Figure 2.2). Kallikrein, a key enzyme for the KKS, circulates in an inactive state (9, 10, 146). Bradykinin is a proteolytic product of activated kallikrein action on its substrate which is a plasma protein known as kininogen (10). Plasma kallikrein activity is increased following exercise in rats (166), and exercise can elevate circulating bradykinin concentration (12, 98, 109, 151, 154, 170).

Although exercise can activate bradykinin production, and bradykinin can positively affect muscle glucose uptake, a role of bradykinin on the post-exercise increase in insulin-stimulated glucose transport has not been established. Dumke et al. (52) studied the role of various proteins in the KKS in an attempt to elucidate their role on insulin-stimulated glucose transport after muscle contractions. The KKS has several characteristics that suggest it may include the serum factor(s) believed to be required for the increase in insulin-stimulated glucose transport after in vitro contraction of isolated skeletal muscle in serum (25, 62). Bradykinin is derived from a circulating protein (kininogen) with a molecular mass greater than 10 kDa (10, 114), consistent with the results of Gao et al. (62) who provided evidence that a serum protein larger than 10 kDa was essential for the post-contraction increase in insulin-stimulated glucose transport. Dumke et al. (52) reported that contraction of isolated rat epitrochlearis muscles in the presence of either kallikrein inhibitors in rat serum or kallikrein deficient human plasma

led to modest attenuation of the post-contraction increase in insulin-stimulated glucose transport compared to muscles performing contraction in untreated rat serum or normal human plasma. These results suggested that kallikrein may be essential for the full effect of prior contraction on insulin-stimulated glucose transport. However, they also found that muscles contracted in serum-free buffer supplemented with bradykinin did not have enhanced insulin-stimulated glucose transport 3 hours post-contraction. Additionally, supplementing rat serum with a bradykinin receptor antagonist did not reduce the insulin-stimulated glucose transport three hours after contraction. Interpretation of the results with bradykinin supplementation or bradykinin antagonists must be interpreted with caution because bradykinin is very labile, so the bradykinin concentration in the incubation media was uncertain.

Regardless, it is important to consider recent evidence suggesting that the mechanism for increased insulin-stimulated glucose transport after in vitro contraction may not be identical to the mechanism for enhanced insulin-mediated glucose transport after in vivo exercise. Funai et al. (60) using both an exercise protocol and an electrical stimulation isolated muscle contraction protocol that alone increased insulin-stimulated glucose transport ~3 hours later showed that the combination of prior exercise followed by electrical stimulation had an additive effect on the subsequent increase in insulin-stimulated glucose transport. This result suggested that the in vivo exercise and in vitro electrical stimulation protocols used by Funai et al. (60) may amplify insulin-stimulated GT through distinct mechanisms. In this context, and given that in vivo exercise can activate the KKS and that the KKS is implicated in improved insulin sensitivity provides

a rationale for evaluating the effects of in vivo exercise on insulin-stimulated glucose transport in genetic models in which key elements of the KKS are modified.

AS160's Regulation and Role in Glucose Metabolism

The gene encoding AS160 was discovered and named TBC1D4 during the creation of a cDNA library for the human brain and comparing the gene products based on homology to known proteins and sequence motifs in various species, including mice (124). The regulation of AS160 by insulin and AS160's functional role for controlling glucose transport remained unknown until they were recognized by Gus Lienhard's group who used the Akt consensus phosphorylation motif RXRXXpS/T (X is any amino acid) to identify novel Akt substrates that were responsive to insulin. Kane et al. (94) used the phospho (Ser/Thr)-Akt substrate (PAS) antibody, which recognizes the Akt consensus motif to detect previously unknown Akt substrates in 3T3-L1 adipocytes stimulated with insulin. They identified Akt Substrate of 160 kDa (AS160) as a protein that becomes phosphorylated in response to insulin and demonstrated that AS160 was identical to TBC1D4. Using 3T3-L1 adipocytes, they also provided evidence of insulin-stimulated AS160's role for increasing cell-surface GLUT4. This protein is found not only in adipose tissue, but it is also expressed in other tissues including brain, pancreas, and skeletal muscle (95). Furthermore, AS160 in rat skeletal muscle was shown to be phosphorylated in response to either insulin or contractile activity (18). Phosphomotifs and domain regions in AS160 are conserved across mouse, rat, and human skeletal muscle and include six putative Akt phosphomotifs (Ser318, Ser341, Ser570, Ser588,

Thr642, and Thr751), two phospho-tyrosine domains (PTB), a Rab GTPase activating protein (RabGAP) domain, and a calmodulin-binding domain (CBD) (94).

AS160 contains a RabGAP domain that can regulate Rab-GTP formation (94, 117, 143). Rab proteins are involved in membrane vesicular trafficking and can exist either in an active GTP-bound form or in an inactive GDP-bound form (47, 129, 130, 182). Unphosphorylated AS160 has an active RabGAP domain that leads to GTP hydrolysis and therefore, inhibits Rab activation. Phosphorylation of AS160 inactivates the RabGAP domain leading to an inhibition of GTP hydrolysis and therefore, activates target Rabs and causes Rab-mediated GLUT4 translocation. In other words, AS160's effect on GLUT4 may be compared to a "brake," in which unphosphorylated, activated AS160 restrains GLUT4 and insulin-stimulated phosphorylation of AS160 inactivates the protein, "releasing the brake" (26, 139). Insulin-stimulated AS160 phosphorylation was shown to occur in a PI3K-dependent manner in adipocytes (143) and later in rat skeletal muscle (18) using the PI3K inhibitor wortmannin.

Later studies expressed AS160 containing point mutations on one or more of the Akt phosphomotifs (with Ser/Thr substituted by Ala to prevent phosphorylation) in 3T3-L1 cells. These studies provided evidence that some of these Akt-phosphosites were important for insulin-stimulated GLUT4 translocation (143). Overexpression of AS160 containing mutations at four Akt phosphomotifs (AS160-4P; Ser318, Ser588, Thr642, Thr751) reduced insulin-mediated GLUT4 translocation by ~80% in adipocytes. The inclusion of mutations on two additional Akt phosphomotifs (Ser341 and Ser570; AS160-6P) did not result in further reduction in GLUT4 translocation (143). The PAS antibody has high affinity for phosphorylated Thr642, lower affinity for phosphorylated Ser588,

and very little, if any, affinity for any of the other insulin-regulated Akt phosphomotifs in AS160 (64, 143). Preventing insulin-stimulated phosphorylation of AS160 on phosphosites targeted by AS160-4P partially blocked insulin-stimulated glucose uptake and this has been confirmed in other models using AS160-4P (40% reduction in L6 cells (90) and 50% reduction in skeletal muscle (102)). These results suggest that other mechanisms in addition to AS160 phosphorylation are involved in insulin-stimulated glucose uptake.

An AS160 mutation that targeted the GAP domain in which a key amino acid was changed (Arg973 substituted for a Lys; AS160-R/K) resulted in elimination of the GAP activity of AS160. Overexpression of AS160-R/K in 3T3-L1 adipocytes (143) exhibited no significant difference between basal and insulin-stimulated GLUT4 translocation compared to WT AS160. Additionally, overexpression of an AS160-4P and R/K double mutant in adipocytes, compared to AS160-R/K, exhibited similar GLUT4 translocation upon insulin stimulation (143). Goodyear's group followed up this study by overexpressing AS160-R/K in mouse skeletal muscle and found that the mutant caused increased insulin-stimulated glucose uptake compared to AS160-4P upon a glucose challenge (102). Overexpression of an AS160-4P and R/K double mutant in mouse skeletal muscle, compared to AS160-R/K, exhibited similar glucose uptake upon glucose challenge (102). These results suggest that the double mutant rescues insulin-stimulated glucose uptake of the AS160-4P, and that the activity of AS160's GAP domain acts to restrain or inhibit glucose uptake.

AS160 has been shown to be associated with 14-3-3 proteins (132). 14-3-3 proteins interact with phosphorylated serine and threonine residues, and they have a

variety of functions including altering protein conformation, protect from protein degradation, affect subcellular distribution of target proteins, inhibit interaction with other proteins, and provide a cellular scaffolding (17). In 3T3-L1 adipocytes (132) and skeletal muscle (85, 86, 161), 14-3-3 association with AS160 increases in response to insulin stimulation. Mutation studies in 3T3-L1 adipocytes using AS160-4P or AS160-Thr642 mutation to alanine (T642A), but not Ser318, Ser341, or Ser588, resulted in a lack of association of 14-3-3 to AS160, which was accompanied with decreased insulin-stimulated GLUT4 translocation. These data suggested that 14-3-3 binding is important for insulin-stimulated glucose transport and that 14-3-3 appears to interact with the Thr642 site of AS160. These findings were supported by various studies in human skeletal muscle from before and after the use of a hyperinsulinameic-euglycemic clamp showing 14-3-3 binding capacity of AS160 is increased in insulin-stimulated muscle (85, 86, 161).

In summary, the AS160 mutation studies suggest the following: 1) the active RabGAP domain of AS160 inhibits GLUT4 translocation, 2) phosphorylation of AS160 induces insulin-stimulated inactivation of the RabGAP domain, possibly secondary to 14-3-3 binding to AS160, and 3) phosphorylation-mediated inactivation of the RabGAP domain of AS160 is essential for insulin-stimulated GLUT4 translocation and glucose uptake.

Effect of Exercise on Insulin-independent Glucose Transport

In 1953, Goldstein and Levine showed in dogs and rats that, independent of insulin, contracting muscles can lead to subsequent muscle glucose uptake (65). Today,

it is well known that a single bout of exercise can increase glucose transport during and immediately after exercise, independent of insulin. The exercise-induced glucose transport that occurs in the absence of insulin, insulin-independent glucose transport, begins to reverse after the completion of in vivo exercise or in vitro muscle contraction. Most of the effects of insulin-independent glucose transport are lost by 1-3 hours post-exercise.

The mechanism of action of insulin-independent glucose transport from exercise has been suggested to result from the cumulative effects of several exercise-induced events. Results from a number of studies have supported the idea that the increase in cytosolic calcium in the contracting muscle fibers (81-84) and activation of 5'-AMP-activated protein kinase (AMPK) (66, 74, 75, 106, 174, 175) are key elements of this insulin-independent effect of exercise. Experiments using isolated muscles from mice expressing dominant negative AMPK had a ~30% decrease, versus wildtype, in contraction-stimulated glucose uptake suggesting that AMPK only partially accounted for the increased glucose uptake from contraction (122). Another study using a muscle-specific LKB1 (an AMPK kinase) knockout found that contraction-stimulated activation was completely eliminated, and glucose uptake (in situ) was reduced by ~50% compared to wildtype littermate controls (140). Wright et al. (179) provided evidence from experiments using isolated rat skeletal muscle that calcium, acting through calcium-calmodulin dependent protein kinase (CAMK), accounted for half of the contraction-stimulated increase in glucose transport, with AMPK-related mechanisms accounting for the remainder of the increase.

Post-Exercise Effect on Insulin-stimulated Glucose Transport

A single bout of exercise can enhance insulin-stimulated glucose transport (insulin-dependent glucose transport). This post-exercise increase in insulin-stimulated glucose transport can last 1-48 hours after cessation of exercise. The post-exercise increase in insulin-stimulated glucose transport 3 hours after acute exercise is attributed to increased insulin-stimulated GLUT4 cell surface localization (72) without increased total GLUT4 abundance (23).

The results of many studies have indicated that the increase in insulin-stimulated glucose transport after acute exercise occurs without elevation of key upstream insulin signaling events (Figure 2.3). Prior exercise has been reported to not affect the following insulin signaling steps: 1) IR binding (14-16, 186), 2) IR tyrosine phosphorylation (30, 72, 87, 160, 178), 3) IR tyrosine kinase activity (159, 160, 176, 178), 4) IRS tyrosine phosphorylation (72, 87, 177, 178, 183), 5) IRS-PI3K association (37, 56, 159, 177, 178), 6) Akt serine phosphorylation (5, 30, 56, 71, 159, 176), 7) Akt threonine phosphorylation (61, 71), and 8) Akt activity (61).

In contrast to upstream insulin signaling steps, AS160 phosphorylation on sites recognized by the PAS antibody (PAS-AS160) is greater in epitrochlearis muscles from exercised rats (3-4 hours post-exercise) compared to sedentary controls (5). Funai et al. (61) later showed that Thr642 phosphorylation on AS160, which previous studies have showed accounts for a majority of PAS signaling (143), is greater in epitrochlearis muscles from exercised rats (~3 hours post-exercise) compared to sedentary controls. In both cases, this post-exercise compared to sedentary increase of PAS-AS160 was evident both in muscles that were not incubated with insulin (i.e. basal incubation) and that were

incubated with insulin. When the insulin-stimulated increase above basal was estimated by subtracting basal values from insulin-stimulated values, this calculated delta insulin value was not different for exercised versus sedentary muscles. These results indicated that the sustained increase in PAS-AS160 was evident in the absence of insulin, and was not because of increased insulin-induced AS160 phosphorylation. Funai et al. (61) later extended these results by demonstrating that the increased AS160 phosphorylation (using either anti-PAS or a pT642-site specific antibody) was increased concomitant with enhanced insulin-stimulated glucose transport up to 27 hours after exercise. It has been speculated that the insulin-independent increase in AS160 after exercise may eliminate AS160's restraint on intracellular GLUT4 exocytosis and make the GLUT4 vesicles more susceptible to subsequent insulin-stimulated recruitment and fusion with cell surface membranes (24).

AS160 phosphorylation after exercise has also been studied in humans by analysis of biopsies of recruited muscles. Two studies in humans found increased PAS-AS160 several hours after acute exercise (50, 150). In another study, PAS-AS160 was not observed to be increased after an exercise protocol that did not elicit an improvement in insulin sensitivity three hours post-exercise, consistent with the idea that the sustained increase in AS160 phosphorylation is important for the post-exercise increase in insulin action (85). Finally, 4 hours after one-legged exercise, AS160 phosphorylation at Ser 318, Ser341, and Ser751 (and Ser588 tended to increase) were all increased compared to the resting control muscles from the unexercised leg. Following a euglycemic-hyperinsulinemic clamp at ~5.5 hours post-exercise, insulin-stimulated glucose uptake

was increased, and the same AS160 phosphosites showed increased phosphorylation compared to the resting control muscle.

The reversal of the post-exercise increase in insulin sensitivity appears to be altered by dietary carbohydrate consumption after exercise. The post-exercise increase in insulin sensitivity in epitrochlearis muscle can be maintained for 48 hours if the rat does not eat carbohydrate. Decreased muscle glycogen has been suggested to contribute to the increased insulin sensitivity following exercise (187). However when rats were fed a high-fat, carbohydrate-free diet for 48 hours after exercise, the exercised rats showed increased insulin-stimulated glucose transport in their epitrochlearis muscles even though the muscle glycogen levels had returned to levels similar to unexercised controls (27). Therefore, a sustained reduction in muscle glycogen concentration below sedentary values was not essential for the persistent post-exercise increase in insulin-stimulated glucose transport under these conditions. However, if rats consumed a high carbohydrate diet for 18 hours after exercise, resulting in a muscle glycogen concentration 50% greater than normal resting concentration (supercompensation of muscle glycogen), the enhanced insulin-stimulated glucose transport was completely reversed (27). Therefore, glycogen supercompensation may be involved in the mechanism related to the reversal of the post-exercise increase in muscle insulin sensitivity. There is also evidence for a relationship between post-exercise diet and sustained post-exercise AS160 phosphorylation. Upon refeeding of high-carbohydrate rat chow after exercise for 3-4 hours, the increased AS160 phosphorylation seen at either 3-4 hours or 27 hours post-exercise time-points was reversed to levels found in sedentary controls, concomitant with reversal of the post-exercise increase in insulin-stimulated glucose transport (61).

In summary, while much has been discovered about the conditions that lead to the sustained increase in insulin-stimulated glucose transport following exercise, the specific mechanisms involved in this action are unclear and extremely complex. The increased insulin sensitivity that results occurs in the absence of increases in upstream insulin-signaling. Recent studies have found that AS160 phosphorylation is sustained after exercise and implicated this persistent effect as a possible contributor to the improved insulin sensitivity after a single bout of exercise.

Protein Kinases that Regulate AS160 Phosphorylation

AS160 was originally identified as an Akt substrate, but subsequent studies have demonstrated that it is also a substrate for several other kinases, including AMPK, RSK, and SGK (Figure 2.4). Bruss et al. (18) found that incubation of isolated rat epitrochlearis muscle with the AMPK-activator AICAR caused an increase in PAS-AS160. Since AICAR did not affect Akt phosphorylation, this result suggested that AMPK leads (directly or indirectly through other kinases) to an increase in PAS-AS160. The idea that AMPK acts directly on AS160 is supported by the observation that recombinant AMPK can PAS-phosphorylate AS160 in a cell-free assay (162).

RSK has been shown to be activated by mechanical stress and by muscle contraction (104, 137). SGK's role in exercise has not yet been established. However, SGK is activated by insulin and growth factors via PI3K, 3-phosphoinositide dependent kinase PDK1 and mTOR (100, 101, 127). Both RSK and SGK have been shown to phosphorylate AS160 at Ser318, Ser588, and Thr642 in cell-free assays (64). The protein kinase p70S6K is a candidate to participate in the regulation of exercise-induced AS160

phosphorylation on the basis of the similarity of its consensus motif for substrates (p70S6K on Thr642 and Ser588 based on p70S6K's similarity to RSK and SGK consensus motifs (64, 128). In addition, exercise can activate p70S6K in skeletal muscle (7, 32). However, the ability p70S6K to phosphorylate AS160 remains to be demonstrated. Furthermore, their activation several hours after exercise has not previously been evaluated concomitant with the determination of insulin-stimulated glucose transport.

Protein Phosphatases that Regulate AS160 Phosphorylation

Although four protein kinases that can phosphorylate AS160 are known, to date, none of the phosphatases that dephosphorylate AS160 have been identified. In contrast to the hundreds of genes that encode protein kinases, there are far fewer that encode the various catalytic subunits of protein phosphatases (34). Therefore, the functional diversity of serine/threonine protein phosphatases may lie in mechanisms that affect the formation of a large number of regulatory subunits that could affect the substrate specificity and subcellular localization of the phosphatases (35).

Protein phosphatases have classically been categorized based on inhibition studies and were often studied with the use of phosphatase inhibitors that either selectively or indiscriminately target the various classes of phosphatases. Using this system, there are two major types of protein serine/threonine phosphatases: type-1 protein phosphatases (PP1) and type-2 protein phosphatases (PP2) (88, 147). Continued research led to the discovery of diverse PP2 proteins and was therefore further divided into subgroups based on unique inhibitors selectively altering their activity: PP2A, PP2B, and PP2C.

Molecular cloning of mammalian phosphatase catalytic subunits further expanded the list of phosphatases to include PP2A-like phosphatases (PP3, PP4, PP5, and PP6) that share a conserved catalytic site and common inhibitors can alter activity of these phosphatases. An additional phosphatase that was discovered, PP7, has been shown to be insensitive to the known phosphatase inhibitors of PP2A and PP2B, similar to PP2C. Based on much of the work accomplished using inhibitors, it is estimated that PP1 and PP2A account for over 90% of the serine/threonine protein phosphatases activity (171).

With the advent of genome sequencing, classification of protein phosphatases has evolved. Protein phosphatases are now broadly defined by their ability to dephosphorylate either tyrosine residues, Tyr phosphatases, or by their ability to dephosphoyrlate serine/threonine residues, Ser/Thr phosphatases. Furthermore, Ser/Thr phosphatases can be organized into three major families: phosphoprotein phosphatases (PPPs), metal-dependent protein phosphatases (PPMs), and aspartate-based phosphatases (148). PPPs include protein phosphatases 1 (PP1), PP2A, PP2B (also known as calcineurin), PP4, PP5, PP6, and PP7 (148). PPMs include phosphatases dependent on manganese or magnesium ions (Mn^{2+}/Mg^{2+}), such as PP2C and pyruvate dehydrogenase kinase (148). PPPs also have regulatory domains whereas PPMs do not. Finally, PP1 and PP2A content are nearly equivalent in most tissues, only skeletal muscle appears to have a disproportionately greater amount of PP1 compared to PP2A (171).

PP1

PP1 is a dimer protein consisting of a catalytic and regulatory subunit. There are four known isoforms of the catalytic subunit (alpha, beta, gamma, and delta) and at least 100 known regulatory subunits (120). PP1 activity is regulated by many different

endogenous inhibitory proteins such as inhibitor-1 (126), inhibitor-2 (57), CPI-17 (53), and DARPP-32 (125, 167). Relatively high concentrations of fostriecin (>100μM) and okadaic acid (>10nM) have been shown to inhibit PP1, but these concentrations also inhibit PP2A and PP2A-like phosphatases (33).

PP2A

PP2A is a heterotrimer consisting of a catalytic, scaffolding, and regulatory subunit. The role(s) of each subunit is incompletely understood. There are two isoforms for each of the catalytic (alpha and beta) and scaffolding (alpha and beta) subunits. There are four families of the regulatory subunit (B, B', B'', and B''') and each of these families have different isoforms with some of these isoforms containing splice variants (93). The activity of the catalytic subunit (both isoforms) is regulated by post-translational modifications via tyrosine phosphorylation of the 307 site and methylation of the 309 site (55, 184). Additionally, methylation of the 309 site has been shown to preferentially allow the association of particular regulatory subunits (19, 45, 121, 184).

PP2B/Calcineurin

Calcineurin is a heterodimer consisting of a catalytic and a regulatory subunit. Calcineurin is inactive alone and acquires phosphatase activity upon association with calcium-calmodulin binding (49). Calcineurin appears to recognize substrate proteins (including NFAT) on a consensus recognition motif (20, 38, 135). Cyclosporin A and FK506 are known specific inhibitors and do not target other phosphatases including PP2A-like phosphatases and PP2C (171). EGTA, a calcium chelator, also inhibits calcineurin activity by virtue of inhibiting calcium-calmodulin binding (171).

PP2C

PP2C, unlike PP1, PP2A, and PP2B, is a monomeric protein that is a manganese and/or magnesium-dependent protein phosphatase. There are 16 distinct genes in the human genome coding for 22 different isoforms of PP2C (107). PP2C is insensitive to the inhibitors of PP2A and PP2B and much of the available research seeking to inhibit PP2C involves using gene-targeting such as siRNA (108). The two most highly expressed isoforms, alpha and beta, are both expressed in skeletal muscle (115, 116). Several lines of evidence implicate PP2C as a phosphatase of AMPK (44, 113, 119, 142, 153).

Rationale for Models Used in this Research

Two rodent species (mice and rats) were used in the proposed research. A large body of research has used rats to examine the post-exercise increase in insulin-stimulated glucose uptake. Previous research using rats has characterized: 1) the post-exercise increase in insulin-stimulated GLUT4 translocation (72), 2) the time-course for the post-exercise increase in insulin-stimulated glucose transport (27, 134), 3) the insulin-dose response for the post-exercise increase in insulin-stimulated glucose transport (63, 69, 91, 134), 4) the lack of increased proximal insulin signaling post-exercise (56, 72, 160, 186), 5) an increased AS160 phosphorylation in skeletal muscle post-exercise (5), and 6) the time-course for AS160 phosphorylation in skeletal muscle post-exercise (5).

The genetic rat model that will be used in Study 2 is the Brown Norway Katholiek (BNK) rat. These rats are plasma kininogen-deficient rats because of a spontaneous point mutation in the kininogen gene that causes serum kininogen deficiency by interfering with secretion of the liver synthesized kininogen (39-42). BNK rats compared to Brown

Norway (BN) control rats have been reported to have moderate in vivo insulin resistance during a hyperinsulinemic-euglycemic clamp and fasting normoglycemia (41).

The genetic mouse model used in Study 1 was the B2 receptor of bradykinin knockout (B2RKO) mouse. The B2RKO mice was compared to normal wildtype controls. Using a hyperinsulinemic-euglycemic clamp, Duka et al. (51) reported a lower glucose infusion rate for B2RKO compared to WT mice, indicating that the B2RKO mice are more insulin resistant than WT mice.

The number of publications discussing research using the mouse to study the post-exercise increase in insulin-stimulated glucose uptake is much smaller than for rats. However, previous studies have evaluated the: 1) post-exercise increase in insulin-stimulated glucose uptake (14, 16, 71), 2) insulin-dose response for glucose uptake (14, 16, 71), and 3) Akt phosphorylation in skeletal muscle post-exercise (71).

Insulin-stimulated glucose transport in rat skeletal muscle can be increased after in vivo exercise (27, 61, 72, 134, 169), in situ contractions (62, 97) and in vitro contractions performed in serum (52, 56, 58-60, 62). Each of these models can provide useful information, but this proposal will exclusively use in vivo exercise which is relevant for translation to understanding how exercise benefits human health.

Rationale for Exercise Protocols

Swim exercise will be used in the rat experiments. Swim exercise by rats has been well characterized as a model for studying the effects of the post-exercise increase in insulin-stimulated glucose transport. Swimming exercise by rats has been shown to recruit the epitrochlearis muscle as evidenced by glycogen depletion, AMPK phosphorylation, insulin-independent glucose transport immediately after exercise, and

AS160 phosphorylation (immediately, 3 hours, and 27 hours after exercise) (5, 61, 158, 168). An advantage of swim exercise compared to treadmill running exercise in rats is that swimming does not require prior familiarization, whereas, treadmill exercise typically requires several familiarization sessions on at least two days prior to the terminal experiment.

Treadmill exercise will be used in mouse experiments. The protocol was developed as described in Hamada et al. and consists of 60 minutes of running (15–25 m/min at 0% slope). This treadmill exercise has been shown to recruit the mouse soleus and EDL on the basis of glycogen depletion, increased AMPK phosphorylation, and increased insulin-independent glucose transport (71). Bonen and colleagues (14, 16) showed increased glucose uptake in isolated mouse soleus muscle after acute treadmill running compared with sedentary controls. Swim exercise will not be used for mice because a recent study reported that several different swim exercise protocols did not cause a subsequent increase insulin-stimulated glucose uptake in skeletal muscles (epitrochlearis, soleus, or EDL) from mice (96). The explanation for these results is not obvious, but treadmill exercise compared to swim exercise appears to be a better model for studying post-exercise insulin sensitivity in mice.

Rationale for Rodent Muscles to Be Used

For experiments using rats, the epitrochlearis muscle will be used. The epitrochlearis muscle originates from the tendon of insertion of m. latissimus dorsi and inserting into the medial epicondyle of the humerus and functions to assist in the extension of the antebrachium (29). It is innervated by the ulnar nerve and is supplied with blood via the muscular branch of a. profunda brachii (68). The rat epitrochlearis is

very thin (only 21-24 fibers in thickness) (168), which allows for a small diffusion distance to maintain adequate and rapidly attained tissue levels of oxygen, nutrients, and 3-O-methylglucose for muscle incubation and glucose transport measurement. Long-term incubation studies (30 hours) of epitrochlearis reveal no metabolic effects on ATP, PCr, glycogen, or lactate levels, indicative of adequate oxygenation and energy status of the muscles during incubation (168). The fiber type proportions of the rat epitrochlearis (75% Type IIb, 17% Type IIa, and 8% Type I) are very similar to the average fiber type composition of the 32 muscles of the rat hindlimb (76% Type IIb, 19% Type IIa, and 5% Type I) (6, 168). The epitrochlearis is also highly recruited during swim exercise based on glycogen depletion, AMPK phosphorylation and activation, and increased glucose transport after exercise compared to sedentary controls (5, 168). Studies in rats have revealed that insulin stimulation and/or acute exercise does not result in a change in GLUT4 abundance, but rather in the cell-surface GLUT4 content. Several hours after swim exercise by rats, there is no change in key proximal insulin signaling steps, but there is increased AS160 phosphorylation.

For experiments using mice, the soleus and extensor digitorum longus (EDL) muscles will be used. The soleus and EDL are often used in incubation and glucose uptake studies (13, 16, 80, 87, 136, 138, 156, 178). In mice, fiber type composition for soleus is reported to be 0% Type IIb, 39% Type IIa, and 61% Type I (43) and the EDL is reported to be 39% Type IIb, 60% Type IIa, and 1% Type I (31). The soleus and EDL are both recruited during treadmill exercise based on glycogen depletion, AMPK phosphorylation and activation, and increased insulin-independent glucose uptake after exercise compared to sedentary controls (71). Treadmill exercise led to a significant

increase in glucose uptake of insulin-stimulated soleus muscle (71). Bonen and coworkers have also found that prior treadmill exercise led to increased glucose uptake by isolated EDL and soleus muscles from mice (14, 16).

Gaps to Be Filled by this Research

In the early 1980s Erik Richter and colleagues showed that exercise is followed by an increase in muscle insulin sensitivity (134). While their observation has been confirmed in many previous studies in various species (including rats, mice, and humans) during the subsequent 30 years, the precise cellular mechanisms that are involved have not been fully elucidated.

The KKS is activated by exercise, and its activation has been reported to favor improved insulin sensitivity. Therefore, Studies 1 and 2 will explore the relationship between the KKS and the post-exercise increase in insulin-stimulated glucose transport. In Study 1, the necessity of the B2 receptor of bradykinin (B2R), an element of the KKS, to the post-exercise increase in insulin-stimulated glucose transport will be determined. Potential triggers leading to the increase in insulin-stimulated glucose transport that have not been evaluated with in vivo exercise are elements of the Kallikrein-Kininogen System (KKS) which can be modulated by exercise and which can enhance muscle glucose uptake under some conditions. Earlier studies have shown that in vitro contraction performed in the presence of bradykinin did not lead to a subsequent increase in insulin-stimulated glucose transport. However, because bradykinin is degraded rapidly by kininases and recent evidence has suggested that increased skeletal muscle glucose uptake after in vitro contraction and in vivo exercise appear to occur through different

mechanisms, it would be useful to evaluate the role of the B2R with in vivo exercise. Accordingly, B2R knock-out mice will be compared to normal controls, to see if B2R is required for the post-exercise increase in insulin-stimulated glucose uptake. In Study 2, the necessity of normal amounts of plasma kininogen, an element of the KKS that is the precursor for bradykinin formation during exercise, for the post-exercise increase in insulin-stimulated glucose transport will be determined. Therefore, rats with a spontaneous mutation of the kininogen gene leading to plasma kininogen deficiency will be compared to normal rats to assess if, upon exercise, plasma kininogen is essential for the post-exercise increase in insulin-stimulated glucose transport.

In Study 3, the post-exercise activation states of muscle protein kinases and phosphatases that may contribute to the long-lasting increase in AS160 phosphorylation after exercise will be studied. The increase in insulin-stimulated glucose transport after acute exercise occurs without enhanced upstream insulin signaling. However, elevated AS160 phosphorylation in skeletal muscle is sustained up to 27 hours after exercise and this increase appears to track with the increased insulin sensitivity. AS160 is an attractive target to study its involvement in enhanced insulin action, and presumably, sustained AS160 phosphorylation would result from sustained kinase signaling and/or phosphatase inhibition. However, the kinases and phosphatases involved in sustained AS160 phosphorylation after exercise are unknown. Therefore, to understand the events leading to sustained post-exercise AS160 phosphorylation, this study will determine: 1) if known kinases of AS160 are activated concomitant with prolonged phosphorylation of AS160 after exercise, 2) what protein phosphatases (PP1, PP2A, PP2B, and/or PP2C) can dephosphorylate phosphorylated AS160 from rat skeletal muscle stimulated with insulin

under cell-free conditions, 3) if phosphatases co-immunoprecipitate (PP1, PP2A, PP2B, and/or PP2C) with AS160, and 4) if exercise alters co-immunoprecipitation of phosphatases (PP1, PP2A, PP2B, and/or PP2C) with AS160 from rat skeletal muscle three hours after an acute bout of exercise.

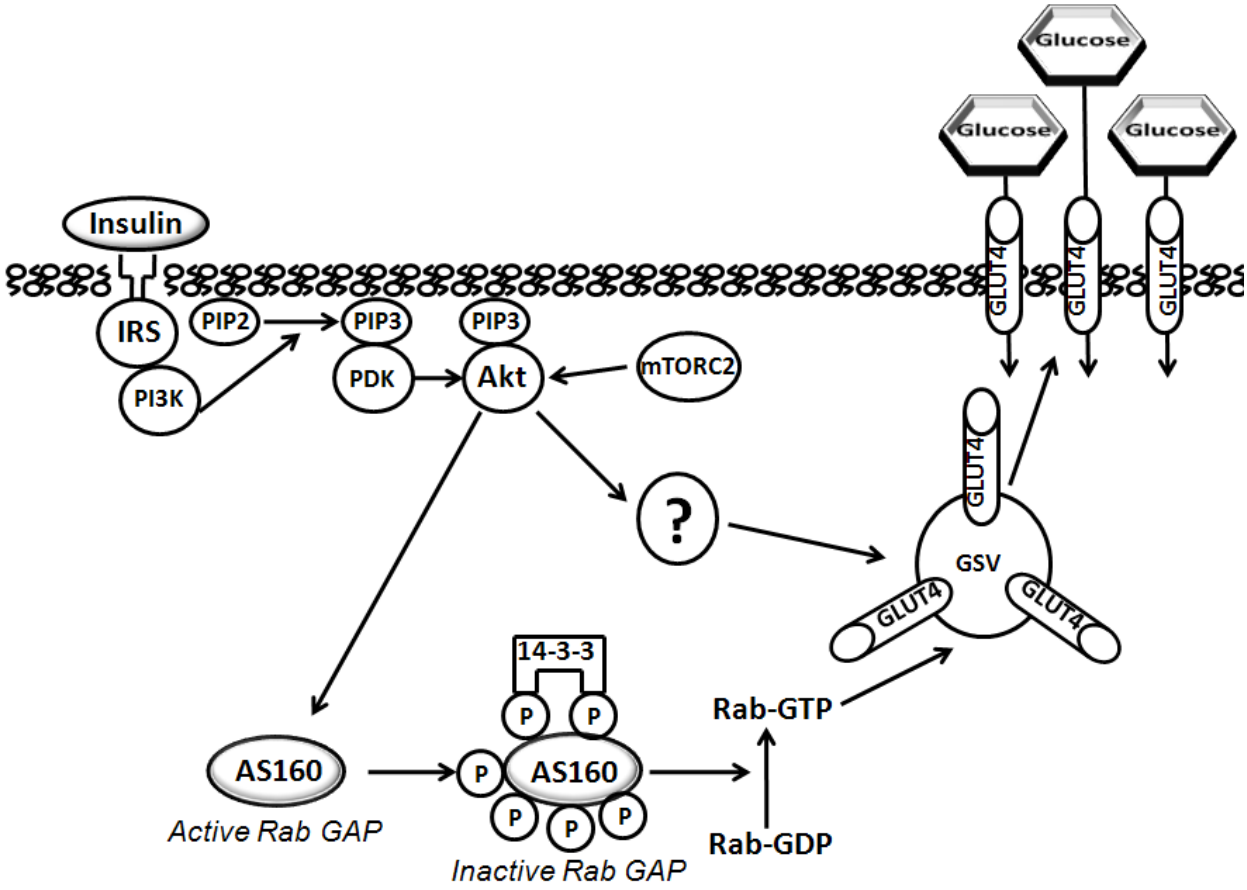

Figure 2.1
Insulin Signaling Cascade. Insulin binding to its receptor triggers a downstream signaling cascade through insulin receptor substrate (IRS), phosphatidylinsolitol 3-kinase (PI3K), phosphatidylinsolitol-(3,4,5)- trisphosphate (PIP3), phosphoinositide-dependent kinase-1 (PDK1), mammalian target of rapamycin complex-2 (mTORC2) and Akt. Akt phosphorylates AS160 on multiple sites, including Thr642 and Ser588 and, coincident with binding to 14-3-3 protein, leading to inhibition of AS160's RabGAP activity and allowing subsequent Rab-dependent GLUT4 translocation to the cell-surface membrane from GLUT Storage Vesicles (GSV). There is also evidence that insulin can increase GLUT4 translocation by AS160-independent mechanisms (e.g., by Akt's phosphorylation of other substrates, depicted by the question mark).

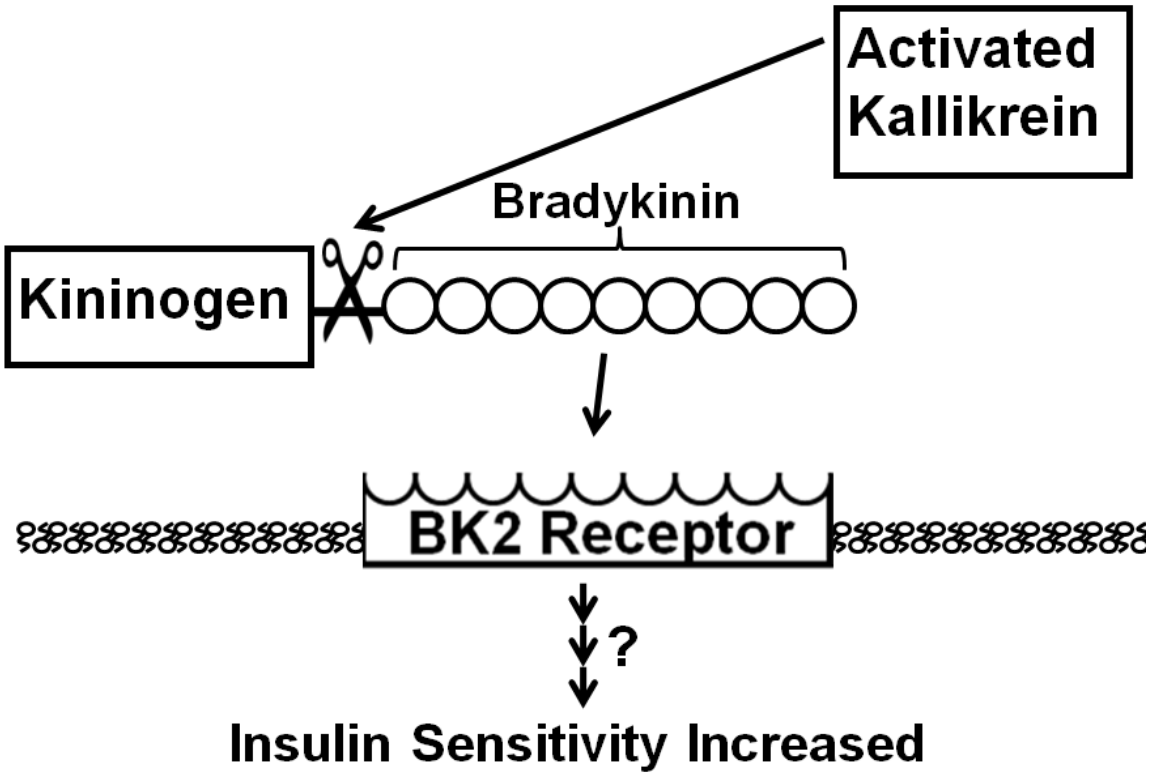

Figure 2.2
The Kallikrein-Kininogen System (KKS). Kallikrein is a plasma enzyme that, when activated, cleaves the nine amino acid peptide bradykinin from kininogen, another plasma protein. Bradykinin binds to the Bradykinin-2 Receptor (BK2) which is expressed by skeletal muscle cells. Previous evidence has implicated bradykinin action on its receptor as being able to improve insulin sensitivity. However, a role of the KKS in the post-exercise insulin sensitivity has not been established.

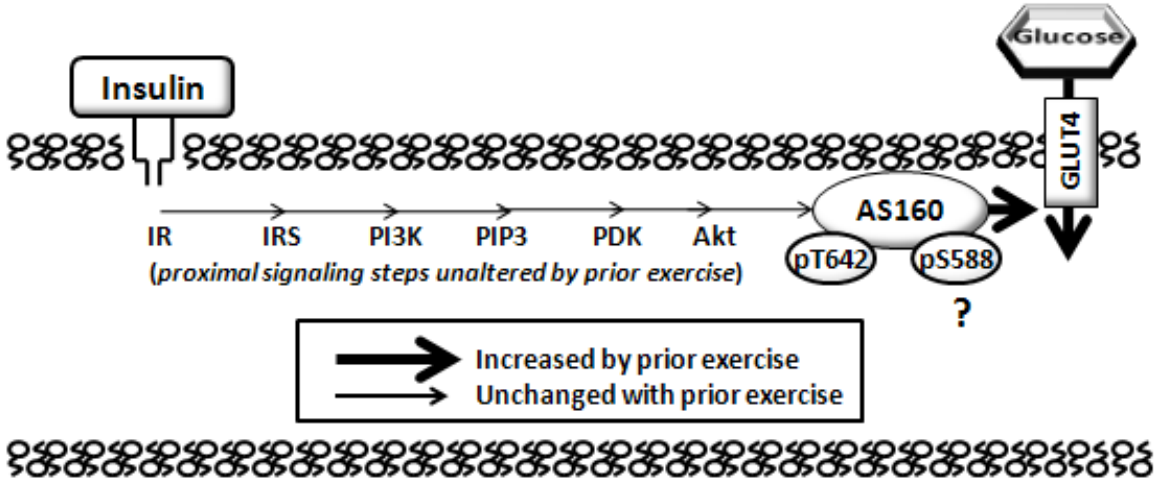

Figure 2.3
Proposed Role of Sustained AS160 Phosphorylation in the Post-Exercise Increase in Insulin-stimulated Glucose Transport. The post-exercise increase in insulin-stimulated glucose transport occurs as a result of a post-exercise increase in insulin-stimulated GLUT4 translocation. However, proximal insulin signaling steps (IR binding, pIR, IRTK, pIRS, IRS-PI3K, pAkt) that lead to insulin-stimulated GLUT4 translocation are not enhanced by prior exercise. In contrast, AS160 phosphorylation at Thr642 (pT642) in exercised rats remains elevated above sedentary controls 4 hours post-exercise. It seems conceivable that the sustained increase in AS160 phosphorylation at Thr642 (and possibly also Ser588) post-exercise plays a role in post-exercise increase in insulin-stimulated glucose transport. (Figure is modified from Cartee and Funai *Exercise and Sport Sciences Reviews* 37: 188-95, 2009.)

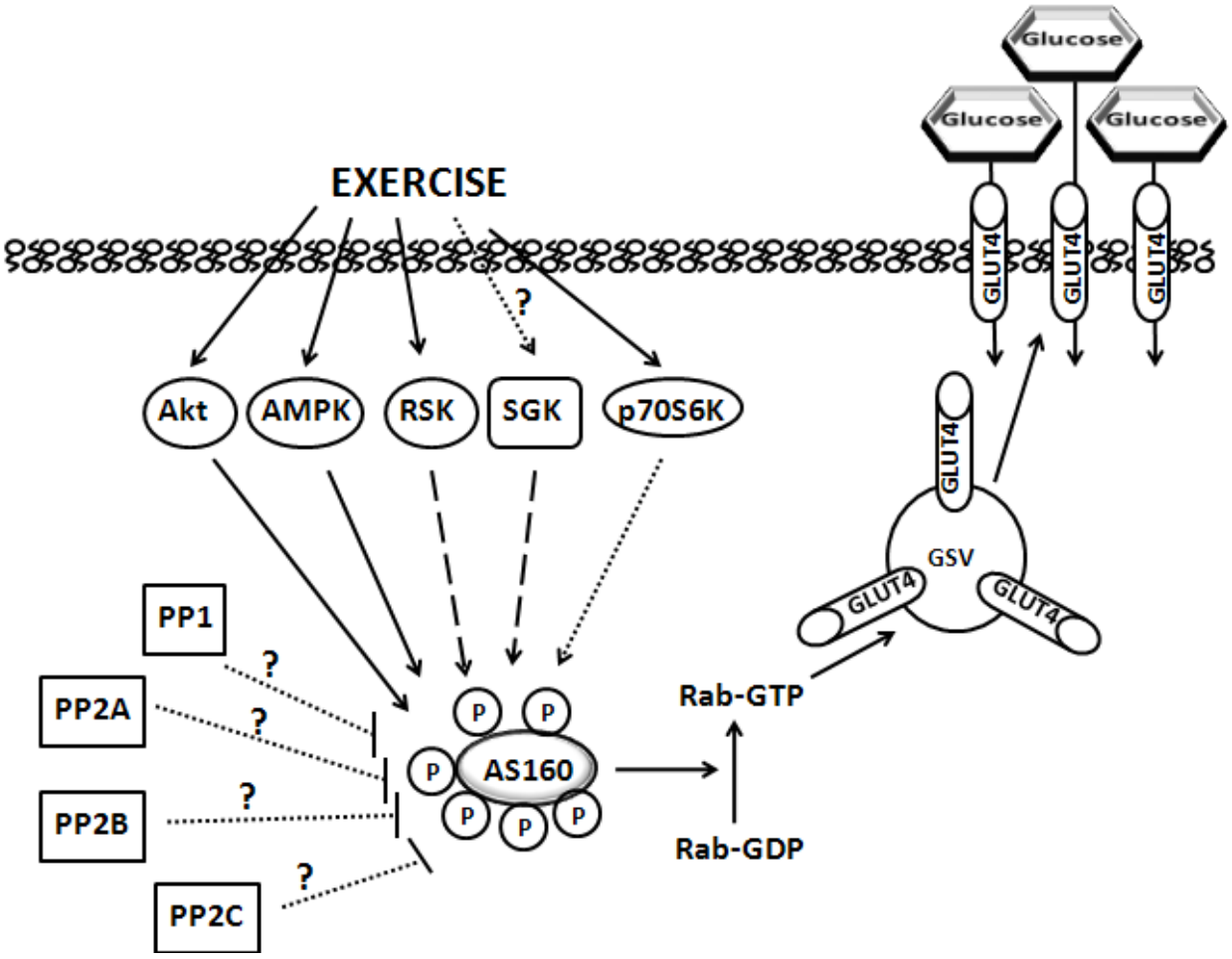

Figure 2.4
Kinases and Phosphatases Proposed to Potentially Regulate AS160 Phosphorylation at Thr642 and Ser588. The figure includes 5 possible kinases of AS160. Akt and AMPK (solid arrow) have been supported by multiple lines of evidence to be physiologically relevant AS160 kinases in skeletal muscle. RSK and SGK (dashed arrow) can phosphorylate AS160 in a cell-free assay, but neither has been tested using more physiological approaches. The consensus motif of p70S6K (dotted arrow) are similar to AS160's Thr642 and Ser588 sites, but there is no direct evidence regarding whether p70S6K is a physiologically relevant AS160 kinase. Exercise has been shown to activate 4 of the 5 kinases (only SGK has not yet been evaluated post-exercise). AS160 phosphatases have not been evaluated, but PP1, PP2A, PP2B, and PP2C are Ser/Thr protein phosphatases that are highly expressed by rat skeletal muscle.

REFERENCES

1. Economic costs of diabetes in the U.S. In 2007. *Diabetes Care* 31: 596-615, 2008.
2. **Alessi DR, Deak M, Casamayor A, Caudwell FB, Morrice N, Norman DG, Gaffney P, Reese CB, MacDougall CN, Harbison D, Ashworth A, and Bownes M.** 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase. *Curr Biol* 7: 776-789, 1997.
3. **Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, and Cohen P.** Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr Biol* 7: 261-269, 1997.
4. **Andjelkovic M, Alessi DR, Meier R, Fernandez A, Lamb NJ, Frech M, Cron P, Cohen P, Lucocq JM, and Hemmings BA.** Role of translocation in the activation and function of protein kinase B. *The Journal of biological chemistry* 272: 31515-31524, 1997.
5. **Arias EB, Kim J, Funai K, and Cartee GD.** Prior exercise increases phosphorylation of Akt substrate of 160 kDa (AS160) in rat skeletal muscle. *American journal of physiology* 292: E1191-1200, 2007.
6. **Armstrong RB and Phelps RO.** Muscle fiber type composition of the rat hindlimb. *Am J Anat* 171: 259-272, 1984.
7. **Baar K and Esser K.** Phosphorylation of p70(S6k) correlates with increased skeletal muscle mass following resistance exercise. *Am J Physiol* 276: C120-127, 1999.
8. **Beard KM, Lu H, Ho K, and Fantus IG.** Bradykinin Augments Insulin-Stimulated Glucose Transport in Rat Adipocytes via Endothelial Nitric Oxide Synthase-Mediated Inhibition of Jun NH2-Terminal Kinase. *Diabetes %R 102337/db05-1538* 55: 2678-2687, 2006.
9. **Beaubien G, Rosinski-Chupin I, Mattei MG, Mbikay M, Chretien M, and Seidah NG.** Gene structure and chromosomal localization of plasma kallikrein. *Biochemistry* 30: 1628-1635, 1991.
10. **Bhoola KD, Figueroa CD, and Worthy K.** Bioregulation of kinins: kallikreins, kininogens, and kininases. *Pharmacological reviews* 44: 1-80, 1992.
11. **Birnbaum MJ.** The insulin-sensitive glucose transporter. *Int Rev Cytol* 137: 239-297, 1992.
12. **Blais C, Jr., Adam A, Massicotte D, and Peronnet F.** Increase in blood bradykinin concentration after eccentric weight-training exercise in men. *J Appl Physiol* 87: 1197-1201, 1999.
13. **Bonen A, Clune PA, and Tan MH.** Chronic exercise increases insulin binding in muscles but not liver. *Am J Physiol* 251: E196-203, 1986.
14. **Bonen A and Tan MH.** Dissociation between insulin binding and glucose utilization after intense exercise in mouse skeletal muscles. *Horm Metab Res* 21: 172-178, 1989.
15. **Bonen A, Tan MH, Clune P, and Kirby RL.** Effects of exercise on insulin binding to human muscle. *Am J Physiol* 248: E403-408, 1985.
16. **Bonen A, Tan MH, and Watson-Wright WM.** Effects of exercise on insulin binding and glucose metabolism in muscle. *Can J Physiol Pharmacol* 62: 1500-1504, 1984.

17. **Bridges D and Moorhead GB.** 14-3-3 proteins: a number of functions for a numbered protein. *Sci STKE* 2005: re10, 2005.
18. **Bruss MD, Arias EB, Lienhard GE, and Cartee GD.** Increased phosphorylation of Akt substrate of 160 kDa (AS160) in rat skeletal muscle in response to insulin or contractile activity. *Diabetes* 54: 41-50, 2005.
19. **Bryant JC, Westphal RS, and Wadzinski BE.** Methylated C-terminal leucine residue of PP2A catalytic subunit is important for binding of regulatory Balpha subunit. *The Biochemical journal* 339 (Pt 2): 241-246, 1999.
20. **Bultynck G, Heath VL, Majeed AP, Galan JM, Haguenauer-Tsapis R, and Cyert MS.** Slm1 and slm2 are novel substrates of the calcineurin phosphatase required for heat stress-induced endocytosis of the yeast uracil permease. *Molecular and cellular biology* 26: 4729-4745, 2006.
21. **Cann AD and Kohanski RA.** Cis-autophosphorylation of juxtamembrane tyrosines in the insulin receptor kinase domain. *Biochemistry* 36: 7681-7689, 1997.
22. **Cantley LC.** The phosphoinositide 3-kinase pathway. *Science (New York, NY* 296: 1655-1657, 2002.
23. **Cartee GD, Briggs-Tung C, and Kietzke EW.** Persistent effects of exercise on skeletal muscle glucose transport across the life-span of rats. *J Appl Physiol* 75: 972-978, 1993.
24. **Cartee GD and Funai K.** Exercise and Insulin: Convergence or Divergence at AS160 and TBC1D1? *Exercise and Sport Sciences Reviews* 37: 188-195, 2009.
25. **Cartee GD and Holloszy JO.** Exercise increases susceptibility of muscle glucose transport to activation by various stimuli. *Am J Physiol* 258: E390-393, 1990.
26. **Cartee GD and Wojtaszewski JF.** Role of Akt substrate of 160 kDa in insulin-stimulated and contraction-stimulated glucose transport. *Appl Physiol Nutr Metab* 32: 557-566, 2007.
27. **Cartee GD, Young DA, Sleeper MD, Zierath J, Wallberg-Henriksson H, and Holloszy JO.** Prolonged increase in insulin-stimulated glucose transport in muscle after exercise. *Am J Physiol* 256: E494-499, 1989.
28. **Carvalho CR, Thirone AC, Gontijo JA, Velloso LA, and Saad MJ.** Effect of captopril, losartan, and bradykinin on early steps of insulin action. *Diabetes* 46: 1950-1957, 1997.
29. **Chiasson RB.** *Laboratory Anatomy of the White Rat*. Dubuque, Iowa, U.S.A. : W. M. C. Brown Company Publishers, 1969.
30. **Chibalin AV, Yu M, Ryder JW, Song XM, Galuska D, Krook A, Wallberg-Henriksson H, and Zierath JR.** Exercise-induced changes in expression and activity of proteins involved in insulin signal transduction in skeletal muscle: differential effects on insulin-receptor substrates 1 and 2. *Proceedings of the National Academy of Sciences of the United States of America* 97: 38-43, 2000.
31. **Chleboun GS, Patel TJ, and Lieber RL.** Skeletal muscle architecture and fiber-type distribution with the multiple bellies of the mouse extensor digitorum longus muscle. *Acta Anat (Basel)* 159: 147-155, 1997.
32. **Coffey VG, Zhong Z, Shield A, Canny BJ, Chibalin AV, Zierath JR, and Hawley JA.** Early signaling responses to divergent exercise stimuli in skeletal muscle from well-trained humans. *Faseb J* 20: 190-192, 2006.

33. **Cohen P, Klumpp S, and Schelling DL.** An improved procedure for identifying and quantitating protein phosphatases in mammalian tissues. *FEBS letters* 250: 596-600, 1989.
34. **Cohen PT.** Novel protein serine/threonine phosphatases: variety is the spice of life. *Trends in biochemical sciences* 22: 245-251, 1997.
35. **Cohen PT.** Protein phosphatase 1--targeted in many directions. *J Cell Sci* 115: 241-256, 2002.
36. **Coyle EF.** Timing and method of increased carbohydrate intake to cope with heavy training, competition and recovery. *J Sports Sci* 9 Spec No: 29-51; discussion 51-22, 1991.
37. **Cusi K, Maezono K, Osman A, Pendergrass M, Patti ME, Pratipanawatr T, DeFronzo RA, Kahn CR, and Mandarino LJ.** Insulin resistance differentially affects the PI 3-kinase- and MAP kinase-mediated signaling in human muscle. *J Clin Invest* 105: 311-320, 2000.
38. **Czirjak G and Enyedi P.** Targeting of calcineurin to an NFAT-like docking site is required for the calcium-dependent activation of the background K+ channel, TRESK. *The Journal of biological chemistry* 281: 14677-14682, 2006.
39. **Damas J.** The brown Norway rats and the kinin system. *Peptides* 17: 859-872, 1996.
40. **Damas J and Adam A.** The kallikrein-kininogens-kinins system in the Brown-Norway rat. *Biomedicine* 31: 249, 1979.
41. **Damas J, Bourdon V, and Lefebvre PJ.** Insulin sensitivity, clearance and release in kininogen-deficient rats. *Exp Physiol* 84: 549-557, 1999.
42. **Damas J, Garbacki N, and Lefebvre PJ.** The kallikrein-kinin system, angiotensin converting enzyme inhibitors and insulin sensitivity. *Diabetes Metab Res Rev* 20: 288-297, 2004.
43. **Davidson YS, Clague JE, Horan MA, and Pendleton N.** The effect of aging on skeletal muscle capillarization in a murine model. *J Gerontol A Biol Sci Med Sci* 54: B448-451, 1999.
44. **Davies SP, Helps NR, Cohen PT, and Hardie DG.** 5'-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2C alpha and native bovine protein phosphatase-2AC. *FEBS letters* 377: 421-425, 1995.
45. **De Baere I, Derua R, Janssens V, Van Hoof C, Waelkens E, Merlevede W, and Goris J.** Purification of porcine brain protein phosphatase 2A leucine carboxyl methyltransferase and cloning of the human homologue. *Biochemistry* 38: 16539-16547, 1999.
46. **DeFronzo RA, Jacot E, Jequier E, Maeder E, Wahren J, and Felber JP.** The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. *Diabetes* 30: 1000-1007, 1981.
47. **Deneka M, Neeft M, and van der Sluijs P.** Regulation of membrane transport by rab GTPases. *Critical reviews in biochemistry and molecular biology* 38: 121-142, 2003.
48. **Dietze GJ, Wicklmayr M, Rett K, Jacob S, and Henriksen EJ.** Potential role of bradykinin in forearm muscle metabolism in humans. *Diabetes* 45 Suppl 1: S110-114, 1996.

49. **Dodge KL and Scott JD.** Calcineurin anchoring and cell signaling. *Biochemical and biophysical research communications* 311: 1111-1115, 2003.
50. **Dreyer HC, Drummond MJ, Glynn EL, Fujita S, Chinkes DL, Volpi E, and Rasmussen BB.** Resistance exercise increases human skeletal muscle AS160/TBC1D4 phosphorylation in association with enhanced leg glucose uptake during postexercise recovery. *J Appl Physiol* 105: 1967-1974, 2008.
51. **Duka I, Shenouda S, Johns C, Kintsurashvili E, Gavras I, and Gavras H.** Role of the B(2) receptor of bradykinin in insulin sensitivity. *Hypertension* 38: 1355-1360, 2001.
52. **Dumke CL, Kim J, Arias EB, and Cartee GD.** Role of kallikrein-kininogen system in insulin-stimulated glucose transport after muscle contractions. *J Appl Physiol* 92: 657-664, 2002.
53. **Eto M, Senba S, Morita F, and Yazawa M.** Molecular cloning of a novel phosphorylation-dependent inhibitory protein of protein phosphatase-1 (CPI17) in smooth muscle: its specific localization in smooth muscle. *FEBS letters* 410: 356-360, 1997.
54. **Facchini FS, Hua N, Abbasi F, and Reaven GM.** Insulin resistance as a predictor of age-related diseases. *J Clin Endocrinol Metab* 86: 3574-3578, 2001.
55. **Favre B, Zolnierowicz S, Turowski P, and Hemmings BA.** The catalytic subunit of protein phosphatase 2A is carboxyl-methylated in vivo. *The Journal of biological chemistry* 269: 16311-16317, 1994.
56. **Fisher JS, Gao J, Han DH, Holloszy JO, and Nolte LA.** Activation of AMP kinase enhances sensitivity of muscle glucose transport to insulin. *American journal of physiology* 282: E18-23, 2002.
57. **Foulkes JG and Cohen P.** The regulation of glycogen metabolism. Purification and properties of protein phosphatase inhibitor-2 from rabbit skeletal muscle. *European journal of biochemistry / FEBS* 105: 195-203, 1980.
58. **Funai K and Cartee GD.** Contraction-stimulated glucose transport in rat skeletal muscle is sustained despite reversal of increased PAS-phosphorylation of AS160 and TBC1D1. *J Appl Physiol* 105: 1788-1795, 2008.
59. **Funai K and Cartee GD.** Inhibition of contraction-stimulated AMP-activated protein kinase inhibits contraction-stimulated increases in PAS-TBC1D1 and glucose transport without altering PAS-AS160 in rat skeletal muscle. *Diabetes* 58: 1096-1104, 2009.
60. **Funai K, Schweitzer GG, Castorena CM, Kanzaki M, and Cartee GD.** In vivo exercise followed by in vitro contraction additively elevates subsequent insulin-stimulated glucose transport by rat skeletal muscle. *American journal of physiology* 298: E999-1010, 2010.
61. **Funai K, Schweitzer GG, Sharma N, Kanzaki M, and 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.
62. **Gao J, Gulve EA, and Holloszy JO.** Contraction-induced increase in muscle insulin sensitivity: requirement for a serum factor. *Am J Physiol* 266: E186-192, 1994.

63. **Garetto LP, Richter EA, Goodman MN, and Ruderman NB.** Enhanced muscle glucose metabolism after exercise in the rat: the two phases. *American journal of physiology* 246: E471-475, 1984.
64. **Geraghty KM, Chen S, Harthill JE, Ibrahim AF, Toth R, Morrice NA, Vandermoere F, Moorhead GB, Hardie DG, and MacKintosh C.** Regulation of multisite phosphorylation and 14-3-3 binding of AS160 in response to IGF-1, EGF, PMA and AICAR. *The Biochemical journal* 407: 231-241, 2007.
65. **Goldstein MS, Mullick V, Huddlestun B, and Levine R.** Action of muscular work on transfer of sugars across cell barriers; comparison with action of insulin. *Am J Physiol* 173: 212-216, 1953.
66. **Goodyear LJ.** AMP-activated protein kinase: a critical signaling intermediary for exercise-stimulated glucose transport? *Exerc Sport Sci Rev* 28: 113-116, 2000.
67. **Goodyear LJ and Kahn BB.** Exercise, glucose transport, and insulin sensitivity. *Annu Rev Med* 49: 235-261, 1998.
68. **Greene EC.** *Anatomy of the Rat*. New York and London: Hafner Publishing Company, 1963.
69. **Gulve EA, Cartee GD, Zierath JR, Corpus VM, and Holloszy JO.** Reversal of enhanced muscle glucose transport after exercise: roles of insulin and glucose. *Am J Physiol* 259: E685-691, 1990.
70. **Haffner SM.** Epidemiology of insulin resistance and its relation to coronary artery disease. *Am J Cardiol* 84: 11J-14J, 1999.
71. **Hamada T, Arias EB, and Cartee GD.** Increased submaximal insulin-stimulated glucose uptake in mouse skeletal muscle after treadmill exercise. *J Appl Physiol* 101: 1368-1376, 2006.
72. **Hansen PA, Nolte LA, Chen MM, and Holloszy JO.** Increased GLUT-4 translocation mediates enhanced insulin sensitivity of muscle glucose transport after exercise. *J Appl Physiol* 85: 1218-1222, 1998.
73. **Haring HU, Tippmer S, Kellerer M, Mosthaf L, Kroder G, Bossenmaier B, and Berti L.** Modulation of insulin receptor signaling. Potential mechanisms of a cross talk between bradykinin and the insulin receptor. *Diabetes* 45 Suppl 1: S115-119, 1996.
74. **Hayashi T, Hirshman MF, Fujii N, Habinowski SA, Witters LA, and Goodyear LJ.** Metabolic stress and altered glucose transport: activation of AMP-activated protein kinase as a unifying coupling mechanism. *Diabetes* 49: 527-531, 2000.
75. **Hayashi T, Hirshman MF, Kurth EJ, Winder WW, and Goodyear LJ.** Evidence for 5' AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. *Diabetes* 47: 1369-1373, 1998.
76. **Henriksen EJ and Jacob S.** Angiotensin converting enzyme inhibitors and modulation of skeletal muscle insulin resistance. *Diabetes Obes Metab* 5: 214-222, 2003.
77. **Henriksen EJ, Jacob S, Augustin HJ, and Dietze GJ.** Glucose transport activity in insulin-resistant rat muscle. Effects of angiotensin-converting enzyme inhibitors and bradykinin antagonism. *Diabetes* 45 Suppl 1: S125-128, 1996.
78. **Henriksen EJ, Jacob S, Fogt DL, and Dietze GJ.** Effect of chronic bradykinin administration on insulin action in an animal model of insulin resistance. *Am J Physiol* 275: R40-45, 1998.

79. **Henriksen EJ, Jacob S, Kinnick TR, Youngblood EB, Schmit MB, and Dietze GJ.** ACE inhibition and glucose transport in insulinresistant muscle: roles of bradykinin and nitric oxide. *Am J Physiol* 277: R332-336, 1999.
80. **Higaki Y, Wojtaszewski JF, Hirshman MF, Withers DJ, Towery H, White MF, and Goodyear LJ.** Insulin receptor substrate-2 is not necessary for insulin- and exercise-stimulated glucose transport in skeletal muscle. *The Journal of biological chemistry* 274: 20791-20795, 1999.
81. **Holloszy JO.** A forty-year memoir of research on the regulation of glucose transport into muscle. *American journal of physiology* 284: E453-467, 2003.
82. **Holloszy JO and Narahara HT.** Enhanced permeability to sugar associated with muscle contraction. Studies of the role of Ca++. *J Gen Physiol* 50: 551-562, 1967.
83. **Holloszy JO and Narahara HT.** Nitrate ions: Potentiation of increased permeability to sugar associated with muscle contraction. *Science (New York, NY* 155: 573-575, 1967.
84. **Holloszy JO and Narahara HT.** Studies of tissue permeability. X. Changes in permeability to 3-methylglucose associated with contraction of isolated frog muscle. *The Journal of biological chemistry* 240: 3493-3500, 1965.
85. **Howlett KF, Mathews A, Garnham A, and Sakamoto K.** The effect of exercise and insulin on AS160 phosphorylation and 14-3-3 binding capacity in human skeletal muscle. *American journal of physiology* 294: E401-407, 2008.
86. **Howlett KF, Sakamoto K, Garnham A, Cameron-Smith D, and Hargreaves M.** Resistance exercise and insulin regulate AS160 and interaction with 14-3-3 in human skeletal muscle. *Diabetes* 56: 1608-1614, 2007.
87. **Howlett KF, Sakamoto K, Hirshman MF, Aschenbach WG, Dow M, White MF, and Goodyear LJ.** Insulin signaling after exercise in insulin receptor substrate-2-deficient mice. *Diabetes* 51: 479-483, 2002.
88. **Ingebritsen TS and Cohen P.** The protein phosphatases involved in cellular regulation. 1. Classification and substrate specificities. *European journal of biochemistry / FEBS* 132: 255-261, 1983.
89. **Isami S, Kishikawa H, Araki E, Uehara M, Kaneko K, Shirotani T, Todaka M, Ura S, Motoyoshi S, Matsumoto K, Miyamura N, and Shichiri M.** Bradykinin enhances GLUT4 translocation through the increase of insulin receptor tyrosine kinase in primary adipocytes: evidence that bradykinin stimulates the insulin signalling pathway. *Diabetologia* 39: 412-420, 1996.
90. **Ishikura S, Bilan PJ, and Klip A.** Rabs 8A and 14 are targets of the insulin-regulated Rab-GAP AS160 regulating GLUT4 traffic in muscle cells. *Biochemical and biophysical research communications* 353: 1074-1079, 2007.
91. **Ivy JL and Holloszy JO.** Persistent increase in glucose uptake by rat skeletal muscle following exercise. *Am J Physiol* 241: C200-203, 1981.
92. **Ivy JL, Katz AL, Cutler CL, Sherman WM, and Coyle EF.** Muscle glycogen synthesis after exercise: effect of time of carbohydrate ingestion. *J Appl Physiol* 64: 1480-1485, 1988.
93. **Janssens V and Goris J.** Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *The Biochemical journal* 353: 417-439, 2001.

94. **Kane S, Sano H, Liu SC, Asara JM, Lane WS, Garner CC, and Lienhard GE.** A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain. *The Journal of biological chemistry* 277: 22115-22118, 2002.
95. **Karlsson HK, Zierath JR, Kane S, Krook A, Lienhard GE, and Wallberg-Henriksson H.** Insulin-Stimulated Phosphorylation of the Akt Substrate AS160 Is Impaired in Skeletal Muscle of Type 2 Diabetic Subjects. *Diabetes* 54: 1692-1697, 2005.
96. **Kim J, Arias EB, and Cartee GD.** Effects of gender and prior swim exercise on glucose uptake in isolated skeletal muscles from mice. *J Physiol Sci* 56: 305-312, 2006.
97. **Kim J, Solis RS, Arias EB, and Cartee GD.** Postcontraction insulin sensitivity: relationship with contraction protocol, glycogen concentration, and 5' AMP-activated protein kinase phosphorylation. *J Appl Physiol* 96: 575-583, 2004.
98. **Kishi K, Muromoto N, Nakaya Y, Miyata I, Hagi A, Hayashi H, and Ebina Y.** Bradykinin directly triggers GLUT4 translocation via an insulin-independent pathway. *Diabetes* 47: 550-558, 1998.
99. **Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, and Nathan DM.** Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N Engl J Med* 346: 393-403, 2002.
100. **Kobayashi T and Cohen P.** Activation of serum- and glucocorticoid-regulated protein kinase by agonists that activate phosphatidylinositide 3-kinase is mediated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and PDK2. *The Biochemical journal* 339 (Pt 2): 319-328, 1999.
101. **Kobayashi T, Deak M, Morrice N, and Cohen P.** Characterization of the structure and regulation of two novel isoforms of serum- and glucocorticoid-induced protein kinase. *The Biochemical journal* 344 Pt 1: 189-197, 1999.
102. **Kramer HF, Witczak CA, Taylor EB, Fujii N, Hirshman MF, and Goodyear LJ.** AS160 regulates insulin- and contraction-stimulated glucose uptake in mouse skeletal muscle. *The Journal of biological chemistry* 281: 31478-31485, 2006.
103. **Kudoh A and Matsuki A.** Effects of angiotensin-converting enzyme inhibitors on glucose uptake. *Hypertension* 36: 239-244, 2000.
104. **Kumar A, Chaudhry I, Reid MB, and Boriek AM.** Distinct signaling pathways are activated in response to mechanical stress applied axially and transversely to skeletal muscle fibers. *The Journal of biological chemistry* 277: 46493-46503, 2002.
105. **Kumari M, Brunner E, and Fuhrer R.** Minireview: mechanisms by which the metabolic syndrome and diabetes impair memory. *J Gerontol A Biol Sci Med Sci* 55: B228-232, 2000.
106. **Kurth-Kraczek EJ, Hirshman MF, Goodyear LJ, and Winder WW.** 5' AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. *Diabetes* 48: 1667-1671, 1999.
107. **Lammers T and Lavi S.** Role of type 2C protein phosphatases in growth regulation and in cellular stress signaling. *Critical reviews in biochemistry and molecular biology* 42: 437-461, 2007.
108. **Lammers T, Peschke P, Ehemann V, Debus J, Slobodin B, Lavi S, and Huber P.** Role of PP2Calpha in cell growth, in radio- and chemosensitivity, and in tumorigenicity. *Molecular cancer* 6: 65, 2007.

109. **Langberg H, Bjorn C, Boushel R, Hellsten Y, and Kjaer M.** Exercise-induced increase in interstitial bradykinin and adenosine concentrations in skeletal muscle and peritendinous tissue in humans. *The Journal of physiology* 542: 977-983, 2002.
110. **Lavan BE and Lienhard GE.** Insulin signalling and the stimulation of glucose transport. *Biochem Soc Trans* 22: 676-680, 1994.
111. **Lawlor MA and Alessi DR.** PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *J Cell Sci* 114: 2903-2910, 2001.
112. **Levine R and Goldstein MS.** Effect of insulin on rate of transfer of sugars across cell barriers. *Brookhaven Symp Biol* 5: 73-80; discussion, 80-74, 1952.
113. **Lindgren K, Ormestad M, Persson M, Martinsson S, Svensson LT, and Mahlapuu M.** Regulation of the muscle-specific AMP-activated protein kinase alpha2beta2gamma3 complexes by AMP and implications of the mutations in the gamma3-subunit for the AMP dependence of the enzyme. *The FEBS journal* 274: 2887-2896, 2007.
114. **Margolius HS.** Kallikreins and kinins. Molecular characteristics and cellular and tissue responses. *Diabetes* 45 Suppl 1: S14-19, 1996.
115. **McGowan CH, Campbell DG, and Cohen P.** Primary structure analysis proves that protein phosphatases 2C1 and 2C2 are isozymes. *Biochimica et biophysica acta* 930: 279-282, 1987.
116. **McGowan CH and Cohen P.** Identification of two isoenzymes of protein phosphatase 2C in both rabbit skeletal muscle and liver. *European journal of biochemistry / FEBS* 166: 713-721, 1987.
117. **Miinea CP, Sano H, Kane S, Sano E, Fukuda M, Peranen J, Lane WS, and Lienhard GE.** AS160, the Akt substrate regulating GLUT4 translocation, has a functional Rab GTPase-activating protein domain. *The Biochemical journal* 391: 87-93, 2005.
118. **Miyata T, Taguchi T, Uehara M, Isami S, Kishikawa H, Kaneko K, Araki E, and Shichiri M.** Bradykinin potentiates insulin-stimulated glucose uptake and enhances insulin signal through the bradykinin B2 receptor in dog skeletal muscle and rat L6 myoblasts. *Eur J Endocrinol* 138: 344-352, 1998.
119. **Moore F, Weekes J, and Hardie DG.** Evidence that AMP triggers phosphorylation as well as direct allosteric activation of rat liver AMP-activated protein kinase. A sensitive mechanism to protect the cell against ATP depletion. *European journal of biochemistry / FEBS* 199: 691-697, 1991.
120. **Moorhead GB, Trinkle-Mulcahy L, and Ulke-Lemee A.** Emerging roles of nuclear protein phosphatases. *Nature reviews* 8: 234-244, 2007.
121. **Moreno CS, Park S, Nelson K, Ashby D, Hubalek F, Lane WS, and Pallas DC.** WD40 repeat proteins striatin and S/G(2) nuclear autoantigen are members of a novel family of calmodulin-binding proteins that associate with protein phosphatase 2A. *The Journal of biological chemistry* 275: 5257-5263, 2000.
122. **Mu J, Brozinick JT, Jr., Valladares O, Bucan M, and Birnbaum MJ.** A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. *Molecular cell* 7: 1085-1094, 2001.
123. **Myers MG, Jr., Sun XJ, and White MF.** The IRS-1 signaling system. *Trends in biochemical sciences* 19: 289-293, 1994.

124. **Nagase T, Ishikawa K, Miyajima N, Tanaka A, Kotani H, Nomura N, and Ohara O.** Prediction of the coding sequences of unidentified human genes. IX. The complete sequences of 100 new cDNA clones from brain which can code for large proteins in vitro. *DNA Res* 5: 31-39, 1998.
125. **Nairn AC, Hemmings HC, Jr., Walaas SI, and Greengard P.** DARPP-32 and phosphatase inhibitor-1, two structurally related inhibitors of protein phosphatase-1, are both present in striatonigral neurons. *Journal of neurochemistry* 50: 257-262, 1988.
126. **Nimmo GA and Cohen P.** The regulation of glycogen metabolism. Phosphorylation of inhibitor-1 from rabbit skeletal muscle, and its interaction with protein phosphatases-III and -II. *European journal of biochemistry / FEBS* 87: 353-365, 1978.
127. **Park J, Leong ML, Buse P, Maiyar AC, Firestone GL, and Hemmings BA.** Serum and glucocorticoid-inducible kinase (SGK) is a target of the PI 3-kinase-stimulated signaling pathway. *The EMBO journal* 18: 3024-3033, 1999.
128. **Pearce LR, Komander D, and Alessi DR.** The nuts and bolts of AGC protein kinases. *Nature reviews* 11: 9-22, 2010.
129. **Pfeffer S.** Filling the Rab GAP. *Nature cell biology* 7: 856-857, 2005.
130. **Pfeffer S and Aivazian D.** Targeting Rab GTPases to distinct membrane compartments. *Nature reviews* 5: 886-896, 2004.
131. **Rabito SF, Minshall RD, Nakamura F, and Wang LX.** Bradykinin B2 receptors on skeletal muscle are coupled to inositol 1,4,5-trisphosphate formation. *Diabetes* 45 Suppl 1: S29-33, 1996.
132. **Ramm G, Larance M, Guilhaus M, and James DE.** A role for 14-3-3 in insulin-stimulated GLUT4 translocation through its interaction with the RabGAP AS160. *The Journal of biological chemistry* 281: 29174-29180, 2006.
133. **Rett K, Wicklmayr M, Dietze GJ, and Haring HU.** Insulin-induced glucose transporter (GLUT1 and GLUT4) translocation in cardiac muscle tissue is mimicked by bradykinin. *Diabetes* 45 Suppl 1: S66-69, 1996.
134. **Richter EA, Garetto LP, Goodman MN, and Ruderman NB.** Muscle glucose metabolism following exercise in the rat: increased sensitivity to insulin. *J Clin Invest* 69: 785-793, 1982.
135. **Roy J, Li H, Hogan PG, and Cyert MS.** A conserved docking site modulates substrate affinity for calcineurin, signaling output, and in vivo function. *Molecular cell* 25: 889-901, 2007.
136. **Ryder JW, Chibalin AV, and Zierath JR.** Intracellular mechanisms underlying increases in glucose uptake in response to insulin or exercise in skeletal muscle. *Acta Physiol Scand* 171: 249-257, 2001.
137. **Ryder JW, Fahlman R, Wallberg-Henriksson H, Alessi DR, Krook A, and Zierath JR.** Effect of contraction on mitogen-activated protein kinase signal transduction in skeletal muscle. Involvement Of the mitogen- and stress-activated protein kinase 1. *The Journal of biological chemistry* 275: 1457-1462, 2000.
138. **Ryder JW, Kawano Y, Chibalin AV, Rincon J, Tsao TS, Stenbit AE, Combatsiaris T, Yang J, Holman GD, Charron MJ, and Zierath JR.** In vitro analysis of the glucose-transport system in GLUT4-null skeletal muscle. *The Biochemical journal* 342 (Pt 2): 321-328, 1999.

139. **Sakamoto K and Holman GD.** Emerging role for AS160/TBC1D4 and TBC1D1 in the regulation of GLUT4 traffic. *American journal of physiology* 295: E29-37, 2008.
140. **Sakamoto K, McCarthy A, Smith D, Green KA, Grahame Hardie D, Ashworth A, and Alessi DR.** Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction. *The EMBO journal* 24: 1810-1820, 2005.
141. **Saltiel AR and Kahn CR.** Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* 414: 799-806, 2001.
142. **Sanders MJ, Grondin PO, Hegarty BD, Snowden MA, and Carling D.** Investigating the mechanism for AMP activation of the AMP-activated protein kinase cascade. *The Biochemical journal* 403: 139-148, 2007.
143. **Sano H, Kane S, Sano E, Miinea CP, Asara JM, Lane WS, Garner CW, and Lienhard GE.** Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *The Journal of biological chemistry* 278: 14599-14602, 2003.
144. **Sarbassov DD, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF, Markhard AL, and Sabatini DM.** Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Molecular cell* 22: 159-168, 2006.
145. **Sarbassov DD, Guertin DA, Ali SM, and Sabatini DM.** Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science (New York, NY* 307: 1098-1101, 2005.
146. **Seidah NG, Sawyer N, Hamelin J, Mion P, Beaubien G, Brachpapa L, Rochemont J, Mbikay M, and Chretien M.** Mouse plasma kallikrein: cDNA structure, enzyme characterization, and comparison of protein and mRNA levels among species. *DNA Cell Biol* 9: 737-748, 1990.
147. **Shenolikar S and Nairn AC.** Protein phosphatases: recent progress. *Advances in second messenger and phosphoprotein research* 23: 1-121, 1991.
148. **Shi Y.** Serine/threonine phosphatases: mechanism through structure. *Cell* 139: 468-484, 2009.
149. **Skyler JS and Oddo C.** Diabetes trends in the USA. *Diabetes Metab Res Rev* 18 Suppl 3: S21-26, 2002.
150. **Sriwijitkamol A, Coletta DK, Wajcberg E, Balbontin GB, Reyna SM, Barrientes J, Eagan PA, Jenkinson CP, Cersosimo E, DeFronzo RA, Sakamoto K, and Musi N.** Effect of acute exercise on AMPK signaling in skeletal muscle of subjects with type 2 diabetes: a time-course and dose-response study. *Diabetes* 56: 836-848, 2007.
151. **Stebbins CL and Bonigut S.** Endogenous bradykinin in the thoracic spinal cord contributes to the exercise pressor reflex. *J Appl Physiol* 81: 1288-1294, 1996.
152. **Stokoe D, Stephens LR, Copeland T, Gaffney PR, Reese CB, Painter GF, Holmes AB, McCormick F, and Hawkins PT.** Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science (New York, NY* 277: 567-570, 1997.
153. **Suter M, Riek U, Tuerk R, Schlattner U, Wallimann T, and Neumann D.** Dissecting the role of 5'-AMP for allosteric stimulation, activation, and deactivation of AMP-activated protein kinase. *The Journal of biological chemistry* 281: 32207-32216, 2006.

154. **Taguchi T, Kishikawa H, Motoshima H, Sakai K, Nishiyama T, Yoshizato K, Shirakami A, Toyonaga T, Shirontani T, Araki E, and Shichiri M.** Involvement of bradykinin in acute exercise-induced increase of glucose uptake and GLUT-4 translocation in skeletal muscle: studies in normal and diabetic humans and rats. *Metabolism* 49: 920-930, 2000.
155. **Tamemoto H, Kadowaki T, Tobe K, Yagi T, Sakura H, Hayakawa T, Terauchi Y, Ueki K, Kaburagi Y, Satoh S, and et al.** Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. *Nature* 372: 182-186, 1994.
156. **Tan MH and Bonen A.** Effect of exercise training on insulin binding and glucose metabolism in mouse soleus muscle. *Can J Physiol Pharmacol* 65: 2231-2234, 1987.
157. **Taniguchi CM, Emanuelli B, and Kahn CR.** Critical nodes in signalling pathways: insights into insulin action. *Nature reviews* 7: 85-96, 2006.
158. **Terada S, Goto M, Kato M, Kawanaka K, Shimokawa T, and Tabata I.** Effects of low-intensity prolonged exercise on PGC-1 mRNA expression in rat epitrochlearis muscle. *Biochemical and biophysical research communications* 296: 350-354, 2002.
159. **Thong FS, Derave W, Kiens B, Graham TE, Urso B, Wojtaszewski JF, Hansen BF, and Richter EA.** Caffeine-induced impairment of insulin action but not insulin signaling in human skeletal muscle is reduced by exercise. *Diabetes* 51: 583-590, 2002.
160. **Treadway JL, James DE, Burcel E, and Ruderman NB.** Effect of exercise on insulin receptor binding and kinase activity in skeletal muscle. *Am J Physiol* 256: E138-144, 1989.
161. **Treebak JT, Frosig C, Pehmoller C, Chen S, Maarbjerg SJ, Brandt N, MacKintosh C, Zierath JR, Hardie DG, Kiens B, Richter EA, Pilegaard H, and Wojtaszewski JF.** Potential role of TBC1D4 in enhanced post-exercise insulin action in human skeletal muscle. *Diabetologia* 52: 891-900, 2009.
162. **Treebak JT, Glund S, Deshmukh A, Klein DK, Long YC, Jensen TE, Jorgensen SB, Viollet B, Andersson L, Neumann D, Wallimann T, Richter EA, Chibalin AV, Zierath JR, and Wojtaszewski JF.** AMPK-mediated AS160 phosphorylation in skeletal muscle is dependent on AMPK catalytic and regulatory subunits. *Diabetes* 55: 2051-2058, 2006.
163. **Tuomilehto J, Lindstrom J, Eriksson JG, Valle TT, Hamalainen H, Ilanne-Parikka P, Keinanen-Kiukaanniemi S, Laakso M, Louheranta A, Rastas M, Salminen V, and Uusitupa M.** Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. *N Engl J Med* 344: 1343-1350, 2001.
164. **Vanhaesebroeck B and Alessi DR.** The PI3K-PDK1 connection: more than just a road to PKB. *The Biochemical journal* 346 Pt 3: 561-576, 2000.
165. **Vanhaesebroeck B, Leevers SJ, Panayotou G, and Waterfield MD.** Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends in biochemical sciences* 22: 267-272, 1997.
166. **Vettor R, De Palo C, Calo L, De Carlo E, Sicolo N, Martini C, and Federspil G.** Effect of exercise on plasma kallikrein and muscular phospholipase A2 activity in rats. *Molecular and cellular endocrinology* 45: 65-70, 1986.

167. **Walaas SI and Greengard P.** Protein phosphorylation and neuronal function. *Pharmacological reviews* 43: 299-349, 1991.
168. **Wallberg-Henriksson H.** Glucose transport into skeletal muscle. Influence of contractile activity, insulin, catecholamines and diabetes mellitus. *Acta Physiol Scand Suppl* 564: 1-80, 1987.
169. **Wallberg-Henriksson H, Constable SH, Young DA, and Holloszy JO.** Glucose transport into rat skeletal muscle: interaction between exercise and insulin. *J Appl Physiol* 65: 909-913, 1988.
170. **Wang X and Fiscus RR.** Lactic acid potentiates bradykinin- and low-pH-induced release of CGRP from rat spinal cord slices. *Am J Physiol* 273: E92-98, 1997.
171. **Weiser DC and Shenolikar S.** Use of protein phosphatase inhibitors. *Curr Protoc Mol Biol* Chapter 18: Unit 18 10, 2003.
172. **White MF.** Insulin signaling in health and disease. *Science (New York, NY* 302: 1710-1711, 2003.
173. **White MF and Kahn CR.** The insulin signaling system. *The Journal of biological chemistry* 269: 1-4, 1994.
174. **Winder WW.** Energy-sensing and signaling by AMP-activated protein kinase in skeletal muscle. *J Appl Physiol* 91: 1017-1028, 2001.
175. **Winder WW and Hardie DG.** AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. *Am J Physiol* 277: E1-10, 1999.
176. **Wojtaszewski JF, Hansen BF, Gade, Kiens B, Markuns JF, Goodyear LJ, and Richter EA.** Insulin signaling and insulin sensitivity after exercise in human skeletal muscle. *Diabetes* 49: 325-331, 2000.
177. **Wojtaszewski JF, Hansen BF, Kiens B, and Richter EA.** Insulin signaling in human skeletal muscle: time course and effect of exercise. *Diabetes* 46: 1775-1781, 1997.
178. **Wojtaszewski JF, Higaki Y, Hirshman MF, Michael MD, Dufresne SD, Kahn CR, and Goodyear LJ.** Exercise modulates postreceptor insulin signaling and glucose transport in muscle-specific insulin receptor knockout mice. *J Clin Invest* 104: 1257-1264, 1999.
179. **Wright DC, Hucker KA, Holloszy JO, and Han DH.** Ca(2+) and AMPK Both Mediate Stimulation of Glucose Transport by Muscle Contractions. *Diabetes* 53: 330-335, 2004.
180. **Yeh JI, Gulve EA, Rameh L, and Birnbaum MJ.** The effects of wortmannin on rat skeletal muscle. Dissociation of signaling pathways for insulin- and contraction-activated hexose transport. *The Journal of biological chemistry* 270: 2107-2111, 1995.
181. **Youngren JF.** Regulation of insulin receptor function. *Cell Mol Life Sci* 64: 873-891, 2007.
182. **Zerial M and McBride H.** Rab proteins as membrane organizers. *Nature reviews* 2: 107-117, 2001.
183. **Zhou Q and Dohm GL.** Treadmill running increases phosphatidylinositol 3-kinase activity in rat skeletal muscle. *Biochemical and biophysical research communications* 236: 647-650, 1997.
184. **Zhu T, Matsuzawa S, Mizuno Y, Kamibayashi C, Mumby MC, Andjelkovic N, Hemmings BA, Onoe K, and Kikuchi K.** The interconversion of protein phosphatase 2A between PP2A1 and PP2A0 during retinoic acid-induced granulocytic differentiation

and a modification on the catalytic subunit in S phase of HL-60 cells. *Archives of biochemistry and biophysics* 339: 210-217, 1997.
185. **Ziel FH, Venkatesan N, and Davidson MB.** Glucose transport is rate limiting for skeletal muscle glucose metabolism in normal and STZ-induced diabetic rats. *Diabetes* 37: 885-890, 1988.
186. **Zorzano A, Balon TW, Garetto LP, Goodman MN, and Ruderman NB.** Muscle alpha-aminoisobutyric acid transport after exercise: enhanced stimulation by insulin. *Am J Physiol* 248: E546-552, 1985.
187. **Zorzano A, Balon TW, Goodman MN, and Ruderman NB.** Glycogen depletion and increased insulin sensitivity and responsiveness in muscle after exercise. *Am J Physiol* 251: E664-669, 1986.

CHAPTER III

STUDY 1

The B2 receptor of Bradykinin is Not Essential for the Post-exercise Increase in Glucose Uptake by Insulin-stimulated Mouse Skeletal Muscle

ABSTRACT

Bradykinin can enhance skeletal muscle glucose uptake (GU), and exercise increases both bradykinin production and muscle insulin sensitivity, but bradykinin's relationship with post-exercise insulin action is uncertain. Our primary aim was to determine if the B2 receptor of bradykinin (B2R) is essential for the post-exercise increase in GU by insulin-stimulated mouse soleus and extensor digitorum longus (EDL) muscles. Wildtype (WT) and B2R knockout (B2RKO) mice were sedentary or performed 60 minutes of treadmill exercise that has been shown to increase glucose uptake in the insulin-stimulated soleus. Two experiments were conducted. For Experiment 1, paired soleus muscles were excised from WT and B2RKO mice (SED or EX) and incubated with [^{3}H]-2-deoxyglucose in the absence or presence of submaximally effective insulin (60μU/ml) to measure glucose uptake. For Experiment 2, paired soleus and EDL muscles were excised and incubated with [^{3}H]-2-deoxyglucose in the absence or presence of submaximally effective insulin (100μU/ml) and were used for measurement of glucose

uptake and Akt threonine phosphorylation (pThrAkt). In each experiment, glucose uptake was measured approximately 70-90 minutes after exercise. Data were analyzed by two-way ANOVA. GU tended to be greater for WT vs. B2RKO soleus with 60μU/mL insulin ($P=0.166$) and was significantly greater for soleus muscles with 100μU/mL insulin ($P<0.05$), but there was no difference in GU in the EDL. Both genotypes had significant exercise-induced reductions ($P<0.05$) in glycemia and insulinemia, and the decrements for glucose (~14%) and insulin (~55%) were similar between genotypes. GU tended to be greater for exercised vs. sedentary soleus with 60μU/mL insulin ($P=0.063$) and was significantly greater for muscles with 100μU/mL insulin ($P<0.05$). There was no significant effect of exercise on glucose uptake by the EDL, with or without insulin, in either muscle. There were no significant interactions between genotype and exercise for blood glucose, plasma insulin or GU. There were also no significant effects (exercise, genotype or interaction) on pThrAkt in either muscle, regardless of insulin concentration. These results indicate that the B2R is not essential for the exercise-induced decrements in blood glucose or plasma insulin or for the post-exercise increase in GU by insulin-stimulated mouse soleus muscle.

INTRODUCTION

A single exercise bout can lead to subsequently improved whole body insulin sensitivity, and skeletal muscle is the major tissue that accounts for this exercise-induced improvement in glucose disposal (22, 37). The increased glucose uptake by muscle is evident several hours after exercise cessation *in vivo* (38), and persists when rodent skeletal muscles are dissected out after exercise and studied *in vitro* (8, 9, 21).

The mechanisms whereby prior exercise initiates the improvement in skeletal muscle insulin sensitivity remain incompletely understood. Presumably, events that occur during exercise trigger the processes that subsequently lead to increased insulin-stimulated glucose transport. Exercise induces increased production of a circulating nonapeptide bradykinin (2, 3, 34, 42, 43). Bradykinin can favor a subsequent increase in insulin-stimulated glucose uptake (1, 15, 23-26, 35). These observations raised the possibility that bradykinin may participate in the post-exercise increase in skeletal muscle insulin sensitivity.

Skeletal muscle cells express the B2 receptor of bradykinin, B2R (14, 18, 36), which is important for bradykinin's influence on glucose uptake (1, 15, 18, 20). We studied mice that were null for B2R (B2 receptor of bradykinin knockout, B2RKO) and normal, wildtype (WT) mice under sedentary and post-exercise conditions to test the hypothesis that the B2R is essential for the post-exercise increase in glucose uptake by insulin-stimulated skeletal muscle.

METHODS

Materials. Human recombinant insulin was from Eli Lilly (Indianapolis, IN). 2-Deoxy-[^{3}H]glucose and [^{14}C]mannitol were from Perkin-Elmer (Boston, MA). Reagents and apparatus for SDS-PAGE, nonfat dry milk, and nitrocellulose membranes were from Bio-Rad Laboratories (Hercules, CA). A bicinchoninic acid assay kit for total protein determination and SuperSignal WestDura Extended Duration Substrate for immunodetection were from Pierce Biotechnology (Rockford, IL). Anti-phospho-Akt Thr^{308} and secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG) was

from Cell Signaling Technology. Other reagents were from Sigma-Aldrich (St. Louis, MO).

Animals. Animal care was approved by the University of Michigan Committee on Use and Care for Animals. Male mice null for the B2 receptor of bradykinin (B2RKO; strain 002641) and wildtype (WT) control mice (strain 101045; B6129SF2/J) were from the Jackson Laboratory (Bar Harbor, ME). All mice were housed in plastic cages and provided a standard diet (Lab Diet, PMI Nutrition International, Richmond, IN) and water ad libitum.

Treadmill exercise protocol. All mice (8–18 wk old) were familiarized with treadmill (Columbus Instruments, Columbus, OH) running for 10min on 2 consecutive days (1st day at 12–22m/min; 2nd day at 12–25 m/min). On the morning after the 2nd familiarization day, the WT and B2RKO mice were assigned to either a sedentary or exercised group. The exercise protocol consisted of 3 consecutive 20min-periods of progressive interval exercise (5min at 15m/min, 10min at 20m/min, and 5min at 25m/min with 0% slope) totaling 60min of running (21). Access to food was removed for all mice at the time that the protocol began. The approximate percentages of maximum O_2 uptake required for untrained mice running at the speeds used in this exercise protocol, based on the prediction equation of Fernando et al. (17), would be expected to be ~80% at 15 m/min, ~86% at 20 m/min, and ~91% at 25 m/min. All of the exercised mice completed the 60min protocol, after which exercised and sedentary mice were immediately anesthetized (intraperitoneal injection of pentobarbital sodium, 50mg/kg body wt) and rapidly dissected for muscle incubation. Therefore, glucose uptake was measured approximately 70-90 minutes after completion of exercise bout.

Blood glucose and plasma insulin. Blood was collected from the tail using heparinized capillary tubes, prior to anesthetization, in sedentary mice and in exercise mice immediately following the 60min treadmill protocol. Blood glucose was determined using an Accu-Check® Aviva (Roche Diagnostics, Indianapolis, IN) hand-held blood glucose meter. Blood was transferred to microcentrifuge tubes, centrifuged, and the plasma collected was used to assay insulin with the ALPCO Diagnostics™ Insulin (Mouse) Ultrasensitive EIA kit, catalog no. 80-INSMSU-E01 (Alpco Diagostics, Salem, NH).

Tissue dissection and muscle incubation. Paired soleus (Experiments 1 and 2) and extensor digitorum longus (EDL; Experiment 2) muscles from anesthetized mice were excised and incubated using a 2-step incubation protocol. During the 1st step, muscles were placed in vials containing 1.5mL of Krebs-Henseleit Buffer (KHB) supplemented with 0.1% bovine serum albumin (BSA), 2mM sodium pyruvate, and 6mM mannitol in the absence or presence of insulin (60μU/mL insulin was used for mice in Experiment 1; 100μU/mL insulin was used for mice in Experiment 2) for 60min. During all incubation steps, vials were placed in a heated (35°C), shaking water bath and continuously gassed from above (95% O_2-5% CO_2).

After the 1st incubation step, muscles were transferred to a 2nd vial containing KHB with 0.1% BSA, 1mM 2-deoxyglucose (2-deoxy-[^{3}H]glucose, 6 mCi/mmol), 9mM mannitol ([^{14}C]mannitol, 0.053 mCi/mmol), and the same insulin concentration as the previous step. Muscles were incubated at 35°C for 15min and then rapidly blotted on ice-cold filter paper, trimmed, freeze clamped, and stored at –80°C until processed.

Epididymal fat pads and gastrocnemius muscles were removed from some mice and weighed.

Muscle homogenization. Frozen muscles were weighed, transferred to prechilled glass tubes and homogenized in: 1) 0.5ml of perchloric acid (muscles from Experiment 1) or 2) ice-cold lysis buffer (0.5mL) containing 20mM Tris-HCl, 150mM NaCl, 1% NP-40, 1mM activated Na_3VO_4, 2mM EDTA, 2mM EGTA, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1μg/ml leupeptin (muscles from Experiment 2). Lysis buffer was used for the muscles analyzed for both glucose uptake and immunoblotting. Samples homogenized in perchloric acid were centrifuged (15,000g, 15min), and samples homogenized in the lysis buffer were rotated for ~1hr before being centrifuged (15,000g, 15min). Aliquots from supernatants were quantified for [^{3}H] and [^{14}C] using a liquid scintillation counter, and glucose uptake was calculated (7).

Immunoblotting. Portions of samples processed in lysis buffer were analyzed by immunoblotting for phospho-Akt Thr^{308}. Total protein concentration of the supernatants used for immunoblotting was determined by the bicinchoninic acid assay (41). Samples were resolved on a 10% SDS-PAGE gel and transferred to nitrocellulose in electrotransfer buffer overnight at 4°C. Blots were incubated in blocking solution [Tris-buffered saline (TBS) with 0.1% Tween 20 (TBST) and 5% nonfat dry milk] for 1hr at room temperature, washed with TBST and then incubated with anti-phospho-Akt Thr^{308} overnight at 4°C. Blots were washed with TBST and incubated with secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG). Blots were washed of excess antibody with TBST and then subjected to SuperSignal enhanced chemiluminesence (Thermo Scientific, Rockford, IL). Immunoreactive protein was quantified by

densitometry (Alpha Innotech, San Leandro, CA). The mean value for WT sedentary samples on each immunoblot, expressed in densitometry units relative to total protein, was adjusted to equal 1.0. Each sample value was expressed relative to the adjusted mean value for the WT sedentary control.

Statistical analyses. Statistical analyses used Sigma Stat version 2.0 (San Rafael, CA). Data are expressed as means ± SE. Two-way ANOVA was used to determine significant differences, and a Tukey post hoc test was used to identify the source of significant variance. A P value ≤ 0.05 was considered statistically significant.

RESULTS

There were no statistically significant interactions (exercise x genotype) for any of the measurements that were made.

Body and tissue masses. Body mass was similar between genotypes, but the epididymal fat pad/ body mass ratio was ~30-37% lower ($P<0.001$) for B2RKO compared to WT mice (Table 1). The soleus/body mass ratio was ~10% greater ($P<0.05$) and the EDL/body mass ratio was 15% greater ($P<0.05$) for B2RKO vs. WT mice, but there was not a significant effect of genotype on gastrocnemius/body mass ratio (Table 1).

Blood glucose and plasma insulin. Glycemia was 14% greater in B2RKO vs. WT mice under both sedentary and exercised conditions ($P<0.01$; Table 1). Within each genotype, exercise caused a similar ~14% decrease in blood glucose ($P<0.05$). Plasma insulin was ~32% lower ($P<0.05$) in the B2RKO vs. WT mice in sedentary and exercised

conditions. In both genotypes, exercise caused a 55% decrease in plasma insulin ($P<0.001$).

Muscle glucose uptake. In the soleus (Fig. 3.1A and 3.1B) and EDL (Fig. 3.2), there was not an effect of genotype on glucose uptake without insulin in either experiment or when the data from both soleus experiments were pooled (data not shown). Soleus glucose uptake with insulin was lower for B2RKO vs. WT mice with 100µU/mL insulin ($P<0.05$), and a non-significant trend ($P=0.166$) for lower values in the B2RKO mice was also evident with 60µU/mL insulin. For the pooled data from the insulin-treated soleus, glucose uptake was significantly lower ($P<0.05$) in the B2RKO compared to WT mice. EDL glucose uptake with insulin was not different between genotypes. Paired muscles were used for glucose uptake measurements, with one muscle from each mouse incubated without insulin and the contralateral muscle incubated with insulin. We calculated the insulin-stimulated increase in glucose uptake (delta insulin) by subtracting the basal value from the insulin-stimulated value. Soleus delta insulin was significantly lower for B2RKO vs. WT mice with 60µU/mL ($P<0.05$), 100µU/mL ($P<0.05$) and when the data from both experiments were pooled ($P<0.001$). EDL delta insulin was not different between genotypes.

Soleus glucose uptake without insulin tended to be slightly higher for exercised vs. sedentary mice in both Experiment 1 ($P=0.090$; Fig. 3.1A) and Experiment 2 ($P=0.087$; Fig. 3.1B). The pooled values for soleus glucose uptake without insulin from both experiments were significantly greater for the exercised vs. sedentary ($P<0.05$). In the insulin-stimulated soleus, glucose uptake tended to be greater for the exercised vs. sedentary mice in Experiment 1 ($P=0.063$ with 60µU/ml; Fig. 3.1A) and was significantly

greater in Experiment 2 ($P<0.05$ with 100µU/ml; Fig. 3.1B). When data from both experiments were pooled, soleus glucose uptake of insulin-stimulated muscles was significantly greater ($P<0.005$) for exercised vs. sedentary mice. Soleus delta insulin tended to be greater for exercised vs. sedentary mice with 60µU/mL ($P=0.150$) or 100µU/mL ($P=0.094$), and the delta insulin values were significantly greater after exercise when the data from both experiments were pooled ($P<0.05$).

Glucose uptake by the EDL, determined only for Experiment 2, was not altered by exercise or genotype without or with 100µU/mL of insulin (Fig. 3.2).

Akt^{Thr308} phosphorylation. In both soleus (Fig 3.3) and EDL (Fig 3.4), there was a significant increase in Akt^{Thr308} phosphorylation when incubating muscles with insulin compared to incubating muscles without insulin. Soleus (Fig. 3.3) and EDL Akt^{Thr308} phosphorylation (Fig. 3.4) were not significantly altered by exercise or genotype, with or without insulin, or for delta insulin (Fig. 3.3).

DISCUSSION

Improved whole body insulin sensitivity is a hallmark-effect of prior exercise. A post-exercise elevation in insulin-stimulated glucose uptake by skeletal muscle has been found in humans (38, 46), rats (8, 9, 37), and mice (4, 5, 21). The primary aim of this study was to determine if the B2R is essential for the post-exercise increase in glucose uptake in insulin-stimulated skeletal muscle. Prior exercise resulted in an increased glucose uptake by soleus muscles incubated with 100µU/mL insulin and a trend ($P=0.063$) for increased glucose uptake by soleus muscles incubated with 60µU/mL insulin. The lack of a significant interaction between genotype and exercise on glucose

uptake by soleus muscles incubated with either insulin concentration indicates that the B2R is not essential for the post-exercise increase in glucose uptake. There was not an exercise effect on glucose uptake by the isolated EDL. Whereas earlier research has consistently indicated that treadmill exercise by mice can induce a subsequent increase in glucose uptake by the soleus, the results in the EDL have been more variable (4, 5, 21). The explanation for the apparently more robust exercise effect in soleus compared to EDL muscle of mice remains to be identified, e.g., whether it is related to fiber type (44), muscle recruitment (31), contractile properties (6), or something else. This study focused on glucose uptake, which is a rate-controlling process for skeletal muscle glucose metabolism. However, the current results do not eliminate the possibility that the B2R may influence other aspects of muscle metabolism, including glycogen synthesis, glucose oxidation, lipid synthesis, and fatty acid esterification that we did not assess.

Earlier research demonstrated that circulating bradykinin can be increased by exercise or muscle contraction (2, 3, 34, 42, 43). Furthermore, there is substantial evidence that bradykinin can elevate glucose uptake in both intact animals (11, 15, 32, 45) and isolated cells or tissues (1, 12, 26, 30, 33, 35). It seemed possible that bradykinin could be involved in the post-exercise increase in insulin-stimulated glucose transport. However, previous studies indicated that electrically stimulated contractions by isolated rat epitrochlearis in the presence of exogenous bradykinin did not cause a subsequent increase in glucose transport of insulin-stimulated muscles (16). Furthermore, HOE-140, a B2R inhibitor, did not reduce the ability of prior contraction in serum to increase insulin-dependent glucose transport (16). These results did not support a role for bradykinin in the post-contraction increase in insulin sensitivity, but because bradykinin

is very rapidly degraded by kininases, experiments using exogenous bradykinin should be interpreted cautiously. Accordingly, in the current study, we used *in vivo* exercise by mice lacking the B2R as a novel approach to assess bradykinin's potential importance for the persistent increase in insulin-stimulated glucose uptake after *in vivo* exercise. The current results for *in vivo* exercise extend the earlier findings using exogenous bradykinin or B2 receptor inhibitors with ex vivo muscle contractions. These results using several different approaches do not support the idea that exercise or contraction lead to subsequent elevation in insulin sensitivity as the result of a B2R-dependent mechanism.

The ~14% decline in blood glucose in both genotypes compares to an ~12-29% decrease in glycemia previously reported for normal mice after 60min of treadmill exercise (19, 28, 29, 47). Treadmill exercise caused an ~32% decline in plasma insulin concentration in both genotypes, which compares to published results indicating an ~33-52% reduction in circulating insulin after 60min of treadmill exercise by normal mice (19, 21, 27, 28). The absence of significant exercise x genotype interactions in the current study suggests that the expression of the B2R was not essential for exercise effects on either glycemia or insulinemia.

Glucose uptake by isolated soleus and EDL muscles in the absence of insulin was not different for B2RKO compared to WT mice. However, for soleus muscles with 100μU/mL insulin there was a genotype-associated reduction in glucose uptake for B2RKO vs. WT mice, which was not seen in the EDL. A previous study in mouse soleus and EDL muscle using HOE-140 with a maximal insulin challenge *in vivo* revealed a non-significant trend for a decrease in glucose uptake in the soleus, but not the EDL, in HOE-140 vs. saline injected mice (40). There is evidence from experiments using either

a genetic or pharmacologic approach suggesting that the B2R may be more important for controlling the insulin-stimulated glucose uptake of the soleus versus the EDL, but the explanation for the difference between muscles is uncertain.

The reduced glucose uptake seen in the soleus of B2RKO vs. WT mice are reminiscent of the previously reported results of a study using the euglycemic-hyperinsulinemic-clamp in which Duka et al. (15) reported a lower glucose infusion rate for B2RKO compared to WT mice. Duka et al. (15) reported a non-significant trend for a 13% increase in fasting glycemia for B2RKO vs. WT mice which corresponds to the 14% increase in blood glucose that we found in B2RKO mice. We found a lower value for plasma insulin concentration in the B2RKO vs. WT mice, whereas Duka et al. (15) did not find a significant difference between the genotypes for fasting insulin. Rather they found a non-significant trend for higher fasting insulin for B2RKO mice. The mice in the earlier study had been treated for 3 days prior to the clamp with captopril, an ACE inhibitor which also inhibits kininase II (the enzyme which degrades bradykinin). Furthermore, the mice in the current study were only fasted ~1hr prior to sampling plasma for the insulin assay. Duka et al. (15) did not explicitly describe the duration of the fast, but they cited the method used in an earlier euglycemic-hyperinsulinemic clamp study in which the animals underwent an overnight fast. The age and sex of the mice in the earlier study were not described. It is uncertain if these or other differences in experimental design account for the different effect of B2RKO on circulating insulin concentration. The lower plasma insulin concentration for B2RKO vs. WT mice suggests that there may have been decreased insulin secretion in the B2RKO animals.

The current study provides the first insulin signaling data for B2RKO mice. Muscle Akt^{Thr308} phosphorylation for the soleus and the EDL muscles was similar between B2RKO and WT mice implicating an Akt-independent mechanism for the insulin resistance found in the B2RKO soleus muscles. Muscle (soleus or EDL) Akt^{Thr308} phosphorylation was also not different for exercised compared to sedentary groups, consistent with earlier data after exercise by normal mice (21) and providing evidence that the increased insulin sensitivity after exercise, regardless of B2R expression, does not require enhanced Akt^{Thr308} phosphorylation.

The current results are consistent with previous studies (10, 39) in which body mass was not different between B2RKO and WT mice. Schanstra et al. (39) also found no difference between B2RKO and WT mice for food intake. A more recent study reported that B2RKO vs. WT mice had greater energy intake and energy expenditure concomitant with a ~25% reduction in total body fat content (per g body mass) determined by carcass analysis (13). The results for lower body fat are similar to the reduction in epididymal fat pad/body mass ratio for the B2RKO compared to WT mice in the current study. They also found the gastrocnemius mass (per g body mass) of the B2RKO mice to be ~19% greater than control mice. Although gastrocnemius mass was not significantly different between genotypes in the current study, the soleus/body mass ratio and the EDL/body mass ratio were greater for B2RKO versus WT mice. The metabolic phenotype of B2RKO mice occurs despite moderate decrements in body fat and increments in skeletal muscle mass which are typically expected to favor improved insulin sensitivity.

In conclusion, mice lacking the B2R compared to WT controls had a small reduction in glucose uptake by insulin-stimulated soleus muscles concomitant with an undiminished insulin-stimulated increase in Akt phosphorylation. Earlier *in vivo* results from B2RKO mice undergoing a hyperinsulinemic clamp demonstrated insulin resistance, but they had not directly evaluated skeletal muscle or assessed insulin signaling. The current results demonstrate that insulin resistance of the soleus muscles, but not the EDL muscles, lacking the B2R persists ex vivo and suggest this defect is secondary to an Akt-independent mechanism. The explanation for genotype having different effects on insulin-stimulated glucose uptake in the soleus compared to the EDL is unknown. The absence of the B2R also did not alter the effects of exercise on circulating glucose or insulin concentrations *in vivo* indicating that the B2R is not a major modulator of the important effects of acute exercise on either glycemia or insulinemia. Finally, the lack of a significant interaction between exercise and genotype for glucose uptake by insulin-stimulated skeletal muscle after exercise demonstrates that the B2R is not essential for the increased glucose uptake in insulin-stimulated soleus muscles after exercise by mice.

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	WT Sedentary	WT Exercise	B2RKO Sedentary	B2RKO Exercise
Body mass, g	26.4±0.6	26.3±0.7	26.8±0.5	26.4±0.6
Fat pad/body mass ratio (mg/g)	6.5±0.4	7.0±0.4	4.6±0.3*	4.4±0.3*
Soleus/body mass ratio (mg/g)	0.36±0.02	0.34±0.1	0.39±0.2*	0.38±0.2*
EDL/body mass ratio (mg/g)	0.37±0.02	0.39±0.01	0.45±0.02*	0.45±0.02*
Gastrocnemius/body mass ratio (mg/g)	4.7±0.1	4.7±0.1	4.9±0.3	5.0±0.1
Blood glucose, $mmol \cdot L^{-1}$	7.85±0.51	6.74±0.48†	8.91±0.25*	7.65±0.31†*
Plasma insulin, $ng \cdot mL^{-1}$	0.673±0.12	0.307±0.06†	0.455±.06*	0.205±.04†*

Table 3.1
Body mass and tissue/body mass ratios and glucose and insulin concentrations.
Values are means ± SE, n = 21-22 for body mass; n = 21-22 for soleus/body mass ratio; n = 16 for EDL/body mass ratio; n = 8 for gastrocnemius/body mass ratio; and n= 14-16 for epididymal fat pad/body mass ratio and glucose and insulin concentrations. *$P<0.05$ (WT vs. B2RKO). †$P<0.05$ (Sedentary vs. Exercise).

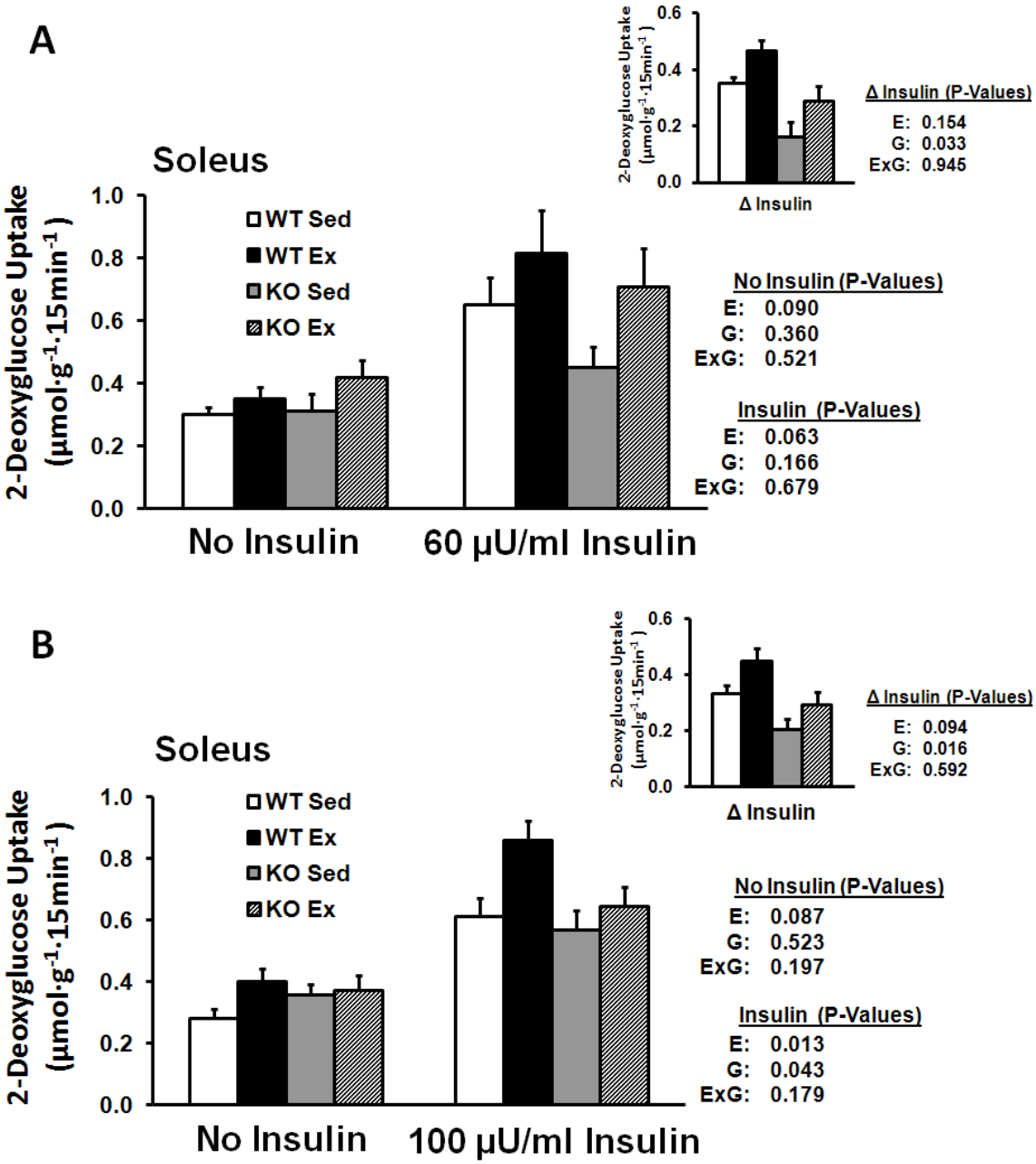

Figure 3.1

Glucose uptake by isolated soleus muscles. Experiment 1: without or with 60µU/mL insulin (A); Experiment 2: without or with 100µU/mL insulin (B) from WT and B2RKO mice that were sedentary (SED) or exercised (EX). Insets of (A) and (B) represent the insulin-stimulated increase in glucose uptake (delta insulin) calculated by subtracting the basal value from the insulin-stimulated value of paired muscles. Values are mean ± SE for 9 or 15-16 muscles per group (A and B, respectively). $P<0.05$. E, main effect of exercise treatment; G, main effect of genotype; E x G, interaction between main effects.

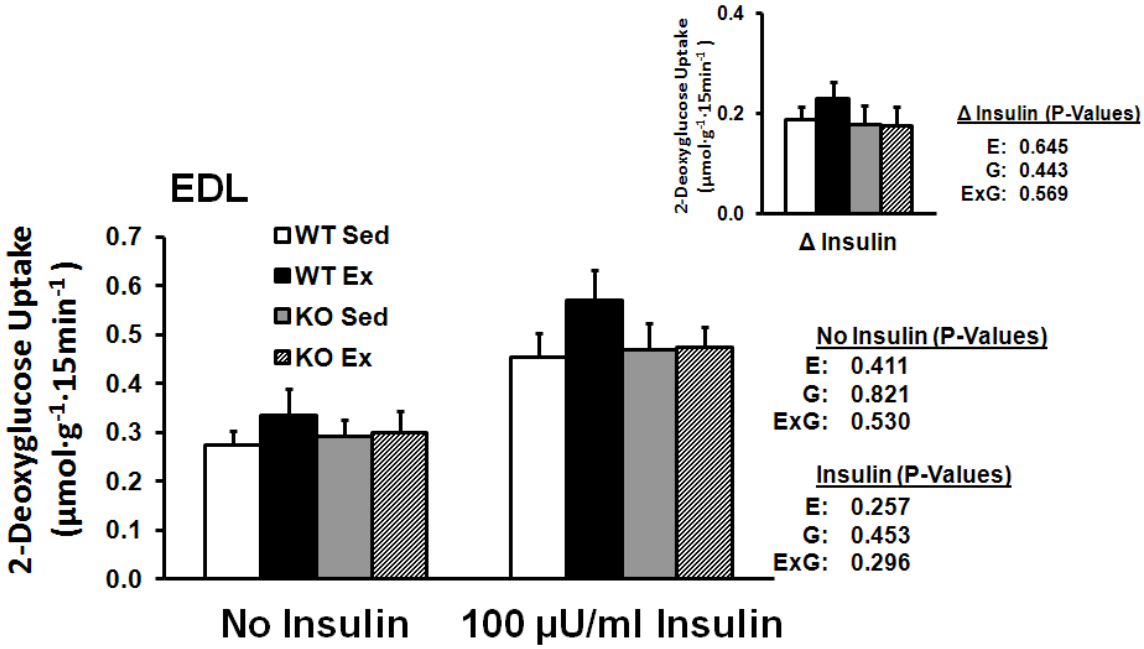

Figure 3.2
Glucose uptake by isolated extensor digitorum longus (EDL) muscles. Glucose uptake by isolated EDL muscles (Experiment 2) without or with 100µU/mL insulin (B) from WT and B2RKO mice that were sedentary (SED) or exercised (EX). Inset represents the insulin-stimulated increase in glucose uptake (delta insulin) calculated by subtracting the basal value from the insulin-stimulated value of paired muscles. Values are mean ± SE for 15-16 muscles per group. $P<0.05$. E, main effect of exercise treatment; G, main effect of genotype; E x G, interaction between main effects.

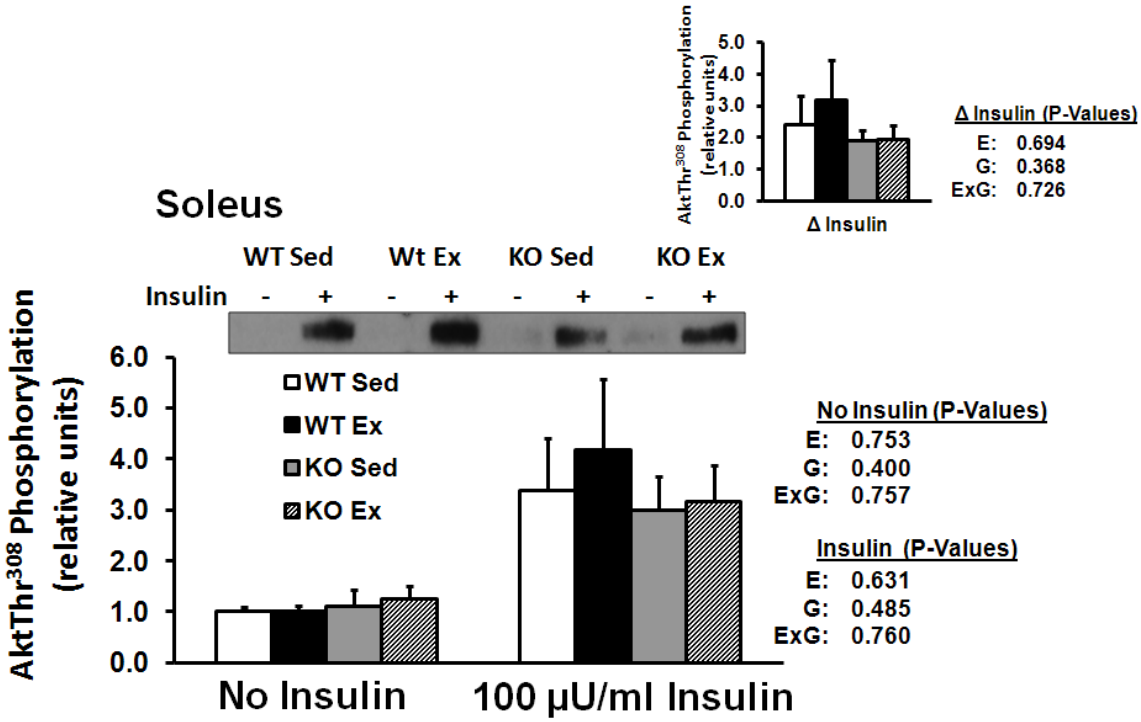

Figure 3.3
Akt^{Thr308} phosphorylation in isolated soleus. Akt^{Thr308} phosphorylation in isolated soleus from WT and B2RKO mice that were SED or EX. The insets represents the insulin-stimulated increase Akt^{Thr308} (delta insulin) calculated by subtracting the basal value from the insulin-stimulated value of paired muscles. Values are mean ± SE for 8 muscles per group.

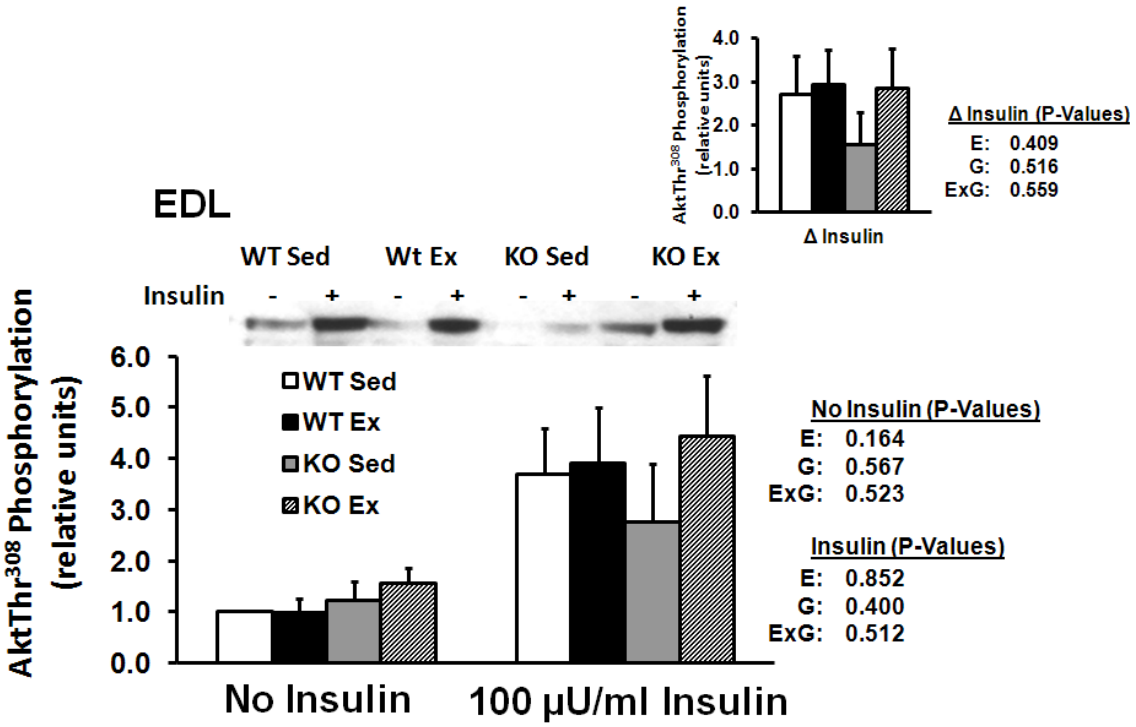

Figure 3.4
Akt^{Thr308} phosphorylation in isolated EDL. Akt^{Thr308} phosphorylation in isolated EDL from WT and B2RKO mice that were SED or EX. The insets represents the insulin-stimulated increase Akt^{Thr308} (delta insulin) calculated by subtracting the basal value from the insulin-stimulated value of paired muscles. Values are mean ± SE for 8 muscles per group.

REFERENCES

1. **Beard KM, Lu H, Ho K, and Fantus IG.** Bradykinin augments insulin-stimulated glucose transport in rat adipocytes via endothelial nitric oxide synthase-mediated inhibition of Jun NH2-terminal kinase. *Diabetes* 55: 2678-2687, 2006.
2. **Blais C, Jr., Adam A, Massicotte D, and Peronnet F.** Increase in blood bradykinin concentration after eccentric weight-training exercise in men. *J Appl Physiol* 87: 1197-1201, 1999.
3. **Boix F, Roe C, Rosenborg L, and Knardahl S.** Kinin peptides in human trapezius muscle during sustained isometric contraction and their relation to pain. *J Appl Physiol* 98: 534-540, 2005.
4. **Bonen A and Tan MH.** Dissociation between insulin binding and glucose utilization after intense exercise in mouse skeletal muscles. *Horm Metab Res* 21: 172-178, 1989.
5. **Bonen A, Tan MH, and Watson-Wright WM.** Effects of exercise on insulin binding and glucose metabolism in muscle. *Can J Physiol Pharmacol* 62: 1500-1504, 1984.
6. **Brooks SV and Faulkner JA.** Contractile properties of skeletal muscles from young, adult and aged mice. *The Journal of physiology* 404: 71-82, 1988.
7. **Cartee GD and Bohn EE.** Growth hormone reduces glucose transport but not GLUT-1 or GLUT-4 in adult and old rats. *Am J Physiol* 268: E902-909, 1995.
8. **Cartee GD and Holloszy JO.** Exercise increases susceptibility of muscle glucose transport to activation by various stimuli. *Am J Physiol* 258: E390-393, 1990.
9. **Cartee GD, Young DA, Sleeper MD, Zierath J, Wallberg-Henriksson H, and Holloszy JO.** Prolonged increase in insulin-stimulated glucose transport in muscle after exercise. *Am J Physiol* 256: E494-499, 1989.
10. **Cervenka L, Harrison-Bernard LM, Dipp S, Primrose G, Imig JD, and El-Dahr SS.** Early onset salt-sensitive hypertension in bradykinin B(2) receptor null mice. *Hypertension* 34: 176-180, 1999.
11. **Damas J, Bourdon V, and Lefebvre PJ.** Insulin sensitivity, clearance and release in kininogen-deficient rats. *Exp Physiol* 84: 549-557, 1999.
12. **Damas J, Garbacki N, and Lefebvre PJ.** The kallikrein-kinin system, angiotensin converting enzyme inhibitors and insulin sensitivity. *Diabetes Metab Res Rev* 20: 288-297, 2004.
13. **de Picoli Souza K, Batista EC, Silva ED, Reis FC, Silva SMA, Araujo RC, Luz J, Santos EL, and Pesquero JB.** Effect of kinin B2 receptor ablation on skeletal muscle development and myostatin gene expression. *Neuropeptides* 44: 209-214, 2010.
14. **Duka A, Duka I, Gao G, Shenouda S, Gavras I, and Gavras H.** Role of bradykinin B1 and B2 receptors in normal blood pressure regulation. *Am J Physiol Endocrinol Metab* 291: E268-274, 2006.
15. **Duka I, Shenouda S, Johns C, Kintsurashvili E, Gavras I, and Gavras H.** Role of the B(2) receptor of bradykinin in insulin sensitivity. *Hypertension* 38: 1355-1360, 2001.
16. **Dumke CL, Kim J, Arias EB, and Cartee GD.** Role of kallikrein-kininogen system in insulin-stimulated glucose transport after muscle contractions. *J Appl Physiol* 92: 657-664, 2002.

17. **Fernando P, Bonen A, and Hoffman-Goetz L.** Predicting submaximal oxygen consumption during treadmill running in mice. *Can J Physiol Pharmacol* 71: 854-857, 1993.
18. **Figueroa CD, Dietze G, and Muller-Esterl W.** Immunolocalization of bradykinin B2 receptors on skeletal muscle cells. *Diabetes* 45 Suppl 1: S24-28, 1996.
19. **Fritsche L, Hoene M, Lehmann R, Ellingsgaard H, Hennige AM, Pohl AK, Haring HU, Schleicher ED, and Weigert C.** IL-6 deficiency in mice neither impairs induction of metabolic genes in the liver nor affects blood glucose levels during fasting and moderately intense exercise. *Diabetologia* 53: 1732-1742, 2010.
20. **Goldman J, Pfister D, and Vukmirovich R.** Potentiation of insulin stimulation of hexose transport by kallikrein and bradykinin in isolated rat adipocytes. *Molecular and cellular endocrinology* 50: 183-191, 1987.
21. **Hamada T, Arias EB, and Cartee GD.** Increased submaximal insulin-stimulated glucose uptake in mouse skeletal muscle after treadmill exercise. *J Appl Physiol* 101: 1368-1376, 2006.
22. **Henriksen EJ.** Invited review: Effects of acute exercise and exercise training on insulin resistance. *J Appl Physiol* 93: 788-796, 2002.
23. **Henriksen EJ and Jacob S.** Effects of captopril on glucose transport activity in skeletal muscle of obese Zucker rats. *Metabolism* 44: 267-272, 1995.
24. **Henriksen EJ, Jacob S, Augustin HJ, and Dietze GJ.** Glucose transport activity in insulin-resistant rat muscle. Effects of angiotensin-converting enzyme inhibitors and bradykinin antagonism. *Diabetes* 45 Suppl 1: S125-128, 1996.
25. **Henriksen EJ, Jacob S, Fogt DL, and Dietze GJ.** Effect of chronic bradykinin administration on insulin action in an animal model of insulin resistance. *Am J Physiol* 275: R40-45, 1998.
26. **Henriksen EJ, Jacob S, Kinnick TR, Youngblood EB, Schmit MB, and Dietze GJ.** ACE inhibition and glucose transport in insulinresistant muscle: roles of bradykinin and nitric oxide. *Am J Physiol* 277: R332-336, 1999.
27. **Higaki Y, Wojtaszewski JF, Hirshman MF, Withers DJ, Towery H, White MF, and Goodyear LJ.** Insulin receptor substrate-2 is not necessary for insulin- and exercise-stimulated glucose transport in skeletal muscle. *J Biol Chem* 274: 20791-20795, 1999.
28. **Hoene M, Lehmann R, Hennige AM, Pohl AK, Haring HU, Schleicher ED, and Weigert C.** Acute regulation of metabolic genes and insulin receptor substrates in the liver of mice by one single bout of treadmill exercise. *The Journal of physiology* 587: 241-252, 2009.
29. **Howlett KF, Sakamoto K, Hirshman MF, Aschenbach WG, Dow M, White MF, and Goodyear LJ.** Insulin signaling after exercise in insulin receptor substrate-2-deficient mice. *Diabetes* 51: 479-483, 2002.
30. **Isami S, Kishikawa H, Araki E, Uehara M, Kaneko K, Shirotani T, Todaka M, Ura S, Motoyoshi S, Matsumoto K, Miyamura N, and Shichiri M.** Bradykinin enhances GLUT4 translocation through the increase of insulin receptor tyrosine kinase in primary adipocytes: evidence that bradykinin stimulates the insulin signalling pathway. *Diabetologia* 39: 412-420, 1996.

31. **James RS, Altringham JD, and Goldspink DF.** The mechanical properties of fast and slow skeletal muscles of the mouse in relation to their locomotory function. *The Journal of experimental biology* 198: 491-502, 1995.
32. **Kohlman O, Jr., Neves Fde A, Ginoza M, Tavares A, Cezaretti ML, Zanella MT, Ribeiro AB, Gavras I, and Gavras H.** Role of bradykinin in insulin sensitivity and blood pressure regulation during hyperinsulinemia. *Hypertension* 25: 1003-1007, 1995.
33. **Kudoh A, Dietze GJ, and Rabito SF.** Insulin enhances the bradykinin response in L8 rat skeletal myoblasts. *Diabetes* 49: 190-194, 2000.
34. **Langberg H, Bjorn C, Boushel R, Hellsten Y, and Kjaer M.** Exercise-induced increase in interstitial bradykinin and adenosine concentrations in skeletal muscle and peritendinous tissue in humans. *The Journal of physiology* 542: 977-983, 2002.
35. **Miyata T, Taguchi T, Uehara M, Isami S, Kishikawa H, Kaneko K, Araki E, and Shichiri M.** Bradykinin potentiates insulin-stimulated glucose uptake and enhances insulin signal through the bradykinin B2 receptor in dog skeletal muscle and rat L6 myoblasts. *Eur J Endocrinol* 138: 344-352, 1998.
36. **Rabito SF, Minshall RD, Nakamura F, and Wang LX.** Bradykinin B2 receptors on skeletal muscle are coupled to inositol 1,4,5-trisphosphate formation. *Diabetes* 45 Suppl 1: S29-33, 1996.
37. **Richter EA, Garetto LP, Goodman MN, and Ruderman NB.** Muscle glucose metabolism following exercise in the rat: increased sensitivity to insulin. *J Clin Invest* 69: 785-793, 1982.
38. **Richter EA, Mikines KJ, Galbo H, and Kiens B.** Effect of exercise on insulin action in human skeletal muscle. *J Appl Physiol* 66: 876-885, 1989.
39. **Schanstra JP, Duchene J, Praddaude F, Bruneval P, Tack I, Chevalier J, Girolami J-P, and Bascands J-L.** Regulation of Cardiovascular Signaling by Kinins and Products of Similar Converting Enzyme Systems: Decreased renal NO excretion and reduced glomerular tuft area in mice lacking the bradykinin B2 receptor. *Am J Physiol Heart Circ Physiol %R 101152/ajpheart011502002* 284: H1904-1908, 2003.
40. **Shiuchi T, Nakagami H, Iwai M, Takeda Y, Cui T, Chen R, Minokoshi Y, and Horiuchi M.** Involvement of bradykinin and nitric oxide in leptin-mediated glucose uptake in skeletal muscle. *Endocrinology* 142: 608-612, 2001.
41. **Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, and Klenk DC.** Measurement of protein using bicinchoninic acid. *Anal Biochem* 150: 76-85, 1985.
42. **Stebbins CL, Carretero OA, Mindroiu T, and Longhurst JC.** Bradykinin release from contracting skeletal muscle of the cat. *J Appl Physiol* 69: 1225-1230, 1990.
43. **Taguchi T, Kishikawa H, Motoshima H, Sakai K, Nishiyama T, Yoshizato K, Shirakami A, Toyonaga T, Shirontani T, Araki E, and Shichiri M.** Involvement of bradykinin in acute exercise-induced increase of glucose uptake and GLUT-4 translocation in skeletal muscle: studies in normal and diabetic humans and rats. *Metabolism* 49: 920-930, 2000.
44. **Taylor EB, An D, Kramer HF, Yu H, Fujii NL, Roeckl KS, Bowles N, Hirshman MF, Xie J, Feener EP, and Goodyear LJ.** Discovery of TBC1D1 as an insulin-, AICAR-, and contraction-stimulated signaling nexus in mouse skeletal muscle. *J Biol Chem* 283: 9787-9796, 2008.

45. **Uehara M, Kishikawa H, Isami S, Kisanuki K, Ohkubo Y, Miyamura N, Miyata T, Yano T, and Shichiri M.** Effect on insulin sensitivity of angiotensin converting enzyme inhibitors with or without a sulphydryl group: bradykinin may improve insulin resistance in dogs and humans. *Diabetologia* 37: 300-307, 1994.
46. **Wojtaszewski JF, Hansen BF, Gade, Kiens B, Markuns JF, Goodyear LJ, and Richter EA.** Insulin signaling and insulin sensitivity after exercise in human skeletal muscle. *Diabetes* 49: 325-331, 2000.
47. **Wojtaszewski JF, Higaki Y, Hirshman MF, Michael MD, Dufresne SD, Kahn CR, and Goodyear LJ.** Exercise modulates postreceptor insulin signaling and glucose transport in muscle-specific insulin receptor knockout mice. *J Clin Invest* 104: 1257-1264, 1999.

CHAPTER IV

STUDY 2

Post-exercise Skeletal Muscle Glucose Transport is Undiminished in Kininogen-Deficient Rats

ABSTRACT

A single exercise bout stimulates skeletal muscle glucose transport (GT) in the absence or presence of insulin. It has been suggested that the Kallikrein-Kininogen System (KKS) may contribute to exercise effects on both insulin-independent and insulin-dependent glucose transport. Plasma kininogen, a key KKS component, is a protein substrate for the enzyme kallikrein and the source of the peptide bradykinin. The purpose of the study was to determine if the post-exercise (PEX) increase in insulin-dependent or insulin-independent GT is reduced in rats deficient in plasma kininogen vs. normal rats. Male Brown Norway (BN) and Brown Norway Katholiek (BNK; plasma kininogen deficient) rats were studied. BN and BNK rats were assigned to exercise (4 x 30 minute swim) or sedentary (SED) groups. Rats were anesthetized immediately (0hPEX) or 3 hours (3hPEX) after exercise. For 0hPEX and 0hSED rats, one epitrochlearis muscle per rat was used for AMPK phosphorylation and muscle glycogen analyses. The contralateral muscle was incubated with [^{3}H]-3-O-methylglucose (3MG) for GT assay. For 3hPEX

and 3hSED rats, one muscle from each rat was incubated without insulin, and the contralateral muscle was incubated with 60μU/ml insulin and both muscles were incubated with 3MG for GT measurement. For 0hPEX vs. 0hSED, both BN and BNK rats had greater insulin-independent GT and AMPK phosphorylation with reduced glycogen. No genotype effects were found 0hPEX. There was a significant main effect of exercise (3hPEX > 3hSED) and no interaction between exercise and genotype for basal or insulin-stimulated GT. Plasma kininogen deficiency did not alter insulin-independent GT, AMPK phosphorylation or glycogen depletion 0hPEX or insulin-dependent GT 3hPEX suggesting that normal plasma kininogen is not essential for these important exercise effects.

INTRODUCTION

Skeletal muscle glucose transport is stimulated both by insulin and exercise. A single exercise session can stimulate glucose transport in the absence of insulin (insulin-independent), and it can also increase insulin's ability to increase glucose transport (insulin-dependent) (9, 10, 12, 43, 45, 53). Most of the insulin independent effect is lost ~3 hours post-exercise in rat muscle, at which time glucose transport in the presence of physiological insulin concentration is elevated, i.e., insulin sensitivity is increased (9, 10, 45, 53).

The mechanisms leading to the increased insulin-independent glucose transport immediately after exercise and increased insulin-dependent glucose transport several hours after exercise are not fully understood. Several lines of evidence have led to the proposal that the Kallikrein-Kininogen System (KKS) might play a role in the effects of

exercise on skeletal muscle glucose transport (19, 33, 50). Exercise or skeletal muscle contraction can activate the KKS as evidenced by increased circulating levels of bradykinin, a nonapeptide that is produced as the result of the interaction between two plasma proteins (kallikrein and kininogen) which are essential elements of the KKS (4, 35, 49). Exercise has been shown to increase the activity of kallikrein (52), the enzyme which catalyzes the proteolytic cleavage of bradykinin from plasma kininogen (3). Bradykinin binding to the B2 receptor of bradykinin has been implicated as a mediator of increased glucose transport and improved insulin sensitivity (11, 20, 27-29, 34, 38).

Gao et al. (24) demonstrated that electrically stimulated contraction by isolated rat epitrochlearis muscle in the presence of serum, but not in the absence of serum, can lead to a subsequent improvement in insulin sensitivity. They also provided convincing evidence that the enhanced insulin-stimulated glucose transport in this model requires the presence of a serum protein with a molecular mass greater than 10 kDa. The molecular masses of kallikrein and kininogen are greater than 10 kDa, raising the possibility that KKS proteins might be required for the post-contraction increase in glucose transport. Dumke et al. (21) found that muscles stimulated to contract in kallikrein-deficient plasma compared to muscles that performed contractions in normal plasma had reduced insulin-stimulated glucose transport. Although electrically stimulated contraction is a valuable model, it is not a perfect replica of *in vivo* exercise. Accordingly, we used *in vivo* exercise for the current study.

Brown Norway Katholiek (BNK) rats have a spontaneous point mutation in the kininogen gene which causes circulating kininogen deficiency by interfering with secretion of the liver synthesized kininogen (14-17). Plasma kininogen values of BNK

rats have been reported to be less than 1% of the normal plasma values for Brown Norway (BN) rats (41). Urinary kinin release is readily measured in normal rats and barely detectable in BNK rats (36, 37, 39). BNK rats have also been reported to have moderate *in vivo* insulin resistance and fasting normoglycemia compared to BN rats (16). The primary hypothesis tested by this study was that BNK compared to BN rats are characterized by a reduction in post-exercise insulin-stimulated glucose transport. It has also been suggested that activation of the KKS may play a role in the exercise-induced increase in insulin-independent glucose transport (33, 50). Therefore, a secondary aim was to compare the post-exercise increase in insulin-independent glucose transport in skeletal muscles from BN and BNK rats.

METHODS

Materials. Human recombinant insulin was obtained from Eli Lilly (Indianapolis, IN). Reagents and apparatus for SDS-PAGE and immunoblotting were purchased from Bio-Rad (Hercules, CA). Bicinchoninic acid protein assay reagent (no. 23227), T-PER tissue protein extraction reagent (no. 78510) and West Dura Extended Duration Substrate (no. 34075) were purchased from Pierce Biotechnology (Rockford, IL). Goat anti-rabbit IgG horseradish peroxidase conjugate (no. 7074) and Anti-phospho-Thr172 AMPKα (pAMPK-Thr^{172}; no. 2531) were purchased from Cell Signaling Technology (Danvers, MA). 3-O-methyl-[^{3}H]glucose ([^{3}H]3-MG) was purchased from Sigma-Aldrich (St. Louis, MO). [^{14}C]Mannitol was from PerkinElmer (Waltham, MA). Other reagents were purchased from Sigma-Aldrich and Fisher Scientific (Pittsburgh, PA).

Animal treatment. Procedures for animal care were approved by the University of Michigan Committee on Use and Care of Animals, and the experiments were performed under adherence to American College of Sports Medicine animal care standards. Male Brown Norway (BN) rats (7-10 wk-old; 160 ± 3g; Charles River Laboratories, Wilmington, MA) and male Brown Norway Katholiek (BNK) rats (7-10 wk-old; 168 ± 8g; generously provided by Dr. Oscar Carretero) had ad libitum access to rodent chow (PMI Nutritional International, Brentwood, MO) and water until 1700 on the night before the experiments, when food was removed from their cages, and all of the rats remained fasted for the remainder of the study. On the following day, rats were randomly assigned to a post-exercise (PEX) or sedentary (SED) treatment. Beginning at ~0900, PEX rats swam in a barrel filled with water (35°C) to a depth of ~60 cm (7 or 8 rats/barrel) for 4 x 30 minute bouts, with a 5 minute rest period between each bout.

Experimental Design. PEX rats of each genotype (BN and BNK) were studied either immediately after exercise (0hPEX; n=7 for BN and n=7 for BNK) or 3 hours after exercise (3hPEX; n=16 for BN and n=15 for BNK). Sedentary rats remained in their cages while the PEX rats were swimming and were studied at times that matched either the 0hPEX group (0hSED; n=8 for BN and n=8 for BNK) or the 3hPEX group (3hSED; n=15 for BN and n=15 for BNK).

Muscle incubations. Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (5mg/100 g body wt) either immediately post-exercise (0hPEX) or 3-4 hours post-exercise (3hPEX) along with time-matched sedentary rats. For the 0hPEX experiment, following anesthetization, one epitrochlearis muscle from each rat was rapidly dissected out, trimmed, freeze-clamped using aluminum clamps cooled to the

temperature of liquid N_2, and stored at -80°C until analyzed. After dissection, the contralateral muscle in the 0hPEX study was incubated for 10 minutes in flasks containing Krebs-Henseleit Buffer (KHB) with 0.1% bovine serum albumin (BSA), 2 mM pyruvate, and 6 mM mannitol for 10 minutes. The muscles were then transferred to flasks containing KHB, 0.1% BSA, 8 mM 3-MG (including 0.25 mCi/mmol [^{3}H]3-MG), and 2 mM mannitol (including 0.1 mCi/mmol [^{14}C]mannitol). For both incubation steps, flasks were continuously gassed from above with 95% O_2-5% CO_2 and shaken in a heated water bath (30°C).

For the 3hPEX experiment, rats were dried following the final exercise bout and returned to their cage for 3 hours before being anesthetized. After anesthetization, both epitrochlearis muscles from each animal were dissected out. One muscle from each rat was incubated in KHB, 0.1% BSA, 8 mM glucose, 2 mM mannitol supplemented with 60µU/ml insulin (a physiological concentration), and the contralateral muscle was incubated in the same solution without insulin. Both muscles were incubated for 30 minutes in a shaking water bath at 35°C. Insulin remained present at the same concentration throughout all subsequent incubations. After the initial incubation, muscles were transferred to vials containing KHB, 0.1% BSA, 2 mM pyruvate, and 6mM mannitol with or without 60µU/ml insulin at 30°C for 10 minutes. Finally, muscles were transferred to flasks containing KHB, 0.1% BSA with 8 mM 3-MG (including 0.25 mCi/mmol [^{3}H]3-MG), and 2 mM mannitol (including 0.1 mCi/mmol [^{14}C]mannitol) with or without 60µU/ml insulin for determination of glucose transport rate. After incubation with 3-MG for 15 minutes, the muscles were rapidly blotted on filter paper dampened with incubation medium, trimmed, freeze-clamped, and stored at -80°C.

Homogenization, glucose transport measurement, and protein concentration determination. Frozen muscles used for glucose transport and immunoblotting (pAMPK-Thr^{172}) were homogenized in 1 ml ice-cold homogenization buffer (2 mM activated Na_3VO_4, 2 mM EDTA, 2 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM phenylmethanesulfonyl fluoride, and 1 µg/ml leupeptin in T-PER) using tissue grinder tubes (Kontes, Vineland, NJ). Homogenates were subsequently rotated at 4°C at 50 rpm for 1 hour before being centrifuged (15,000 g for 15 minutes at 4°C). Aliquots of the supernatant from muscles used for the 3-MG transport measurement were pipetted into vials with scintillation cocktail for scintillation counting, and 3-MG transport was determined as previously described (8). A portion of the supernatant was used to determine protein concentration by the bicinchoninic acid assay (48) according to the manufacturer's instructions (Pierce Biotechnology catalog no. 23227). The remaining supernatant was stored at -80°C until further analyzed.

Immunoblotting. Aliquots of supernatants from homogenized muscle lysates were combined with sodium dodecyl sulfate (SDS) loading buffer and separated by SDS-PAGE before being electrophoretically transferred to nitrocellulose. Samples were then rinsed with Tris-buffered saline plus Tween-20 (TBST) (20mM Tris base, 150mM NaCl, pH 7.6, and 0.1% Tween-20), blocked with 5% nonfat dry milk in TBST for 1 hour at room temperature, washed 3 x 5 minutes at room temperature, and treated with the primary antibody (anti-pAMPK-Thr^{172} at 1:1,000 in TBST with 5% BSA) overnight at 4°C. Blots were then washed 3 x 5 minutes with TBST, incubated with the secondary antibody, goat anti-rabbit IgG horseradish peroxidase conjugate (1:20,000 in TBST with 5% milk), for 1 hour at room temperature, washed again 3 x 5 minutes with TBST, and

developed with West Dura SuperSignal enhanced chemiluminescence reagent (Thermo Fisher Scientific, Waltham, MA). Protein bands were quantified by densitometry (Cell Biosciences, Santa Clara, CA). The mean values for sedentary samples without insulin on each blot were normalized to equal 1.0, and then all samples on the blot were expressed relative to the normalized sedentary without insulin value.

Muscle glycogen concentration. Muscles used for measurement of glycogen were frozen immediately after dissection, weighed, and homogenized in ice-cold 0.3 M perchloric acid. An aliquot of the homogenate was stored at -80°C for later determination of glycogen concentration by the amyloglucosidase method (40).

Statistical analyses. Statistical analyses used Sigma Stat version 2.0 (San Rafael, CA). Data are expressed as means ± SE. Two-way ANOVA was used to determine significant differences between the main effects of exercise and genotype, and a Tukey post hoc test was used to identify the source of significant variance. A P value ≤ 0.05 was considered statistically significant.

RESULTS

Muscle Glycogen. Epitrochlearis muscle glycogen was reduced in 0hPEX vs. 0hSED rats, and it was not significantly different for BNK compared to BN rats (Fig. 4.1).

AMPK Thr^{172} Phosphorylation. Epitrochlearis AMPK Thr^{172} phosphorylation was increased in 0hPEX vs. 0hSED rats, and it was not significantly different in BNK vs. BN rats (Fig. 4.2).

Muscle Glucose Transport. There was an increase in glucose transport in 0hPEX vs. 0hSED in both BN and BNK rats (Fig. 4.3). There was not a difference between

BNK and BN rats for the exercise-induced increase in insulin-independent glucose transport.

Glucose transport without insulin was significantly greater for 3hPEX vs. 3hSED rats in ($P < 0.05$; Fig. 4.4). There was not a significant effect of genotype (BNK vs. BN) on glucose transport without insulin in 3hSED and 3hPEX rats (Fig. 4.4).

In the insulin-stimulated epitrochlearis, glucose transport was significantly greater for 3hPEX vs. 3hSED rats ($P < 0.001$; Fig. 4.4). Glucose transport with insulin was not different for BNK vs. BN rats in 3hSED and 3hPEX groups. Paired muscles were used for glucose transport measurements, with one muscle from each rat incubated without insulin and the contralateral muscle incubated with insulin. We calculated the insulin-stimulated increase in glucose transport (delta insulin) by subtracting the basal value from the insulin-stimulated value for each rat. Delta insulin was also significantly greater for 3hPEX vs. 3hSED rats ($P < 0.05$). Delta insulin was not different for BNK compared to BN rats in the 3hSED and 3hPEX groups.

There were no significant interactions (exercise x genotype) for any of the measurements made.

DISCUSSION

A single exercise session can cause an increase in both insulin-dependent and insulin-independent glucose uptake by skeletal muscle in mice (6, 7, 26), rats (9, 10, 42, 54), and humans (44, 56). Several previous studies have demonstrated that the Kallikrein-Kininogen System (KKS) can be activated by exercise or muscle contractions (33, 49, 52), and it has been suggested that this activation might play a role in elevated

glucose uptake by skeletal muscle (19, 21, 50). The most important new findings of this study were that unexercised BN and BNK rats (which are known to be deficient in plasma kininogen) did not differ for insulin-stimulated glucose transport, and that the effects of exercise on both insulin-independent and insulin-dependent glucose transport were undiminished in the BNK rats.

Results of several published studies suggest that the KKS is linked to insulin-dependent glucose transport in the absence of exercise, but the results of other published studies are not consistent with this idea. In support, incubation with bradykinin has been reported to increase insulin-stimulation of GLUT4 translocation and glucose transport of L6 myocytes, 3T3-L1 adipocytes, or isolated dog adipocytes (2, 31, 38). In addition, previous studies found that insulin-stimulated glucose transport was greater in isolated epitrochlearis muscles that were dissected from obese Zucker rats that had been injected with bradykinin either acutely (2 hours prior to muscle dissection) or chronically (twice daily for 14 days) (29, 30). However, *in vivo* bradykinin treatment did not enhance insulin-stimulated glucose uptake by isolated epitrochlearis muscles from lean Zucker rats (29). Most relevant to the results of the current study, BNK vs. BN rats had a significantly reduced glucose disposal rate during a hyperinsulinemic-euglycemic clamp (16). In contrast, the current study did not provide evidence for insulin resistance in the isolated epitrochlearis of BNK vs. BN rats. The results from the *in vivo* clamp procedure would be directly influenced by systemic factors that were absent from the isolated muscle preparation, including blood flow, circulating hormones, and neural regulation. In addition, glucose disposal *in vivo* depends on multiple tissues, including the liver, adipose tissue, and skeletal muscle. It is also possible that the epitrochlearis is not

representative of the glucose uptake by all of the skeletal muscles in the body. However, the fiber type composition of the epitrochlearis (predominantly type IIb) corresponds closely to that reported for 32 muscles of the rat hindlimb (1), suggesting it is reasonable to use the epitrochlearis as a representative muscle based on fiber type. It is notable that Shimojo et al. (47) also did not find an effect of bradykinin on *in vitro*, insulin-stimulated glucose uptake by the predominantly type I soleus muscle. The current results clearly demonstrate that there is not intrinsic insulin resistance of the isolated epitrochlearis (composed of predominantly type II fibers) of BNK rats.

A number of studies have assessed the possible role of the KKS system in insulin-independent increase in skeletal muscle glucose transport with exercise or contraction. Dietze and Wicklmayr (19) found that most of the isometric exercise-induced increase in glucose uptake by the human forearm could be blocked by infusion of aprotinin (a kallikrein inhibitor). They also found that bradykinin infusion could eliminate aprotinin's inhibition of exercise-induced glucose uptake. Bradykinin can elevate blood flow, so the results for forearm glucose uptake during *in vivo* exercise were likely partly attributable to changes in muscle blood flow which was observed to be decreased by aprotinin and increased by bradykinin infusion. Consistent with this interpretation, studying isolated rat epitrochlearis muscles, Constable et al. (13) found that a range of bradykinin concentrations did not result in increased glucose transport, nor was contraction-stimulated glucose transport attenuated in the presence of aprotinin. Insulin-independent glucose uptake by L6 myotubes, 3T3-L1 adipocytes, and Chinese hamster ovary cells was greater for cells that stably overexpressed both GLUT4 and B2 receptor of bradykinin compared to cells that overexpressed only GLUT4 (33). However, in L6 cells

or isolated muscle strips from dogs without overexpression of B2 receptors of bradykinin, insulin-independent glucose transport was not elevated by bradykinin (38). Taguchi et al. (50) measured the sarcolemmal GLUT4 content and glucose uptake by sarcolemmal vesicles prepared from skeletal muscle that was sampled immediately post-exercise (1 hour swim by rats). Exercised rats were studied with or without 5 days of infusion of a B2 receptor of bradykinin inhibitor. The inhibitor caused an ~50% decline in the immediate post-exercise increases in membrane GLUT4 and glucose uptake. It seems possible that chronic treatment with a bradykinin B2 receptor inhibitor might induce adaptations that altered the effects of exercise on GLUT4 and glucose uptake. In the current study, using isolated epitrochlearis muscles, BNK vs. BN rats were not different for insulin-independent glucose transport measured immediately post-exercise. Although the KKS can influence blood flow and thereby indirectly alter *in vivo* glucose uptake during exercise, data from studies that have evaluated glucose transport by isolated skeletal muscle (in which blood flow is not a factor) after *in vivo* exercise or *in vitro* contractions provide no support for the idea that KKS is responsible for increased insulin-independent glucose transport after exercise or contractions.

Because exercise can activate the KKS leading to bradykinin production (4, 5, 35, 50), and because of multiple lines of evidence linking the KKS to enhanced insulin sensitivity in intact animals (16, 20, 34, 51) and isolated cells or tissues (2, 17, 30, 31, 38), it seemed possible that kininogen-deficient rats might not have a normal post-exercise elevation in insulin-stimulated glucose transport. However, the current results indicated that exercise effects on subsequent insulin-stimulated glucose transport are essentially the same for BN and BNK rats. We recently performed a study using the bradykinin B2

receptor null mouse as a different model to assess the possible role of the KKS in post-exercise glucose transport (46). Isolated skeletal muscles from the mice that were null for the B2 receptor of bradykinin and normal control mice did not differ for their post-exercise increase in insulin-dependent glucose transport after exercise. The results of experiments using two different animal models for important components of the KKS provide evidence that a normal KKS is not essential to attain a normal elevation in insulin-stimulated glucose transport by isolated skeletal muscles after *in vivo* exercise.

Exercise induces AMPK activation and glycogen depletion, and these effects have been suggested to be important for both increased insulin-independent and insulin-dependent glucose transport after exercise (18, 22, 25, 32, 55). The current results were apparently the first to evaluate exercise effects on AMPK phosphorylation or glycogen concentration in a KKS model. Kininogen-deficient rats, compared to normal rats, did not have altered glycogen depletion or AMPK activation as indicated by AMPK Thr172 phosphorylation after exercise. These observations are consistent with the lack of difference seen between the BN and BNK rats for insulin-independent glucose transport immediately post-exercise or insulin-dependent glucose transport 3 hours post-exercise.

Electrical stimulation of isolated skeletal muscle has often been used as model for *in vivo* exercise. Gao et al. (24) demonstrated that electrically stimulated contraction by isolated rat epitrochlearis muscle in the presence of serum, but not in the absence of serum, can lead to a subsequent improvement in insulin sensitivity. They also provided convincing evidence that the enhanced insulin-stimulated glucose transport in this model requires the presence of a serum protein with molecular mass greater than 10 kDa. The molecular masses of kallikrein and kininogen are greater than 10 kDa, and we (21)

previously hypothesized that KKS proteins might be required for the post-contraction increase in glucose transport. In support of this idea, Dumke et al. (21) reported that rat epitrochlearis muscles electrically stimulated to contract in kallikrein-deficient plasma compared to muscles stimulated to contract in normal plasma had lower insulin-stimulated glucose transport. However, *in vivo* exercise and *in vitro* contractions in serum may not lead to increased insulin-stimulated glucose transport by an identical mechanism. Funai et al. (23) found that when *in vivo* exercise is followed by rapid removal of the epitrochlearis and *in vitro* contraction of the isolated muscle, the two stimuli induced an additive effect on the subsequent increase in insulin-stimulated glucose transport. These results suggested that the increase in insulin-stimulated glucose transport induced after *in vitro* contraction and after *in vivo* exercise may be caused by distinct mechanisms. The current study focused on the effects of *in vivo* exercise because this intervention is more physiologically relevant than electrical stimulation.

In conclusion, insulin-stimulated glucose transport by isolated epitrochlearis muscles was similar for sedentary BN and BNK rats. Rats deficient in plasma kininogen compared to normal rats had similar post-exercise increases in both insulin-independent and insulin-dependent glucose transport by isolated epitrochlearis muscle. It is well-established that exercise has robust effects on insulin-independent and insulin-dependent glucose transport that persist in isolated muscles, and the current data indicate that these important exercise effects are undiminished in plasma kininogen-deficient rats.

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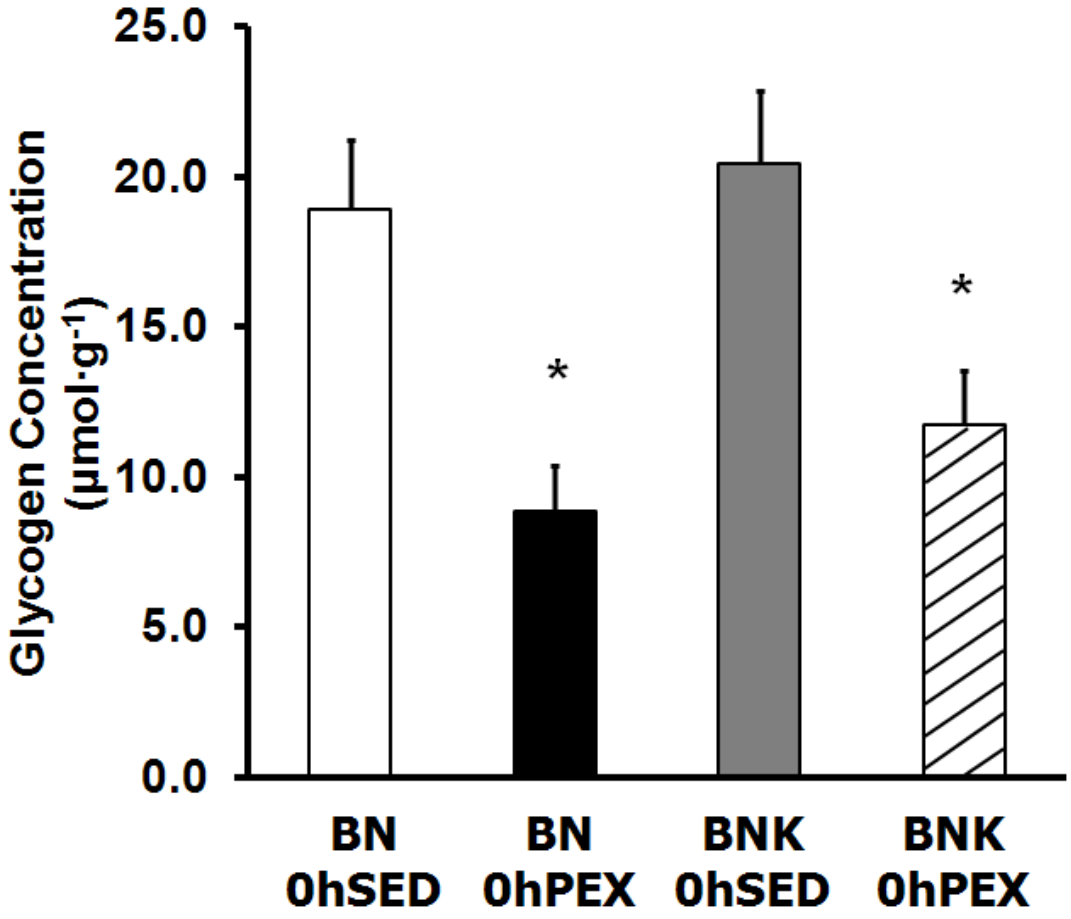

Figure 4.1
Muscle glycogen concentration in isolated epitrochlearis. Muscle glycogen concentration in isolated epitrochlearis from BN and BNK rats that were sedentary (0hSED) or exercised (0hPEX). Values are mean ±SE for 7-8 muscles per group. *P < 0.05 for exercised rats having lesser muscle glycogen than sedentary rats.

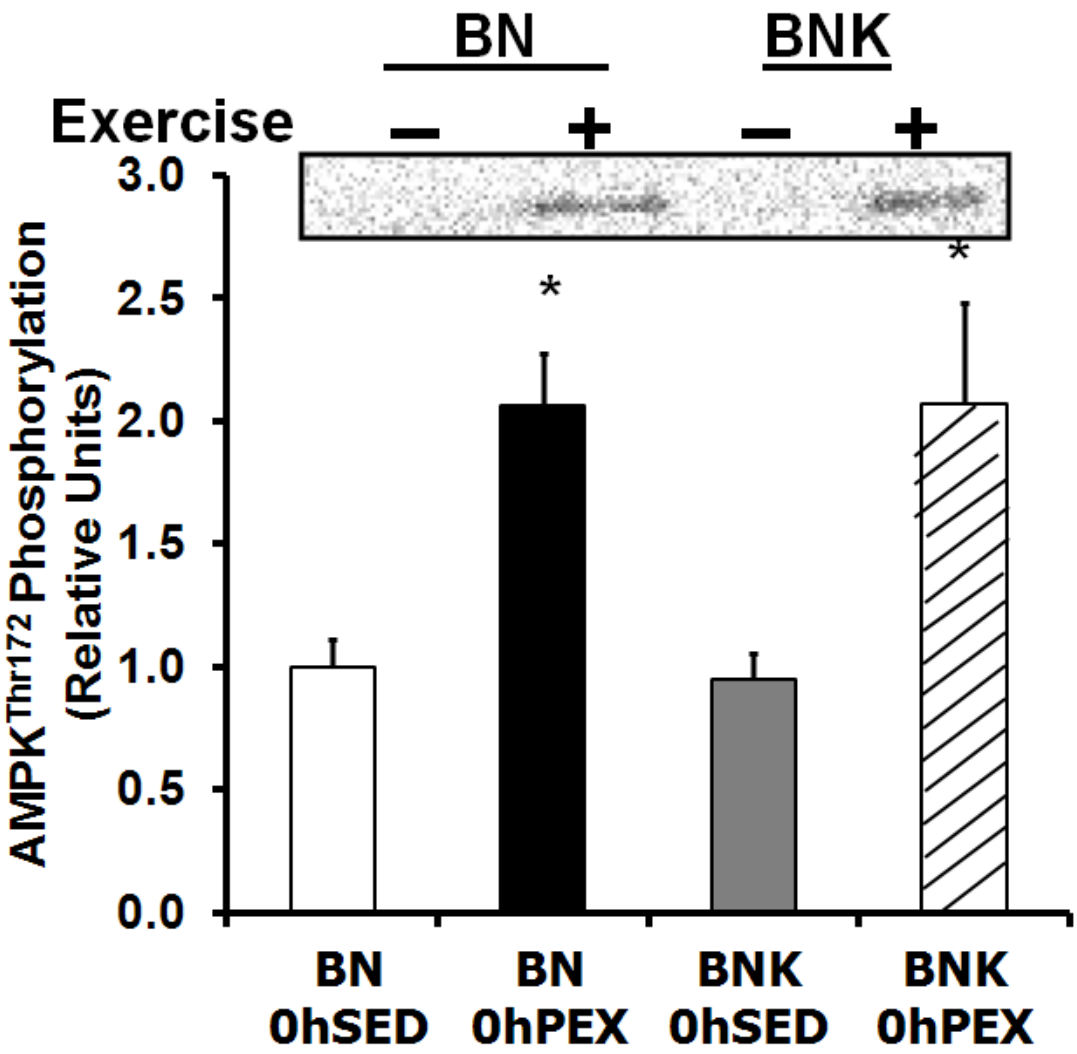

Figure 4.2
AMPK^{Thr172} phosphorylation in isolated epitrochlearis. AMPK^{Thr172} phosphorylation in isolated epitrochlearis from BN and BNK rats that were sedentary (0hSED) or exercised (0hPEX). Values are mean ±SE for 7-8 muscles per group. *$P < 0.05$ for exercised rats having greater phosphorylation than sedentary rats.

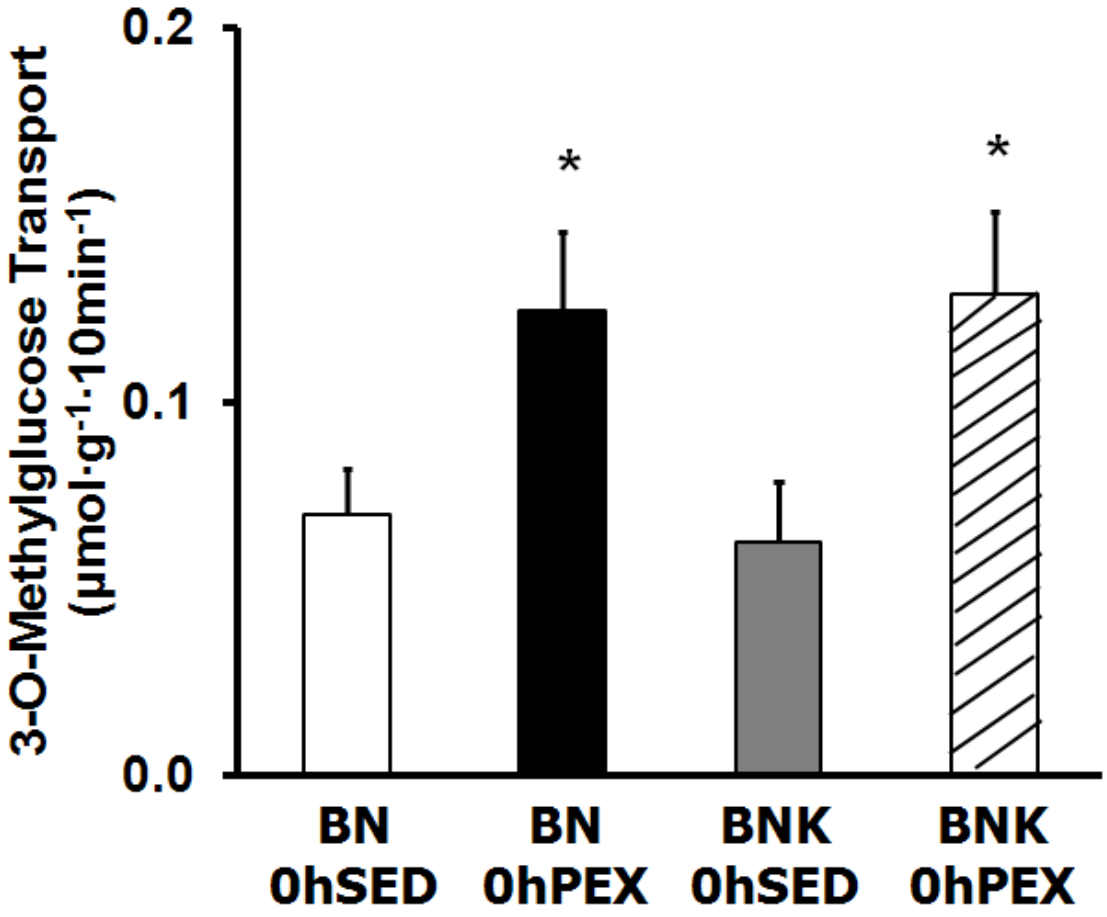

Figure 4.3
Immediate post-exercise glucose transport by isolated epitrochlearis muscle.
Glucose transport by isolated epitrochlearis muscles without insulin from BN and BNK rats that were sedentary (0hSED) or exercised (0hPEX). Values are mean ± SE for 7-8 muscles per group. *$P < 0.05$ for exercised rats having greater glucose transport than sedentary rats.

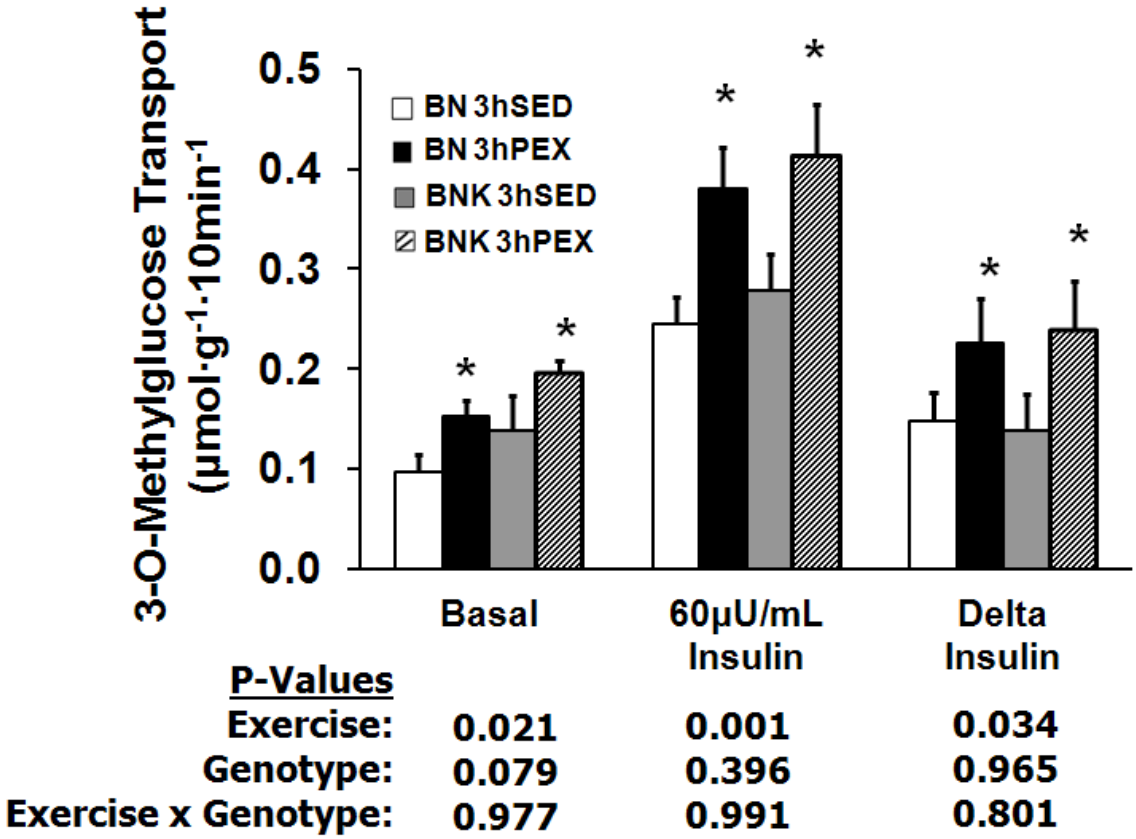

Figure 4.4
Three hour post-exercise insulin-stimulated glucose transport by isolated epitrochlearis muscle. Glucose transport by isolated epitrochlearis muscles without or with 60µU/mL insulin from BN and BNK rats that had undergone two hours of swimming exercise and rested for three hours (3hPEX) or time-matched sedentary controls (3hSED). Delta insulin represents the insulin-stimulated increase above basal in glucose transport calculated by subtracting the basal value from the insulin-stimulated value of paired muscles. Values are mean ± SE for 15-16 muscles per group. *$P < 0.05$. Exercise, main effect of exercise treatment; Genotype, main effect of genotype; Exercise x Genotype, interaction between main effects.

REFERENCES

1. **Armstrong RB and Phelps RO.** Muscle fiber type composition of the rat hindlimb. *Am J Anat* 171: 259-272, 1984.
2. **Beard KM, Lu H, Ho K, and Fantus IG.** Bradykinin augments insulin-stimulated glucose transport in rat adipocytes via endothelial nitric oxide synthase-mediated inhibition of Jun NH2-terminal kinase. *Diabetes* 55: 2678-2687, 2006.
3. **Bhoola KD, Figueroa CD, and Worthy K.** Bioregulation of kinins: kallikreins, kininogens, and kininases. *Pharmacol Rev* 44: 1-80, 1992.
4. **Blais C, Jr., Adam A, Massicotte D, and Peronnet F.** Increase in blood bradykinin concentration after eccentric weight-training exercise in men. *J Appl Physiol* 87: 1197-1201, 1999.
5. **Boix F, Roe C, Rosenborg L, and Knardahl S.** Kinin peptides in human trapezius muscle during sustained isometric contraction and their relation to pain. *J Appl Physiol* 98: 534-540, 2005.
6. **Bonen A and Tan MH.** Dissociation between insulin binding and glucose utilization after intense exercise in mouse skeletal muscles. *Horm Metab Res* 21: 172-178, 1989.
7. **Bonen A, Tan MH, and Watson-Wright WM.** Effects of exercise on insulin binding and glucose metabolism in muscle. *Can J Physiol Pharmacol* 62: 1500-1504, 1984.
8. **Cartee GD and Bohn EE.** Growth hormone reduces glucose transport but not GLUT-1 or GLUT-4 in adult and old rats. *Am J Physiol* 268: E902-909, 1995.
9. **Cartee GD and Holloszy JO.** Exercise increases susceptibility of muscle glucose transport to activation by various stimuli. *Am J Physiol* 258: E390-393, 1990.
10. **Cartee GD, Young DA, Sleeper MD, Zierath J, Wallberg-Henriksson H, and Holloszy JO.** Prolonged increase in insulin-stimulated glucose transport in muscle after exercise. *Am J Physiol* 256: E494-499, 1989.
11. **Carvalho CR, Thirone AC, Gontijo JA, Velloso LA, and Saad MJ.** Effect of captopril, losartan, and bradykinin on early steps of insulin action. *Diabetes* 46: 1950-1957, 1997.
12. **Constable SH, Favier RJ, Cartee GD, Young DA, and Holloszy JO.** Muscle glucose transport: interactions of in vitro contractions, insulin, and exercise. *J Appl Physiol* 64: 2329-2332, 1988.
13. **Constable SH, Favier RJ, Uhl J, and Holloszy JO.** Bradykinin does not mediate activation of glucose transport by muscle contraction. *J Appl Physiol* 61: 881-884, 1986.
14. **Damas J.** The brown Norway rats and the kinin system. *Peptides* 17: 859-872, 1996.
15. **Damas J and Adam A.** The kallikrein-kininogens-kinins system in the Brown-Norway rat. *Biomedicine* 31: 249, 1979.
16. **Damas J, Bourdon V, and Lefebvre PJ.** Insulin sensitivity, clearance and release in kininogen-deficient rats. *Exp Physiol* 84: 549-557, 1999.
17. **Damas J, Garbacki N, and Lefebvre PJ.** The kallikrein-kinin system, angiotensin converting enzyme inhibitors and insulin sensitivity. *Diabetes Metab Res Rev* 20: 288-297, 2004.

18. **Derave W, Ai H, Ihlemann J, Witters LA, Kristiansen S, Richter EA, and Ploug T.** Dissociation of AMP-activated protein kinase activation and glucose transport in contracting slow-twitch muscle. *Diabetes* 49: 1281-1287, 2000.
19. **Dietze G and Wicklmayr M.** Evidence for a participation of the kallikrein-kinin system in the regulation of muscle metabolism during muscular work. *FEBS Lett* 74: 205-208, 1977.
20. **Duka I, Shenouda S, Johns C, Kintsurashvili E, Gavras I, and Gavras H.** Role of the B(2) receptor of bradykinin in insulin sensitivity. *Hypertension* 38: 1355-1360, 2001.
21. **Dumke CL, Kim J, Arias EB, and Cartee GD.** Role of kallikrein-kininogen system in insulin-stimulated glucose transport after muscle contractions. *J Appl Physiol* 92: 657-664, 2002.
22. **Fisher JS, Gao J, Han DH, Holloszy JO, and Nolte LA.** Activation of AMP kinase enhances sensitivity of muscle glucose transport to insulin. *Am J Physiol Endocrinol Metab* 282: E18-23, 2002.
23. **Funai K, Schweitzer GG, Castorena CM, Kanzaki M, and Cartee GD.** In vivo exercise followed by in vitro contraction additively elevates subsequent insulin-stimulated glucose transport by rat skeletal muscle. *Am J Physiol Endocrinol Metab* 298: E999-1010, 2010.
24. **Gao J, Gulve EA, and Holloszy JO.** Contraction-induced increase in muscle insulin sensitivity: requirement for a serum factor. *Am J Physiol* 266: E186-192, 1994.
25. **Garetto LP, Richter EA, Goodman MN, and Ruderman NB.** Enhanced muscle glucose metabolism after exercise in the rat: the two phases. *Am J Physiol Endocrinol Metab* 246: E471-475, 1984.
26. **Hamada T, Arias EB, and Cartee GD.** Increased submaximal insulin-stimulated glucose uptake in mouse skeletal muscle after treadmill exercise. *J Appl Physiol* 101: 1368-1376, 2006.
27. **Haring HU, Tippmer S, Kellerer M, Mosthaf L, Kroder G, Bossenmaier B, and Berti L.** Modulation of insulin receptor signaling. Potential mechanisms of a cross talk between bradykinin and the insulin receptor. *Diabetes* 45 Suppl 1: S115-119, 1996.
28. **Henriksen EJ, Jacob S, Augustin HJ, and Dietze GJ.** Glucose transport activity in insulin-resistant rat muscle. Effects of angiotensin-converting enzyme inhibitors and bradykinin antagonism. *Diabetes* 45 Suppl 1: S125-128, 1996.
29. **Henriksen EJ, Jacob S, Fogt DL, and Dietze GJ.** Effect of chronic bradykinin administration on insulin action in an animal model of insulin resistance. *Am J Physiol* 275: R40-45, 1998.
30. **Henriksen EJ, Jacob S, Kinnick TR, Youngblood EB, Schmit MB, and Dietze GJ.** ACE inhibition and glucose transport in insulinresistant muscle: roles of bradykinin and nitric oxide. *Am J Physiol* 277: R332-336, 1999.
31. **Isami S, Kishikawa H, Araki E, Uehara M, Kaneko K, Shirotani T, Todaka M, Ura S, Motoyoshi S, Matsumoto K, Miyamura N, and Shichiri M.** Bradykinin enhances GLUT4 translocation through the increase of insulin receptor tyrosine kinase in primary adipocytes: evidence that bradykinin stimulates the insulin signalling pathway. *Diabetologia* 39: 412-420, 1996.

32. **Kim J, Solis RS, Arias EB, and Cartee GD.** Postcontraction insulin sensitivity: relationship with contraction protocol, glycogen concentration, and 5' AMP-activated protein kinase phosphorylation. *J Appl Physiol* 96: 575-583, 2004.
33. **Kishi K, Muromoto N, Nakaya Y, Miyata I, Hagi A, Hayashi H, and Ebina Y.** Bradykinin directly triggers GLUT4 translocation via an insulin-independent pathway. *Diabetes* 47: 550-558, 1998.
34. **Kohlman O, Jr., Neves Fde A, Ginoza M, Tavares A, Cezaretti ML, Zanella MT, Ribeiro AB, Gavras I, and Gavras H.** Role of bradykinin in insulin sensitivity and blood pressure regulation during hyperinsulinemia. *Hypertension* 25: 1003-1007, 1995.
35. **Langberg H, Bjorn C, Boushel R, Hellsten Y, and Kjaer M.** Exercise-induced increase in interstitial bradykinin and adenosine concentrations in skeletal muscle and peritendinous tissue in humans. *J Physiol* 542: 977-983, 2002.
36. **Majima M, Mizogami S, Kuribayashi Y, Katori M, and Oh-ishi S.** Hypertension induced by a nonpressor dose of angiotensin II in kininogen-deficient rats. *Hypertension* 24: 111-119, 1994.
37. **Majima M, Yoshida O, Mihara H, Muto T, Mizogami S, Kuribayashi Y, Katori M, and Oh-ishi S.** High sensitivity to salt in kininogen-deficient brown Norway Katholiek rats. *Hypertension* 22: 705-714, 1993.
38. **Miyata T, Taguchi T, Uehara M, Isami S, Kishikawa H, Kaneko K, Araki E, and Shichiri M.** Bradykinin potentiates insulin-stimulated glucose uptake and enhances insulin signal through the bradykinin B2 receptor in dog skeletal muscle and rat L6 myoblasts. *Eur J Endocrinol* 138: 344-352, 1998.
39. **Nakajima S, Ito H, Hayashi I, Kuribayashi Y, Okumura T, Yajima Y, Katori M, and Majima M.** Inhibition of kinin degradation on the luminal side of renal tubules reduces high blood pressure in deoxycorticosterone acetate salt-treated rats. *Clin Exp Pharmacol Physiol* 27: 80-87, 2000.
40. **Passoneau JV and Lauderdale VR.** A comparison of three methods of glycogen measurement. *Anal Biochem* 60: 405-412, 1974.
41. **Rhaleb NE, Yang XP, Nanba M, Shesely EG, and Carretero OA.** Effect of Chronic Blockade of the Kallikrein-Kinin System on the Development of Hypertension in Rats. *Hypertension* 37: 121-128, 2001.
42. **Richter EA, Garetto LP, Goodman MN, and Ruderman NB.** Muscle glucose metabolism following exercise in the rat: increased sensitivity to insulin. *J Clin Invest* 69: 785-793, 1982.
43. **Richter EA, Hansen BF, and Hansen SA.** Glucose-induced insulin resistance of skeletal-muscle glucose transport and uptake. *Biochem J* 252: 733-737, 1988.
44. **Richter EA, Mikines KJ, Galbo H, and Kiens B.** Effect of exercise on insulin action in human skeletal muscle. *J Appl Physiol* 66: 876-885, 1989.
45. **Ruderman NB, Balon T, Zorzano A, and Goodman M.** The postexercise state: altered effects of insulin on skeletal muscle and their physiologic relevance. *Diabetes Metab Rev* 1: 425-444, 1986.
46. **Schweitzer GG, Castorena CM, Hamada T, Funai K, Arias EB, and Cartee GD.** The B2 receptor of bradykinin is not essential for the post-exercise increase in glucose uptake by insulin-stimulated mouse skeletal muscle. *Physiological research / Academia Scientiarum Bohemoslovaca*, 2011.

47. **Shimojo N, Pickens TG, Margolius HS, and Mayfield RK.** Tissue kallikrein and bradykinin do not have direct insulin-like actions on skeletal muscle glucose utilization. *Biol Chem Hoppe Seyler* 368: 1355-1361, 1987.
48. **Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, and Klenk DC.** Measurement of protein using bicinchoninic acid. *Anal Biochem* 150: 76-85, 1985.
49. **Stebbins CL, Carretero OA, Mindroiu T, and Longhurst JC.** Bradykinin release from contracting skeletal muscle of the cat. *J Appl Physiol* 69: 1225-1230, 1990.
50. **Taguchi T, Kishikawa H, Motoshima H, Sakai K, Nishiyama T, Yoshizato K, Shirakami A, Toyonaga T, Shirontani T, Araki E, and Shichiri M.** Involvement of bradykinin in acute exercise-induced increase of glucose uptake and GLUT-4 translocation in skeletal muscle: studies in normal and diabetic humans and rats. *Metabolism* 49: 920-930, 2000.
51. **Uehara M, Kishikawa H, Isami S, Kisanuki K, Ohkubo Y, Miyamura N, Miyata T, Yano T, and Shichiri M.** Effect on insulin sensitivity of angiotensin converting enzyme inhibitors with or without a sulphydryl group: bradykinin may improve insulin resistance in dogs and humans. *Diabetologia* 37: 300-307, 1994.
52. **Vettor R, De Palo C, Calo L, De Carlo E, Sicolo N, Martini C, and Federspil G.** Effect of exercise on plasma kallikrein and muscular phospholipase A2 activity in rats. *Molecular and cellular endocrinology* 45: 65-70, 1986.
53. **Wallberg-Henriksson H.** Glucose transport into skeletal muscle. Influence of contractile activity, insulin, catecholamines and diabetes mellitus. *Acta Physiol Scand Suppl* 564: 1-80, 1987.
54. **Wallberg-Henriksson H, Constable SH, Young DA, and Holloszy JO.** Glucose transport into rat skeletal muscle: interaction between exercise and insulin. *J Appl Physiol* 65: 909-913, 1988.
55. **Winder WW and Thomson DM.** Cellular energy sensing and signaling by AMP-activated protein kinase. *Cell Biochem Biophys* 47: 332-347, 2007.
56. **Wojtaszewski JF, Hansen BF, Gade, Kiens B, Markuns JF, Goodyear LJ, and Richter EA.** Insulin signaling and insulin sensitivity after exercise in human skeletal muscle. *Diabetes* 49: 325-331, 2000.

CHAPTER V

STUDY 3

Mechanisms for the Prolonged Post-Exercise Increase in AS160 Phosphorylation: Roles of Protein Kinases and Phosphatases

ABSTRACT

A single exercise session can induce a persistent increase in insulin-stimulated glucose transport that can last for three or more hours post-exercise. Sustained AS160 phosphorylation after exercise has been demonstrated to track with and possibly contribute to the persistent post-exercise increase in insulin-stimulated glucose transport in a variety of models including rat skeletal muscle. Two plausible mechanisms that potentially lead to sustained AS160 phosphorylation post-exercise are: 1) increased activation of kinases that regulate AS160 phosphorylation, and/or 2) decreased activation of phosphatases that regulate AS160 dephosphorylation. Male Wistar rats were randomly assigned to exercise (4 x 30 minute swim) or sedentary (3hSED) groups. Rats were anesthetized 3 hours post-exercise (3hPEX), and epitrochlearis muscles were isolated. Muscles from 3hPEX versus 3hSED rats had greater AS160^{Thr642} and AS160^{Ser588} phosphorylation, but none of the known kinases of AS160 (Akt, AMPK, RSK, and SGK) had greater phosphorylation in 3hPEX versus 3hSED rats. As the

AS160 phosphatases are unknown, we evaluated the most abundant Ser/Thr phosphatases in skeletal muscle (PP1, PP2A, PP2A, and PP2C) for their ability to dephosphorylate AS160. Each of these phosphatases was found to dephosphorylate AS160 on both the Thr642 and Ser588 phospho-sites in cell-free conditions. Furthermore, PP1 and PP2C, but not PP2A or PP2B, were found to co-immunoprecipitate with AS160. Basal or insulin-stimulated muscles from 3hPEX versus 3hSED muscles did not differ for the amount of PP1, PP2A, PP2B, or PP2C that co-immunoprecipitated with AS160, nor did these muscles have altered PP2A Tyr307 phosphorylation or Leu309 methylation. These novel results demonstrated that: 1) the sustained AS160 phosphorylation at three hours post-exercise on Thr642 and S588 was not attributable to persistent phosphorylation of any of the kinases that are known to phosphorylate AS160; 2) PP1, PP2A, PP2B, and PP2C were each able to dephosphorylate the two AS160 phospho-sites that are most important for regulating insulin-stimulated glucose transport; 3) Post-translational modifications important for PP2A's activity were not altered by exercise; 4) PP1 and PP2C were associated with AS160, while there was no detectable AS160 binding of PP2A and PP2B; and 5) the co-immunoprecipitation of each of these phosphatases with AS160 was not different in 3hSED versus 3hPEX rat muscles incubated with or without insulin. Our working hypothesis is that the regulation of PP1 and/or PP2C by mechanisms other than reduced association with AS160 may contribute to the persistent increase in AS160 phosphorylation in muscle after acute exercise.

INTRODUCTION

A single exercise session can induce a persistent increase in insulin-stimulated glucose transport that can last up to 3-48 hours post-exercise (13, 14, 55, 56, 70). The increase in insulin-stimulated glucose transport after acute exercise is attributed to increased ability of insulin to recruit GLUT4 to the cell surface after exercise (34) without increased total GLUT4 abundance (12). Many studies have provided evidence that greater cell-surface GLUT4 is not attributable to key proximal insulin signaling steps including insulin receptor (IR) binding (5-7, 78), IR tyrosine phosphorylation (18, 34, 36, 67, 74), IR tyrosine kinase activity (66, 67, 72, 74), insulin receptor substrate (IRS) tyrosine phosphorylation (34, 36, 73, 74, 76), IRS-associated phosphatidylinositol 3 kinase (PI3K) activity (22, 27, 66, 73, 74), Akt serine phosphorylation (2, 18, 27, 33, 66, 72), Akt threonine phosphorylation (31, 33), and Akt activity (31).

Sano et al. (59) identified an Akt substrate of 160 kDa (also known as AS160 or TBC1D4) as a link between insulin's activation of Akt and the subsequent increase in cell-surface GLUT4 in 3T3-L1 adipocytes. Insulin can also lead to increased AS160 phosphorylation in skeletal muscle (9), and this increase is important for insulin-stimulated glucose transport (43). Additionally, Sano et al. (59) found that Thr642 and Ser588 of AS160 are the two primary sites that regulate cell-surface GLUT4 content upon insulin stimulation in 3T3-L1 adipocytes. In rats, the sustained increase in phosphorylated AS160 was first recognized using the phospho-Ser/Thr Akt substrate (PAS) antibody designed to recognize Akt phosphomotif peptide sequences (41). The PAS antibody was subsequently shown to have differing immunoreactivities for different

sites on AS160: highest for Thr642, modest for Ser588, and little to no immunoreactivity for Ser318, Ser341, Ser570, and Ser751 (32, 59).

Our group was the first to report that sustained AS160 phosphorylation (using the PAS antibody and an antibody against the Thr642 site) closely tracked with the post-exercise increase in insulin-stimulated glucose transport in rat skeletal muscle (2, 30, 31). Our working hypothesis is that the persistent AS160 phosphorylation post-exercise may be important for the increase in insulin-stimulated glucose transport after acute exercise. Sustained AS160 phosphorylation after exercise was later found in humans. In human skeletal muscle samples collected during a hyperinsulinemic-euglycemic clamp four hours after one-legged treadmill exercise versus non-exercised muscle, AS160 phosphorylation has been shown to be elevated on several sites including Ser318, Ser341, Ser751, and a strong trend for elevation at Ser588, but no elevation for Thr642 (68). As phosphorylation of the Ser588 site of AS160 had not yet been demonstrated in rat skeletal muscle, the first aim of this study was to determine if AS160 Ser588 phosphorylation is enhanced post-exercise in rat skeletal muscle as was previously shown on AS160 Thr642 (31).

The mechanism for the persistent elevation in AS160 phosphorylation after acute exercise is unknown, but two plausible mechanisms that might lead to sustained phosphorylation of a protein are: 1) increased kinase activity on the substrate and/or 2) decreased phosphatase activity on the substrate. Geraghty et al. (32), using a cell-free assay, identified several kinases that are capable of phosphorylating AS160: AMPK and several AGC (named after protein kinase A, protein kinase G, and protein kinase C, the original members of this class) protein kinase family members (Akt, SGK, RSK).

However, Akt and AMPK have been shown to not be phosphorylated on sites that are required for greater enzyme activity in epitrochlearis muscles with increased AS160 phosphorylation three to four hours after exercise (2, 31). Another AGC protein kinase that has been reported to be activated by some exercise protocols, p70S6K, has not been previously evaluated for its ability to phosphorylate AS160 (3, 15, 20, 28) in spite of its consensus motif implicating AS160 as a possible target (25, 53). Therefore, a second aim was to determine if SGK, RSK, and/or p70S6K are phosphorylated three hours post-exercise in skeletal muscles with elevated AS160 phosphorylation.

The protein phosphatases that are able to dephosphorylate AS160 have not been identified. Serine/threonine protein phosphatase 1 (PP1), 2A (PP2A), 2B (PP2B), and 2C (PP2C) are the most highly expressed serine/threonine protein phosphatases by skeletal muscle (21, 35, 38, 61, 71). Therefore, the third aim was to determine if PP1, PP2A, PP2B, and/or PP2C, using cell-free assays, are able to dephosphorylate AS160 from rat skeletal muscle that had been phosphorylated via prior insulin stimulation**.**

It would be valuable to elucidate the mechanisms at which PP1, PP2A, PP2B, and/or PP2C are regulated in skeletal muscle. A potential mechanism for regulating dephosphorylation of AS160 would be to alter the amount of phosphatase(s) associated with AS160. Therefore, the fourth aim was to determine if AS160 co-immunoprecipitates with PP1, PP2A, PP2B, and/or PP2C in rat skeletal muscle from sedentary rats. Furthermore, the fifth aim was to determine if prior exercise or insulin treatment alters AS160 association with any of these protein phosphatases.

Finally, PP2A has been shown to be regulated via post-translational modifications on its catalytic subunit. The activity of the catalytic subunit of PP2A can be altered with

PP2A tyrosine phosphorylation at the 307 site, so a change in phosphorylation on the Tyr307 site is a possible mechanism for reducing PP2A effects on substrates (40, 46, 65). Additionally, methylation on the Leu309 site of the catalytic subunit of PP2A has been reported to play a role in the assembly of the heterotrimeric enzyme, specifically by influencing which regulatory subunit is associated with the PP2A complex (10, 24, 48, 77). There is also some evidence that methylation of this site may regulate PP2A activity (24, 26, 49, 77). Neither of these sites have been evaluated in skeletal muscle and the final aim was to determine if tyrosine phosphorylation or leucine methylation on PP2A's catalytic site were altered immediately or 3h post-exercise, concomitant with sustained AS160 phosphorylation.

METHODS

Materials. Human recombinant insulin was obtained from Eli Lilly (Indianapolis, IN). Reagents and apparatus for SDS-PAGE and immunoblotting were purchased from Bio-Rad (Hercules, CA). Bicinchoninic acid protein assay reagent (no. 23227), T-PER tissue protein extraction reagent (no. 78510), and West Dura Extended Duration Substrate (no. 34075) were purchased from Pierce Biotechnology (Rockford, IL). Goat anti-rabbit IgG horseradish peroxidase conjugate (no. 7074), anti-phospho-Thr308 Akt (no. 9275), anti-p70 ribosomal S6 Kinase (p70S6K; no. 9202), anti-phospho-Thr389 p70S6K (no. 9205), anti-ribosomal S6 kinase 1 antibody (RSK1; no.9333), anti-phospho-Ser380 RSK1 (no. 9341), anti-AMPKα (no. 2532), anti-phospho-Thr172 AMPKα (no. 2531) were purchased from Cell Signaling Technology (Danvers, MA). Anti-phospho-Thr642 AS160 (no.07-802), anti-serum- and glucocorticoid-induced protein kinase 1

(SGK1; no. 07-315), anti-phospho-Thr256 SGK1 (no.36-002), anti-PP1α (no. 07-273), anti-calcineurin pan A [anti-PP2B pan A] (no. 07-1491), anti-AS160-serum (no. 07-741), anti-AS160-purified (no. ABS54), and protein G agarose beads (no. 16-266) were purchased from Millipore (Billerica, MA). Protein A Sepharose Beads 6MB (17-0469-01) were purchased from GE Healthcare (Piscataway, NJ). Anti-PP2A catalytic α (no. 610555) was purchased from BD Biosciences (San Jose, CA). Recombinant PP2A (no. V6311) was purchased from Promega (Madison, WI). Recombinant PP1 (no. P0754) was purchased from New England Biolabs (Ipswich, MA). Recombinant PP2B/calcineurin and calmodulin was included in the Calcineurin Activity Kit (no. BML-AK816-0001) and was purchased from Enzo Life Sciences (Farmingdale, NY). Recombinant PP2Cα isoform (no. 539569) was purchased from EMD Chemical (Gibbstown, NJ). Anti-PP2Cα/β (no. ab27267) and anti-phospho-Thr642 AS160 (no. ab65753) used in cell-free assay experiments was purchased from Abcam (Cambridge, MA). Anti-phospho-Tyr307 PP2A-Cα/β (no. sc-12615), Anti-methyl-PP2A-Cα/β (no.sc-81603), Anti-PP2Cα (no. sc-32402), and donkey anti-goat IgG-HRP (no. sc-2020) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Ser588 AS160 (no. 3028-P2) was purchased from B-Bridge International (Mountain View, CA). 3-O-methyl-[^{3}H]glucose ([^{3}H]3-MG) was purchased from Sigma-Aldrich (St. Louis, MO). [^{14}C]Mannitol was purchased from PerkinElmer (Waltham, MA). Other reagents were purchased from Sigma-Aldrich or Fisher Scientific (Pittsburgh, PA).

Animal treatment. Procedures for animal care were approved by the University of Michigan Committee on Use and Care of Animals. Male Wistar rats (~135–200 g; Harlan, Indianapolis, IN) were provided with rodent chow (Lab Diet; PMI Nutritional

International, Brentwood, MO) and water ad libitum. Starting at 1700 on the night before the experiments, rats were fasted. On the following day, rats were randomly assigned to a post-exercise (PEX) or sedentary (SED) treatment. Beginning at ~0900, PEX rats swam in a barrel filled with water (35°C) to a depth of ~60 cm (7 or 8 rats/barrel) for 4 x 30 min bouts, with a 5-min rest period between each bout. Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (5mg/100 g body wt) either immediately post-exercise (0h PEX) or 3-4h post-exercise (3h PEX) along with time-matched sedentary rats. While the rats were under deep anesthesia, both epitrochlearis muscles were rapidly dissected out and either freeze-clamped immediately or transferred to vials for subsequent incubation.

Muscle incubations. For the 0hPEX experiment, following anesthetization, one epitrochlearis muscle from each rat was rapidly dissected out, trimmed, freeze-clamped using aluminum clamps cooled to the temperature of liquid N_2, and stored at -80°C until analyzed. After dissection, the contralateral muscle from the 0hPEX group and the time-matched SED group were incubated for 10 min at 30°C in flasks containing in KHB + BSA + 2 mM pyruvate + 6mM mannitol (*solution 2*) at 30°C for 10 min and then transferred to flasks containing KHB, 0.1% BSA with 8 mM 3-MG (including 0.25 mCi/mmol [^{3}H]3-MG), and 2 mM mannitol (including 0.1 mCi/mmol [^{14}C]mannitol) (*solution 3*). For all incubation steps, flasks were continuously gassed from above with 95% O_2-5% CO_2 and shaken in a heated water bath.

For the 3hPEX experiment, rats were dried following the final exercise bout and returned to their cage for 3h before being anesthetized. Time-matched SED rats were also anesthetized and then epitrochlearis muscles were dissected out. Both epitrochlearis

muscles from each animal was incubated in Krebs-Henseleit buffer (KHB) + 0.1% bovine serum albumin (BSA) + 8 mM glucose + 2 mM mannitol (*solution 1*) for 30 min in a water bath at 35°C. During this step, one muscle from each rat was incubated in *solution 1* supplemented with 50 µU/ml of insulin, and the contralateral muscle was incubated in *solution 1* without insulin. The same insulin concentration was used for each muscle during all subsequent incubations. After the initial incubation, muscles were transferred to vials containing *solution 2* with or without 50 µU/ml insulin at 30°C for 10 min. Finally, muscles were transferred to flasks containing *solution 3* with or without 50 µU/ml insulin for determination of glucose transport rate. After incubation with 3-MG for 15 min, the muscles were rapidly blotted on filter paper dampened with incubation medium, trimmed, freeze-clamped, and stored at -80°C until being processed as described below.

Homogenization and glucose transport measurement. Frozen muscles were homogenized in 1 ml ice-cold homogenization buffer (1% Triton X-100, 1 mM activated Na_3VO_4, 20mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM phenylmethanesulfonyl fluoride, and 1 µg/ml leupeptin in water) using glass-on-glass tubes (Kontes, Vineland, NJ) or high-speed tissue disruption with the TissueLyser II (Qiagen, Valencia, CA). Homogenates were subsequently solubilized by rotating at 4°C at 50 rpm for 1 h before being centrifuged (15,000 g for 15 min at 4°C). Aliquots of the supernatant from muscles used for the 3-MG transport measurement were pipetted into vials with scintillation cocktail for scintillation counting, and 3-MG transport were determined as previously described (11). A portion of the supernatant were used to determine protein concentration

by the bicinchoninic acid assay (63) according to the manufacturer's instructions (Pierce Biotechnology; no. 23227). The remaining supernatant was stored at -80°C until further analyzed.

Immunoprecipitation. Aliquots of the homogenates prepared as described above (300 µg protein) were brought to the same volume to equalize the concentration of muscle lysates and were precleared with 100 µL of protein G agarose beads or protein A sepharose beads for 1 h. When using protein G agarose beads for the immunoprecipitation of SGK, the muscle lysates were mixed with an immuno-matrix of 100 µL of protein G agarose beads that had been gently rotated with 3 µg/µL of anti-AS160 for 1 h. The lysate-antibody-protein G mixture was gently rotated overnight at 4°C at 5 rpm. When using protein A sepharose beads were used for immunoprecipitation of AS160, the supernatant from the muscle lysates were gently rotated with 2 µg for 3 hours and then 25 µL of a 50/50 slurry of protein A sepharose beads were added. The lysate-antibody-protein A mixture was gently rotated overnight. Following overnight rotation in both protein G agarose and protein A sepharose immunoprecipiation assays, the immunoprecipitation mix was centrifuged (4000g) and supernatant were aspirated. An aliquot of the aspirated supernatant was used to assess immunoprecipitation efficiency by comparing against immunoprecipated samples bound to the beads via densitometry analysis. After washing (4 times with 500 µL PBS), immunoprecipitated proteins were eluted with 2X Laemmli sample buffer, boiled, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). For the cell-free assays, after washing (3 times with 500 µL PBS and 2 times with the appropriate phosphatases buffer [described

below]), immunoprecipitated AS160 was eluted with 2X Laemmli sample buffer (1:1 volume), boiled, and subjected to SDS-PAGE.

Immunoblotting and protein phosphorylation. Homogenized muscle lysates were subjected to SDS-PAGE and immunoblotted to nitrocellulose membranes. Blots were then rinsed with Tris-buffered saline plus Tween (TBST) (0.14 mol/l NaCl, 0.02 mol/l Tris base, pH 7.6, and 0.1% Tween), blocked with 5% BSA in TBST for 1 h at room temperature, washed 3 x 5 min at room temperature, and incubated with the relevant primary antibody (1:1,000 in TBST + 5% BSA) overnight at 4°C. Blots were then washed 3 x 5 min with TBST, incubated with the relevant secondary antibody (goat anti-rabbit, goat anti-mouse, goat-anti-sheep) IgG horseradish peroxidase conjugate (1:20,000 in TBST + 5% BSA), for 1 h at room temperature, washed again 3 x 5 min with TBST, and developed with SuperSignal reagent. Protein bands were quantified by digital densitometry (ProteinSimple, Santa Clara, CA). The mean values for sedentary samples without insulin on each blot were normalized to equal 1.0, and then all samples on the blot were expressed relative to the normalized sedentary without insulin value.

Cell-free dephosphorylation assay using recombinant PP1, PP2A, PP2B, or PP2C. AS160 that had been immunoprecipitated from lysates prepared from epitrochlearis muscles that had been incubated with insulin (2000 μU/ml for 30min to induce high levels of AS160 phosphorylation) were used to assess the ability of recombinant PP1, PP2A, PP2B, and PP2C to dephosphorylate $AS160^{Thr642}$ and $AS160^{Ser588}$. The immunoprecipitated AS160 (as described above) was incubated for 2 hr at 30°C with gentle shaking in the appropriate buffer for each phosphatase. PP1 buffer was 50mM HEPES, 100nM NaCl, 2mM DTT, 1mM $MnCl_2$ in 0.01% Brij35 (pH 7.5).

PP2A buffer was 50mM Tris-HCl (pH 8.5), 20mM $MgCl_2$, and 1mM DTT. PP2B buffer was 50mM Tris (ph7.5), 100mM NaCl, 6mM $MgCl_2$, and 0.5mM DTT, 1mM $CaCl_2$, 0.05% NP-40, and 0.5μM calmodulin. PP2C buffer was 20mM Tris-HCL (pH 7.4), 10mM $MnCl_2$, 0.5mM EGTA (pH 8.0), 3mM 2-mercaptoethanol in 0.2 mg/ml BSA. The reactions were terminated by adding SDS loading buffer (2X) to the reaction and boiling the sample for 5 min. Samples were subsequently subjected to SDS-PAGE, imunoblotted to nitrocellulose membranes, and blots were probed with anti-phospho-Thr642 AS160 and anti-phospho-Ser588 AS160 (17). Following $AS160^{Thr642}$ and $AS160^{Ser588}$ phosphorylation detection, blots were stripped (20 min of shaking in 0.2M glycine in 0.05% Tween 20 at 70°C) and reprobed with an antibody against total AS160 to verify equal loading.

Statistical analyses. Statistical analyses were done using Sigma Stat version 2.0 (San Rafael, CA). Data were expressed as means ± SE. When an analysis comparing four groups was required, a two-way ANOVA was used to determine significant differences, and a Tukey post hoc test was used to identify the source of significant variance. When an analysis comparing 2 groups was required, a t-test was used to determine significant differences. A P value ≤ 0.05 was considered statistically significant.

RESULTS

3-MG transport. Insulin-independent glucose transport measured immediately post-exercise was 3-4 fold greater than time-matched sedentary controls ($P < 0.05$; Fig. 5.1A). This effect was reversed in muscles dissected from rats that were returned to their cage following exercise without access to food for 3 hours (3hPEX). Muscles stimulated

with a submaximally effective insulin dose (50μU/ml) had greater glucose transport than paired muscles incubated without insulin for both 3hSED (~2 fold greater; $P < 0.05$) and 3hPEX (3-4 fold greater; $P < 0.05$; Fig. 5.1B) rats. 3hPEX rats versus 3hSED rats had significantly greater glucose transport for insulin-stimulated muscle ($P < 0.05$; Fig. 5.1B).

AS160 phosphorylation. $AS160^{Thr642}$ and $AS160^{Ser588}$ phosphorylation were increased in 0hPEX versus 0hSed rats ($P < 0.05$; Fig. 5.2A-B). $AS160^{Thr642}$ and $AS160^{Ser588}$ were increased with insulin in both 3hSED and 3hPEX rats ($P < 0.05$; Fig. 5.2C-D). Additionally, for 3hPEX rats versus 3hSED rats in the absence of insulin, there were residual and significant increases in $AS160^{Thr642}$ and $AS160^{Ser588}$ phosphorylation ($P < 0.05$; Fig. 5.2C-D).

Kinases. Among all of the kinases that were assessed for phosphorylation in the absence of insulin, there was only a significant increase in $AMPK^{Thr172}$ at 0hPEX versus 0hSED ($P < 0.05$; Fig. 3A), and there were no exercise-related differences in Akt^{Thr308}, RSK^{Ser380}, SGK^{Thr256}, and $p70S6K^{Thr389}$ at 0hPEX (Fig. 5.3B-E).

Akt^{Thr308} phosphorylation was increased with insulin in both 3hSED and 3hPEX rats ($P < 0.05$), and Akt^{Thr308} phosphorylation in muscles incubated with insulin was greater for 3hPEX versus 3hSED ($P < 0.05$; Fig. 5.4B), consistent with our previous results (2, 31). However, there was no residual increase in Akt^{Thr308} phosphorylation in 3hPEX versus 3hSED rats in muscles incubated without insulin (Fig. 5.4B). Akt^{Ser473} phosphorylation was increased with insulin in both 3hSED and 3hPEX rats ($P < 0.05$), but there was no difference in insulin-stimulated Akt^{Ser473} phosphorylation in 3hPEX rats versus 3hSED rats (Fig. 5.4C).

RSK^{Ser380} phosphorylation was not significantly altered with insulin in either 3hSED or 3hPEX rats, and there was a trend for a decrease in RSK^{Ser380} phosphorylation in 3hPEX versus 3hSED rats (P = 0.15; Fig. 5.4D). AMPK^{Thr172}, SGK^{Thr256}, and p70S6K^{Thr389} phosphorylation were not significantly different with or without insulin in either 3hSED or 3hPEX rats, and there was no significant difference for each of these kinases at 3hPEX versus 3hSED (Fig 5.4A, 5.4E, and 5.4F, respectively).

Cell-free phosphatase assays with AS160. PP1, PP2A, PP2B, PP2C are expressed in skeletal muscle (21, 35, 61, 71). Recombinant PP1, PP2A, PP2B, and PP2C were each able to dephosphorylate insulin-stimulated AS160^{Thr642} and AS160^{Ser588} phosphorylation in rat epitrochleraris muscle under cell-free conditions (Table 5.1 and Fig. 5.5A-D).

Co-immunoprecipitation of AS160 and phosphatases catalytic subunits. Duplicate aliquots for each sample were loaded in lanes 1 and 2 and had been treated identically except that an antibody against total AS160 was present for the sample loaded in lane 1 and absent for the sample loaded in lane 2 (Fig. 5.6A). As seen in lane 1 of the immunoprecipitation (IP) blot, the AS160 antibody was highly efficient for precipitating AS160 as there is little to no detectable AS160 in the post-immunoprecipitation supernatant (PIPS). As seen in lane 2, the lack of an antibody against total AS160 resulted in no detectable AS160 in the immunoprecipitation, but rather AS160 was detected in the PIPS.

Immunoblotting for PP1 revealed detectable AS160-PP1 association on the IP blot and non-AS160-bound PP1 in the PIPS blot (Lane 1, Fig. 5.6B). PP1 was not detected in the IP blot when the total AS160 antibody was not included, but PP1 was found in the PIPS (Lane 2, Fig. 5.6B).

Immunoblotting for PP2A revealed detectable AS160-PP2A association on the IP blot and non-AS160-bound PP2A in the PIPS blot (Lane 1, Fig. 5.6C). However, PP2A was also detected when the total AS160 antibody was not included, and the amount of PP2A was not significantly different when the results were compared with anti-AS160 included (Lane 1, Fig. 5.6C) versus without the AS160 antibody included (Lane 2, Fig. 5.6C) indicating that the AS160 antibody was not specifically pulling down AS160-bound-PP2A. Additionally, PP2A was also found in the PIPS when the AS160 antibody was not included (Lane 2, Fig. 5.6C).

Immunoblotting for PP2B revealed no detectable AS160-PP2B association on the IP blot, and non-AS160-bound PP2B in the PIPS blot (Lane 1, Fig. 5.6D).

Immunoblotting for PP2C revealed detectable AS160-PP2C association on the IP blot and non-AS160-bound PP2C in the PIPS blot (Lane 1, Fig. 5.6E). PP2C was not detected when the total AS160 antibody was not included, but was found in the PIPS (Lane 2, Fig. 5.6E).

Exercise effects on co-immunoprecipitation of AS160 and phosphatase catalytic subunits. The amount of PP1alpha catalytic subunit-AS160 association was not altered with or without insulin in 3hSED and 3hPEX rats (Fig. 5.6B). The absence of any detectable PP2Aalpha catalytic subunit-AS160 association above the non-specific association of PP2A with protein A sepharose beads in the absence of anti-AS160 was found with or without insulin in 3hSED and 3hPEX rats (Fig. 5.6C). PP2B catalytic subunit using a pan-PP2B-isoform antibody was not found to be associated with AS160 regardless of prior exercise of incubation of muscles with insulin (Fig. 5.6D).

PP2Calpha/beta-AS160 association was not altered with or without insulin in 3hSED and 3hPEX rats (Fig. 5.6E).

Post-translational modifications of PP2A. There was no difference in $PP2A^{Tyr307}$ phosphorylation or $PP2A^{Leu309}$ methylation in 0hSED versus 0hPEX rats (Fig.. 5.7A-B). $PP2A^{Tyr307}$ phosphorylation was not altered by incubation with insulin or by prior exercise (Fig. 5.7C). $PP2A^{Leu309}$ methylation was also not altered by incubation with insulin or by prior exercise (Fig. 5.7D).

DISCUSSION

An acute bout of exercise has long been known to enhance insulin's ability to increase glucose transport in skeletal muscle after the initial insulin-independent glucose transport effects of exercise have worn off. This has been attributed, not to an increase in GLUT4 abundance, but rather to the amount of GLUT4 translocating to the cell membranes. However, the mechanism for this increase in GLUT4 translocation is unknown. This effect does not appear to be due to increased activation of proximal steps in the insulin signaling pathway, but AS160, a distal signaling step, has been reported to have a sustained increase in phosphorylation for hours after acute exercise (2, 30, 31).

Lienhard's group investigated the contribution of specific AS160 phospho-sites to insulin-stimulated GLUT4 translocation in adipocytes. Mutation of the Thr642 (to Ala642) site and Ser588 (to Ala588) resulted in a ~50% decrease and a ~25% decrease, respectively, in insulin-stimulated GLUT4 translocation compared to normal vector controls. Mutating both sites simultaneously resulted in an even greater (~75%) decrease in insulin-stimulated GLUT4 translocation compared to normal vector controls, but

mutating additional phospho-sites did not result in a further decrease in insulin-stimulated GLUT4 translocation suggesting that Thr642 and Ser588 sites account for most of AS160's effects on insulin-stimulated GLUT4 translocation (59). We confirmed earlier results (31) showing that AS160^{Thr642} phosphorylation is sustained three hours post-exercise (Fig. 5.2A), and demonstrated for the first time that AS160^{Ser588} phosphorylation is also sustained three hours post-exercise in rat skeletal muscle (Fig. 5.2B). Therefore, it is notable that the two phospho-sites of AS160 that are known to be important for insulin-stimulated GLUT4 translocation (Thr642 and Ser588) have sustained phosphorylation in rat skeletal muscle long after acute exercise.

Increased activation of kinases that regulate AS160 phosphorylation is one plausible mechanism to explain sustained AS160 phosphorylation after exercise. Therefore, all the known AS160 kinases were investigated. Four kinases (Akt, AMPK, RSK, and SGK) have been shown to phosphorylate AS160 in cell free assays (32). AMPK phosphorylation on the site essential for its kinase activity (64) was increased immediately post-exercise. Because there was no immediate post-exercise increase in the phosphorylation of other known AS160 kinases, these results implicate AMPK as the leading candidate to account for the exercise-induced increase in AS160 phosphorylation. Supporting the idea that AMPK is important for exercise effects on AS160 phosphorylation, prior studies reported that incubation of isolated skeletal muscle with an AMPK inhibitor resulted in decreased contraction-stimulated AS160 phosphorylation (29), and AMPK kinase dead-mice had decreased contraction-stimulated AS160 phosphorylation in skeletal muscle (42).

The exercise effect on AMPK phosphorylation observed immediately after exercise had reversed at three hours post-exercise indicating that sustained AMPK phosphorylation does not explain sustained AS160 phosphorylation. However, one scenario could be that while AMPK phosphorylation was reversed at three hours post-exercise, it may not yet be completely reversed at ~1-2 hours post-exercise. Two lines of evidence are not consistent with the idea that incompletely reversed AMPK activation at 1-2 hours after completion of the exercise is a likely mechanism for the sustained AS160 thereafter. The first line of evidence is that, immediately post-exercise, the phosphorylation of each of the 3 AMPK substrates that were evaluated (AS160, ACC and TBC1D1) is increased, but there is sustained phosphorylation at 3-4 hours post-exercise only for AS160 (i.e., not for ACC and TBC1D1) (2, 31). The second line of evidence is that elevated AS160 phosphorylation in the epitrochlearis muscles persists for at least 27 hours post-exercise versus sedentary controls (31). The simplest interpretation of the available evidence is that the sustained phosphorylation of AS160 is not attributable to persistent activation of AMPK.

In addition, we found no evidence that phosphorylation of Akt, RSK, and SGK on sites known to be required for increased kinase activity (1, 23, 44, 51) was increased either immediately or three hours after exercise. Additionally, we evaluated p70S6K as a possible AS160 kinase because its consensus motif for AS160 phospho-sites is similar to that of SGK and RSK (25, 53). However, we found no changes in phosphorylation of p70S6K on sites known to be required for its increased kinase activity (54) either immediately or three hours post-exercise when compared to sedentary controls.

While phosphorylation of the 5 kinases on the phospho-sites that were studied is essential for increased kinase activity of these enzymes, it remains possible that one or more of these kinases can influence AS160 phosphorylation by other mechanisms [e.g., by altered subcellular localization of the kinase(s)]. However, it is notable that although the same exercise protocol used in the current study caused increased phosphorylation of TBC1D1 [a paralog protein of AS160, and substrate of both AMPK and Akt (16)] immediately post-exercise (31), there is not a sustained increase in TBC1D1 phosphorylation in rat epitrochlearis muscle 3-4 h post-exercise, arguing against sustained activation of these kinases. Nonetheless, it remains possible that other unknown kinases are also able to phosphorylate AS160, and these unidentified kinases may have a sustained increase in activity after exercise.

In the absence of any evidence supporting increased phosphorylation of AS160 by any of the known AS160 kinases, it was logical to consider that decreased phosphatase activity may contribute to the sustained AS160 phosphorylation after acute exercise. As there are no known AS160 phosphatases and a number of phosphatase candidates to consider, we began investigating the phosphatases that account for over 99% of the serine/threonine phosphatase activity in skeletal muscle (PP1, PP2A, PP2B, and PP2C) to see if they have the ability to dephosphorylate AS160 (21, 35, 38, 61, 71). Under cell-free conditions, each of the four phosphatases was found to dephosphorylate insulin-stimulated AS160 at both the Thr642 and Ser588 phospho-sites. These results provide the first information about the specific phosphatases that are able to dephosphorylate AS160.

Because the cell-free conditions do not represent normal physiology in the skeletal muscle, it was important to find endogenous physiological evidence supporting regulation of AS160 phosphorylation by phosphatases. Multiple studies have shown that protein phosphatases can associate with target substrates as assessed by co-immunoprecipitation. PP2A is the most well-studied in this regard as it known to associate with AGC protein kinases, such as Akt (8, 39, 47, 75), p70S6K (19), and protein kinase C (45). Additionally, PP1, PP2B, and PP2C have been reported to associate with substrates, and PP1 and PP2A both have been shown to associate with the same target substrates indicating multiple phosphatases can associate with the same protein (4, 58, 60). Accordingly, we investigated each of the phosphatases to find out if AS160 from rat epitrochlearis muscle was associated with these highly expressed serine/threonine phosphatases. Furthermore, we tested if this association is reduced in epitrochlearis muscles from rats that had undergone prior exercise and/or incubation with insulin. With the exception of PP2C, the phosphatases investigated are proteins consisting of multiple subunits. There is great diversity in the number of scaffolding (for PP2A) and regulatory subunits (for PP1, PP2A, and PP2B). The catalytic subunit for each phosphatase was investigated because this subunit is essential for dephosphorylating proteins.

PP1 specifically co-immunoprecipitated with AS160 in rat epitrochlearis muscle providing evidence that PP1 is associated with AS160. This association was not altered in samples from 3hPEX versus 3hSED rats, with or without insulin incubation. These results suggest that reduced PP1-AS160 association does not account for sustained increase in AS160^{Thr642} and AS160^{Ser588} phosphorylation after exercise.

PP2A did not specifically co-immunoprecipitate with AS160 in rat epitrochlearis muscle as the detection of PP2A without total AS160 antibody indicates that PP2A in the lysate is binding to the beads. There was no detectable evidence for specific PP2A-AS160 association above the large amount of non-specific PP2A-bead binding in the absence of the AS160 antibody. Furthermore, we were unable to detect any evidence for specific PP2A-AS160 association in samples from exercised rats, with or without insulin incubation.

The activity of PP2A can be regulated by post-translational modifications on the catalytic subunit. Multiple lines of evidence have shown that $PP2A^{Tyr307}$ phosphorylation results in decreased activity of the catalytic subunit (40, 46, 65). Additionally, $PP2A^{Leu309}$ methylation has been reported to result in decreased (49, 77), increased (26), or no changes (24) in PP2A activity. $PP2A^{Tyr307}$ phosphorylation and $PP2A^{Leu309}$ methylation were not altered in epitrochlearis muscles dissected immediately after exercise versus sedentary controls, or in muscles incubated with or without insulin after being dissected from rats dissected three hours after exercise or from sedentary controls. These results suggest that these post-translational modifications of PP2A's catalytic subunit do not explain sustained AS160 phosphorylation under these conditions.

PP2B, also known as calcineurin, was not found to co-immunoprecipitate with AS160 in rat epitrochlearis muscle. We also were unable to detect evidence for PP2B-AS160 association in samples from exercised rats, with or without insulin incubation, in spite of detecting PP2B present in the skeletal muscle lysate.

PP2C specifically co-immunoprecipitated with AS160 in rat epitrochlearis muscle providing evidence that PP2C is associated with AS160. However, this association was

not altered in samples from 3hPEX versus 3hSED rats, with or without insulin incubation. These results suggest that reduced PP2C-AS160 association does not account for the sustained increase in AS160^{Thr642} and AS160^{Ser588} phosphorylation after exercise.

Considering all of the phosphatase data together, PP1 and/or PP2C appear to be the most promising candidate phosphatases of AS160 as each can dephosphorylate AS160 on both phospho-sites important for insulin-stimulated GLUT4 translocation and each can associate with AS160 based on co-immunoprecipitation. However, it is important to note that these results do not eliminate the possibility of PP2A or PP2B having a role in the regulation of AS160 as both were also capable of dephosphorylating AS160, and their regulation of sustained AS160 phosphorylation may be independent of co-immunoprecipitation with AS160. For example, AS160 association with these phosphatases may be a transient process, and the analysis therefore missed the time in which to detect the association. Alternatively, the treatments used in the co-immunoprecipitation procedure may have caused disassociation of AS160 from these particular phosphatases.

Given that the amount of PP1 and PP2C associated with AS160 is unaltered after exercise, what are possible mechanisms whereby these serine/threonine phosphatases might contribute to sustained AS160 phosphorylation after exercise? One possibility is that the activity of PP1 and/or PP2C is attenuated by protein-protein interaction with a regulatory protein. Consistent with this idea, PP1 activity has been shown to be regulated by proteins that target the catalytic subunit, altering phosphatase activity (37, 50, 57), and PP1 catalytic activity has been reported to be altered by proteins targeting the regulatory subunit (52, 62, 69). A second possibility is that AS160 itself is somehow

modified, e.g., by a post-translational modification, allosteric processes, and/or binding to a regulatory protein. In this second speculative model, the putative modification of AS160 might induce a conformational change that makes its phospho-sites less susceptible to dephosphorylation by the PP1 and/or PP2C that is bound to AS160.

The main findings in rat epitrochlearis muscles indicate that: 1) both phospho-sites of AS160 that are important for insulin-stimulated GLUT4 translocation (Thr642 and Ser588) have increased phosphorylation both immediately and three hours post-exercise; 2) of the known AS160 kinases, only AMPK phosphorylation was increased immediately post-exercise implicating that AMPK in the exercise-induced increase in AS160 phosphorylation at this time; 3) sustained phosphorylation of the known kinases of AS160 does not appear to explain greater AS160 phosphorylation at three hours post-exercise; 4) all four of the Ser/Thr phosphatases that were studied have the ability to dephosphorylate AS160 on both the Thr642 and Ser588 phospho-sites; 5) PP1 and PP2C, but not PP2A or PP2B, are specifically associated with AS160 based on co-immunoprecipitation; 6) prior exercise or incubation with insulin did not alter post-translational modifications of PP2A; and 7) prior exercise or incubation with insulin did not alter the association of AS160 with the phosphatases.

Protein phosphorylation status represents the balance between the actions of kinases and phosphatases. Although the prevailing view in signaling research has focused more heavily on kinases than on phosphatases, neither the current study nor earlier research provides evidence for sustained post-exercise activation of any of the known AS160 kinases. Furthermore, the sustained phosphorylation of AS160 is not typical, i.e., there is no evidence that prior exercise leads to a global increase in the

phosphorylation of other substrates of these kinases. These results suggest the mechanism may be relatively specific to AS160. In view of the lack of evidence implicating sustained kinase activation, a logical alternative is to consider a possible role for attenuated dephosphorylation of AS160 by the relevant serine/threonine phosphatases. In this context, the current results include the first evidence about the serine/threonine phosphatases that regulate AS160 dephosphorylation and also provide unique information about the influence of prior exercise on these important enzymes. Based on the results of this study, our working model suggests that the mechanism leading to sustained AS160 phosphorylation post-exercise requires regulation by phosphatases by means independent of altering the amount of phosphatase catalytic subunits that are associated with AS160. It will be important to investigate mechanisms whereby phosphatases may regulate AS160 phosphorylation, independent of association, and to determine if phosphatase inhibition contributes to the sustained post-exercise increase in AS160 phosphorylation.

	AS160 Thr642		AS160 Ser588	
	Without Recombinant Phosphatase	**With Recombinant Phosphatase**	**Without Recombinant Phosphatase**	**With Recombinant Phosphatase**
rPP1	1.00±0.00	0.33±0.06*	1.00±0.00	0.53±0.01*
rPP2A	1.00±0.00	0.55±0.12*	1.00±0.00	0.62±0.10*
rPP2B	1.00±0.00	0.73±0.06*	1.00±0.00	0.70±0.12*
rPP2C	1.00±0.00	0.22±0.04*	1.00±0.00	0.13±0.03*

Table 5.1
Quantitative values and statistical analysis for results of cell-free AS160 dephosphorylation assay. AS160 was immunoprecipitated from rat epitrochlearis muscles incubated with 5000μU/ml insulin. Immunoblotting with $AS160^{Thr642}$ and $AS160^{Ser588}$ antibodies results in $AS160^{Thr642}$ and $AS160^{Ser588}$ phosphorylation in the absence of the phosphatase (Without Recombinant Phosphatase) and was normalized to 1.00. Upon incubation with recombinant PP1, PP2A, PP2B, or PP2C (With Recombinant Phosphatase), $AS160^{Thr642}$ and $AS160^{Ser588}$ phosphorylation was reduced. Data are means ± SE; n = 3-7/group. *$P < 0.05$ (recombinant phosphatase effect). rPP1: recombinant PP1, rPP2A: recombinant PP2A, rPP2B: recombinant PP2B, rPP2C: recombinant PP2C.

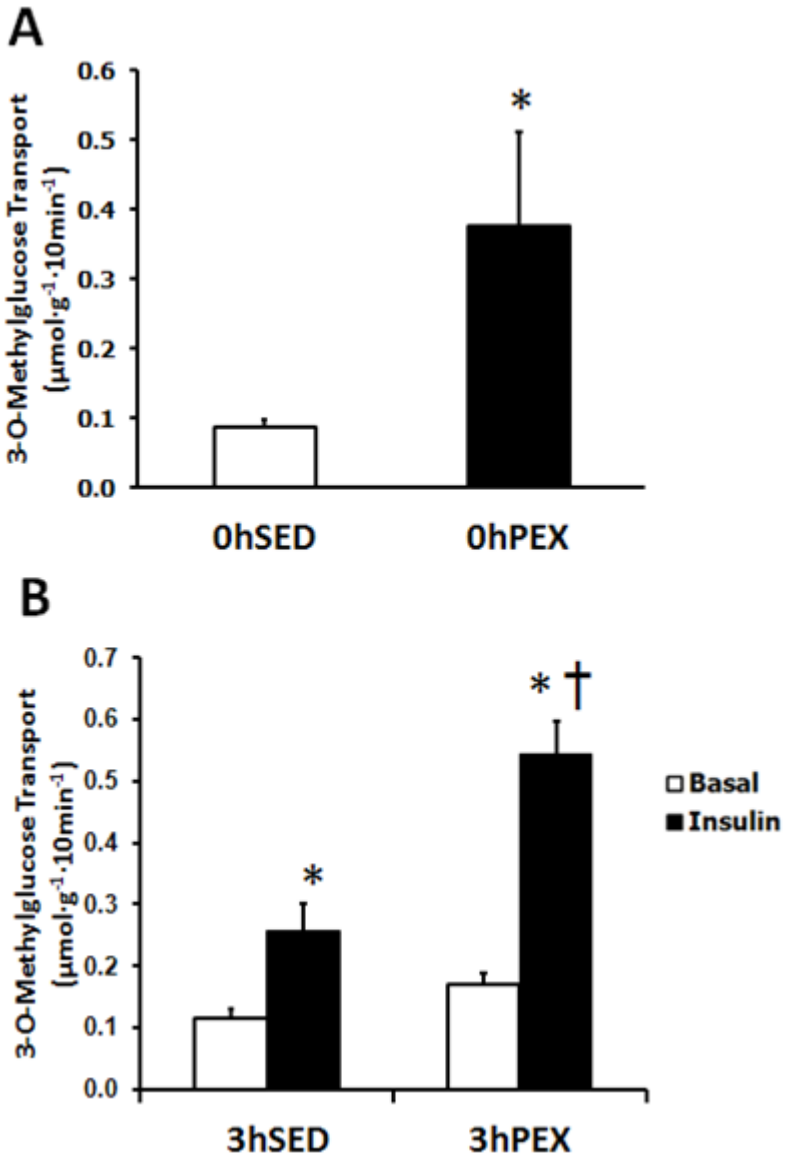

Figure 5.1
Rate of 3-O-methylglucose (3-MG) transport in isolated rat epitrochlearis muscles. A: rats anesthetized immediately post-exercise (0hPEX) with time-matched sedentary controls (0hSED); B: rats anesthetized 3h post-exercise (3hPEX). For rats in the 0hPEX and 0hSED groups, all muscles that were used to measure 3-MG transport were incubated without insulin to determine the insulin-independent effect of exercise. For rats in the 3hPEX groups and sedentary controls (3hSED), one of the paired muscles was incubated without insulin, and the contralateral muscle was incubated with 50μU/ml insulin. A: data are means ± SE; n = 8/group. *$P < 0.05$ (exercise effect, t-test). Open bars, 0hSED; filled bars, 0hPEX. B: data are means ± SE; n = 15-16/group. *$P < 0.05$ (insulin effect, post hoc test); †$P < 0.05$ (exercise effect, post hoc test). In panel B: open bars, without insulin; filled bars, with insulin.

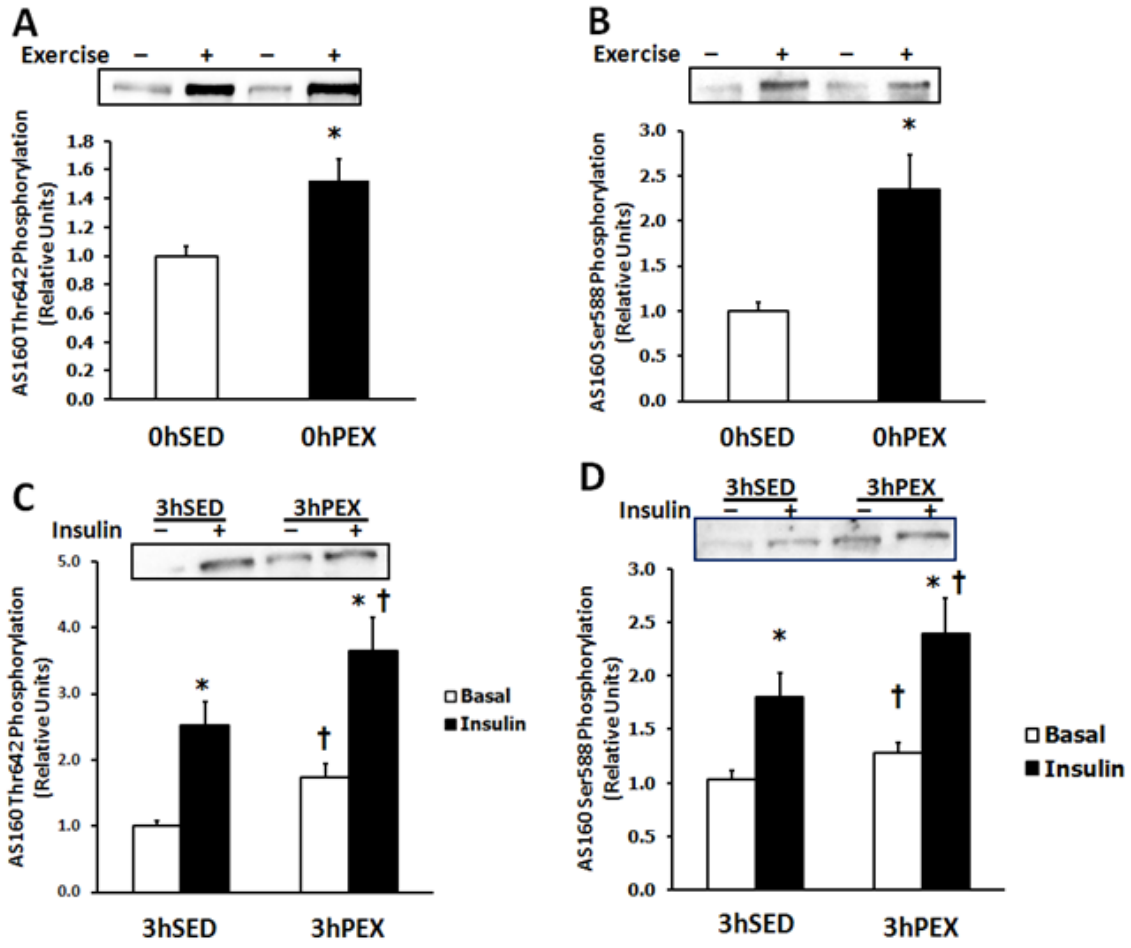

Figure 5.2
AS160^{Thr642} and AS160^{Ser588} phosphorylation in isolated rat epitrochlearis muscles. A-B: rats anesthetized immediately post-exercise (0hPEX) with time-matched sedentary controls (0hSED); C-D: rats anesthetized 3h post-exercise (3hPEX). A, C: samples immunoblotted for AS160^{Thr642} phosphorylation; B, D: samples immunoblotted for AS160^{Ser588} phosphorylation. For rats in the 0hPEX and 0hSED groups, all muscles that were used to measure AS160 phosphorylation were frozen immediately after dissection to determine the insulin-independent effect of exercise. For rats in the 3hPEX groups and sedentary controls (3hSED), one of the paired muscles was incubated without insulin, and the contralateral muscle was incubated with 50μU/ml insulin. A-B: data are means ± SE; n = 19-24/group. *P < 0.05 (exercise effect, t-test). Open bars, 0hSED; filled bars, 0hPEX. C-D: data are means ± SE; n = 19–21/group. *P < 0.05 (insulin effect, post hoc test); †P < 0.05 (exercise effect, post hoc test). In panels C and D: open bars, without insulin; filled bars, with insulin.

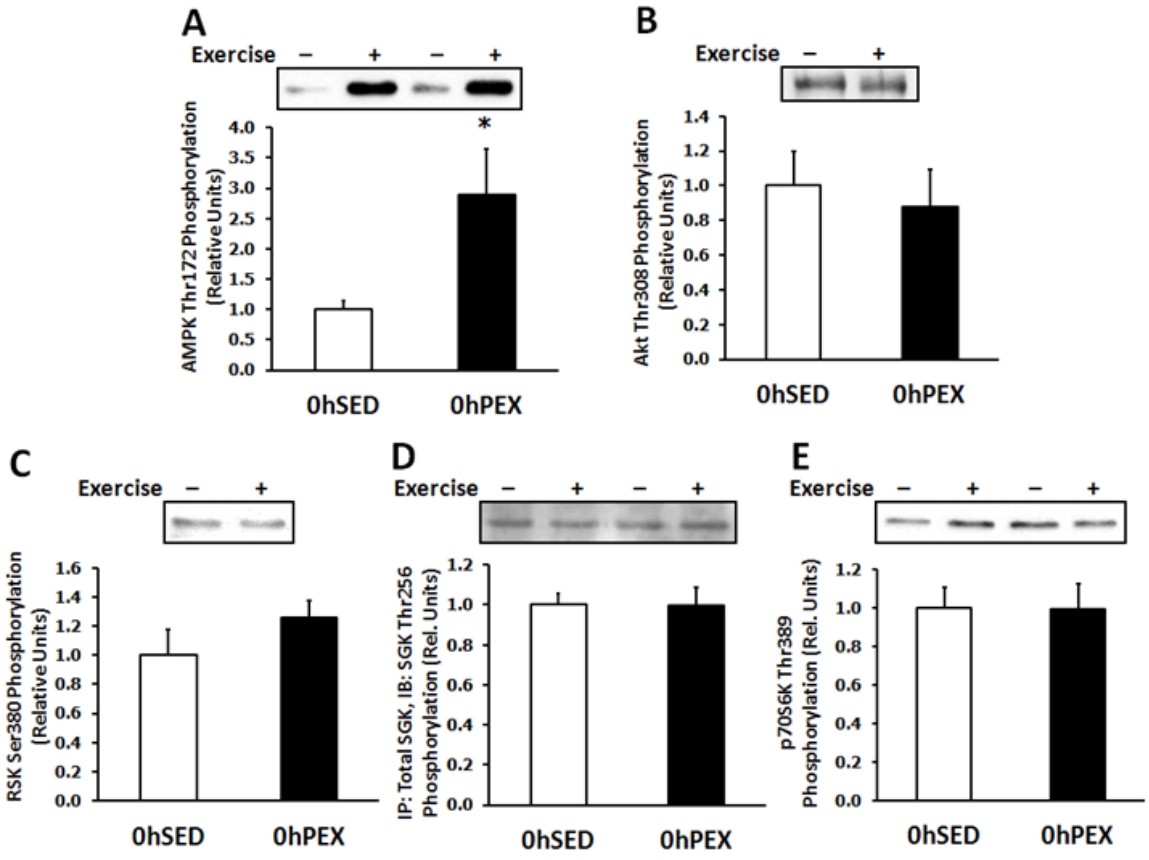

Figure 5.3
Immediate post-exercise kinase phosphorylation in isolated rat epitrochlearis muscles. Rats anesthetized immediately post-exercise (0hPEX) with time-matched sedentary controls (0hSED) and measuring phosphorylation of the following kinases: (A) AMPK^{Thr172}, (B) Akt^{Thr308}, (C) RSK^{Ser380}, (D) SGK^{Thr256}, and (E) p70S6K^{Thr389}. All muscles that were used to measure kinase phosphorylation were frozen immediately after dissection to determine the insulin-independent effect of exercise. A-E: data are means ± SE; n = 5-12/group. *P < 0.05 (exercise effect, t-test).

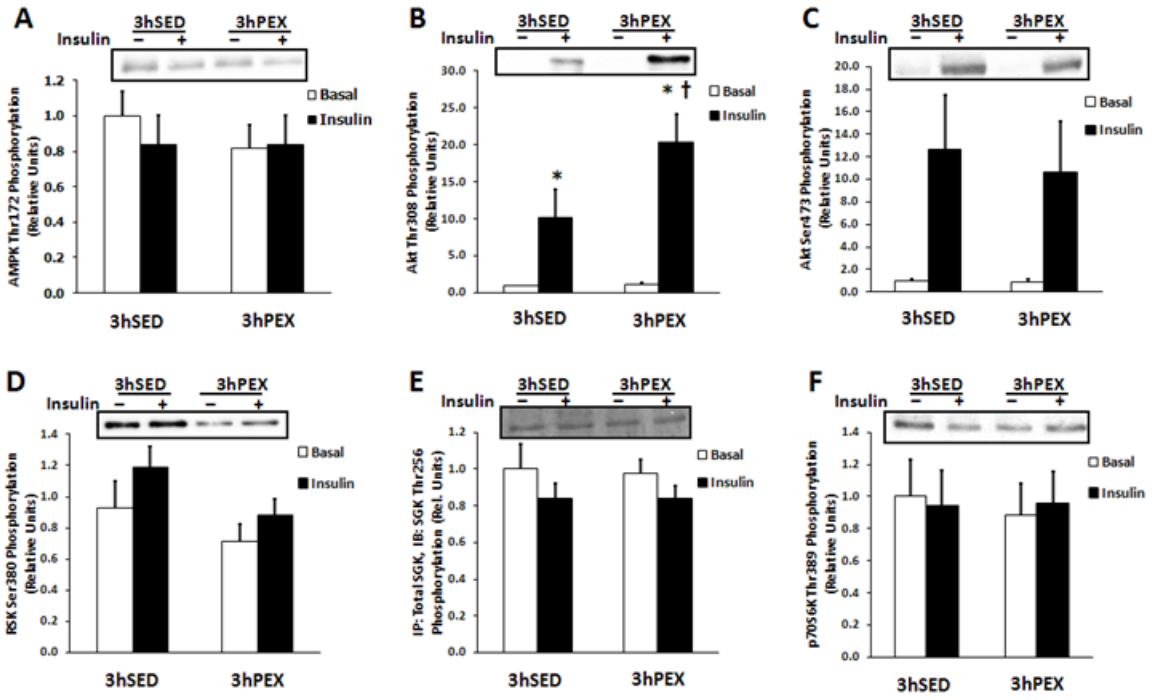

Figure 5.4
Three hour post-exercise kinase phosphorylation in isolated rat epitrochlearis muscles. Phosphorylation was measured for the following kinase phospho-sites: (A) AMPK^{Thr172}, (B) Akt^{Thr308}, (C) Akt^{Ser437}, (D) RSK^{Ser380}, (E) SGK^{Thr256}, and (F) p70S6K^{Thr389}. For rats in the 3hPEX groups and sedentary controls (3hSED), one of the paired muscles was incubated without insulin, and the contralateral muscle was incubated with 50μU/ml insulin. A-F: data are means ± SE; n = 8-12/group. *$P < 0.05$ (insulin effect, post hoc test); †$P < 0.05$ (exercise effect, post hoc test). Open bars, without insulin; filled bars, with insulin.

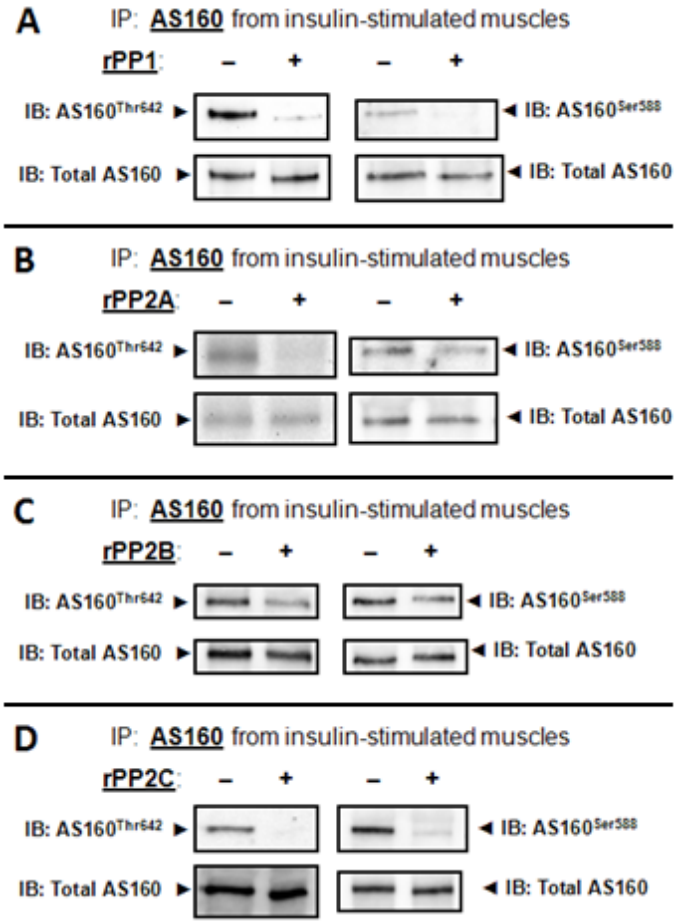

Figure 5.5
Cell-free dephosphorylation assay of AS160 from insulin-stimulated epitrochlearis muscles. AS160 was immunoprecipitated (IP) from rat epitrochlearis muscles incubated with 5000μU/ml insulin. Immunoblotting (IB) with AS160^{Thr642} and AS160^{Ser588} antibodies results in AS160^{Thr642} and AS160^{Ser588} phosphorylation in the absence of the phosphatase. Upon incubation with recombinant (A) PP1, (B) PP2A, (C) PP2B, or (D) PP2C, AS160^{Thr642} and AS160^{Ser588} phosphorylation was reduced. Blots were stripped and reprobed with an antibody against Total AS160 to show equal loading. See Table 5.1 for a summary of the quantitative (Means ± SE) and statistical analysis of the cell-free assays. IB: immunoblot, IP: immunoprecipitation, rPP1: recombinant PP1, rPP2A: recombinant PP2A, rPP2B: recombinant PP2B, rPP2C: recombinant PP2C. See Table 5.1 for values and statistical analysis.

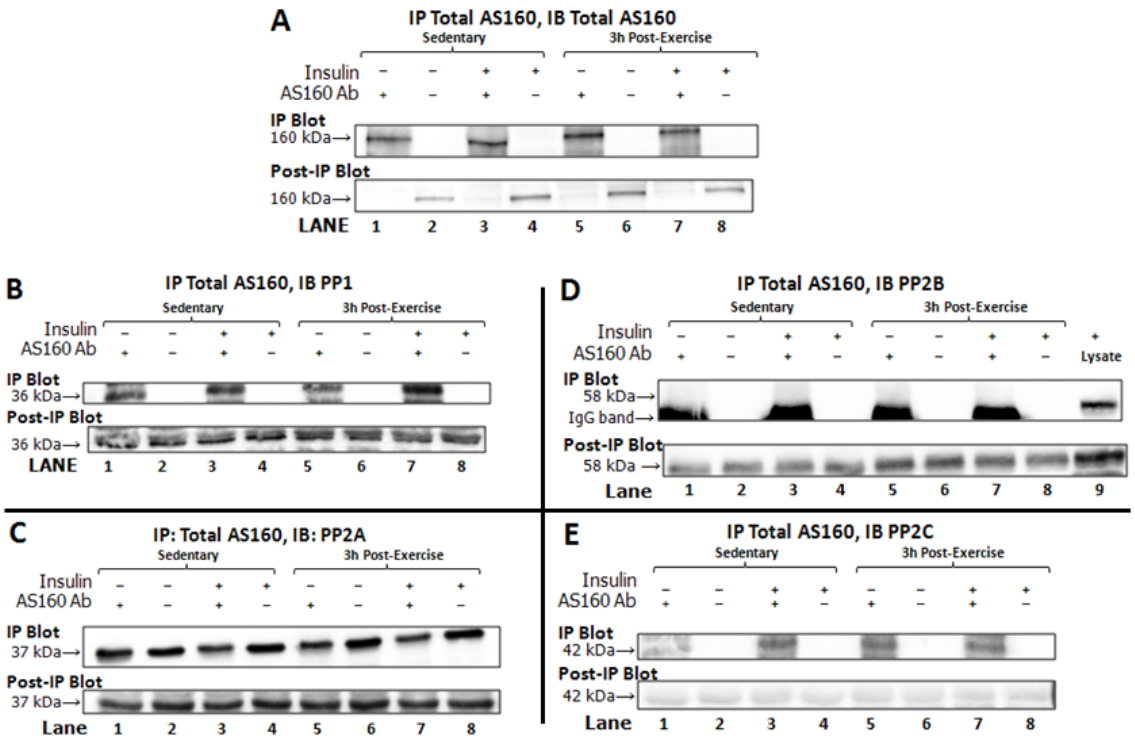

Figure 5.6

Effects of prior exercise and insulin incubation on co-immunoprecipitation of AS160 and phosphatase catalytic subunits. Duplicate epitrochlearis muscle lysate aliquots for each sample were loaded in lanes 1-2, 3-4, 5-6, and 7-8. Muscles from duplicate aliquots had been treated identically (with/without prior exercise and with/without insulin) except for an antibody against total AS160 was present in the immunoprecipitation (IP) cocktail for the sample loaded in the odd-numbered lanes (1, 3, 5, 7) and absent for the sample loaded in the even numbered lanes (2, 4, 6, 8). An immunoblot of the IP is shown in the upper blot. Post-IP supernatant (PIPS) was loaded in a second gel, and an immunoblot of the PIPS is shown in the lower blot. Immunoblot with (A) Total AS160, (B) PP1, (C) PP2A, (D) PP2B, and (E) PP2C. Lane 9 in panel D is PP2B from 20ug of skeletal muscle lysate to show that immunoblotting reveals PP2B in spite of a lack of AS160-PP2B association in Lanes 1, 3, 5, and 7. Ab: antibody, IB: immunoblot, IgG: immunoglobulin G, IP: immunoprecipitation.

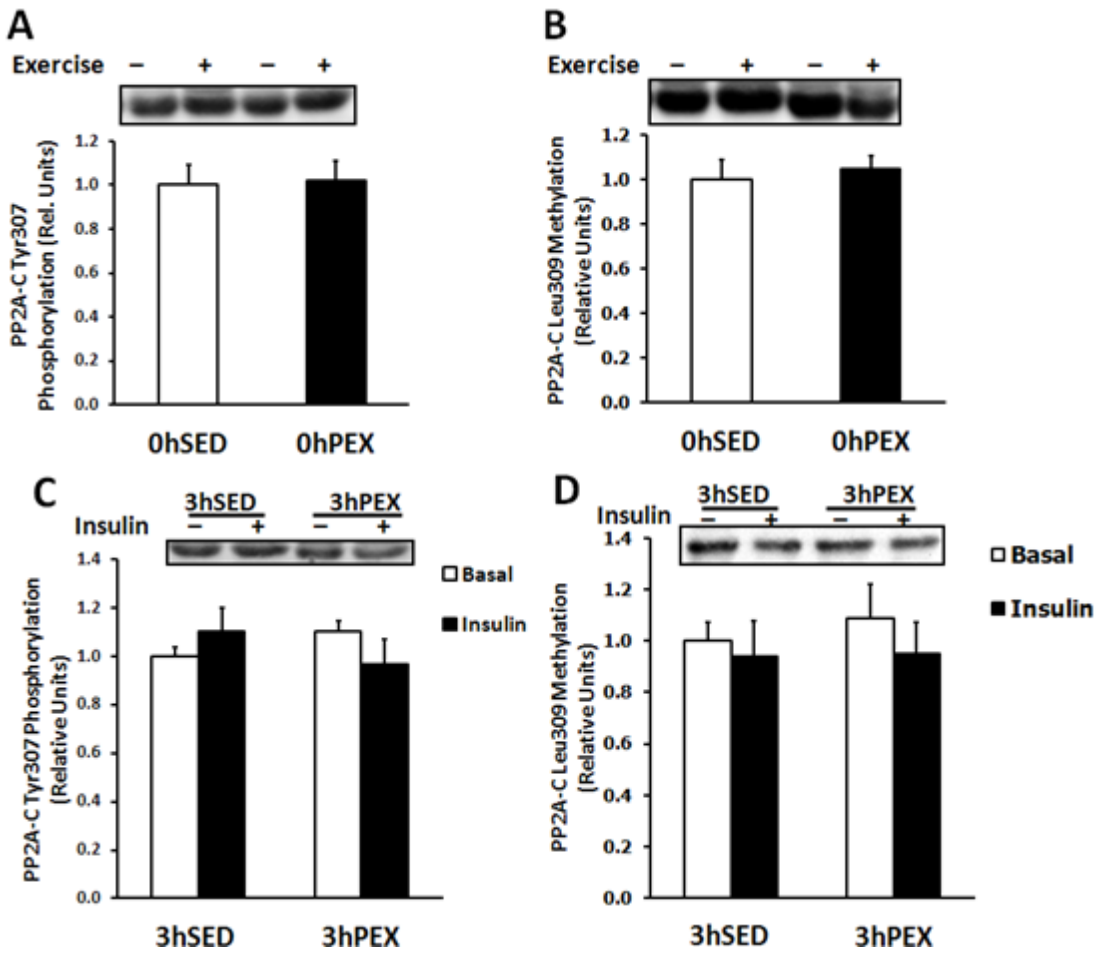

Figure 5.7
PP2A^{Tyr307} phosphorylation and PP2A^{Leu309} methylation in isolated rat epitrochlearis muscles. A-B: rats anesthetized immediately post-exercise (0hPEX) with time-matched sedentary controls (0hSED); C-D: rats anesthetized 3h post-exercise (3hPEX). A, C: samples immunoblotted for PP2A^{Tyr307} phosphorylation; B, D: samples immunoblotted for PP2A^{Leu309} methylation. For rats in the 0hPEX and 0hSED groups, all muscles that were used to measure AS160 phosphorylation were frozen immediately after dissection to determine the insulin-independent effect of exercise. For rats in the 3hPEX groups and sedentary controls (3hSED), one of the paired muscles was incubated without insulin, and the contralateral muscle was incubated with 50μU/ml insulin. A-B: data are means ± SE; n = 7/group. Open bars, 0hSED; filled bars, 0hPEX. C-D: data are means ± SE; n = 8-12/group. For panels C and D: open bars, without insulin; filled bars, with insulin.

REFERENCES

1. **Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, and Hemmings BA.** Mechanism of activation of protein kinase B by insulin and IGF-1. *The EMBO journal* 15: 6541-6551, 1996.
2. **Arias EB, Kim J, Funai K, and Cartee GD.** Prior exercise increases phosphorylation of Akt substrate of 160 kDa (AS160) in rat skeletal muscle. *American journal of physiology* 292: E1191-1200, 2007.
3. **Baar K and Esser K.** Phosphorylation of p70(S6k) correlates with increased skeletal muscle mass following resistance exercise. *Am J Physiol* 276: C120-127, 1999.
4. **Bao Y, Li R, Jiang J, Cai B, Gao J, Le K, Zhang F, Chen S, and Liu P.** Activation of peroxisome proliferator-activated receptor gamma inhibits endothelin-1-induced cardiac hypertrophy via the calcineurin/NFAT signaling pathway. *Molecular and cellular biochemistry* 317: 189-196, 2008.
5. **Bonen A and Tan MH.** Dissociation between insulin binding and glucose utilization after intense exercise in mouse skeletal muscles. *Horm Metab Res* 21: 172-178, 1989.
6. **Bonen A, Tan MH, Clune P, and Kirby RL.** Effects of exercise on insulin binding to human muscle. *Am J Physiol* 248: E403-408, 1985.
7. **Bonen A, Tan MH, and Watson-Wright WM.** Effects of exercise on insulin binding and glucose metabolism in muscle. *Can J Physiol Pharmacol* 62: 1500-1504, 1984.
8. **Borgatti P, Martelli AM, Tabellini G, Bellacosa A, Capitani S, and Neri LM.** Threonine 308 phosphorylated form of Akt translocates to the nucleus of PC12 cells under nerve growth factor stimulation and associates with the nuclear matrix protein nucleolin. *Journal of cellular physiology* 196: 79-88, 2003.
9. **Bruss MD, Arias EB, Lienhard GE, and Cartee GD.** Increased phosphorylation of Akt substrate of 160 kDa (AS160) in rat skeletal muscle in response to insulin or contractile activity. *Diabetes* 54: 41-50, 2005.
10. **Bryant JC, Westphal RS, and Wadzinski BE.** Methylated C-terminal leucine residue of PP2A catalytic subunit is important for binding of regulatory Balpha subunit. *The Biochemical journal* 339 (Pt 2): 241-246, 1999.
11. **Cartee GD and Bohn EE.** Growth hormone reduces glucose transport but not GLUT-1 or GLUT-4 in adult and old rats. *Am J Physiol* 268: E902-909, 1995.
12. **Cartee GD, Briggs-Tung C, and Kietzke EW.** Persistent effects of exercise on skeletal muscle glucose transport across the life-span of rats. *J Appl Physiol* 75: 972-978, 1993.
13. **Cartee GD and Holloszy JO.** Exercise increases susceptibility of muscle glucose transport to activation by various stimuli. *Am J Physiol* 258: E390-393, 1990.
14. **Cartee GD, Young DA, Sleeper MD, Zierath J, Wallberg-Henriksson H, and Holloszy JO.** Prolonged increase in insulin-stimulated glucose transport in muscle after exercise. *Am J Physiol* 256: E494-499, 1989.
15. **Chen HC, Bandyopadhyay G, Sajan MP, Kanoh Y, Standaert M, Farese RV, Jr., and Farese RV.** Activation of the ERK pathway and atypical protein kinase C isoforms in exercise- and aminoimidazole-4-carboxamide-1-beta-D-riboside (AICAR)-

stimulated glucose transport. *The Journal of biological chemistry* 277: 23554-23562, 2002.
16. **Chen S, Murphy J, Toth R, Campbell DG, Morrice NA, and Mackintosh C.** Complementary regulation of TBC1D1 and AS160 by growth factors, insulin and AMPK activators. *The Biochemical journal* 409: 449-459, 2008.
17. **Chiang CW, Yan L, and Yang E.** Phosphatases and regulation of cell death. *Methods Enzymol* 446: 237-257, 2008.
18. **Chibalin AV, Yu M, Ryder JW, Song XM, Galuska D, Krook A, Wallberg-Henriksson H, and Zierath JR.** Exercise-induced changes in expression and activity of proteins involved in insulin signal transduction in skeletal muscle: differential effects on insulin-receptor substrates 1 and 2. *Proceedings of the National Academy of Sciences of the United States of America* 97: 38-43, 2000.
19. **Cho DH, Choi YJ, Jo SA, Ryou J, Kim JY, Chung J, and Jo I.** Troglitazone acutely inhibits protein synthesis in endothelial cells via a novel mechanism involving protein phosphatase 2A-dependent p70 S6 kinase inhibition. *Am J Physiol Cell Physiol* 291: C317-326, 2006.
20. **Coffey VG, Zhong Z, Shield A, Canny BJ, Chibalin AV, Zierath JR, and Hawley JA.** Early signaling responses to divergent exercise stimuli in skeletal muscle from well-trained humans. *Faseb J* 20: 190-192, 2006.
21. **Cohen P.** The structure and regulation of protein phosphatases. *Annual review of biochemistry* 58: 453-508, 1989.
22. **Cusi K, Maezono K, Osman A, Pendergrass M, Patti ME, Pratipanawatr T, DeFronzo RA, Kahn CR, and Mandarino LJ.** Insulin resistance differentially affects the PI 3-kinase- and MAP kinase-mediated signaling in human muscle. *J Clin Invest* 105: 311-320, 2000.
23. **Dalby KN, Morrice N, Caudwell FB, Avruch J, and Cohen P.** Identification of regulatory phosphorylation sites in mitogen-activated protein kinase (MAPK)-activated protein kinase-1a/p90rsk that are inducible by MAPK. *The Journal of biological chemistry* 273: 1496-1505, 1998.
24. **De Baere I, Derua R, Janssens V, Van Hoof C, Waelkens E, Merlevede W, and Goris J.** Purification of porcine brain protein phosphatase 2A leucine carboxyl methyltransferase and cloning of the human homologue. *Biochemistry* 38: 16539-16547, 1999.
25. **Dubois F, Vandermoere F, Gernez A, Murphy J, Toth R, Chen S, Geraghty KM, Morrice NA, and Mackintosh C.** Differential 14-3-3-affinity capture reveals new downstream targets of PI 3-kinase signaling. *Mol Cell Proteomics*, 2009.
26. **Favre B, Zolnierowicz S, Turowski P, and Hemmings BA.** The catalytic subunit of protein phosphatase 2A is carboxyl-methylated in vivo. *The Journal of biological chemistry* 269: 16311-16317, 1994.
27. **Fisher JS, Gao J, Han DH, Holloszy JO, and Nolte LA.** Activation of AMP kinase enhances sensitivity of muscle glucose transport to insulin. *American journal of physiology* 282: E18-23, 2002.
28. **Frosig C, Sajan MP, Maarbjerg SJ, Brandt N, Roepstorff C, Wojtaszewski JF, Kiens B, Farese RV, and Richter EA.** Exercise improves phosphatidylinositol-3,4,5-trisphosphate responsiveness of atypical protein kinase C and interacts with insulin

signalling to peptide elongation in human skeletal muscle. *The Journal of physiology* 582: 1289-1301, 2007.
29. **Funai K and Cartee GD.** Inhibition of contraction-stimulated AMP-activated protein kinase inhibits contraction-stimulated increases in PAS-TBC1D1 and glucose transport without altering PAS-AS160 in rat skeletal muscle. *Diabetes* 58: 1096-1104, 2009.
30. **Funai K, Schweitzer GG, Castorena CM, Kanzaki M, and Cartee GD.** In vivo exercise followed by in vitro contraction additively elevates subsequent insulin-stimulated glucose transport by rat skeletal muscle. *American journal of physiology* 298: E999-1010, 2010.
31. **Funai K, Schweitzer GG, Sharma N, Kanzaki M, and 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.
32. **Geraghty KM, Chen S, Harthill JE, Ibrahim AF, Toth R, Morrice NA, Vandermoere F, Moorhead GB, Hardie DG, and MacKintosh C.** Regulation of multisite phosphorylation and 14-3-3 binding of AS160 in response to IGF-1, EGF, PMA and AICAR. *The Biochemical journal* 407: 231-241, 2007.
33. **Hamada T, Arias EB, and Cartee GD.** Increased submaximal insulin-stimulated glucose uptake in mouse skeletal muscle after treadmill exercise. *J Appl Physiol* 101: 1368-1376, 2006.
34. **Hansen PA, Nolte LA, Chen MM, and Holloszy JO.** Increased GLUT-4 translocation mediates enhanced insulin sensitivity of muscle glucose transport after exercise. *J Appl Physiol* 85: 1218-1222, 1998.
35. **Herzig S and Neumann J.** Effects of serine/threonine protein phosphatases on ion channels in excitable membranes. *Physiological reviews* 80: 173-210, 2000.
36. **Howlett KF, Sakamoto K, Hirshman MF, Aschenbach WG, Dow M, White MF, and Goodyear LJ.** Insulin signaling after exercise in insulin receptor substrate-2-deficient mice. *Diabetes* 51: 479-483, 2002.
37. **Huang KX and Paudel HK.** Ser67-phosphorylated inhibitor 1 is a potent protein phosphatase 1 inhibitor. *Proceedings of the National Academy of Sciences of the United States of America* 97: 5824-5829, 2000.
38. **Ingebritsen TS, Stewart AA, and Cohen P.** The protein phosphatases involved in cellular regulation. 6. Measurement of type-1 and type-2 protein phosphatases in extracts of mammalian tissues; an assessment of their physiological roles. *European journal of biochemistry / FEBS* 132: 297-307, 1983.
39. **Ivaska J, Nissinen L, Immonen N, Eriksson JE, Kahari VM, and Heino J.** Integrin alpha 2 beta 1 promotes activation of protein phosphatase 2A and dephosphorylation of Akt and glycogen synthase kinase 3 beta. *Molecular and cellular biology* 22: 1352-1359, 2002.
40. **Janssens V, Longin S, and Goris J.** PP2A holoenzyme assembly: in cauda venenum (the sting is in the tail). *Trends in biochemical sciences* 33: 113-121, 2008.
41. **Kane S, Sano H, Liu SC, Asara JM, Lane WS, Garner CC, and Lienhard GE.** A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain. *The Journal of biological chemistry* 277: 22115-22118, 2002.

42. **Kramer HF, Witczak CA, Fujii N, Jessen N, Taylor EB, Arnolds DE, Sakamoto K, Hirshman MF, and Goodyear LJ.** Distinct signals regulate AS160 phosphorylation in response to insulin, AICAR, and contraction in mouse skeletal muscle. *Diabetes* 55: 2067-2076, 2006.
43. **Kramer HF, Witczak CA, Taylor EB, Fujii N, Hirshman MF, and Goodyear LJ.** AS160 regulates insulin- and contraction-stimulated glucose uptake in mouse skeletal muscle. *The Journal of biological chemistry* 281: 31478-31485, 2006.
44. **Kumar A, Chaudhry I, Reid MB, and Boriek AM.** Distinct signaling pathways are activated in response to mechanical stress applied axially and transversely to skeletal muscle fibers. *The Journal of biological chemistry* 277: 46493-46503, 2002.
45. **Lee IH, Lim HJ, Yoon S, Seong JK, Bae DS, Rhee SG, and Bae YS.** Ahnak protein activates protein kinase C (PKC) through dissociation of the PKC-protein phosphatase 2A complex. *The Journal of biological chemistry* 283: 6312-6320, 2008.
46. **Liu R, Zhou XW, Tanila H, Bjorkdahl C, Wang JZ, Guan ZZ, Cao Y, Gustafsson JA, Winblad B, and Pei JJ.** Phosphorylated PP2A (tyrosine 307) is associated with Alzheimer neurofibrillary pathology. *Journal of cellular and molecular medicine* 12: 241-257, 2008.
47. **Liu W, Akhand AA, Takeda K, Kawamoto Y, Itoigawa M, Kato M, Suzuki H, Ishikawa N, and Nakashima I.** Protein phosphatase 2A-linked and -unlinked caspase-dependent pathways for downregulation of Akt kinase triggered by 4-hydroxynonenal. *Cell death and differentiation* 10: 772-781, 2003.
48. **Moreno CS, Park S, Nelson K, Ashby D, Hubalek F, Lane WS, and Pallas DC.** WD40 repeat proteins striatin and S/G(2) nuclear autoantigen are members of a novel family of calmodulin-binding proteins that associate with protein phosphatase 2A. *The Journal of biological chemistry* 275: 5257-5263, 2000.
49. **Palanivel R, Veluthakal R, and Kowluru A.** Regulation by glucose and calcium of the carboxylmethylation of the catalytic subunit of protein phosphatase 2A in insulin-secreting INS-1 cells. *American journal of physiology* 286: E1032-1041, 2004.
50. **Park IK, Roach P, Bondor J, Fox SP, and DePaoli-Roach AA.** Molecular mechanism of the synergistic phosphorylation of phosphatase inhibitor-2. Cloning, expression, and site-directed mutagenesis of inhibitor-2. *The Journal of biological chemistry* 269: 944-954, 1994.
51. **Park J, Leong ML, Buse P, Maiyar AC, Firestone GL, and Hemmings BA.** Serum and glucocorticoid-inducible kinase (SGK) is a target of the PI 3-kinase-stimulated signaling pathway. *The EMBO journal* 18: 3024-3033, 1999.
52. **Paterson J, Kelsall IR, and Cohen PT.** Disruption of the striated muscle glycogen-targeting subunit of protein phosphatase 1: influence of the genetic background. *Journal of molecular endocrinology* 40: 47-59, 2008.
53. **Pearce LR, Komander D, and Alessi DR.** The nuts and bolts of AGC protein kinases. *Nature reviews* 11: 9-22, 2010.
54. **Pearson RB, Dennis PB, Han JW, Williamson NA, Kozma SC, Wettenhall RE, and Thomas G.** The principal target of rapamycin-induced p70s6k inactivation is a novel phosphorylation site within a conserved hydrophobic domain. *The EMBO journal* 14: 5279-5287, 1995.

55. **Richter EA, Hansen BF, and Hansen SA.** Glucose-induced insulin resistance of skeletal-muscle glucose transport and uptake. *The Biochemical journal* 252: 733-737, 1988.
56. **Ruderman NB, Balon T, Zorzano A, and Goodman M.** The postexercise state: altered effects of insulin on skeletal muscle and their physiologic relevance. *Diabetes Metab Rev* 1: 425-444, 1986.
57. **Sahin B, Shu H, Fernandez J, El-Armouche A, Molkentin JD, Nairn AC, and Bibb JA.** Phosphorylation of protein phosphatase inhibitor-1 by protein kinase C. *The Journal of biological chemistry* 281: 24322-24335, 2006.
58. **Saito S, Matsui H, Kawano M, Kumagai K, Tomishige N, Hanada K, Echigo S, Tamura S, and Kobayashi T.** Protein phosphatase 2Cepsilon is an endoplasmic reticulum integral membrane protein that dephosphorylates the ceramide transport protein CERT to enhance its association with organelle membranes. *The Journal of biological chemistry* 283: 6584-6593, 2008.
59. **Sano H, Kane S, Sano E, Miinea CP, Asara JM, Lane WS, Garner CW, and Lienhard GE.** Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *The Journal of biological chemistry* 278: 14599-14602, 2003.
60. **Seth A, Sheth P, Elias BC, and Rao R.** Protein phosphatases 2A and 1 interact with occludin and negatively regulate the assembly of tight junctions in the CACO-2 cell monolayer. *The Journal of biological chemistry* 282: 11487-11498, 2007.
61. **Shenolikar S.** Protein serine/threonine phosphatases--new avenues for cell regulation. *Annual review of cell biology* 10: 55-86, 1994.
62. **Shi Y.** Serine/threonine phosphatases: mechanism through structure. *Cell* 139: 468-484, 2009.
63. **Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, and Klenk DC.** Measurement of protein using bicinchoninic acid. *Anal Biochem* 150: 76-85, 1985.
64. **Stein SC, Woods A, Jones NA, Davison MD, and Carling D.** The regulation of AMP-activated protein kinase by phosphorylation. *The Biochemical journal* 345 Pt 3: 437-443, 2000.
65. **Suryawan A, Escobar J, Frank JW, Nguyen HV, and Davis TA.** Developmental regulation of the activation of signaling components leading to translation initiation in skeletal muscle of neonatal pigs. *American journal of physiology* 291: E849-859, 2006.
66. **Thong FS, Derave W, Kiens B, Graham TE, Urso B, Wojtaszewski JF, Hansen BF, and Richter EA.** Caffeine-induced impairment of insulin action but not insulin signaling in human skeletal muscle is reduced by exercise. *Diabetes* 51: 583-590, 2002.
67. **Treadway JL, James DE, Burcel E, and Ruderman NB.** Effect of exercise on insulin receptor binding and kinase activity in skeletal muscle. *Am J Physiol* 256: E138-144, 1989.
68. **Treebak JT, Frosig C, Pehmoller C, Chen S, Maarbjerg SJ, Brandt N, MacKintosh C, Zierath JR, Hardie DG, Kiens B, Richter EA, Pilegaard H, and Wojtaszewski JF.** Potential role of TBC1D4 in enhanced post-exercise insulin action in human skeletal muscle. *Diabetologia* 52: 891-900, 2009.

69. **Tsaytler P, Harding HP, Ron D, and Bertolotti A.** Selective inhibition of a regulatory subunit of protein phosphatase 1 restores proteostasis. *Science (New York, NY* 332: 91-94, 2011.
70. **Wallberg-Henriksson H.** Glucose transport into skeletal muscle. Influence of contractile activity, insulin, catecholamines and diabetes mellitus. *Acta Physiol Scand Suppl* 564: 1-80, 1987.
71. **Weiser DC and Shenolikar S.** Use of protein phosphatase inhibitors. *Curr Protoc Mol Biol* Chapter 18: Unit 18 10, 2003.
72. **Wojtaszewski JF, Hansen BF, Gade, Kiens B, Markuns JF, Goodyear LJ, and Richter EA.** Insulin signaling and insulin sensitivity after exercise in human skeletal muscle. *Diabetes* 49: 325-331, 2000.
73. **Wojtaszewski JF, Hansen BF, Kiens B, and Richter EA.** Insulin signaling in human skeletal muscle: time course and effect of exercise. *Diabetes* 46: 1775-1781, 1997.
74. **Wojtaszewski JF, Higaki Y, Hirshman MF, Michael MD, Dufresne SD, Kahn CR, and Goodyear LJ.** Exercise modulates postreceptor insulin signaling and glucose transport in muscle-specific insulin receptor knockout mice. *J Clin Invest* 104: 1257-1264, 1999.
75. **Yellaturu CR, Bhanoori M, Neeli I, and Rao GN.** N-Ethylmaleimide inhibits platelet-derived growth factor BB-stimulated Akt phosphorylation via activation of protein phosphatase 2A. *The Journal of biological chemistry* 277: 40148-40155, 2002.
76. **Zhou Q and Dohm GL.** Treadmill running increases phosphatidylinositol 3-kinase activity in rat skeletal muscle. *Biochemical and biophysical research communications* 236: 647-650, 1997.
77. **Zhu T, Matsuzawa S, Mizuno Y, Kamibayashi C, Mumby MC, Andjelkovic N, Hemmings BA, Onoe K, and Kikuchi K.** The interconversion of protein phosphatase 2A between PP2A1 and PP2A0 during retinoic acid-induced granulocytic differentiation and a modification on the catalytic subunit in S phase of HL-60 cells. *Archives of biochemistry and biophysics* 339: 210-217, 1997.
78. **Zorzano A, Balon TW, Garetto LP, Goodman MN, and Ruderman NB.** Muscle alpha-aminoisobutyric acid transport after exercise: enhanced stimulation by insulin. *Am J Physiol* 248: E546-552, 1985.

CHAPTER VI

DISCUSSION

Focus of this Discussion

This chapter of the dissertation will: 1) summarize the key findings from the three studies; 2) identify some questions raised by the results of Studies 1 and 2 and propose general future directions to take with those studies; 3) identify some questions raised by the results of Study 3; 4) provide an integrated interpretation of the results of Studies 1, 2, and 3; 5) provide a brief proposal of possible future experiments related to Study 3; and 6) provide overall conclusions.

Summary of Key Findings

Results from Studies 1 and 2 of the dissertation, which focused on the role of the Kallikrein Kininogen System (KKS) in understanding the mechanisms associated with the post-exercise increase in insulin-stimulated glucose transport, extended the initial *in vitro* observations with electrically stimulated isolated skeletal muscles (7) by investigating the KKS from an *in vivo* physiological standpoint (28, 29). This was accomplished using two separate genetic models: mice with a genetic knockout of the B2

receptor of bradykinin (B2RKO) and rats with a naturally occurring deficiency in kininogen (BNK).

Results from Study 3 of the dissertation provided more insight into the mechanisms that regulate the sustained increase in AS160 phosphorylation after acute exercise, specifically regarding the kinases and phosphatases that participate. Kinases were evaluated by studying epitrochlearis muscles from rats using an exercise protocol known to result in sustained phosphorylation of AS160. Phosphatases were evaluated using this same protocol as well as by employing cell-free dephosphorylation assays and co-immunoprecipiation of AS160 with the phosphatases.

Study 1: *The B2 receptor of bradykinin is not essential for the post-exercise increase in glucose uptake by insulin-stimulated mouse skeletal muscle*

Study 1 used genetically modified mice null for the B2 receptor (B2R) to test the idea that this receptor may be important for the post-exercise increase in insulin action. Isolated soleus and EDL muscles from B2RKO and wildtype (WT) control mice that had previously undergone acute exercise (EX) or remained sedentary (SED) were incubated in a solution without or with a physiological dose of insulin. The insulin-stimulated increase in glucose uptake (delta insulin) calculated by subtracting the basal values from the insulin-stimulated values was also determined. Insulin-stimulated muscles from Experiment 1 were incubated in 60μU/ml insulin, and insulin-stimulated muscles from Experiment 2 muscles were incubated in 100μU/ml insulin.

Genotype Effects of Muscle Glucose Uptake

- Soleus glucose uptake without insulin was not different between genotypes.
- EDL glucose uptake without insulin was not different between genotypes.
- Soleus glucose uptake with insulin tended to be lower for B2RKO vs. WT mice with 60µU/ml insulin, was significantly lower for B2RKO vs. WT mice with 100µU/ml insulin, and was significantly lower for B2RKO vs. WT mice when pooling samples incubated with 60µU/ml and 100µU/ml insulin.
- EDL glucose uptake with insulin was not different between genotypes
- Soleus delta insulin was significantly lower for B2RKO vs. WT mice with 60µU/ml insulin, 100µU/ml insulin, and when data from both experiments were pooled.
- EDL delta insulin was not different between genotypes.

Exercise Effects of Muscle Glucose Uptake

- Soleus glucose uptake without insulin tended to be greater for EX vs. SED mice in Experiment 1, tended to be greater for EX vs. SED mice in Experiment 2, and was significantly greater for EX vs. SED mice when pooling samples from both experiments.
- EDL glucose uptake without insulin was not different between EX and SED mice.
- Soleus glucose uptake with insulin tended to be to be greater for EX vs. SED mice with 60µU/ml, was significantly greater for EX vs. SED mice

with 100µU/ml insulin, and was significantly greater for EX vs. SED mice when pooling samples incubated with 60µU/ml and 100µU/ml insulin.

- EDL glucose uptake with insulin was not different between EX and SED mice.
- Soleus delta insulin tended to be greater for EX vs. SED mice with 60µU/ml, tended to be greater for EX vs. SED mice with 100µU/ml insulin, and was significantly greater for EX vs. SED mice when pooling samples incubated with 60µU/ml and 100µU/ml insulin.
- EDL delta insulin was not different between EX vs. SED mice.

Interactions of Genotype and Exercise of Muscle Glucose Uptake

- There were no statistically significant interactions between genotype (B2RKO vs. WT) and exercise (EX vs. SED) for any of the muscle glucose uptake measurements.

Akt^{Thr308} Phosphorylation

- Soleus and EDL Akt^{Thr308} phosphorylation were not significantly different with genotype or exercise, with or without insulin, or delta insulin.

Study 2: *Post-exercise skeletal muscle glucose transport is normal in kininogen-deficient rats*

Study 2 used rats used BNK to determine if normal circulating kininogen levels are required for the sustained increase in insulin-stimulated glucose transport in skeletal muscle after exercise. Isolated epitrochlearis muscles from BNK and Brown Norway (BN) control rats that had previously undergone acute exercise (3hPEX) or remained

sedentary (3hSED) were incubated in a solution without or with a physiological dose of insulin. The insulin-stimulated increase in glucose uptake (delta insulin) calculated by subtracting the basal values from the insulin-stimulated values was also determined. Furthermore, this study evaluated the insulin-independent effect of exercise on skeletal muscle glucose transport comparing muscles from rats of each genotype that had been dissected immediately post-exercise (0hPEX) or were time-matched sedentary controls (0hSED).

Genotype Effects of Muscle Glucose Transport

- Epitrochlearis glucose transport without insulin was not different between BNK and BN rats.
- Epitrochlearis glucose transport with 60µU/ml insulin was not different between BNK and BN rats.
- Epitrochlearis delta insulin was not different between BNK and BN rats.

Exercise Effects of Muscle Glucose Transport

- Epitrochlearis glucose transport without insulin was significantly greater in 3hPEX vs. 3hSED rats.
- Epitrochlearis glucose transport with 60µU/ml insulin was significantly greater in 3hPEX vs. 3hSED rats.
- Epitrochlearis delta insulin was significantly greater in 3hPEX vs. 3hSED rats.

Interactions of Genotype and Exercise of Muscle Glucose Uptake

- There were no statistically significant interactions between genotype (BNK vs. BN) and exercise (3hPEX vs. 3hSED) for any of the muscle glucose transport measurements.

Insulin-Independent Effects

- There was no difference in BNK vs. BN rats for the exercise-induced increase in insulin-independent glucose transport.
- There was a significant increase in glucose transport in 0hPEX rats vs. 0hSED rats in both BN and BNK rats.
- Muscle glycogen and $AMPK^{Thr172}$ phosphorylation were not different in BNK vs. BN rats.
- Muscle glycogen was significantly reduced and $AMPK^{Thr172}$ phosphorylation was significantly increased in 0hPEX vs. 0hSED to a similar extent in both BNK and BN rats.

Study 3: *Mechanisms for the prolonged post-exercise increase in AS160 phosphorylation: Roles of protein kinases and phosphatases*

Study 3 used epitrochlearis muscles from Wistar rats that had undergone an exercise protocol known to result in sustained AS160 phosphorylation and other experiments required immunoprecipitated AS160 from rat epitrochlearis muscle. There were three overall goals: 1) To establish if $AS160^{Ser588}$ phosphorylation was sustained post-exercise, 2) To evaluate the known kinases of AS160 to determine if greater phosphorylation of these kinases is found after prior exercise, and 3) To conduct the first known investigation of the phosphatases that regulate AS160 dephosphorylation.

AS160 Phosphorylation

- AS160^{Ser588} phosphorylation was increased immediately post-exercise, and greater phosphorylation was sustained three hours post-exercise in rat epitrochlearis muscle. This phospho-site has been reported to be one of the two most important sites for insulin-stimulated GLUT4 translocation (27).
- AS160^{Thr642} phosphorylation was confirmed to be increased immediately post-exercise, and greater phosphorylation was sustained three hours post-exercise in rat epitrochlearis muscle, and this phospho-site has been reported to be one of the two most important sites for insulin-stimulated GLUT4 translocation (27).

Kinases

- The four known kinases of AS160 (Akt, AMPK, RSK, and SGK) did not have greater phosphorylation on phospho-sites known to be essential for increased enzyme activation in muscles from rats that had undergone exercise three hours prior versus sedentary controls.
- Another possible AS160 kinase based on consensus motifs, p70S6K, did not have greater phosphorylation on a phospho-site known to be essential for its function in rats that had undergone exercise three hours prior versus sedentary controls.
- Of the 5 studied kinases, only AMPK had greater phosphorylation immediately post-exercise vs. sedentary control rats.

Phosphatases

- PP1, PP2A, PP2B, and PP2C had the ability to dephosphorylate AS160 on the Thr642 and Ser588 phospho-sites of rat epitrochlearis muscle in a cell-free assay.
- PP1 and PP2C were specifically associated with AS160 from rat epitrochlearis muscles based on results from co-immunoprecipitation.
- PP2A and PP2B were not found to have specific association with AS160 from rat epitrochlearis muscle based on results from co-immunoprecipitation.
- $PP2A^{Tyr307}$ phosphorylation in rat epitrochlearis muscle was not altered by prior exercise or incubation with physiological insulin.
- $PP2A^{Leu309}$ methylation in rat epitrochlearis muscle was not altered by prior exercise or incubation with physiological insulin.
- $PP2A^{Tyr307}$ phosphorylation and $PP2A^{Leu309}$ methylation was not altered immediately post-exercise vs. time-matched sedentary controls.
- The amount of PP1, PP2A, PP2B, and PP2C association with AS160 as measured by co-immunoprecipitation was not altered by prior exercise or incubation with physiological insulin.

Future Directions for Studies 1 and 2

Results from Studies 1 and 2 of the dissertation did not support the idea that the KKS is essential for the post-exercise increase in insulin-stimulated glucose transport in skeletal muscle. There are several caveats of this interpretation. In Study 1, it is

conceivable that the B1 receptor of bradykinin (B1R) may be compensating for the lack of B2R in B2RKO mice. It has been reported that mRNA of B1R is greater in B2RKO mice vs. WT (6). Because skeletal muscle abundance of B1R protein in B2RKO has not been reported, we obtained the only available antibodies for B1R from Alomone Labs and from Dr. Werner Müller-Esterl, a bradykinin researcher at the University of Frankfurt. However, repeated experiments using the B1R antibody revealed multiple bands that defied interpretation. Regardless, it is important to note that B2RKO mice are insulin resistant (6), indicating that B1R is unable to replace the essential role of B2R for normal insulin sensitivity. In Study 2, plasma kininogen values of BNK rats have been reported to be less than 1% of the normal plasma values for Brown Norway (BN) rats (24). Accordingly, it remains formally possible that this very low amount of kininogen in BNK rats may be sufficient to trigger increases in skeletal muscle insulin sensitivity post-exercise.

It is also important to note that following completion of Studies 1 and 2, it was discovered that in vivo exercise and in vitro contractions performed in serum may not lead to increased insulin-stimulated glucose transport by an identical mechanism (9). Funai et al. (9) found that when in vivo exercise is followed by rapid removal of the epitrochlearis and in vitro contraction of the isolated muscle, the two stimuli induced an additive effect on the subsequent increase in insulin-stimulated glucose transport. These results suggested that the increase in insulin-stimulated glucose transport induced after in vitro contraction and after in vivo exercise may be caused by distinct mechanisms. This finding can serve to reconcile the evidence supporting KKS's involvement in the post-contraction increase in insulin-stimulated glucose transport in an *in vitro* model (7) with

the lack of evidence supporting the KKS's involvement in the post-exercise increase in insulin-stimulated glucose transport in *in vivo* models in Studies 1 and 2.

Although the results of Studies 1 and 2 do not support a role for the KKS as a possible trigger for increased insulin-stimulated glucose transport after *in vivo* exercise, there would still be value in understanding the mechanisms that lead to increased insulin-stimulated glucose transport after in vitro contractions in serum. In this context, it would be useful to consider new candidate proteins found in the serum that may be essential for the development of post-contraction increase in skeletal muscle insulin sensitivity. New possibilities may come from a repository of candidates of a new and relatively unstudied area in skeletal muscle physiology, myokines. Myokines are cytokine-like proteins secreted from skeletal muscle that may be responsible for bioeffects on target tissues (22, 23). There are over 600 known myokines (13) that can be screened for the characteristics the serum factor is likely to contain: 1) it should be regulated by muscle contractions, 2) it should improve insulin sensitivity, and 3) it should be composed of proteins greater than 10 kDa.

The following questions that are raised from Studies 1 and 2 which could guide future research:

1) Is the post-exercise increase in insulin-stimulated glucose uptake of muscle found in B2RKO mice attributable to a compensatory effect of greater B1R in the skeletal muscle of B2RKO mice?
2) Is the relatively low amount of circulating plasma kininogen in BNK rats compared to BN rats, sufficient to increase skeletal muscle insulin sensitivity after exercise?

3) If questions 1 and 2 do not implicate the KKS, what other candidate proteins in the serum are responsible for triggering the *in vitro* contraction increase in insulin-stimulated glucose transport with serum retained?
4) Are one or multiple myokines essential for elevated insulin-stimulated glucose transport in skeletal muscle after in vitro contraction in serum and is this also necessary for elevated insulin-stimulated glucose transport after in vivo exercise?

Future Directions for Study 3

Results from Study 3 of the dissertation have provided more insight in the mechanisms that regulate sustained AS160 phosphorylation after exercise. Because the results of Study 3 did not provide evidence implicating the known AS160 kinases as being responsible for sustained AS160 phosphorylation after exercise, serine/threonine protein phosphatases were investigated. These experiments demonstrated that each of the four most abundant skeletal muscle phosphatases (PP1, PP2A, PP2B, and PP2C) were all shown to dephosphorylate AS160 in cell-free conditions. However, catalytic subunits of PP1 and PP2C, but not PP2A or PP2B, were shown to specifically associate with AS160. The amount of association of these phosphatases to AS160 was not altered by prior exercise or insulin incubation. Based on current evidence, PP1 and PP2C appear to be the leading candidates for regulating AS160 dephosphorylation on Thr642 and Ser588, but it is uncertain if they have redundant, complementary, or independent actions on AS160 phosphorylation status. Ultimately, it will be important to study both their

separate and combined roles, but as a practical matter, I will first focus on PP1 for recommended future research because there is a more extensive scientific literature base and more readily available reagents to study PP1 vs. PP2C. The results of Study 3 raise many general questions with regards to AS160 phosphatases, specifically PP1:

1) What is/are the specific PP1 regulatory subunit(s) linking the PP1 catalytic subunit to AS160 in skeletal muscle?
2) Does the PP1 regulatory subunit associated with AS160 in skeletal muscle regulate the activity of PP1 catalytic subunit and therefore AS160 dephosphorylation?
3) Does altering the expression of the PP1 regulatory subunit associated with AS160 alter insulin-stimulated AS160 phosphorylation or AS160 phosphorylation post-exercise in skeletal muscle?
4) Does altering the expression of the PP1 regulatory subunit associated with AS160 alter post-exercise insulin-stimulated glucose transport?
5) Are there any endogenous inhibitors of the catalytic subunit of PP1?
6) Is the activity of the inhibitors of the catalytic subunit of PP1 altered with prior exercise or insulin incubation?

Because current results are consistent with the idea that PP1 inhibition results in sustained AS160 phosphorylation, it will be important to design future studies to demonstrate if this model is valid.

Integrated Interpretation of the Results of Studies 1, 2, and 3

The three studies in this dissertation were each related to specific aspects of the mechanisms whereby acute exercise results in a sustained effect on skeletal muscle glucose transport. Studies 1 and 2 addressed the role of the KKS for the post-exercise increase in insulin-stimulated glucose uptake, and Study 3 investigated the mechanisms whereby protein kinases and phosphatases contributed to sustained AS160 phosphorylation post-exercise.

Figure 6.2 illustrates a simple model for the series of steps that account for the post-exercise increase in insulin-stimulated glucose transport in skeletal muscle. The model includes four components (*triggers, memory elements, mediators,* and the *end-effector*) that lead from prior exercise to the ultimate outcome of increased insulin-stimulated glucose transport. The progression of steps for the post-exercise increase in insulin-stimulated glucose transport includes: 1) *triggers* that are initiating events that activate the next component. 2) The second component is called a *memory element,* and it provides a persistent mechanism for coupling triggers to the subsequent component. 3) This next component is called a *mediator,* and it regulates the function of the final component. 4) The last component is designated the *end-effector*, and it performs the final action (i.e., increased insulin-stimulated glucose transport).

The time-course associated with each of the components in relationship to the exercise is also included in the figure. In addition, the figure indicates candidates for bioeffects corresponding to particular components of the model that were assessed in this dissertation. Finally, the figure also indicates if the results of the experiments were

supportive or not supportive of each of the respective candidate bioeffects that were tested.

For these components in our model for the post-exercise increase in insulin-stimulated glucose transport, it is well-established that increased cell surface GLUT-4 is an *end-effector* accounting for increased insulin-stimulated glucose transport (12, 33). For prior exercise to lead to increased insulin-stimulated glucose transport, exercise must trigger something to initiate the series of events. Furthermore, the muscle needs to "remember" that it had exercised several hours or even days prior. Therefore, some form of *memory element* must be present in order to allow mediators to affect the effector. Physiological insulin must regulate these mediators and effectors in order to lead to increased insulin-stimulated glucose transport after exercise.

Using two different animal models (mice and rats) to study two different components of the KKS (B2R and plasma kininogen), results from Study 1 and Study 2 argued against KKS as an essential *trigger* for the post-exercise increase in insulin sensitivity in skeletal muscle. The original hypothesis implicating KKS as a necessary *trigger* came from in vitro contraction studies that implicated a "serum factor" being necessary for the post-contraction increase in insulin-stimulated glucose transport in skeletal muscle as contraction of the muscle in serum versus contraction in a buffer solution results in a greater insulin-stimulated increase in glucose transport (4, 11). However, recent data revealed that in vivo exercise and in vitro contraction in rat serum versus either stimuli alone additively elevated the subsequent increase in glucose transport in skeletal muscle (9). These data suggest that the mechanisms leading to the post-exercise increase in insulin-stimulated glucose transport in skeletal muscle may be

different from the mechanisms leading to the post-contraction increase in insulin-stimulated glucose transport in skeletal muscle. Therefore, KKS may be an essential *trigger* for the post-contraction increase in insulin-stimulated glucose transport, but as described in Studies 1 and 2, it is not an essential *trigger* for the post-exercise increase in insulin-stimulated glucose transport in skeletal muscle.

Notably, the results in Study 3 indicated that AMPK is a top candidate for a *trigger* as evidenced by this protein being the only known AS160 kinase with increased $AMPK^{Thr172}$ phosphorylation immediately after exercise on a phospho-site essential for its activity concomitant with increased $AS160^{Thr642}$ and $AS160^{Ser588}$ phosphorylation immediately after exercise. Furthermore in Study 3, the results indicated that in addition to sustained phosphorylation of the $AS160^{Thr642}$ phospho-site, $AS160^{Ser588}$ phosphorylation is also sustained after exercise suggesting that this is also a strong candidate for a *memory element*/*mediator*. The evidence did not implicate the known AS160 kinases for sustained AS160 phosphorylation, but studies of serine/threonine phosphatases revealed two top candidate AS160 phosphatases (PP1 and PP2C) that can control AS160 dephosphorylation and associate with AS160 in skeletal muscle. Future research built on the foundation of the results of Study 3 can test new hypotheses related to the possible roles of PP1 and/or PP2C in the sustained exercise effect on AS160 phosphorylation. These future studies can evaluate the possibility that sustained AS160 phosphorylation acts as a memory element/mediator and test how sustained AS160 phosphorylation might contribute to the *end-effector*, increased cell-surface GLUT4, resulting in increased insulin-stimulated glucose transport.

Proposal for Future Research

The results of this dissertation were used to develop the brief proposal for future research that is found below. The proposal focuses on the possible role of PP1 in the regulation of AS160 dephosphorylation.

Specific Aims

Studying the function of PP1 on specific target substrates is a challenge as PP1 is a highly abundant phosphatase in skeletal muscle that is associated with many target substrates. Regulation of PP1 activity can occur via 2 primary mechanisms: 1) changes in the activity of the catalytic subunit whose inhibitory proteins is altered by phosphorylation (a more detailed explanation is below) and 2) modulation of the PP1 regulatory subunit by mechanisms described below. The few known catalytic subunits of PP1 are bound to a diverse number of regulatory subunits (over 100 have been identified) that provide substrate specificity and subcellular localization (31). These PP1 regulatory subunits can, in turn, bind and be regulated by other proteins (21, 34) or can be regulated by post-translational modifications of the regulatory subunit (17) resulting in alterations in PP1's phosphatase activity.

As none of the regulatory subunit(s) of PP1 localized to AS160 have been identified, it will be important to discover the identity of such proteins (via methods such as mass spectrometry) to determine if a specific PP1 regulatory subunit(s) associates with AS160 in vivo.

Specific Aim 1: *Identify the specific PP1 regulatory subunit(s) associated with AS160 (PP1reg-AS160) from rat epitrochlearis muscle.*

Hypothesis 1A: Mass spectrometry analysis will reveal a single PP1 regulatory subunit that is associated with AS160 and is a promising candidate to be the PP1reg-AS160.

If results from Specific Aim 1, reveal a promising candidate, then further analysis of this protein as a regulator of AS160 dephosphorylation will be performed as described below for Specific Aims 2 and 3. If results from Specific Aim 1, do not reveal a promising candidate for the PP1 regulatory subunit, Specific Aims 4, 5, and 6 can still be conducted to study regulation at the site of the PP1 catalytic subunit.

Specific Aim 2: *Determine if the overexpression of the PP1 regulatory subunit associated with AS160 (PP1reg-AS160) regulates AS160 dephosphorylation by PP1.*

Hypothesis 2: Rats transfected with DNA for PP1reg-AS160 in epitrochlearis muscle (versus the contralateral muscle transfected with an empty vector) will have 1) greater abundance of PP1reg-AS160, 2) greater association of PP1reg-AS160 with AS160 (via co-immunoprecipitation), 3) greater association of PP1reg-AS160 with the PP1 catalytic subunit (PP1cat), 4) decreased $AS160^{Thr642}$ and $AS160^{Ser588}$ phosphorylation after incubation with insulin , and 5) a more rapid time course for reversal of $AS160^{Thr642}$ and $AS160^{Ser588}$ phosphorylation after transient incubation with physiological insulin.

Specific Aim 3: *Determine if the reduction of expression of the PP1 regulatory subunit associated with AS160 (PP1reg-AS160) regulates AS160 dephosphorylation by PP1.*

Hypothesis 3: Rats transfected with siRNA targeting PP1reg-AS160 in epitrochlearis muscle (versus the contralateral muscle transfected with an empty vector) will have 1) reduced abundance of PP1reg-AS160, 2) reduced association of PP1reg-AS160 with AS160 (via co-immunoprecipitation), 3) reduced association of PP1reg-AS160 with the PP1cat, 4) increased $AS160^{Thr642}$ and $AS160^{Ser588}$ phosphorylation after incubation with insulin , and 5) a more prolonged time course for reversal of $AS160^{Thr642}$ and $AS160^{Ser588}$ phosphorylation after transient incubation with physiological insulin.

If results from Specific Aim 2 and 3 support PP1's role as a phosphatase of AS160, follow up studies can examine the specific mechanisms by which PP1 functions (via regulation of Inhibitor-1 and/or Inhibitor 2; see Specific Aim 4). If results from Specific Aim 2 do not support PP1's role as a phosphatase of AS160, similar studies will be performed on PP2A, PP2B, or PP2C based on results of the mass spectrometry analysis from Specific Aim 1.

As described above, the phosphatase activity of PP1 is also regulated by the activation of binding proteins to the PP1 catalytic subunit. Several endogenous inhibitory proteins such as Inhibitior-1 (Inh-1), Inhibitor-2 (Inh-2), protein kinase C (PKC)-dependent phosphatase inhibitor of 17kDa (CPI-17), and Dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) have been reported to, upon activation, bind and inhibit PP1 phosphatase activity (31). While DARPP-32 is most abundant in neuronal tissue and CPI-17 is abundant in smooth muscle, Inh-1 and Inh-2 are abundant in skeletal muscle (18, 19, 32). Inhibition of PP1 by Inh-1 requires phosphorylation of either Thr35 or Ser67 site of Inh-1 (8, 14, 15, 25). Inhibition of PP1 by Inh-2 requires phosphorylation of Thr72 primarily, and phosphorylation of Ser86 of Inh-2 enhances the

rate of Inh-2^{Thr72} phosphorylation (20, 35). Various kinases have been identified to phosphorylate each phospho-site of Inh-1 and Inh-2, independently. It will therefore be important to assess if inhibitory proteins targeting the catalytic subunit also regulate PP1-bound AS160 function.

Inh-1 and Inh-2 proteins are also attractive targets for analysis because they provide a logical, simple mechanism in which prior exercise could result in PP1 inhibition thereby leading to the sustained increase in AS160 phosphorylation after exercise.

Specific Aim 4: *Determine if PP1 inhibitory proteins regulate sustained AS160 phosphorylation three hours after exercise via PP1 catalytic subunit inhibition.*

Hypothesis 4A: Inh-1 associated with PP1-AS160 will have increased phosphorylation on Thr35 and/or Ser67 in isolated rat epitrochlearis muscles immediately and three hours post-exercise in the absence of insulin.

Hypothesis 4B: Inh-2 associated with PP1-AS160 will have increased phosphorylation on Thr72 and/or Ser86 in isolated rat epitrochlearis muscles immediately and three hours post-exercise in the absence of insulin.

Hypothesis 4C: Inh-1 and Inh-2 phosphorylation in isolated rat epitrochlearis muscles will not be altered when incubated with physiologic insulin versus without insulin (with or without prior exercise).

If results from Specific Aim 4 support the role of Inh-1 and/or Inh-2 as an inhibitor of PP1 activity after prior exercise, the roles of Inh-1 and/or Inh-2 will be further examined to see if modulating the phosphorylation/activation state of these

proteins results in altered PP1 inhibition and therefore sustained AS160 phosphorylation. If results from Specific Aim 4 do not support the role Inh-1 and/or Inh-2 as an inhibitor of PP1 activity after prior exercise, regulation of the activity of other phosphatase(s) of AS160 can be examined, such as the other top candidate, PP2C.

As Inh-1 and Inh-2 are homogenous proteins that can target PP1 catalytic subunits on skeletal muscle proteins not regulating AS160, altering PP1-AS160-specific Inh-1/Inh-2 proteins presents a challenge. To minimize systemic effects and localize the mutation to a single muscle, vector DNA developed with mutated Inh-1/Inh-2 proteins will be electroporated into rat epitrochlearis muscle (the contralateral epitrochlearis muscle will be electroporated with empty vector and serve as a control). Rats will then be exercised to see if muscles expressing the phospho-mutants no longer have sustained AS160 phosphorylation.

Specific Aim 5: *Determine if overexpressing phospho-mutants of Inh-1 and/or Inh-2 in the rat epitrochlearis muscle results in the impairment of the sustained increase in AS160 phosphorylation three hours after exercise.*

Hypothesis 5: Electroporating DNA vectors of Inh-1/Inh-2 with mutations (via alanine substitution) of the phospho-sites essential for its activity into the rat epitrochlearis will result in the elimination of sustained AS160 phosphorylation three hours after exercise.

If results from Specific Aim 4, reveal that AS160 phosphorylation is no longer sustained three hours post-exercise, in the genetically modified muscles, the post-exercise increase in insulin-stimulated glucose transport skeletal muscle glucose will be assessed

to determine if sustained AS160 phosphorylation after exercise is essential for this functional outcome. We will also assess other relevant proteins (GLUT4 abundance, amount of other serine/threonine phosphatases associated with AS160, activation states of kinases, etc.) to monitor for potential compensatory or indirect effects of phospho-mutants.

Specific Aim 6: *Determine if overexpressing mutated Inh-1 and Inh-2 results in the impairment of the post-exercise increase in insulin-stimulated glucose transport in rat skeletal muscle.*

Hypothesis 6: Mutations of Inh-1/Inh-2 phospho-sites in rat epitrochlearis muscle (via electroporation of DNA containing alanine substitutions) will have reduced glucose transport post-exercise with a physiological insulin concentration vs. empty vector controls.

Research Design and Methods

Research Protocol

Specific Aim 1: AS160 will be immunoprecipitated from rat epitrochlearis lysate and prepared for mass spectrometry. The mass spectrometry analysis will provide a list of candidate proteins associated with AS160 that can be assessed to determine if any of the PP1 regulatory subunit(s) are associated with AS160.

Specific Aim 2: PP1reg-AS160 DNA vectors will be obtained or isolated from rat DNA via PCR. These vectors will be heat-shocked into competent cells, grown en masse in E. Coli cells, and a commercially available DNA preparation kit will be used to isolate

a large quantity of pure DNA from the cells. This DNA containing PP1reg-AS160 will be electroporated into one epitrochlearis muscle of male Wistar rats (6-8 weeks old) and a sham electroporation transfection (using empty vector) will be performed in the contralateral muscle as previously described (2, 3, 5). The epitrochlearis muscles from these rats will be dissected and split into two longitudinal muscle strips with one strip being incubated in a solution with physiological insulin and the other being incubated in a solution without insulin. The muscles will then be frozen and assessed for PP1reg-AS160 abundance, AS160 and PP1reg-AS160 association, PP1cat-PP1reg-AS160 association, and $AS160^{Thr642}$ and $AS160^{Ser588}$ phosphorylation will be measured using immunoprecipitation and immunoblotting. The procedure of the time course of the reversal of $AS160^{Thr642}$ and $AS160^{Ser588}$ phosphorylation after transient physiological insulin incubation will be conducted as previously described (30).

Specific Aim3: PP1reg-AS160 siRNA would be generated either by purchasing from a manufacturer or constructing one using a kit (26). The PP1reg-AS160 siRNA will be electroporated into one epitrochlearis muscle in of male Wistar rats (6-8 weeks old) and a sham electroporation transfection (using empty vector) will be performed in the contralateral muscle as previously described (1, 16). The epitrochlearis muscles from these rats will be dissected and split into two longitudinal muscle strips with one strip being incubated in a solution with physiological insulin and the other being incubated in a solution without insulin. The muscles will then be frozen and assessed for PP1reg-AS160 abundance, AS160 and PP1reg-AS160 association, PP1cat-PP1reg-AS160 association, and $AS160^{Thr642}$ and $AS160^{Ser588}$ phosphorylation will be measured using immunoprecipitation and immunoblotting. The procedure of the time course of the

reversal of AS160^{Thr642} and AS160^{Ser588} phosphorylation after transient physiological insulin incubation will be conducted as previously described (30).

Specific Aim 4: Male (6-8 week old) Wistar rats will be assigned to either remain sedentary or to perform a swimming exercise protocol (10, 28) that is known to result in sustained AS160 phosphorylation after exercise. Following exercise, the rats will be returned to their cage for three hours without access to food. The rats that had exercised and time-matched sedentary controls will be anesthetized and their epitrochlearis muscles will be dissected and contra-lateral muscles will being incubated in a solution with or without a physiologic dose of insulin (50μU/ml). Muscles from another cohort of rats will be dissected immediately after exercise. The muscles from both time points will then be frozen and AS160 will be immunoprecipitated and then immunoblotted to assess phosphorylation of Inh-1 and Inh-2 using commercially available antibodies to specific phospho-sites of these proteins.

Specific Aim 5: Inh-1/Inh-2 DNA vectors will be obtained or isolated from rat DNA via PCR. Primers containing point mutations of the specific serine/threonine residues (to alanine) will be developed and used to amplify mutated Inh-1 and Inh-2 constructs via PCR. These vectors will be heat-shocked into competent cells, grown en masse in E. Coli cells, and a commercially available DNA preparation kit will be used to isolate a large quantity of pure DNA from the cells. This DNA containing mutated forms of Inh-1/Inh-2 will be electroporated into one epitrochlearis muscle of male Wistar rats (6-8 weeks old) and an electroporation transfection will be performed in the contralateral muscle with empty vector. After recovery, these rats will be assigned to either remain sedentary or to perform a swimming exercise protocol (10, 28) that is known to result in

sustained AS160 phosphorylation after exercise. Following exercise, the rats will be returned to their cage for three hours without access to food. The rats that had exercised and time-matched sedentary controls will be dissected, frozen, and AS160 will be immunoprecipitated and then immunoblotted to assess phosphorylation of Inh-1 and Inh-2 using commercially available antibodies to specific phospho-sites of these proteins.

Specific Aim 6: The same protocol will be used as described in Specific Aim 4, except that epitrochlearis muscles (the transfected and empty vector transfected muscle) will be split into two longitudinal muscle strips with one strip being incubated in a solution with physiological insulin and the other being incubated in a solution without insulin. After incubation of the skeletal muscles in insulin, those muscles will be subsequently incubated for the measurement of 3-methylglucose transport and then frozen.

Interpretation

Specific Aim 1: If mass spectrometry analysis of immunoprecipitated AS160 reveals a PP1reg-AS160 candidate, this would suggest that PP1 is a specific phosphatase of AS160. If no PP1reg-AS160 candidates are found, this would suggest that PP1 is either not a phosphatase of AS160 or that the conditions in which AS160 was immunoprecipitated resulted in disassociated PP1reg-AS160.

Specific Aim 2: If overexpression of PP1reg-AS160 in rat epitrochlearis muscle is accompanied by all of the predicted effects, it would be strong evidence that the PP1reg - AS160 is an important determinant of AS160 dephosphorylation by PP1.

If successful overexpression of PP1reg-AS160 resulted in increased PP1reg-AS160 abundance, association with AS160, and association with PP1cat, but did not result in reduced phosphorylation of AS160 or a more rapid time course of reversal, the hypothesis would not be supported and suggest that AS160 dephosphorylation is not dependent on increased PP1 abundance (via PP1reg-AS160 overexpression).

Specific Aim 3: If reduced expression of PP1reg-AS160 in rat epitrochlearis muscle is accompanied by all of the predicted effects, it would be strong evidence that the PP1-reg-AS160 is an important determinant of AS160 dephosphorylation by PP1.

If reduced expression of PP1reg-AS160 that resulted in decreased PP1reg-AS160 abundance, association with AS160, and association with PP1cat, but did not result in increased phosphorylation of AS160 or a prolonged rapid time course of reversal, the hypothesis would not be supported and suggest that AS160 dephosphorylation is not dependent on PP1 (via PP1reg-AS160 reduction), there is compensation from another phosphatase, or that the degree of phosphorylation of AS160 that occurs in normally expressing PP1reg-AS160 has already reached an upper physiological limit (i.e. more AS160 proteins cannot be phosphorylated).

Specific Aim 4: If phosphorylation of Inh-1^{Thr35}, Inh-1^{Ser67}, Inh-2^{Thr72}, and/or Inh-2^{Ser86} from immunoprecipitated AS160 from rat skeletal muscle are increased after prior exercise, the hypothesis would be supported suggesting that PP1's phosphatase activity is inhibited concomitant with sustained AS160^{Thr642} and AS160^{Ser588} phosphorylation. This would support the working model that sustained AS160 phosphorylation results from PP1 inhibition by Inh-1 and/or Inh-2. If phosphorylation of these proteins is not altered with prior exercise, this would suggest that PP1 inhibition of AS160 occurs via an Inh-1 and

Inh-2 phosphorylation independent mechanism. Additionally, if these increases in phosphorylation of Inh-1 and Inh-2 after exercise occur independent of insulin, this would suggest that insulin-stimulation does not regulate Inh-1 and Inh-2 phosphorylation.

Specific Aim 5: If mutating Inh-1/Inh-2 phospho-sites results in diminished skeletal muscle AS160^{Thr642} and AS160^{Ser588} phosphorylation after exercise, the hypothesis would be supported and suggests that inhibiting PP1 phosphatase activity on AS160 abolishes the sustained phosphorylation of AS160 post-exercise. If mutating Inh-1/Inh-2 phospho-sites does not diminish skeletal muscle AS160^{Thr642} and AS160^{Ser588} phosphorylation after insulin treatment, the hypothesis would not be supported and suggest that Inh-1 and Inh-2 phosphorylation is either not important for PP1 regulation of AS160 dephosphorylation or is necessary, but not sufficient for PP1 regulation of AS160 dephosphorylation.

Specific Aim 6: If the post-exercise increase in insulin-stimulated glucose transport is reduced in mutated muscle vs. non-mutated muscle, the hypothesis would be supported and suggest that AS160 dephosphorylation by PP1 (secondary to increased Inh-1/Inh-2 phosphorylation) leading to sustained AS160 phosphorylation results in enhanced insulin-stimulated glucose transport post-exercise. A working model is depicted in Figure 6.1.

If the post-exercise increase in insulin-stimulated glucose transport is similar in mutated muscle vs. non-mutated muscle, the hypothesis would not be supported and suggest that AS160 dephosphorylation by PP1 (secondary to increased Inh-1/Inh-2 phosphorylation) leading to sustained AS160 phosphorylation is independent of the mechanisms leading to enhanced insulin-stimulated glucose transport post-exercise.

Alternative Approaches

PP1 regulatory subunit: If there are problems with the electroporation of the PP1reg-AS160 DNA or siRNA, we can assess the transfection in L6 myotubes.

PP1 catalytic subunit: If there are problems with the electroporation of the Inh-1/Inh-2 phospho-mutants, the kinases of Inh-1 and Inh-2 can be targeted as they are well established. Inhibiting the activity of the kinases on their ability to phosphorylate Inh-1 and Inh-2 may be conducted via genetic manipulation or pharmacological intervention using known inhibitors.

Overall Conclusions

In conclusion, this dissertation investigated the mechanism of two endpoints resulting from the same initial intervention: the long-lasting effects of exercise on 1) insulin-stimulated glucose transport and 2) AS160 phosphorylation in rodent skeletal muscle. The first part of this dissertation focused on the possible triggering mechanisms that lead to the post-exercise increase in insulin-stimulated glucose transport. We hypothesized that mice that lacked the B2 receptor of bradykinin and rats that were deficient in plasma kininogen versus their respective sedentary controls would not develop an increase in insulin-stimulated glucose transport after a single bout of exercise in skeletal muscle. However, these hypotheses were not supported suggesting that the KKS is not essential for development of increased muscle insulin sensitivity after *in vivo* exercise.

The second part of the dissertation focused on the mechanisms regulating a key signaling event that has been reported to occur concomitant with increased post-exercise insulin-stimulated glucose transport, sustained AS160 phosphorylation after acute exercise. The results in this study demonstrated that not only is AS160 phosphorylation increased at the Thr642 site in rat epitrochlearis muscle, but it is also sustained at the Ser588 site. These results are significant because these are the two phopsho-sites important for the full effect of insulin-stimulated GLUT4 translocation (27). The results of the study implicated AMPK as the AS160 kinase that is most likely to be responsible for the increased AS160 phosphorylation found in skeletal muscle immediately after exercise. Additionally, the results of this study provided evidence that sustained phosphorylation of these AS160 phospho-sites after exercise is not likely regulated by a sustained increase in phosphorylation of any of the four known AS160 kinases (Akt, AMPK, RSK, SGK) on sites essential for their activity. The results from this study further revealed that of the four most highly expressed serine/threonine phosphatases in skeletal muscle, PP1 and PP2C are both associated with AS160 and able to dephosphorylate AS160 immunoprecipitated from isolated skeletal muscle. Future studies are needed to clarify the possible roles of these phosphatases for sustained AS160 phosphorylation and to establish a role for sustained AS160 phosphorylation for the increase in insulin-stimulated glucose transport in skeletal muscle after acute exercise.

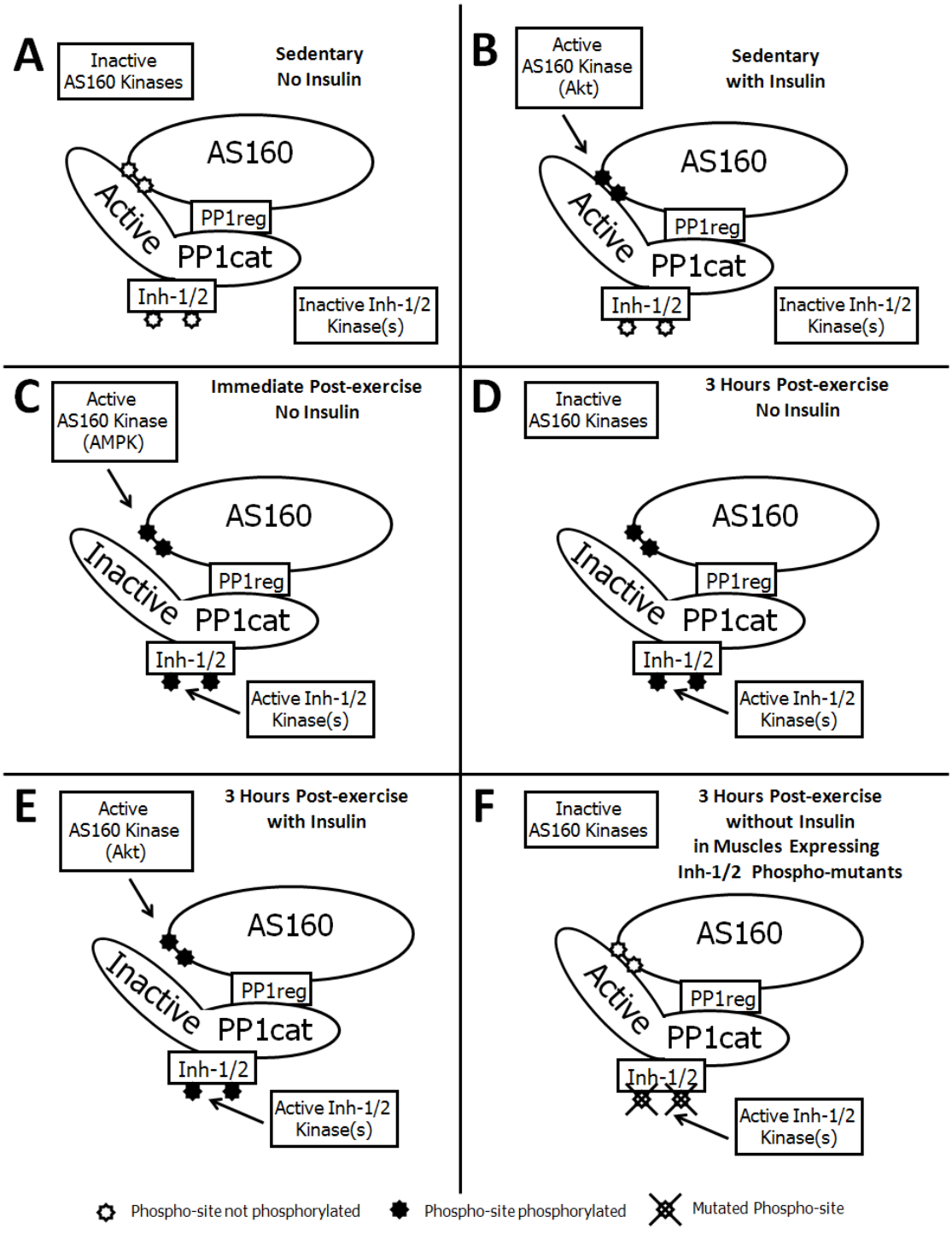

Figure 6.1
PP1 and AS160 Working Model for Research Proposal.

Abbreviations: *PP1cat: PP1 catalytic subunit, PP1reg: PP1 regulatory subunit, Inh-1/2: Inhibitor 1 and/or Inhibitor-2.*

Legend for Figure 6.1 (see preceding page)

A) *PP1 and AS160 under sedentary conditions with no insulin*: Both AS160 kinases and Inh-1/2 kinases are inactive, Inh-1/2 is not phosphorylated, and PP1 is constitutively active favoring dephosphorylated AS160.

B) *PP1 and AS160 under sedentary conditions with insulin*: Inh-1/2 kinases are inactive, therefore Inh-1/2 is not phosphorylated resulting in constitutively active PP1. AS160 kinase activity (Akt via insulin) is increased sufficiently to oppose constitutively active PP1, resulting in increased AS160 phosphorylation.

C) *PP1 and AS160 immediately post-exercise with no insulin:* Exercise causes activation of Inh-1/2 kinases that phosphorylate Inh-1/2, which in turn renders PP1 inactive. Exercise causes activation of AS160 kinase (AMPK) resulting in increased AS160 phosphorylation.

D) *PP1 and AS160 three hours post-exercise with no insulin*: Exercise causes activation of Inh-1/2 kinases that phosphorylate Inh-1/2, which in turn renders PP1 inactive. The immediate effects of exercise on activation of AS160 kinases (AMPK) have reversed, resulting in inactive AS160 kinases. However, PP1 dephosphorylation activity is inhibited, resulting in sustained AS160 phosphorylation that had originally occurred because of the activation of AS160 kinase activity (AMPK) during and immediately after exercise).

E) *PP1 and AS160 three hours post-exercise with insulin*: Exercise causes activation of Inh-1/2 kinases that phosphorylate Inh-1/2, which in turn renders PP1 inactive. The immediate effects of exercise on activation of AS160 kinases (AMPK) have reversed, but insulin results in activation of AS160 kinases (Akt). However, PP1 dephosphorylation activity is inhibited, resulting in sustained and enhanced AS160 phosphorylation because of the cumulative effects of activation of AS160 kinase (AMPK) during and immediately after exercise along with the subsequent activation of AS160 kinase (Akt) induced by insulin.

F) *PP1 and AS160 three hours post-exercise without insulin in muscles expressing Inh-1/2 phospho-mutant:* Exercise causes activation of Inh-1/2 kinases that phosphorylate Inh-1/2, but they are unable to phosphorylate Inh-1/2 phospho-mutants resulting in constitutively active PP1. The immediate effects of exercise on activation of AS160 kinases (AMPK) have reversed resulting in inactive AS160 kinases and, constitutively active PP1 favors dephosphorylated AS160.

An assumption of this simple model is that PP1 is the only Ser/Thr phosphatase regulating AS160 dephosphorylation that is responsive to exercise-induced inactivation.

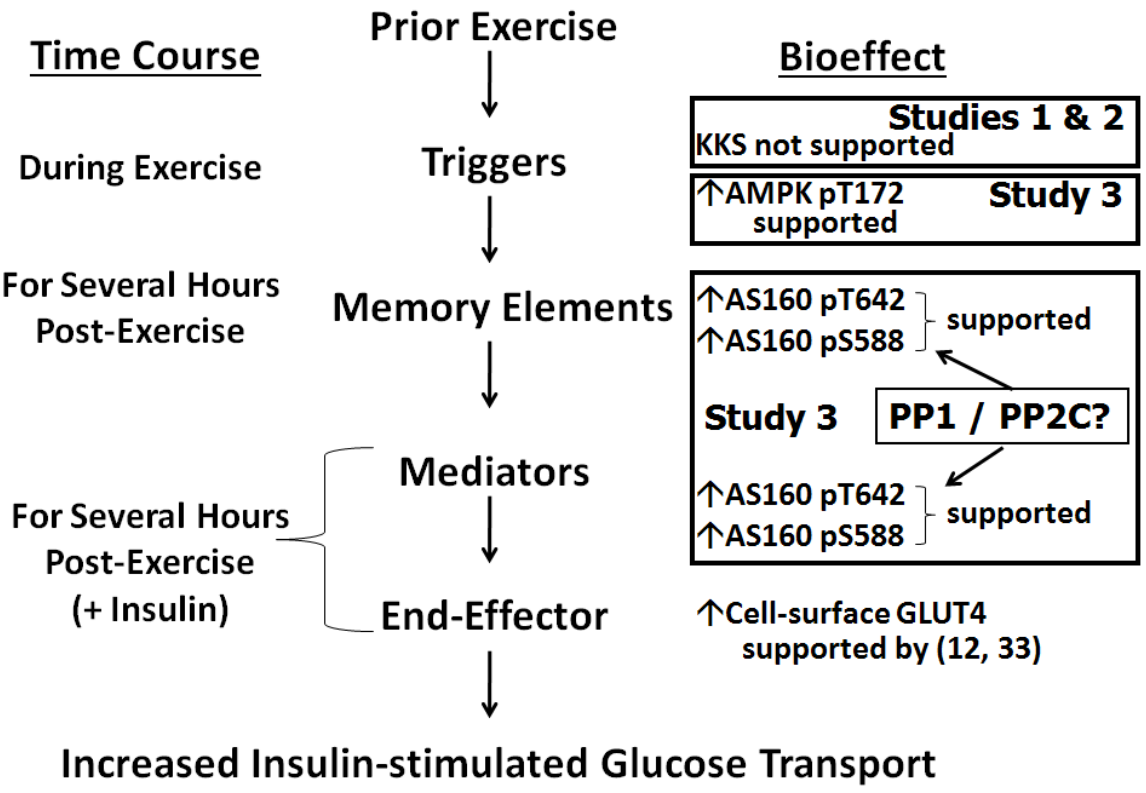

Figure 6.2
Working model for prior exercise resulting in increased insulin-stimulated glucose transport in skeletal muscle. This model describes the time-course and bioeffects associated with each step. Studies 1 & 2 did not support the KKS as an essential *trigger* during exercise, whereas Study 3 provided support for phosphorylated AMPK as a possible *trigger*. Study 3 supported sustained phosphorylation of AS160^{Thr642} and AS160^{Ser588} as *memory element/mediator* in the hours after exercise. PP1 and/or PP2C may regulate AS160^{Thr642} and AS160^{Ser588} phosphorylation. Prior research indicates increased cell-surface GLUT4 as an *end-effector* resulting in increased insulin-stimulated glucose transport. AMPK pT172: phosphorylated AMPK^{Thr172}, AS160 pT642: phosphorylated AS160^{Thr642}, AS160 pS588: phosphorylated AS160^{Ser588}, GLUT4: glucose transporter 4, KKS: Kallikrein-Kininogen System.

REFERENCES

1. **Al-Khalili L, Cartee GD, and Krook A.** RNA interference-mediated reduction in GLUT1 inhibits serum-induced glucose transport in primary human skeletal muscle cells. *Biochemical and biophysical research communications* 307: 127-132, 2003.
2. **An D, Toyoda T, Taylor EB, Yu H, Fujii N, Hirshman MF, and Goodyear LJ.** TBC1D1 regulates insulin- and contraction-induced glucose transport in mouse skeletal muscle. *Diabetes* 59: 1358-1365, 2010.
3. **Benton CR, Holloway GP, Han XX, Yoshida Y, Snook LA, Lally J, Glatz JF, Luiken JJ, Chabowski A, and Bonen A.** Increased levels of peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1alpha) improve lipid utilisation, insulin signalling and glucose transport in skeletal muscle of lean and insulin-resistant obese Zucker rats. *Diabetologia* 53: 2008-2019, 2010.
4. **Cartee GD and Holloszy JO.** Exercise increases susceptibility of muscle glucose transport to activation by various stimuli. *Am J Physiol* 258: E390-393, 1990.
5. **Czibik G, Sagave J, Martinov V, Ishaq B, Sohl M, Sefland I, Carlsen H, Farnebo F, Blomhoff R, and Valen G.** Cardioprotection by hypoxia-inducible factor 1 alpha transfection in skeletal muscle is dependent on haem oxygenase activity in mice. *Cardiovascular research* 82: 107-114, 2009.
6. **Duka I, Shenouda S, Johns C, Kintsurashvili E, Gavras I, and Gavras H.** Role of the B(2) receptor of bradykinin in insulin sensitivity. *Hypertension* 38: 1355-1360, 2001.
7. **Dumke CL, Kim J, Arias EB, and Cartee GD.** Role of kallikrein-kininogen system in insulin-stimulated glucose transport after muscle contractions. *J Appl Physiol* 92: 657-664, 2002.
8. **Foulkes JG, Strada SJ, Henderson PJ, and Cohen P.** A kinetic analysis of the effects of inhibitor-1 and inhibitor-2 on the activity of protein phosphatase-1. *European journal of biochemistry / FEBS* 132: 309-313, 1983.
9. **Funai K, Schweitzer GG, Castorena CM, Kanzaki M, and Cartee GD.** In vivo exercise followed by in vitro contraction additively elevates subsequent insulin-stimulated glucose transport by rat skeletal muscle. *American journal of physiology* 298: E999-1010, 2010.
10. **Funai K, Schweitzer GG, Sharma N, Kanzaki M, and Cartee GD.** Increased AS160 phosphorylation, but not TBC1D1 phosphorylation, with increased postexercise insulin sensitivity in rat skeletal muscle. *American journal of physiology* 297: E242-251, 2009.
11. **Gao J, Gulve EA, and Holloszy JO.** Contraction-induced increase in muscle insulin sensitivity: requirement for a serum factor. *Am J Physiol* 266: E186-192, 1994.
12. **Hansen PA, Nolte LA, Chen MM, and Holloszy JO.** Increased GLUT-4 translocation mediates enhanced insulin sensitivity of muscle glucose transport after exercise. *J Appl Physiol* 85: 1218-1222, 1998.
13. **Henningsen J, Rigbolt KT, Blagoev B, Pedersen BK, and Kratchmarova I.** Dynamics of the skeletal muscle secretome during myoblast differentiation. *Mol Cell Proteomics* 9: 2482-2496, 2010.

14. **Huang FL and Glinsmann WH.** Separation and characterization of two phosphorylase phosphatase inhibitors from rabbit skeletal muscle. *European journal of biochemistry / FEBS* 70: 419-426, 1976.
15. **Huang KX and Paudel HK.** Ser67-phosphorylated inhibitor 1 is a potent protein phosphatase 1 inhibitor. *Proceedings of the National Academy of Sciences of the United States of America* 97: 5824-5829, 2000.
16. **Ishikura S, Bilan PJ, and Klip A.** Rabs 8A and 14 are targets of the insulin-regulated Rab-GAP AS160 regulating GLUT4 traffic in muscle cells. *Biochemical and biophysical research communications* 353: 1074-1079, 2007.
17. **Kuntziger T, Rogne M, Folstad RL, and Collas P.** Association of PP1 with its regulatory subunit AKAP149 is regulated by serine phosphorylation flanking the RVXF motif of AKAP149. *Biochemistry* 45: 5868-5877, 2006.
18. **MacDonald JA, Eto M, Borman MA, Brautigan DL, and Haystead TA.** Dual Ser and Thr phosphorylation of CPI-17, an inhibitor of myosin phosphatase, by MYPT-associated kinase. *FEBS letters* 493: 91-94, 2001.
19. **MacDougall LK, Campbell DG, Hubbard MJ, and Cohen P.** Partial structure and hormonal regulation of rabbit liver inhibitor-1; distribution of inhibitor-1 and inhibitor-2 in rabbit and rat tissues. *Biochimica et biophysica acta* 1010: 218-226, 1989.
20. **Park IK, Roach P, Bondor J, Fox SP, and DePaoli-Roach AA.** Molecular mechanism of the synergistic phosphorylation of phosphatase inhibitor-2. Cloning, expression, and site-directed mutagenesis of inhibitor-2. *The Journal of biological chemistry* 269: 944-954, 1994.
21. **Paterson J, Kelsall IR, and Cohen PT.** Disruption of the striated muscle glycogen-targeting subunit of protein phosphatase 1: influence of the genetic background. *Journal of molecular endocrinology* 40: 47-59, 2008.
22. **Pedersen BK.** Muscles and their myokines. *The Journal of experimental biology* 214: 337-346, 2011.
23. **Pedersen BK, Akerstrom TC, Nielsen AR, and Fischer CP.** Role of myokines in exercise and metabolism. *J Appl Physiol* 103: 1093-1098, 2007.
24. **Rhaleb NE, Yang XP, Nanba M, Shesely EG, and Carretero OA.** Effect of Chronic Blockade of the Kallikrein-Kinin System on the Development of Hypertension in Rats. *Hypertension* 37: 121-128, 2001.
25. **Sahin B, Shu H, Fernandez J, El-Armouche A, Molkentin JD, Nairn AC, and Bibb JA.** Phosphorylation of protein phosphatase inhibitor-1 by protein kinase C. *The Journal of biological chemistry* 281: 24322-24335, 2006.
26. **Sanchez-Elsner T, Gou D, Kremmer E, and Sauer F.** Noncoding RNAs of trithorax response elements recruit Drosophila Ash1 to Ultrabithorax. *Science (New York, NY* 311: 1118-1123, 2006.
27. **Sano H, Kane S, Sano E, Miinea CP, Asara JM, Lane WS, Garner CW, and Lienhard GE.** Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *The Journal of biological chemistry* 278: 14599-14602, 2003.
28. **Schweitzer GG and Cartee GD.** Postexercise skeletal muscle glucose transport is normal in kininogen-deficient rats. *Medicine and science in sports and exercise* 43: 1148-1153, 2011.

29. **Schweitzer GG, Castorena CM, Hamada T, Funai K, Arias EB, and Cartee GD.** The B2 receptor of bradykinin is not essential for the post-exercise increase in glucose uptake by insulin-stimulated mouse skeletal muscle. *Physiological research / Academia Scientiarum Bohemoslovaca*, 2011.
30. **Sharma N, Arias EB, and Cartee GD.** Rapid reversal of insulin-stimulated AS160 phosphorylation in rat skeletal muscle after insulin exposure. *Physiological research / Academia Scientiarum Bohemoslovaca*, 2009.
31. **Shi Y.** Serine/threonine phosphatases: mechanism through structure. *Cell* 139: 468-484, 2009.
32. **Svenningsson P, Nishi A, Fisone G, Girault JA, Nairn AC, and Greengard P.** DARPP-32: an integrator of neurotransmission. *Annual review of pharmacology and toxicology* 44: 269-296, 2004.
33. **Thorell A, Hirshman MF, Nygren J, Jorfeldt L, Wojtaszewski JF, Dufresne SD, Horton ES, Ljungqvist O, and Goodyear LJ.** Exercise and insulin cause GLUT-4 translocation in human skeletal muscle. *Am J Physiol* 277: E733-741, 1999.
34. **Tsaytler P, Harding HP, Ron D, and Bertolotti A.** Selective inhibition of a regulatory subunit of protein phosphatase 1 restores proteostasis. *Science (New York, NY* 332: 91-94, 2011.
35. **Wang QM, Park IK, Fiol CJ, Roach PJ, and DePaoli-Roach AA.** Isoform differences in substrate recognition by glycogen synthase kinases 3 alpha and 3 beta in the phosphorylation of phosphatase inhibitor 2. *Biochemistry* 33: 143-147, 1994.